

CHARLES UNIVERSITY IN PRAGUE

Faculty of Science

Department of Organic Chemistry



- I. Synthesis of acyclic nucleoside phosphonates with a thiadiazole base mimicking 5-azacytosine as compounds with potential biological activities**
- II. Development of small molecules targeting *c-MYC* oncogene**

- I. Syntéza acyklických nukleosidfosfonátů s thiadiazolovou bází mimikujících 5-azacytosin jako látek s potenciálními biologickými účinky*
- II. Vývoj malých molekul cílících onkogen *c-MYC**

PhD Thesis

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V Praze dne

.....

Alice Pomeislová

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Abstract

The first part of the thesis regards synthesis of *N*-[2-(phosphonomethoxy)ethyl] (PME) and (*S*)-3-hydroxy-2-(phosphonomethoxy)propyl ((*S*)-HPMP) derived acyclic nucleoside phosphonate (ANP) analogues carrying 5-amino-1,2,4-thiadiazol-3(2*H*)-one as a nucleobase that is supposed to mimic cytosine or 5-azacytosine. A series of 1,2,4-thiadiazole derivatives bearing at the *N*² position PME- or (*S*)-HPMP-moiety and NH₂-protecting group (benzoyl, ethoxycarbonyl, or Fmoc) were obtained as chemically stable ANP congeners. Their synthesis was performed *via* stepwise construction of the thiadiazole ring and required the use of two newly prepared synthons, PME-amine and (*S*)-HPMP-amine. However, all attempts to prepare the intended PME- and (*S*)-HPMP-thiadiazole phosphonic acids with free amino moiety failed due to instability of the *N*²-substituted thiadiazole ring. Biological evaluation of twenty-one selected thiadiazole compounds towards two human cysteine-dependent enzymes, cathepsin K and glycogen synthase kinase 3β, revealed that several compounds inhibited both enzymes in the low micromolar range. Some of these efficient inhibitors had also favourable toxicity profile at 100 μM, which makes them appropriate for further development of potential drug candidates.

In the second part of the dissertation, design and synthesis of small molecules that would stabilize G-quadruplex (G4) in the promoter region of the protooncogene *c-MYC* is described. Two fragment libraries were constructed utilizing click chemistry. The first library was inspired by the 4-(4-methylpiperazin-1-yl)aniline molecule that was identified as a *c-MYC* ligand in previously performed fragment library screen, while the second fragment library was based on the structure of pyridostatin, a well-established ligand of the human telomeric G4 (H-Telo). Both fragment libraries were evaluated by Förster resonance energy transfer (FRET) melting assay for their *c-MYC* stabilization potential. Moreover, template-assisted formation of individual click product in the pool of azide and alkyne fragments was determined by kinetic target-guided synthesis (KTGS). Compounds of the pyridostatin derived fragment library showed only partially restored activity towards *c-MYC* compared to the original pyridostatin molecule in FRET and did not show any formation increase in the presence of G4 template oligonucleotide Pu24 during KTGS. On the other hand, within the 4-(4-methylpiperazin-1-yl)aniline-derived fragment library, click products containing additional amino moiety in position 2 of the aniline ring (*o*-aminoaniline click products) exhibited increased G4 melting temperature in FRET. Evaluation of this library by polymerase extension assay confirmed *c-MYC* stabilization potential of most *o*-aminoaniline compounds. Accordingly, determination of binding mode using chemical-shift perturbation ¹H NMR revealed that all *o*-aminoaniline derivatives behaved as strong *c-MYC* binders.

Abstrakt

První část této práce se týká syntézy *N*-[2-(phosphonomethoxy)ethyl] (PME) a (*S*)-3-hydroxy-2-(phosphonomethoxy)propyl (HPMP) analogů acyklických nukleosidfosfonátů (ANP) nesoucích 5-amino-1,2,4-thiadiazol-3(2*H*)-onový heterocyklus jako nukleobázi, o níž se předpokládá, že by mohla mimikovat cytosin nebo 5-azacytosin. Byla získána série 1,2,4-thiadiazolových derivátů, nesoucích v pozici *N*² PME- nebo (*S*)-HPMP- zbytek, a s chráněnou aminoskupinou (benzoyl, ethoxykarbonyl nebo Fmoc), jako chemicky stabilní kongenery ANP. Syntéza těchto látek byla provedena postupnou výstavbou thiadiazolového kruhu za použití dvou nově připravených syntonů, PME-aminu a (*S*)-HPMP-aminu. Všechny pokusy o syntézu původně zamýšlených PME- and (*S*)-HPMP-thiadiazolových fosfonových kyselin s volnou aminoskupinou bohužel selhaly z důvodu lability příslušného *N*²-substituovaného thiadiazolového kruhu. Biologické testování jednadvaceti vybraných thiadiazolových látek vůči dvěma lidským cystein-dependentním enzymům, katepsinu K a glykogensynthase kinase 3β, odhalilo inhibiční účinky několika látek při nízké mikromolární koncentraci. Některé z těchto účinných inhibitorů též vykazovaly příznivý profil toxicity při 100 μM, což je činí vhodnými pro jejich další vývoj coby možných léčiv.

Ve druhé části disertační práce je popisován design a syntéza malých molekul potenciálně schopných stabilizovat G-quadruplex (G4) nacházející se v promotorové oblasti protoonkogenu *c-MYC*. S využitím click chemie byly konstruovány dvě fragmentové knihovny. První knihovna byla inspirována molekulou 4-(4-methylpiperazin-1-yl)anilinu, která byla prostřednictvím dříve provedeného screenu fragmentové knihovny identifikována jako *c-MYC* ligand, zatímco základem druhé fragmentové knihovny byl pyridostatin, což je osvědčený ligand lidského telomerického G4 (H-Telo). Schopnost obou fragmentových knihoven stabilizovat *c-MYC* byla testována pomocí denaturační eseye využívající Försterův resonanční přenos energie (FRET). Kromě toho byl prostřednictvím kinetické templátové řízené syntézy (KTGS) určen vliv přítomnosti templátu na míru vzniku jednotlivých click produktů ze směsi fragmentů - alkynů a azidů. Látky fragmentové knihovny odvozené od pyridostatinu vykazovaly ve srovnání s původní pyridostatinovou molekulou pouze částečnou aktivitu vůči *c-MYC* ve FRET a přítomnost oligonukleotidu Pu24 coby G4 templátu nevedla ke zvýšení míry jejich vzniku během KTGS. Oproti tomu v rámci knihovny odvozené od 4-(4-methylpiperazin-1-yl)anilinu click produkty obsahující další aminoskupinu v poloze 2 anilinového kruhu (*o*-aminoanilinové click produkty) vykazovaly zvýšenou teplotu tání G4 ve FRET. Testování této knihovny s využitím polymerasové stop eseye potvrdilo schopnost stabilizace *c-MYC* u většiny *o*-aminoanilinových látek. Na základě určení vazebného modu pomocí perturbace chemického posunu v ¹H NMR bylo zjištěno, že všechny *o*-aminoanilinové látky vystupují jako silné ligandy *c-MYC*, což je v souladu s výše uvedenými poznatky.

Abbreviations

AC	5-azacytidine
Anal	elemental analysis
ANP	acyclic nucleoside phosphonate
ANPpp	acyclic nucleoside phosphonate diphosphate
bs	broad singlet
calcd.	calculated
CatK	cathepsin K
CDI	1,1'-carbonyldiimidazole
CMV	cytomegalovirus
CuAAC	copper(I)-catalyzed azide-alkyne cycloaddition
d	doublet
DAC	5-aza-2'-deoxycytidine
dd	doublet of doublets
ddd	doublet of doublets of doublets
dddd	doublet of doublets of doublets of doublets
DHAC	5,6-dihydro-5-azacytidine
DHDAC	5,6-dihydro-2'-deoxy-5-azacytidine
DIAD	diisopropyl azodicarboxylate
DMA	<i>N,N</i> -dimethylformamidine
DMF-DMA	<i>N,N</i> -dimethylformamide dimethyl acetal
dsept	doublet of septets
dt	doublet of triplets
EI	electron ionization
Eoc	ethoxycarbonyl
eq.	equivalent
ESI	electrospray ionization
6-FAM	6-carboxyfluorescein
FBLD	fragment-based lead discovery

Fmoc	fluorenylmethyloxycarbonyl
FPMP	<i>N</i> -[3-fluoro-2-(phosphonomethoxy)propyl]
FRET	Förster resonance energy transfer
FTIR	Fourier-transform infrared spectroscopy
G4	G-quadruplex
GSK-3	glycogen synthase kinase 3
H-Telo	the human telomeric G4
HBV	hepatitis B virus
HMBC	heteronuclear multiple bond correlation
HOMO	highest occupied molecular orbital
HPMP	<i>N</i> -[3-hydroxy-2-(phosphonomethoxy)propyl]
(S)-cHPMP-5-azaC	cyclic (<i>S</i>)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytidine
(S)-HPMP-5-azaC	(<i>S</i>)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytidine
(S)-HPMPC	((<i>S</i>)-[3-hydroxy-2-(phosphonomethoxy)propyl]-cytosine, cidofovir
HRMS	high-resolution mass spectrometry
HSQC	heteronuclear single quantum correlation
HTS	high-throughput screening
<i>i</i>	<i>ipso</i> ; <i>iso</i>
IOCB	Institute of Organic Chemistry and Biochemistry
<i>J</i>	coupling constant [Hz]
KTGS	kinetic target-guided synthesis
LC-MS	light-chain mass spectrometry
LUMO	lowest unoccupied molecular orbital
m	multiplet
<i>m</i>	<i>meta</i>
Mp	melting point [°C]
<i>M_r</i>	relative molecular weight
MS	mass spectrometry
MW	microwave
NHE III₁	nuclease hypersensitive element III ₁

NMP	<i>N</i> -methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
<i>o</i>	<i>ortho</i>
p	pentet
<i>p</i>	<i>para</i>
PEA	polymerase extension assay
Ph₃P	triphenylphosphine
PME	<i>N</i> -[2-(phosphonomethoxy)ethyl]
PME-Cl	<i>N</i> -[2-(phosphonomethoxy)ethyl] chloride
PMEA	(9-[2-(phosphonomethoxy)ethyl]adenine), adefovir
PMP	<i>N</i> -[2-(phosphonomethoxy)propyl]
MPA	((<i>R</i>)-9-[2-(phosphonomethoxy)propyl]adenine), tenofovir
ppm	parts per million
R_f	retention factor
RPM	revolutions per minute
r.t.	room temperature
RuAAC	ruthenium(II)-catalyzed azide-alkyne cycloaddition
s	singlet
SAR	structure-activity relationship
STDEV	standard deviation
t	triplet
<i>t</i>	<i>tert</i>
6-TAMRA	6-carboxy-tetramethylrhodamine
TGS	target-guided synthesis
TLC	thin-layer chromatography
T_m	melting temperature of G-quadruplex [°C]
TMS-Br	trimethylsilyl bromide
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
vbs	very broad singlet

Table of contents

Part I	12
1 Introduction	13
1.1 Chemistry background	13
1.1.1 Acyclic nucleoside phosphonates	13
1.1.1.1 Development of ANPs	14
1.1.1.2 The overview of ANPs.....	14
1.1.1.3 Mechanism of antiviral activity	16
1.1.1.4 Synthesis of ANPs	16
1.1.2 Azacytosine chemistry: 5-Azacytosine nucleosides and ANPs	17
1.1.2.1 5-Azacytosine nucleosides.....	17
1.1.2.2 5-Azacytosine ANPs.....	19
1.1.3 Thiadiazoles	21
1.1.3.1 General overview of thiadiazoles.....	21
1.1.3.2 1,2,4-Thiadiazoles.....	21
1.1.3.2.1 5-Amino-1,2,4-thiadiazol-3(2 <i>H</i>)-one	23
1.2 Cathepsin K and glycogen synthase kinase 3 in the role of biological target.....	27
1.2.1 Cathepsin K.....	27
1.2.2 Glycogen synthase kinase 3	28
2 Aims of the work	29
2.1 Rationale for the specific aim	29
3 Results and discussion	30
3.1 Synthesis of thiadiazole ANPs <i>via</i> direct alkylation of the base.....	30
3.2 Synthesis of thiadiazole ANPs <i>via</i> gradual construction of the heterocyclic ring	35
3.3 Evaluation of biological activity	42
4 Conclusions	47
5 Experimental part	48
5.1 General information	48
5.2 Synthetic part – reaction procedures and spectroscopic data.....	49
6 References	76

Part II	89
7 Introduction	90
7.1 Methods in drug discovery.....	90
7.1.1 Fragment-based lead discovery.....	90
7.1.1.1 Fragment library screening methods for hit identification.....	91
7.1.1.2 Fragment-to-lead optimization.....	91
7.1.2 Target-guided synthesis and click chemistry	92
7.1.2.1 The Huisgen 1,3-dipolar cycloaddition.....	94
7.1.2.1.1 Copper(I)-catalyzed azide-alkyne cycloaddition	96
7.2 The <i>c-MYC</i> protooncogene G-quadruplex in the role of target.....	99
7.2.1 G-quadruplexes	99
7.2.2 G-quadruplex of the protooncogene <i>c-MYC</i>	101
7.2.3 Ligand-G4 interaction study methods.....	103
7.2.3.1 Förster resonance energy transfer melting assay	104
7.2.3.2 Polymerase extension assay	106
7.2.3.3 Chemical-shift perturbation NMR	108
7.3 Chemistry of fragments used for library construction	109
7.3.1 Piperazine and 4-(4-methylpiperazin-1-yl)aniline	109
7.3.2 Pyridostatin	109
8 Aims of the work	113
9 Results and discussion	114
9.1 The focused fragment library based on 4-(4-methylpiperazin-1-yl)aniline	114
9.1.1 Modification of the 4-(4-methylpiperazin-1-yl)aniline molecule	114
9.1.2 Construction of the fragment library based on the 4-(4-methylpiperazin-1-yl)aniline structure	118
9.1.2.1 The mono-amino series.....	118
9.1.2.1.1 Design and synthesis of the mono-amino series	118
9.1.2.1.2 The structure-activity relationship regarding the mono-amino series.....	122
9.1.2.1.3 KTGS of the mono-amino click products	126
9.1.2.2 The bis-amino series	130
9.1.2.2.1 Design and synthesis of the bis-amino series.....	130
9.1.2.2.2 The structure-activity relationship regarding the bis-amino series	133
9.1.2.3 Structural basis of compound binding by chemical-shift perturbation NMR	140
9.1.3 Outlook.....	142
9.2 The focused fragment library based on pyridostatin.....	143

9.2.1 Design and synthesis of the pyridostatin derived series.....	143
9.2.2 The structure-activity relationship regarding the pyridostatin fragment library	149
9.2.3 KTGS of the pyridostatin derived click products	156
10 Conclusions	160
11 Experimental part	162
11.1 General information	162
11.2 Synthetic part – reaction procedures and spectroscopic data	164
11.2.1 Synthesis of 4-(4-methylpiperazin-1-yl)aniline derivatives alkylated on the aniline amino group	164
11.2.2 Synthesis of the mono-amino series.....	165
11.2.3 Synthesis of the bis-amino series	175
11.2.4 Synthesis of pyridostatin derivatives	181
11.3 Biochemical evaluation.....	215
11.3.1 Control FRET experiments for the bis-amino series.....	215
11.3.2 <i>In situ</i> click experiments	216
11.3.2.1 The mono-amino click products: Experiment X-Z – methodology and results	216
11.3.2.2 The pyridostatin click products: Experiment D-G – methodology and results	226
12 References	234

Part I

Synthesis of acyclic nucleoside phosphonates with a thiadiazole base mimicking 5-azacytosine as compounds with potential biological activities

Results described and discussed in this part of the thesis were published in the following article:

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1 Introduction

This part of the thesis is dedicated to synthesis of acyclic nucleoside phosphonates (ANPs) containing a thiadiazole moiety that would mimic the 5-azacytosine or the 5,6-dihydro-5-azacytosine nucleobase. Our objective was to obtain new compounds with hypomethylating, cytostatic, antiviral, or other biological activity. Here I provide an overview of the chemical background regarding ANPs, 5-azacytosine chemistry, and chemistry of thiadiazoles. Also, cathepsin K and glycogen synthase kinase β are introduced from the perspective of a biological target since the synthesized compounds were tested as inhibitors of these enzymes.

1.1 Chemistry background

1.1.1 Acyclic nucleoside phosphonates

ANPs are nucleotide analogues where the sugar moiety is replaced by an aliphatic chain bearing a phosphonate function, which is bound to the rest of the molecule *via* a P-C bond [1]. These compounds were first introduced in 1980s by E. De Clercq and A. Holý as novel antiviral agents [1-4] and later became established antivirals [1, 4]. Nevertheless, some of them were gradually found to exert also cytostatic [5-15], antibacterial [16-18], and antimalaric [18-20] activity.

From a chemical perspective, ANPs are phosphonomethyl ethers in which the phosphonate residue is bound to the aliphatic chain of the acyclic nucleoside analogue by an enzymatically non-degradable and chemically stable ether bond. Moreover, the acyclic side chain is bound to the heterocyclic base by a chemically stable *N*-alkyl bond instead of a nucleoside bond. As a result, ANPs are completely metabolically stable. There are four basic types of ANPs that vary in their side chain: *N*-[2-(phosphonomethoxy)ethyl] (PME) derivatives, *N*-[2-(phosphonomethoxy)propyl] (PMP) derivatives, *N*-[3-hydroxy-2-(phosphonomethoxy)propyl] (HPMP) derivatives, and *N*-[3-fluoro-2-(phosphonomethoxy)propyl] (FPMP) derivatives [1]; examples for each type are depicted in Figure 1.1.

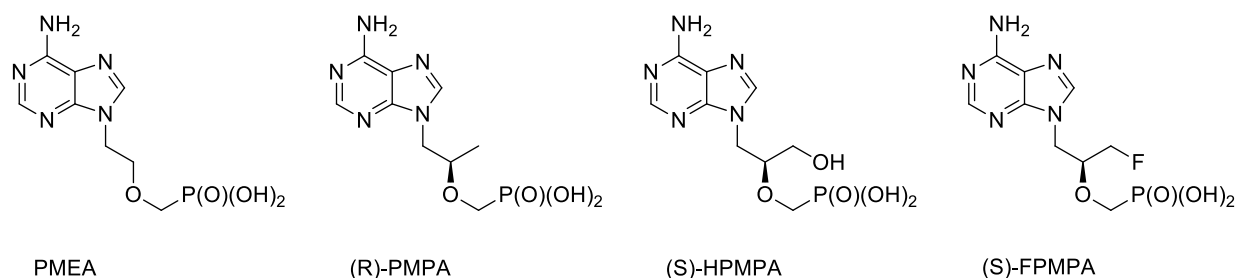


Figure 1.1: Structure of the four basic types of ANP derivatives containing adenine as a nucleobase (drawn according to the ref. [1]).

Apart from the PME derivatives, all other types – PMP, HPMP, and FPMP derived ANPs – contain a stereogenic center in their phosphonate side chain. Among the chiral series, only the (*R*)-PMP, (*S*)-HPMP, and (*S*)-FPMP enantiomers possess biological (antiviral) activity. Important to note, all active enantiomers bear the same absolute configuration as the change from (*R*) to (*S*) is due to change of substituent priority [1, 4, 21, 22].

1.1.1.1 Development of ANPs

At the beginning of ANP discovery, there were efforts to prepare synthetic analogues of natural nucleotides that would serve as antimetabolites in various cell biochemical pathways. These compounds were intended for the treatment of cancer [3, 4, 21], metabolic diseases, or as antiviral or antiparasitic agents. The first generation of such antimetabolites was designed to maximally resemble the natural nucleotides; however, these nucleotide analogues suffered from host toxicity due to their catabolic instability. Therefore, further extensive search regarding substitution of the heterocyclic base and/or the sugar moiety was carried out [4, 21].

All nucleosides are transformed to their active metabolites in three phosphorylation steps by the action of cytosolic enzymes, including nucleoside and nucleotide kinases and nucleoside diphosphate kinases. The first phosphorylation step that affords the 5'-monophosphate is usually the main gridlock of the pathway that leads to biologically active 5'-triphosphate metabolites. Consequently, the first generation of nucleotide analogues, which are dephosphorylated in the blood plasma to enter the cell, exhibited poor activity due to their low phosphorylation rate to the corresponding 5'-monophosphate inside the cell. Moreover, the resulting monophosphorylation products were prone to rapid dephosphorylation in the intracellular space. To avoid the first phosphorylation step and to prepare catabolically stable nucleotide derivatives, the structure of ANPs that contains the phosphonomethoxy arrangement instead of the natural phosphate was proposed. Apart from chemical stability, the phosphonate moiety is isopolar and isosteric with the phosphate functional group as it contains ether oxygen atom in the nearest position adjacent to the α -carbon. The isopolarity as well as flexibility of the aliphatic chain enables recognition of ANPs by phosphorylases and kinases, enzymes that convert ANPs to their biologically active diphosphate (ANPpp) form [4, 21, 22].

1.1.1.2 The overview of ANPs

As outlined previously, ANPs comprise a large number of molecules exerting mainly antiviral activity. The three most important compounds are adefovir, tenofovir, and cidofovir as they are currently used in a clinical practice. To increase their oral bioavailability, adefovir and tenofovir are marketed in their prodrug forms as adefovir dipivoxil, tenofovir disoproxil fumarate [1, 4], and tenofovir alafenamide

[23]. The structure of adefovir dipivoxil, tenofovir disoproxil fumarate, and cidofovir is depicted in Figure 1.2.

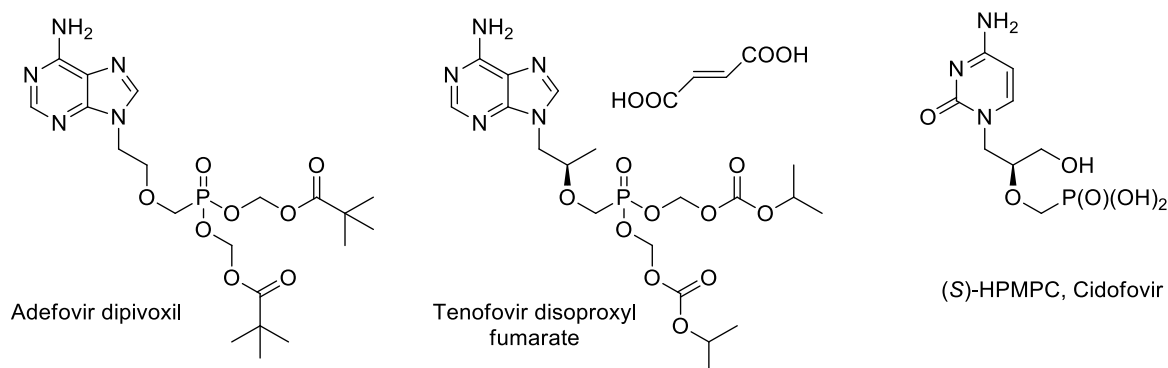


Figure 1.2: Structure of adefovir dipivoxil, tenofovir disoproxil fumarate, and cidofovir (drawn according to the ref. [4]).

Adefovir (9-[2-(phosphonomethoxy)ethyl]adenine), also called "PMEA", exhibits activity against retroviruses as well as DNA viruses – hepadnaviruses and herpesviruses in particular [1, 4, 22]. This compound is used in its dipivoxil prodrug form for the treatment of hepatitis B virus (HBV) infection under the trade name Hepsera® [1, 4, 21, 22]. Adefovir was originally intended as an anti-HIV agent, but clinical trials were terminated as it exhibited side effects at the therapeutic dosage [22, 24]. Apart from antiviral activity, adefovir was also found to have cytostatic effects as it induced differentiation in human erythroleukemia K562 cell cultures [22, 25, 26] and inhibited growth of leukemic lymphoblastic cells KPH-Lw-I [22, 27] and choriocarcinoma in rat [4, 7, 22].

Tenofovir ((*R*)-9-[2-(phosphonomethoxy)propyl]adenine), abbreviated as PMPA, is a potent antiviral agent used against hepadnaviruses and retroviruses, including both the HIV-1 and HIV-2. Its prodrug form, tenofovir disoproxil fumarate, has been launched under the trade name Viread® for treatment of AIDS and HBV infection [1, 4, 22]. In combination with emtricitabine, a dideoxynucleoside analogue with antiretroviral activity, tenofovir disoproxil fumarate was marketed as Truvada® [4, 28]. Although there are other tenofovir therapeutic combinations available on the market, Truvada® is the only one which, besides treatment of HIV-1 infection, serves also for its prevention [28]. In 2016, another tenofovir prodrug form – tenofovir alafenamide fumarate – was launched on the market as Vemlidy® for treatment of HBV infections [23, 29]. Combinations of tenofovir alafenamide with other active substances (e.g., Genvoya® or Odefsey) are used for treatment of HIV-1 [30].

The last ANP-based antiviral drug, cidofovir ((*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-cytosine, (*S*)-HPMPC), is efficient against polyomaviruses, papillomaviruses, adenoviruses, and a wide range of herpesviruses [4]. This compound has been approved under the trade name Vistide® for treatment

of cytomegalovirus (CMV) retinitis in AIDS patients [1, 4, 31] and is also used off-label against papillomatosis infections [32], acyclovir-resistant herpes simplex virus, adenovirus infections and in the case of severe poxvirus infections [33]. In contrast to the above-mentioned ANPs, cidofovir is used in its free phosphonate form as it is administered either intravenously or topically (as a gel, cream, or eye drops) [4]. Besides its antiviral effects, cidofovir served as antitumor agent in animal models representing various human carcinomas, e.g. hemangiosarcoma [4, 12], nasopharyngeal carcinoma [4, 8, 9], or endothelium-derived tumors [4, 13]. The main drawback of cidofovir is its low bioavailability and side-effects, especially nephrotoxicity, which limits a widespread use of this drug [4, 34].

Aside from the above mentioned compounds marketed as drugs, numerous therapeutically promising ANP derivatives have been prepared; however, they never entered clinical trials [35, 36]. As an example, we can mention FPMP derivatives of purines with antiretroviral effects [35, 37], deazapurine analogues of PME derivatives exhibiting activity against DNA viruses and cytostatic effects against L-1210 leukemia [35, 38], and "open-ring" ANP analogues exhibiting significant activity against a broad range of DNA viruses and retroviruses [35, 39-42]. In this last group of compounds, so-called "second generation" ANPs, the naturally occurring base is replaced by either 2,4-diaminopyrimidine or 2-amino-4-hydroxypyrimidine moiety, which is bound to the PME, PMP, or HPMP residue *via* its oxygen atom in position 6, thus mimic a purine base with open imidazole ring [4, 35, 39].

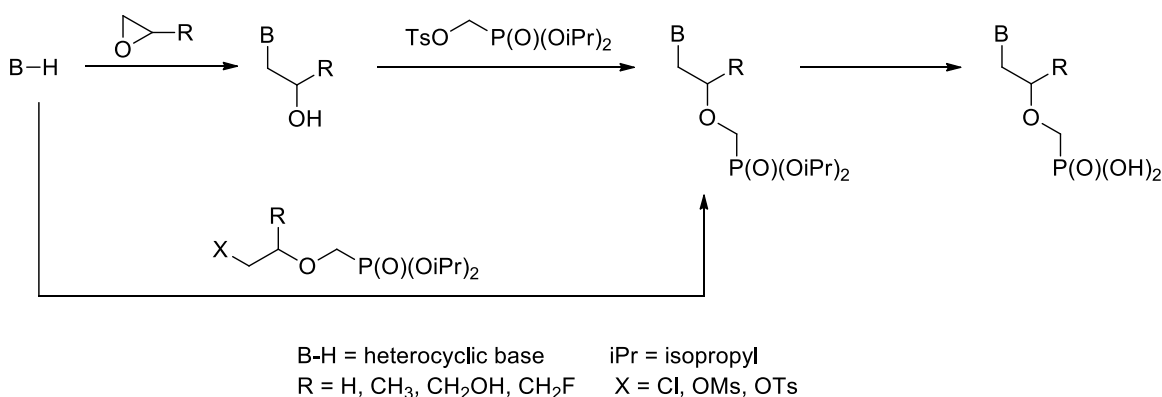
1.1.1.3 Mechanism of antiviral activity

After entering the cell, ANPs are gradually phosphorylated to diphosphate form (ANPpp) that is analogous to the naturally occurring nucleoside triphosphates. ANPpps then serve as inhibitors of both the viral and cellular DNA polymerase and reverse transcriptase in RNA viruses [4, 43, 44, 45]. The antiviral effect is due to higher affinity of the ANPpp towards the viral DNA polymerase or reverse transcriptase compared to the cellular DNA polymerase. Nevertheless, ANPpps act also as substrates of DNA polymerases and are thus incorporated to the growing DNA chain, which leads to DNA chain termination. While PME and PMP derivatives (e.g. adefovir and tenofovir) require incorporation of only one molecule to terminate DNA elongation, two consecutive incorporations of cidofovir (HPMP derivative) are needed for DNA chain termination [3, 4, 44, 45].

1.1.1.4 Synthesis of ANPs

There are two basic approaches for the synthesis of ANPs (Scheme 1.1). The first one regards alkylation of the base with a suitable hydroxyalkyl derivative and subsequent etherification of a free hydroxyl with a phosphonomethoxy moiety. The second option is to alkylate the base with a pre-formed synthon containing all the structural parameters of the intended phosphonate side chain. In both cases, we

obtain a diester that requires deprotection to afford the respective ANP in a free phosphonate state [1, 4, 21, 22].



Scheme 1.1: General scheme for ANP synthesis (drawn according to ref. [22]).

1.1.2 Azacytosine chemistry: 5-Azacytosine nucleosides and ANPs

5-Azacytosine nucleosides and the respective ANP derivatives contain a 1,3,5-triazine, especially 5-azacytosine moiety instead of the cytosine heterocyclic base (Figure 1.3). The compound category of 5-azacytosine nucleosides comprises remarkable biologically active substances mainly with anticancer properties [48]. Azacytosine ANP analogues were developed in order to broaden the spectrum of the cidofovir use and to improve its pharmacological properties. Contrary to 5-azacytosine nucleosides, 5-azacytosine ANPs exert strong antiviral activity [49].

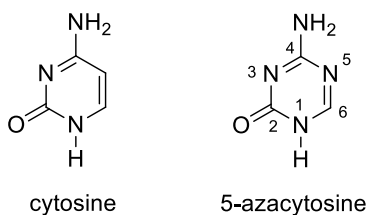


Figure 1.3: Structure of cytosine and 5-azacytosine (drawn according to ref. [50]).

1.1.2.1 5-Azacytosine nucleosides

The first nucleoside derivative containing 5-azacytosine as a nucleobase, 5-azacytidine (AC), was synthesized by A. Piskala in 1963 [48, 51] and in the same year, this compound was found to have antiproliferative activity [48, 52]. Several years later, AC was identified as a naturally occurring metabolite of a Gram-positive bacteria *Streptoverticillium ladakanum* [48, 53]. In 1964, the 2'-deoxy analogue of AC, 5-aza-2'-deoxycytidine (DAC), was synthesized by J. Pliml and F. Šorm [48, 54] and

soon after, this compound was found to have antileukemic activity [48, 55]. Nowadays, both AC (Mylosar, Ladakamycin, Vidaza®) and DAC (Decitabine, Dacogen®) are well-established hypomethylating drugs used for the treatment of myelodysplastic syndromes (Figure 1.4) [48, 56]; besides, they proved to be efficient against acute myeloid leukemia and other types of cancer [48, 57-60].

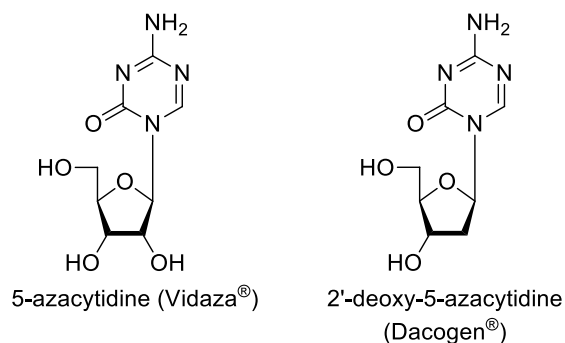


Figure 1.4: Structure of 5-azacytidine and 2'-deoxy-5-azacytidine (drawn according to ref. [48]).

The anticancer effect of AC and DAC is based on their incorporation into DNA upon phosphorylation, which results in inhibition of DNA synthesis in cancer cells [48, 61]. In addition, both AC and DAC are enzymatically deaminated to 5-azauridine and 2'-deoxy-5-azauridine, respectively. These metabolites then interfere with thymidylate synthesis *de novo*, which contributes to cytotoxicity of AC and DAC [48, 62, 63]. Unlike DAC, AC is also incorporated into various types of RNA [48, 62]. While incorporation of AC into mRNA leads to proteosynthesis disruption [48, 62, 64], incorporation into tRNA results in inhibition of tRNA methyltransferases [65] and thus impairment of tRNA acceptor function [66]. Finally, both AC and DAC were found to exhibit DNA demethylating activity [48, 62, 67], which is most apparent at low drug concentrations [48, 62, 68]. After being incorporated into DNA, AC and DAC interact with a thiol residue in the active site of a methyltransferase *via* the 5,6-double bond of its 5-azacytosine ring, thus forming a covalent protein-DNA complex [48, 69]. In relation with its hypomethylating activity, AC and DAC are currently evaluated as epigenetic agents [70-73].

The main drawback of AC and DAC is hydrolytic instability, which limits their pharmaceutical potential. Due to its low electron density, the carbon at position 6 can be easily attacked by a nucleophile (e.g. hydroxyl ion), which leads to a ring opening between the C⁶- and N¹-position [48, 74]. Besides, both AC and DAC are highly cytotoxic at their maximum doses [75-77].

To solve the issue of instability in aqueous solution, 5,6-dihydro- derivatives of AC and DAC were proposed and synthesized (Figure 1.5). Indeed, all compounds containing the 5,6-dihydro-5-azacytosine moiety are hydrolytically stable, as the 5,6-double bond saturation increases electron density at position 6 and thus prevents nucleophilic attack by a water molecule [48, 78]. 5,6-Dihydro-5-

azacytidine (DHAC), the direct 5,6-dihydro-analogue of AC, proved to inhibit RNA synthesis [48, 78-80] as well as DNA methylation [48, 67, 78]; however, serious side effects including cardiotoxicity led to discontinuation of clinical trials [48, 81]. The 5,6-dihydro- analogue of DAC, 5,6-dihydro-2'-deoxy-5-azacytidine (DHDAC) [82], is less toxic than DAC [75] and shows a strong activity against HIV. Nevertheless, the mechanism of DHDAC antiviral activity is different from nucleoside reverse transcriptase inhibitors. After incorporation into the growing DNA chain, DHDAC does not inhibit DNA polymerization, but behaves as an ambiguous base that causes mutations. Increasing number of errors in the viral genome then leads to its disruption and thus may even cause collapse of the viral population [48, 83]. The last remarkable molecule is the α -anomer of DHDAC, (α)-5,6-dihydro-2'-deoxy-5-azacytidine (α -DHDAC). This compound exhibits hypomethylating properties, although weaker than AC and DAC, and low toxicity [48, 75].

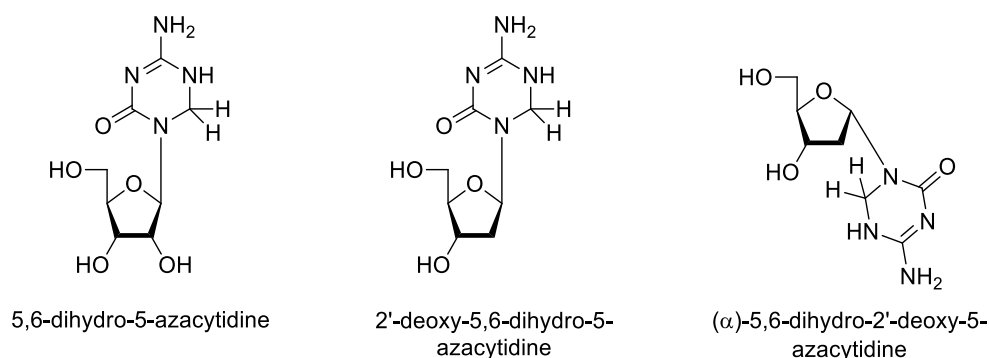


Figure 1.5: Structure of several 5-azacytosine nucleosides (drawn according to ref. [48]).

1.1.2.2 5-Azacytosine ANPs

The close functional resemblance between 5-azacytosine and cytosine nucleobase led to an interest in development and exploration of various 5-azacytosine ANPs that would combine the chemistry of triazines and phosphonates (Figure 1.6) [48, 49]. Among them, the most significant is (*S*)-1-[3-hydroxy-2-(phosphonmethoxy)propyl]-5-azacytidine ((*S*)-HPMP-5-azaC), an 5-azacytidine analogue of cidofovir [84]. This compound shows an extraordinary activity against all DNA viruses, comparable or even higher than cidofovir, and is also more selective and less toxic, which makes it a promising drug candidate [48, 84]. However, similarly as with 5-azacytosine nucleosides, the main drawback of (*S*)-HPMP-5-azaC is hydrolytic instability of the triazine ring (Scheme 1.2). In contrast to the decomposition process of (*S*)-HPMPC (Figure 1.6), the formylamido- derivative is not observed, as it quickly reacts with the hydroxyl moiety in a close proximity upon formiate formation [48, 74]. Its 5,6-dihydro- analogue, 5,6-dihydro-(*S*)-HPMP-5-azaC, exhibited activity against herpesviruses, and to some extent also against vaccinia virus and hepatitis C virus [84].

To improve oral bioavailability of (S)-HPMP-5-azaC, its cyclic prodrug, cyclic (S)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytidine ((S)-cHPMP-5-azaC), and several cyclic ester derivatives were prepared (Figure 1.6) [85]. The (S)-cHPMP-5-azaC molecule showed activity against poxviruses and various herpesviruses, while not inhibiting the cell morphology and growth. Among the cyclic ester prodrugs, the hexadecyloxyethyl derivative exhibited the highest activity and selectivity, followed by the pivaloyloxymethyl ester [48, 85]. Nevertheless, the persisting issue of instability in aqueous solution directed the research towards structural modifications that would increase the electron density in position 6. This was achieved by adding an electron-donating substituent to position 6 (Figure 1.6). While such 6-substituted derivatives were indeed more hydrolytically stable, their antiviral activity was considerably decreased when compared to (S)-HPMP-5-azaC [48, 86]. Therefore, further search for 5-azacytosine derivatives needs to be carried out to solve the problem of the hydrolytic instability and cytotoxicity and, at the same time, to improve their bioavailability by using a suitable ester prodrug form [48].

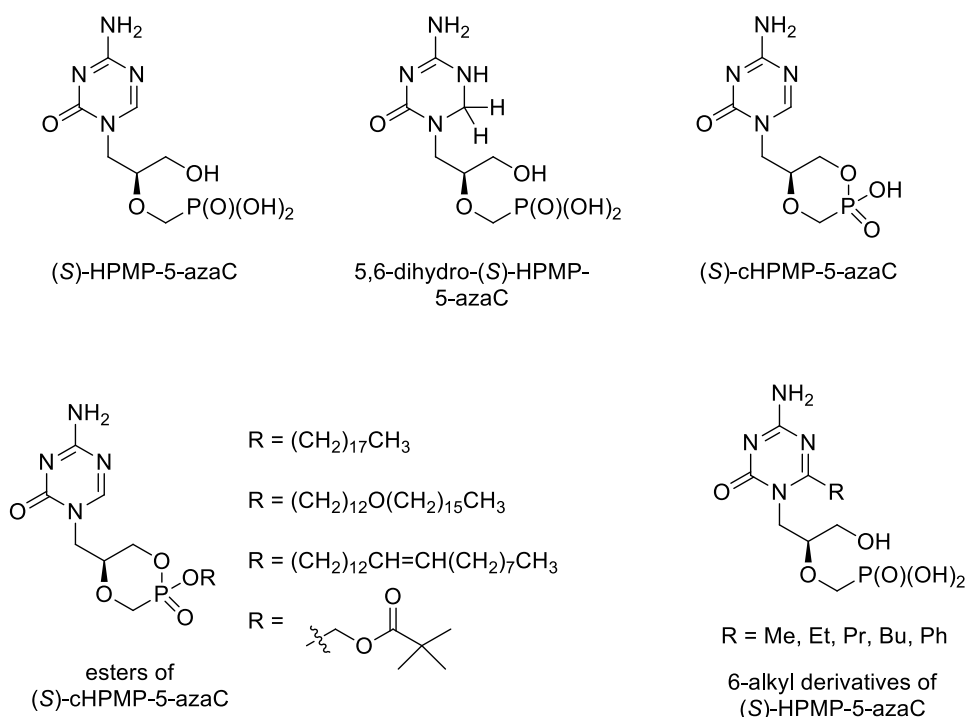
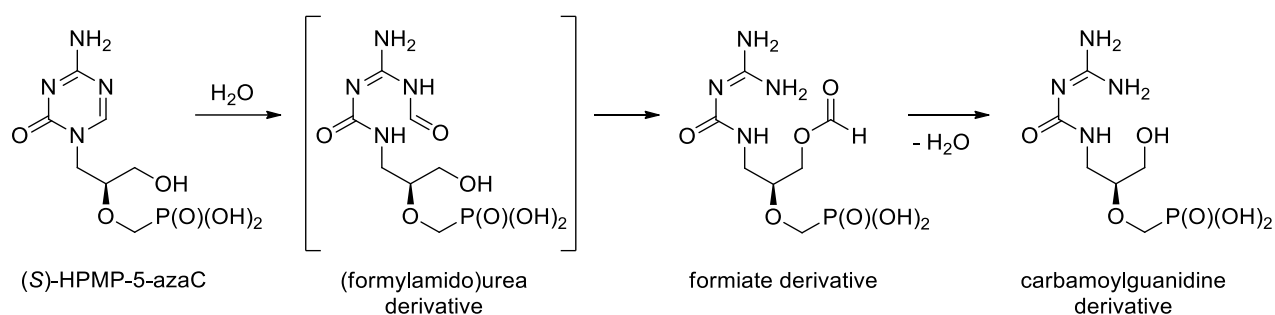


Figure 1.6: Structure of relevant 5-azacytosine ANPs (drawn according to ref. [48]).



Scheme 1.2: Cleavage of the triazine moiety of (S)-HPMP-5-azaC in aqueous solutions (drawn according to ref. [74]).

1.1.3 Thiadiazoles

1.1.3.1 General overview of thiadiazoles

Thiadiazoles are five-membered aromatic heterocycles that contain two nitrogen atoms and one sulfur atom within the cycle. There are four thiadiazole isomers: 1,2,3-thiadiazole, 1,2,4-thiadiazole, 1,2,5-thiadiazole, and 1,3,4-thiadiazole (Figure 1.7) [87].

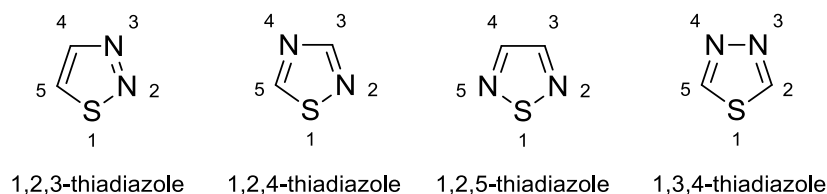


Figure 1.7: Structure of the four thiadiazole isomers (drawn according to ref. [87]).

The structure of thiadiazoles can be found in many natural products and many biologically active compounds, including pharmaceuticals [87]. By far the most common is the 1,3,4-thiadiazole [88], which is a part of many antimicrobial, antitumor, anti-inflammatory, antiviral, neuroprotective, and other biologically active molecules [87]. On the contrary, reported biological activity among 1,2,3- and 1,2,5-thiadiazole derivatives is not that common [88]. The following text will focus on 1,2,4-thiadiazoles and 5-amino-1,2,4-thiadiazol-3(2H)-one in particular, as this compound was used for synthetic elaboration in this research.

1.1.3.2 1,2,4-Thiadiazoles

1,2,4-Thiadiazoles are often found as a part of biologically active molecules [89]. Compounds containing a 1,2,4-thiadiazole moiety exhibit anti-inflammatory [89-91], antihypertensive [89, 92], antibacterial [89, 93, 94], and anticonvulsant activity [89, 95]. As an example, we can name compound SCH-202676 that serves as an allosteric modulator of G-protein coupled receptors [96, 97], 1,2,4-

thiadiazole-derived neuroprotectors that act as inhibitors of glutamate receptors [98], and small heterocyclic thiadiazolidinones that were reported as the first non-ATP competitive inhibitors of glycogen synthase kinase-3 β and have a potential for treatment of Alzheimer's disease [96, 99, 100]. However, the only commercially used 1,2,4-thiadiazole-containing drug is the β -lactam antibiotic Cefozopran [87, 96, 101]. Apart from that, the 1,2,4-thiadiazole scaffold is a part of several biologically active natural products, such as cytotoxic Polycarpathamines A and B isolated from the ascidian *Polycarpa aurata* [87, 102], antiviral Insatindigothiadiazoles A-D isolated from the roots of the herb *Isatis indigotica* [103], cytostatic Penicilliumthiamine B isolated from the fungus *Penicillium oxalicum* [104], and cytotoxic alkaloid Dendrodoin isolated from the tunicate *Dendrodia grossularia* [105]. Structure of several significant 1,2,4-thiadiazole-derived compounds is depicted in Figure 1.8.

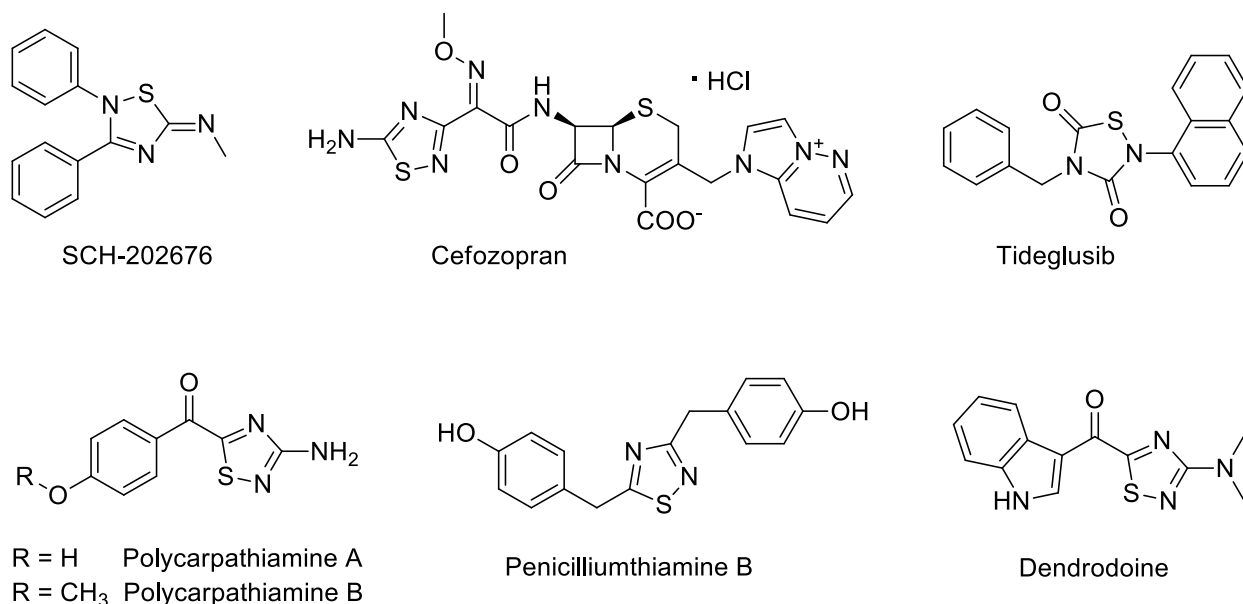
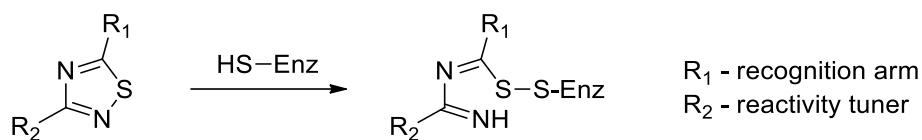


Figure 1.8: Structure of several biologically active 1,2,4-thiadiazole derivatives: SCH-202676 [97], Cefozopran [101], thiadiazolidinone Tideglusib [106], Polycarpathamines A and B [102], Penicilliumthiamine B [104], and Dendrodoin [105].

In addition to the above-mentioned examples, 1,2,4-thiadiazoles were reported as cysteine protease inhibitors [107, 108]. Cysteine proteases contain in the active site a cysteine residue that acts as a nucleophile attacking the sulphur atom of 1,2,4-thiadiazole (Scheme 1.3). Upon thiadiazole ring opening, a disulfidic bond is formed, which leads to irreversible inhibition of the enzyme [108]. The nature of the substituent in position 3 of the thiadiazole ring can fine-tune the reactivity – the rate of ring opening by a thiol. Furthermore, the presence of a peptidomimetic or peptidyl recognition substituent in position 5 can provide the 1,2,4-thiadiazole with enzyme selectivity [108-110]. To date, 1,2,4-thiadiazoles were reported to inhibit guinea pig liver transglutaminase [107], and a lysosomal cysteine protease cathepsin B [108].



Scheme 1.3: Nucleophilic opening of the thiadiazole ring by a cysteine residue in active site of an enzyme leads to disulfide bond formation upon irreversible inhibition of the enzyme. The substituent in position 3 (R_2) serves as a reactivity tuner, while the substituent in position 5 (R_1) acts as a recognition arm (drawn according to ref. [107]).

1.1.3.2.1 5-Amino-1,2,4-thiadiazol-3(2H)-one

In search for efficient nucleoside antimetabolites, the 1,2,4-thiadiazole derivative 5-amino-1,2,4-thiadiazol-3(2H)-one (**1**) has been considered as a suitable analogue of the cytosine nucleobase [111, 112]. Due to similar electronic and spatial properties of the $-\text{CH}=\text{CH}-$ group in aromatic heterocycles and the divalent sulfur atom [111, 113, 114], 5-amino-1,2,4-thiadiazol-3(2H)-one is a base bioisosteric with cytosine (Figure 1.9) [111, 112] and thus could act as its surrogate in construction of intended nucleoside analogues.

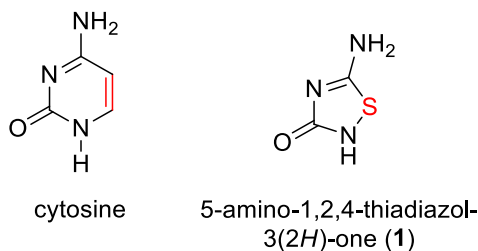
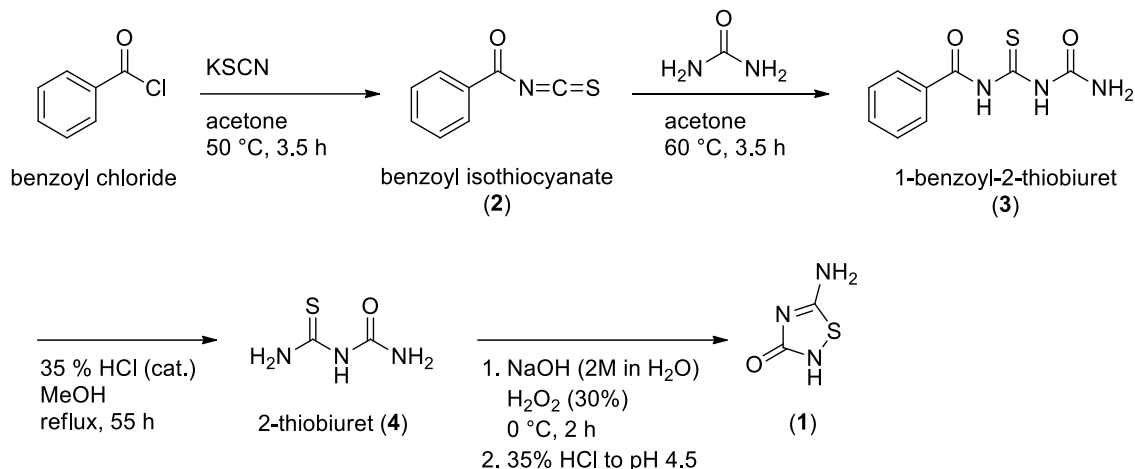


*Figure 1.9: Proposed bioisostericity of the 5,6-double bond of cytosine and the divalent sulfur atom of 5-amino-1,2,4-thiadiazol-3(2H)-one (**1**) (drawn according to ref. [111]).*

Synthesis of 5-amino-1,2,4-thiadiazol-3(2H)-one (**1**) (Scheme 1.4) starts with condensation of benzoyl chloride with potassium thiocyanate in warm acetone upon formation of benzoyl isothiocyanate (**2**). Compound **2** is then reacted with urea upon heating in acetone to obtain 1-benzoyl-2-thiobiuret (**3**). Afterwards, 2-thiobiuret (**4**) is formed *via* removal of the benzol moiety by refluxing of **3** in methanol with a trace of hydrochloric acid as a catalyst. Finally, compound **4** is cyclized in aqueous solution of sodium hydroxide and hydrogen peroxide upon cooling to 0 °C; after subsequent acidifying with hydrochloric acid, the intended compound **1** is obtained [111].



Scheme 1.4: Synthesis of 5-amino-1,2,4-thiadiazol-3(2H)-one (**1**) (drawn according to ref. [111]).

5-Amino-1,2,4-thiadiazol-3(2H)-one (**1**) was tested for cytostatic and antibacterial activity. However, it only showed a slight antibacterial activity against *E. coli* (10% inhibition at 8.5 μ M concentration). Interestingly, the antibacterial activity of **1** was not reversed by the presence of natural pyrimidines. This fact suggests that **1** does not behave as a pyrimidine antimetabolite, but most probably interferes with bacterial proteins and thus inhibits the growth of bacteria [112].

Due to proposed bioisostericity [111-114], there were intentions to use 5-amino-1,2,4-thiadiazol-3(2H)-one (**1**) as a cytosine surrogate [111, 115-117]. In 1989, Párkányi *et al.* synthesized 2-(2,3-dihydroxypropyl)-5-amino-1,2,4-thiadiazol-3(2H)-one (Figure 1.10), an acyclic nucleoside analogue containing the 2,3-dihydroxypropyl moiety bound to N² position of the thiadiazole ring, with expected biological effects. The reported synthesis of this thiadiazole derivative consists in alkylation of **1** with 3-chloropropane-1,2-diol in DMF in the presence of a strong base (NaH) upon heating (Scheme 1.5) [111]. Unfortunately, testing of 2-(2,3-dihydroxypropyl)-5-amino-1,2,4-thiadiazol-3(2H)-one for biological activity has not been mentioned in the literature. Furthermore, there were efforts to prepare various nucleoside analogues with **1** as a nucleobase [115-117]. In 1976 and 1978, Revankar *et al.* reported synthesis of two ribonucleoside derivatives – 5-amino-2-(β -D-ribofuranosyl)-1,2,4-thiadiazol-3(2H)-one and 5-amino-2-(α -2'-deoxy-D-ribofuranosyl)-1,2,4-thiadiazol-3(2H)-one (Figure 1.10) – and their testing for antimicrobial activity [112, 115, 116]. According to Revankar *et al.* [115, 116], these two compounds were obtained *via* alkylation of N⁵-protected 5-amino-1,2,4-thiadiazol-3(2H)-one derivative with the respective *O*-acetyl-protected ribofuranose and SnCl₄ as a Lewis acid, followed by removal of protecting groups. However, when Ling Chwang *et al.* in 1980 unsuccessfully re-attempted synthesis of 5-amino-2-(β -D-ribofuranosyl)-1,2,4-thiadiazol-3(2H)-one and thus improved the experimental procedure [117], they realized that physical properties of their product were different from those reported by Revankar *et*

al. [115, 116]. The structures of the two ribonucleosides prepared by Revankar *et al* are supposed to be incorrect, and their real structure is not known [112, 117].

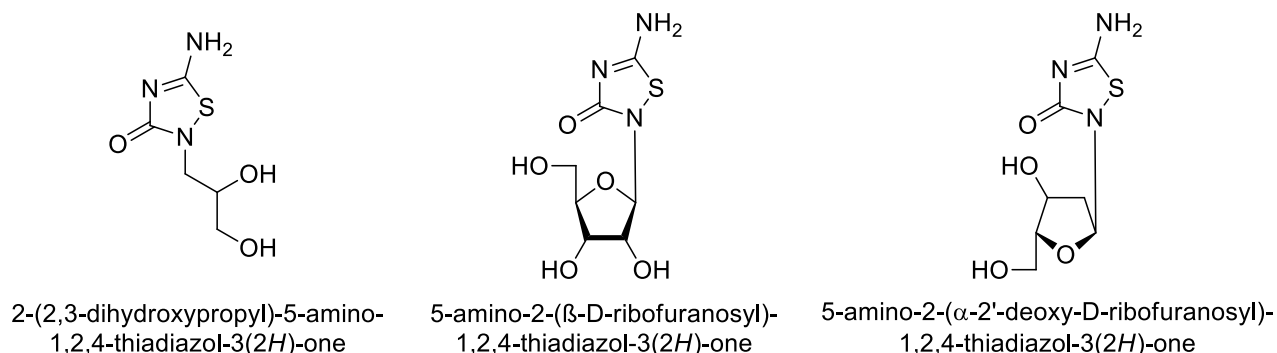
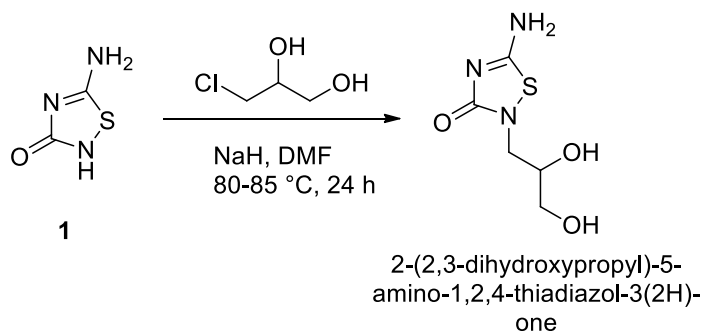
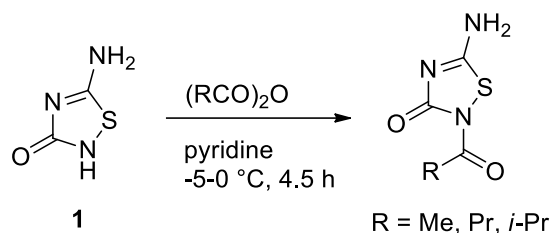


Figure 1.10: Structure of 5-amino-1,2,4-thiadiazol-3(2H)-one-containing nucleosides and acyclic nucleoside analogue: 2-(2,3-dihydroxypropyl)-5-amino-1,2,4-thiadiazol-3(2H)-one (drawn according to ref. [111]), 5-amino-2-(β-D-ribofuranosyl)-1,2,4-thiadiazol-3(2H)-one and 5-amino-2-(α-2'-deoxy-D-ribofuranosyl)-1,2,4-thiadiazol-3(2H)-one (drawn according to ref. [116]).

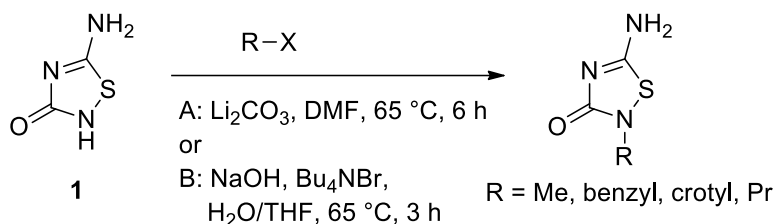


Scheme 1.5: Synthesis of 2-(2,3-dihydroxypropyl)-5-amino-1,2,4-thiadiazol-3(2H)-one (drawn according to ref. [111]).

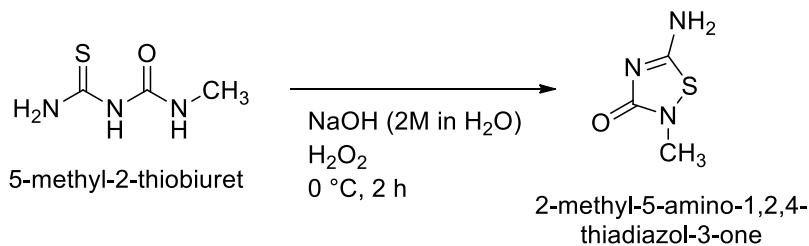
Additionally, several *N*²-acyl derivatives of **1** were synthesized as potentially bioactive compounds and their spectroscopic characteristics were described by Ra *et al.* [118]. The reaction proceeded as direct acylation of **1** using the respective anhydride in pyridine (Scheme 1.6). According to this study, compound **1** is acylated in its lactam form, and the lactam carbonyl in position 3 of the ring of the resulting *N*²-acyl derivatives is in conjugation with the *N*⁴-C⁵ double bond and the 5-amino moiety [118]. In line with these findings, the same authors reported alkylation of compound **1** in *N*² position with various alkyl halides under basic conditions; the reaction was performed either in DMF or H₂O/THF (2:1) solution (Scheme 1.7) [119]. Also, 2-methyl-5-amino-1,2,4-thiadiazol-3-one was prepared by oxidative cyclization of 5-methyl-2-thiobiuret using hydrogen peroxide and sodium hydroxide (Scheme 1.8) [119].



*Scheme 1.6: Synthesis of various N^2 -acyl derivatives of 5-amino-1,2,4-thiadiazol-3(2H)-one (**1**) (drawn according to ref. [118]).*



*Scheme 1.7: Synthesis of various N^2 -substituted derivatives of 5-amino-1,2,4-thiadiazol-3(2H)-one (**1**) either in DMF (A) or in $\text{H}_2\text{O/THF}$ (2:1) solution (B) (drawn according to ref. [119]).*



Scheme 1.8: Synthesis of 2-methyl-5-amino-1,2,4-thiadiazol-3-one via oxidative cyclization of 5-methyl-2-thiobiuret (drawn according to ref. [119]).

1.2 Cathepsin K and glycogen synthase kinase 3 in the role of biological target

1.2.1 Cathepsin K

Cathepsins constitute a large and diverse superfamily of proteolytic enzymes. Based on their catalytic mechanism, we distinguish serine cathepsins, aspartic cathepsins, and cysteine cathepsins [120]. Cathepsin K (CatK), which was targeted in this research, belongs to the family of cysteine cathepsins, lysosomal enzymes with a conserved catalytic Cys-His-Asn triad in the active site [121]. It is expressed mostly in osteoclasts, where it participates on bone resorption during bone remodelling – it degrades type I collagen, the main component of the bone protein matrix, as well as other proteins (osteopontin and osteonectin). Overexpression of CatK leads to disruption of bone homeostatis, which results in bone tissue loss and development of osteoporosis. CatK inhibition thus represents a therapeutic strategy in the treatment of osteoporosis and other diseases associated with increased bone resorption [122-124]. Apart from this, there has been growing evidence that CatK plays a role in developmet of obesity and diabetes [121, 125, 126].

Over the last 20 years, there has been an extensive search for selective CatK inhibitors. The molecule typically possesses an electrophilic functionality (ketone or nitrile) that would covalently bind to the cysteine residue in the active site of CatK, and other moiety that would facilitate enzyme recognition [127]. It was gradually found that the most promising drug candidates contain less reactive functional groups and bind to the active site reversibly, which would allow their chronic medication use [124, 127]. Such compounds include e.g. *N*-cyano-tetrahydro-pyrazine compounds, amides, 2-cyano-pyrimidines, and various biflavones [123]. The compounds that have so far entered clinical trials are Odanacatib (phase III), ONO-5334 (phase II), Balicatib (phase I), and Relacatib (phase I) (Figure 1.11). However, further clinical testing or regulatory considerations of all these drug candidates were abandoned due to serious side effects and safety concerns [128].

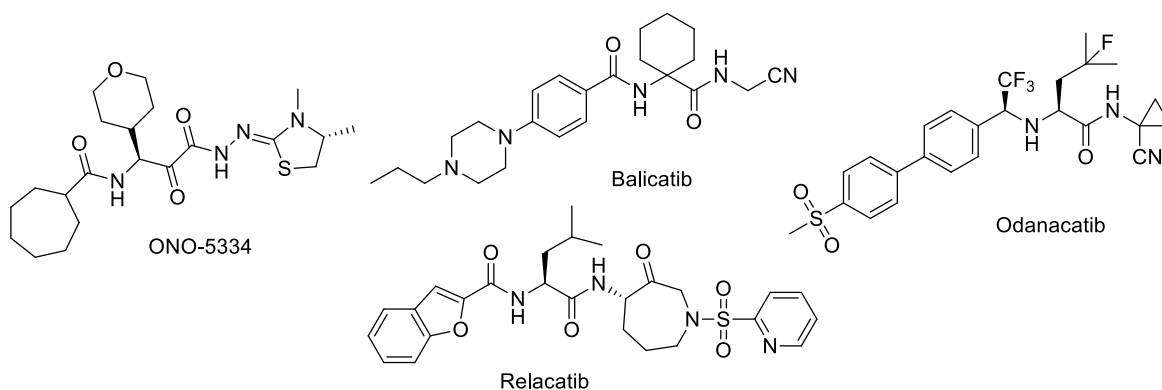


Figure 1.11: Structure of compounds that entered clinical trials: Odanacatib, ONO-5334, Balicatib, and Relacatib (all structures drawn according to ref. [127]).

1.2.2 Glycogen synthase kinase 3

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine protein kinase that is involved in regulation of a wide variety of cellular processes [129]. It was originally identified as a key regulatory enzyme of glycogen metabolism [130], but later it was also found to participate on regulation of many metabolic and signalling pathways including e.g. protein synthesis, mitosis, apoptosis, and several signal transduction pathways [131]. There are two mammalian isoforms of GSK-3 known as α and β that differ in tissue functions and substrate specificity [129, 132]. The isoform β (GSK-3 β), which was originally considered as a tumor suppressor protein, has been eventually shown to positively regulate cancer cell proliferation and to support survival of advanced cancer tissues [129]. When accumulated in cancer cell nucleus, GSK-3 β activates the transcription factor NF- κ B that promotes cancer progression, methastasis, and chemoresistance [129, 133]. Apart from that, GSK-3 β was found to be involved in development of neurodegenerative disorders such as Alzheimer's disease [134, 135].

All these facts raised interest in search of effective GSK-3 β inhibitors. Out of various small molecules tested, 4-benzyl-2-(naphthalen-1-yl)-1,2,4-thiadiazolidine-3,5-dione, referred to as tideglusib (**5**) (Figure 1.12), belongs to the most promising drug candidates [129]. This compound was found to induce apoptosis in human neuroblastoma cells [129, 136] and to support treatment of human glioblastoma [129, 137]. Compound **5** is currently tested in phase II of clinical trials for treatment of congenital myotonic dystrophy [138] and Alzheimer's disease [139]. Moreover, it has been shown to support natural tooth repair as GSK-3 antagonist [140].

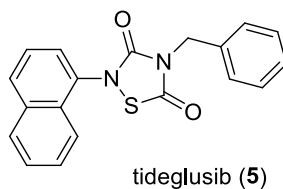


Figure 1.12: Structure of tideglusib (drawn according to ref. [141]).

2 Aims of the work

1. Synthesis of PME- and (*S*)-HPMP-derived ANPs containing 5-amino-1,2,4-thiadiazol-3(2*H*)-one (**1**) as a nucleobase potentially mimicking cytosine or 5-azacytosine.
2. Evaluation of biological activities of the obtained ANPs towards two cysteine-dependent enzymes, CatK and GSK-3 β , and antiviral activities towards various DNA viruses. Also, cytotoxicity of the synthesized compounds against both cancer and non-cancer cell lines should be determined.

2.1 Rationale for the specific aim

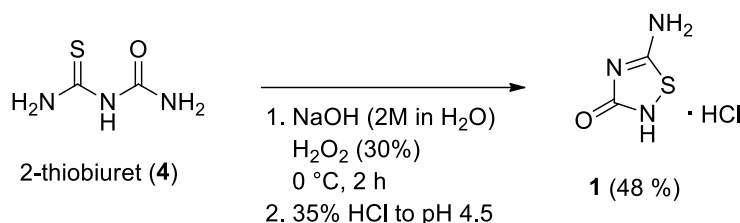
As outlined in the introductory part, 5-azacytosine derived ANPs, e.g. (*S*)-HPMP-5-azaC, suffers from hydrolytic instability [48, 74]. Replacement of the 5-azacytosine moiety with 5-amino-1,2,4-thiadiazol-3(2*H*)-one (**1**), which is supposed to be bioisosteric with cytosine [111, 112] and to mimic 5-azacytosine, can provide the ANP molecule with increased stability and interesting biological effects. These effects may manifest as inhibition of some cysteine-dependent enzymes, such as CatK and GSK-3 β , because the *S-N* bond of the thiadiazole ring can be easily attacked by the cysteine residue in the active site of the enzyme upon its irreversible inhibition [107, 108]. Moreover, investigation of antiviral activities is also desirable.

Our objective was to obtain chemically stable thiadiazole congeners of HPMPC and (*S*)-HPMP-5-azaC and investigation of their biological activities. Besides compounds in the HPMP series, synthesis of the respective PME derivatives was also proposed, especially as a model system for development of appropriate synthetic strategy. Concerning biological activity, it is known that cytosine and 5-azacytosine PME derivatives are almost biologically inactive. On the other hand, their antiviral activity can be increased after transformation to appropriate prodrugs. This fact also substantiates the necessity of detailed biological activity screening of all new thiadiazole PME derivatives.

3 Results and discussion

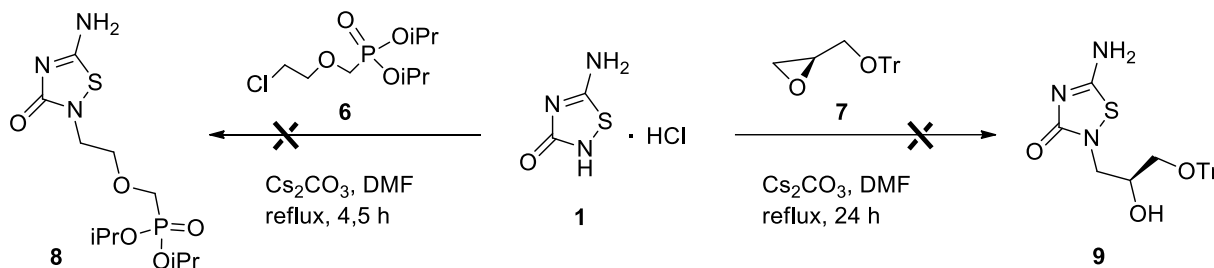
3.1 Synthesis of thiadiazole ANPs *via* direct alkylation of the base

My initial efforts to prepare PME- and HPMP-derived compounds containing 5-amino-1,2,4-thiadiazol-3(2*H*)-one (**1**) as a nucleobase started with direct alkylation of **1**. Compound **1** was synthesized according to the protocol reported by Párkányi *et al* [111] (Scheme 3.1) from 2-thiobiuret (**4**) as a starting material. Compound **4** was subjected to cyclization in aqueous solution of sodium hydroxide and hydrogen peroxide upon cooling to 0 °C. After subsequent acidifying with 35% hydrochloric acid to pH of 4.5, compound **1** was obtained in a moderate yield (48 %).



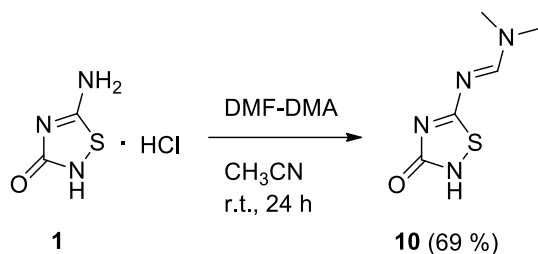
Scheme 3.1: Synthesis of 5-amino-1,2,4-thiadiazol-3(2*H*)-one (**1**) from 2-thiobiuret (**4**).

In order to obtain the respective PME- or HPMP-derivative, compound **1** was subjected to direct alkylation with PME-chloride (PME-Cl) (**6**) or (*S*)-glycidyl trityl ether (**7**), respectively. The reaction was carried out under basic conditions of Cs₂CO₃ in DMF heated to reflux [142] (Scheme 3.2). However, the reaction did not proceed and formation of alkylation product **8** or **9** was not observed.



Scheme 3.2: Efforts to alkylate compound **1** with PME-Cl (**6**) or (*S*)-glycidyl trityl ether (**7**).

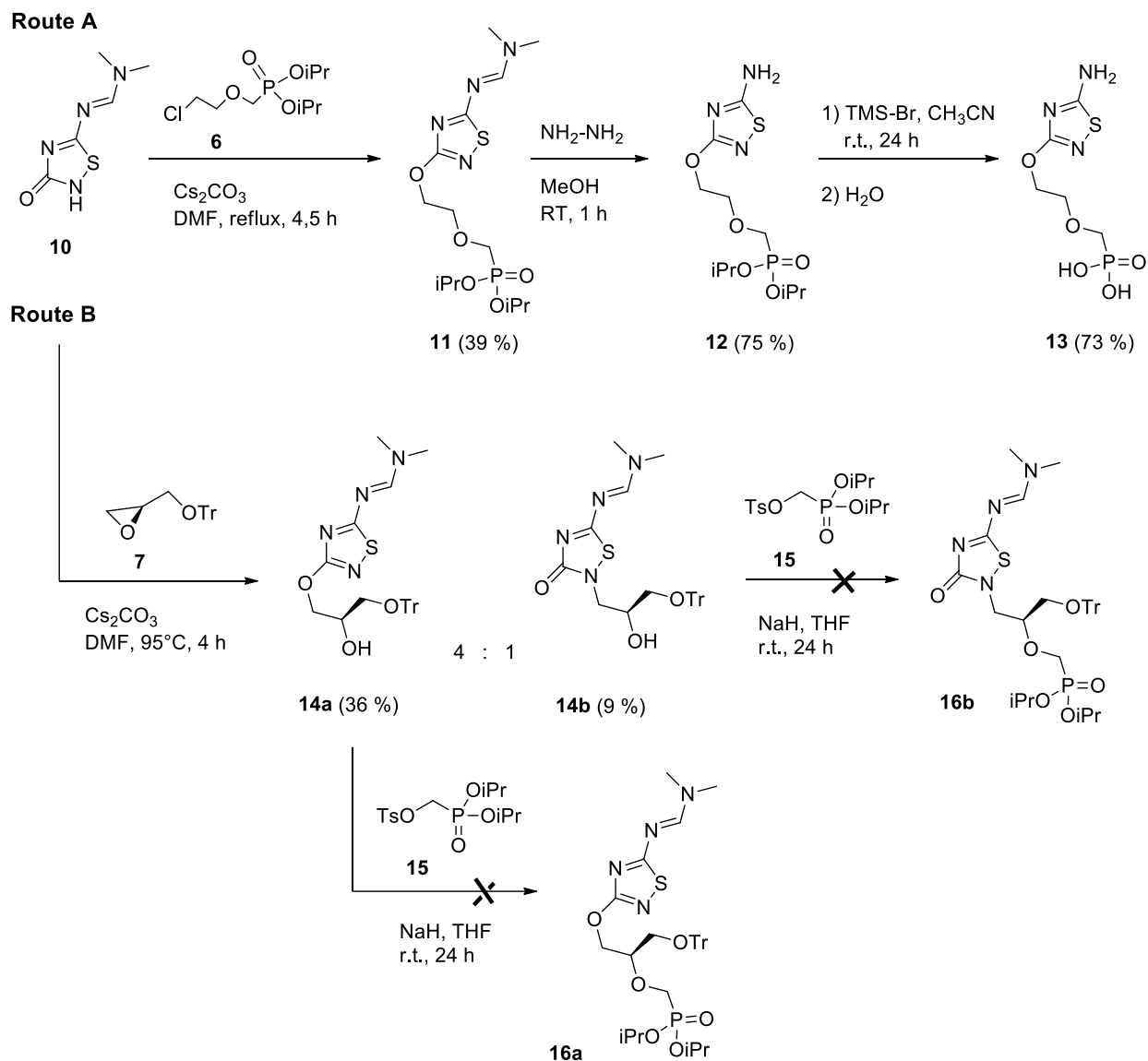
To avoid preferential deprotonation of the NH₂ group in position 5 of the thiadiazole ring, it was protected using *N,N*-dimethylformamide dimethyl acetal (DMF-DMA) to afford the compound **10** (Scheme 3.3). Apart from protection of the NH₂ group, the *N,N*-dimethylformamidine (DMA) moiety serves as an electron-donating substituent that increases electron density on the thiadiazole ring and thus its nucleophilicity.



*Scheme 3.3: Protection of compound **1** with DMF-DMA to obtain compound **10**.*

Compound **10** was alkylated with PME-Cl (**6**) (Scheme 3.4, Route A) or (*S*)-glycidyl trityl ether **7** (Scheme 3.4, Route B); both reactions were performed in DMF under reflux and in the presence of Cs_2CO_3 [142]. Alkylation of **10** with **6** afforded compound **11** in 39% yield; however, alkylation occurred at O^3 position instead of the desired N^2 position. Subsequent removal of the DMA protecting group with hydrazine in methanol gave compound **12** in high yield (75 %), and deprotection of the diisopropyl esters with trimethylsilyl bromide (TMS-Br) in acetonitrile [143] afforded the free phosphonic acid **13** in high yield (73 %). The O^3 -alkylated PME derivative **13**, which represents an analogue of the open-ring ANP series, was tested for biological activity, as well as its precursors **10-12**.

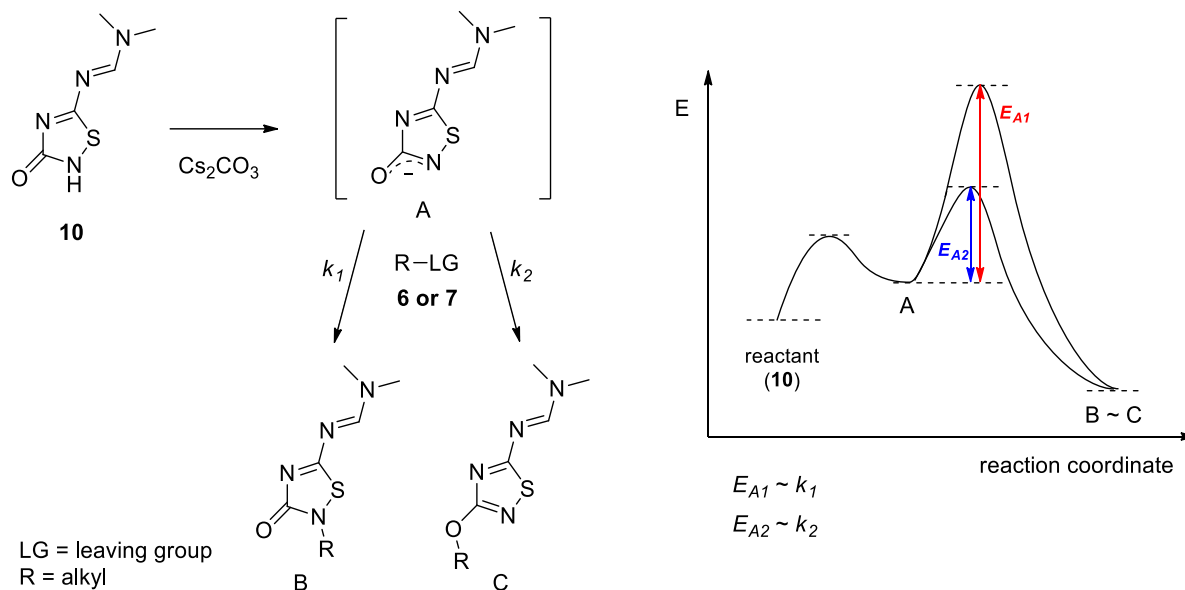
The reaction of **10** with (*S*)-glycidyl trityl ether (**7**) [142] yielded O^3 - (**14a**) and N^2 - (**14b**) alkylation products in the ratio of 4:1 (**14a** – 36 %; **14b** – 9 %). Both isomers were subjected to alkylation with diisopropyl (tosyloxy)methylphosphonate (**15**) in THF using NaH as a base [46], but the reaction did not proceed and no desired product **16a** or **16b** was obtained. Thus, I was unable to prepare the final HPMP O^3 - and N^2 - alkylated phosphonates. Nevertheless, both the alkylation products **14a** and **14b** were selected for biology testing.



*Scheme 3.4: Synthetic efforts in preparation of the PME and HPMP derivatives of 5-amino-1,2,4-thiadiazol-3(2H)-one: Route A – alkylation of **10** with PME-Cl (**6**) to afford O^3 -alkylation product **11** and subsequent deprotection of DMA moiety and diisopropyl esters to obtain the free phosphonic acid **13**; Route B - alkylation of **10** with (*S*)-glycidyl trityl ether (**7**) yielded O^3 - (**14a**) and N^2 - (**14b**) alkylation products, but subsequent efforts to prepare the respective HPMP derivatives failed.*

To explain the regioselectivity during alkylation of NH_2 -protected thiadiazole **10** with PME-Cl, it is useful to consider the reaction kinetics of thiadiazole alkylation (Scheme 3.5). After deprotonation of compound **10** with a base, the corresponding anion A is formed. Obviously, the activation energy E_{A2} of anion A conversion to product C is lower than the activation energy E_{A1} for conversion to product B. This difference between activation energies may be e.g., due to different sterical properties of thiadiazole O^3 - and N^2 -alkylation by PME-Cl (**6**). Interestingly, alkylation of **10** with (*S*)-glycidyl trityl ether (**7**) afforded

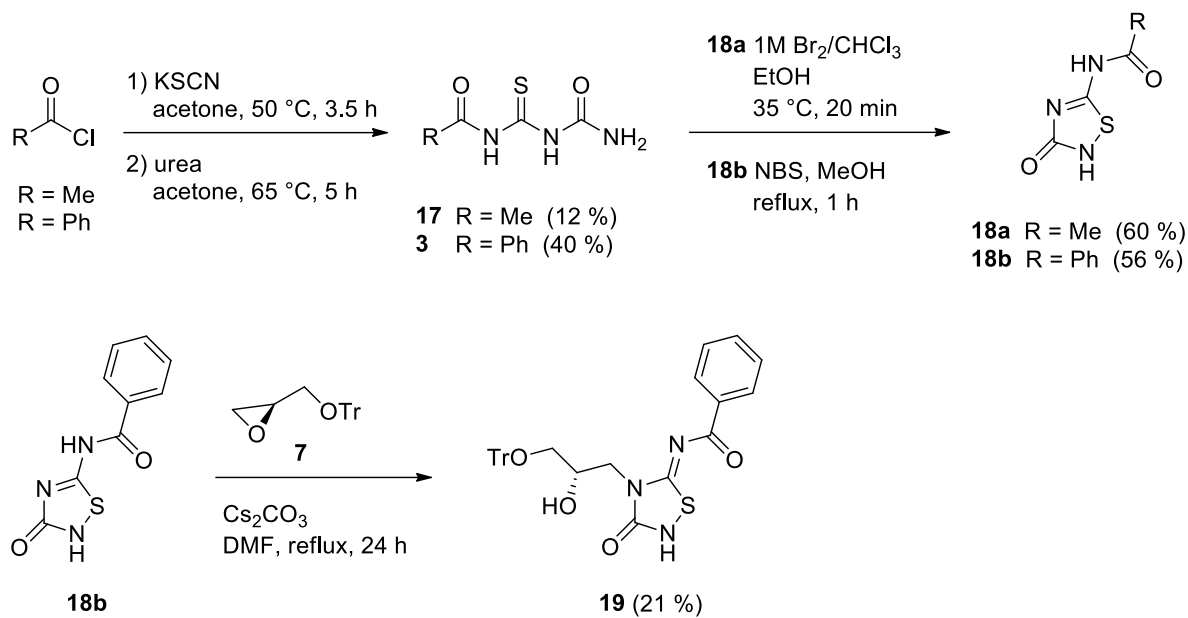
*O*³-regioisomer **14a** and *N*²-regioisomer **14b** in the ratio of 4:1 (Scheme 3.4). The observed partial *N*²-alkylation of anion A by the bulky synthon **7** can be a result of different electronic properties of alkylation agents **6** and **7**.



Scheme 3.5: The course of the thiadiazole alkylation reaction described by the reaction scheme (left) and by the reaction diagram (right).

Another attempt to prepare the desired *N*²-PME and *N*²-HPMP derivatives of 5-amino-1,2,4-thiadiazol-3(2*H*)-one concerned protection of the NH₂ moiety with benzoyl or acetyl group. For this purpose, gradual construction of the thiadiazole ring was employed, and the *N*⁵-protected thiadiazole was intended for direct alkylation with PME-Cl (**6**) or (*S*)-glycidyl trityl ether (**7**) (Scheme 3.6). Firstly, acetyl or benzoyl chloride was reacted with potassium to form the corresponding isothiocyanate (not displayed), which was immediately condensed with urea to yield 1-acetyl-2-thiobiuret **17** [118] or 1-benzoyl-2-thiobiuret **3** [111], respectively.

Cyclization of 1-acyl 2-thiobiurets **17** and **3** to the corresponding thiadiazoles **18a** [118] and **18b** [144] was performed either with bromine in CHCl₃ (for **18a**) or with NBS in ethanol (for **18b**), both according to the protocol described in ref. [144]. Compound **18b** was subsequently alkylated with **7** in DMF in the presence of Cs₂CO₃ [142]. Nevertheless, only the *N*⁴-alkylated product **19** was obtained in a moderate yield. The desired *N*²-substituted derivative was not formed. Therefore, the synthetic approach based on a direct alkylation of *N*⁵-acyl thiadiazoles was abandoned and analogous reactions of compound **18b** with **6** and compound **18a** with **6** and **7** were not attempted. The obtained compounds **18a**, **18b**, and **19** were tested for biological activity as final compounds.

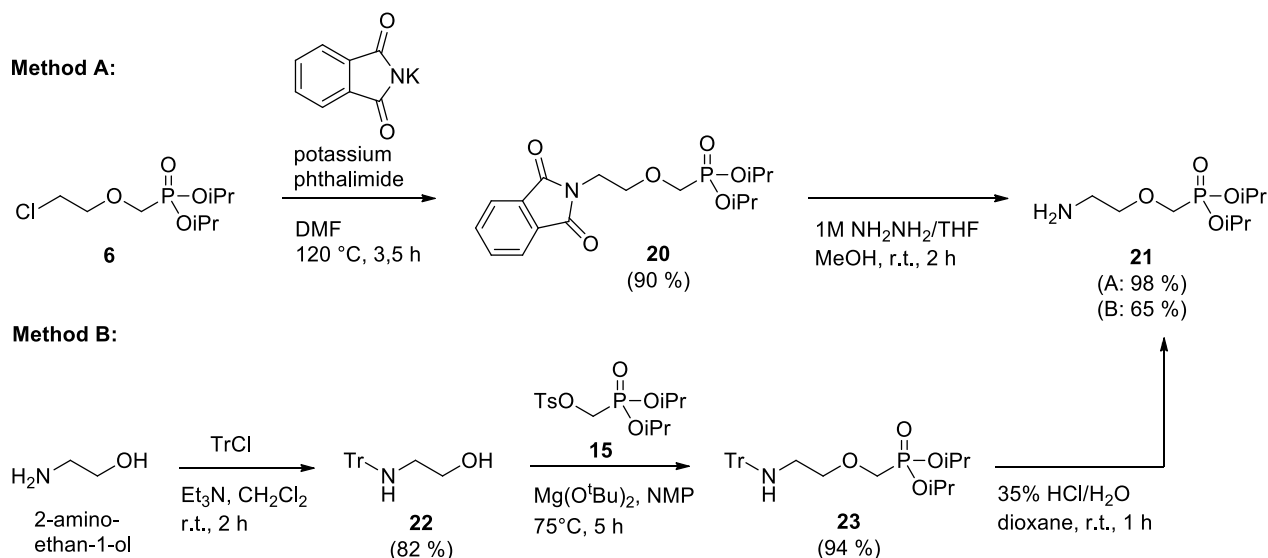


Scheme 3.6: Synthesis of *N*⁵-acyl thiadiazoles **18a** and **18b** and subsequent alkylation of **18b** with (*S*)-glycidyl trityl ether (**7**) to obtain compound **19**.

3.2 Synthesis of thiadiazole ANPs *via* gradual construction of the heterocyclic ring

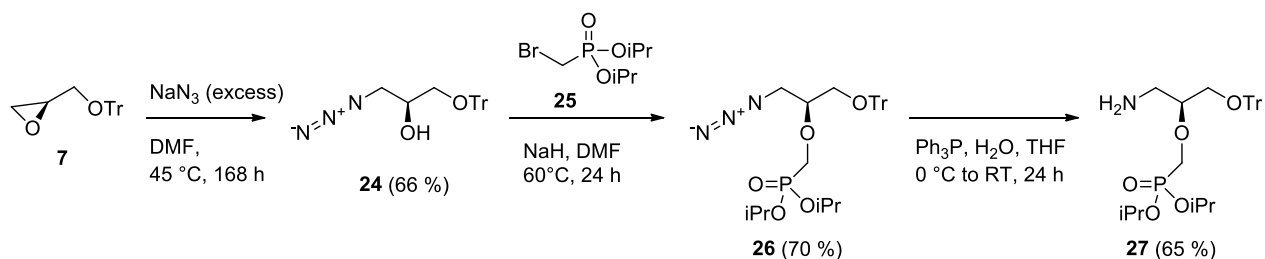
After unsuccessful efforts to prepare N^2 -substituted regioisomers, stepwise construction of the thiadiazole ring was applied. This synthetic strategy was based on cyclization of an appropriate thiobiuret derivative containing PME or HPMP arrangement attached to its N^5 position and protected at the N^1 amino group by an easily removable protecting group.

Initially, it was necessary to prepare diisopropyl ester of 2-[(phosphonomethoxy)ethyl]amine (so-called “PME-amine”, **21**) and an analogous protected diisopropyl ester of 3-hydroxy-2-[(phosphonomethoxy)propyl]amine (so-called “(S)-HPMP-amine”, **27**) as starting synthetic precursors. To prepare compound **21**, two synthetic methods were developed. The first approach (Scheme 3.7, Method A) was based on conversion of PME-Cl (**6**) to **21** *via* Gabriel synthesis. Potassium phthalimide was alkylated with compound **6** in DMF to obtain **20** in a high yield; subsequent removal of the phthalic moiety with hydrazine afforded compound **21** in a high yield. The second method (Scheme 3.7, Method B) started with N^2 -tritylation of 2-aminoethan-1-ol [145]. The resulting 2-(tritylamino)ethan-1-ol (**22**) [146] was *O*-alkylated with diisopropyl (tosyloxy)methylphosphonate (**15**) using $\text{Mg}(\text{O}^i\text{Bu})_2$ as a base [145] to provide compound **23**. Removal of the trityl moiety with hydrochloric acid yielded the final compound **21**.



*Scheme 3.7: Two methods of PME-amine (**21**) synthesis: Method A is based on Gabriel synthesis of primary amines; Method B lays in gradual assembly of the aliphatic chain from N -tritylated 2-aminoethan-1-ol (**22**) via its alkylation with diisopropyl (tosyloxy)methylphosphonate (**15**) and subsequent removal of the trityl moiety.*

Synthesis of compound **27** (Scheme 3.8) started with ring opening of epoxide **7** with sodium azide upon formation of compound **24**. This synthetic step required specific conditions (40-fold excess of sodium azide and heating in DMF to 45 °C for 7 days) to minimize formation of an undesired side product (structure and data not shown). The subsequent *O*-alkylation [46] of **24** with diisopropyl (bromomethyl)phosphonate (**25**) to compound **26**, followed by reduction of its azide functionality *via* Staudinger reaction [147] resulted in formation of the desired diisopropyl ester of 3-(trityloxy)-2-[(phosphonomethoxy)propyl]amine (**27**), a key synthetic precursor for construction of thiadiazole derivatives in the HPMP series.

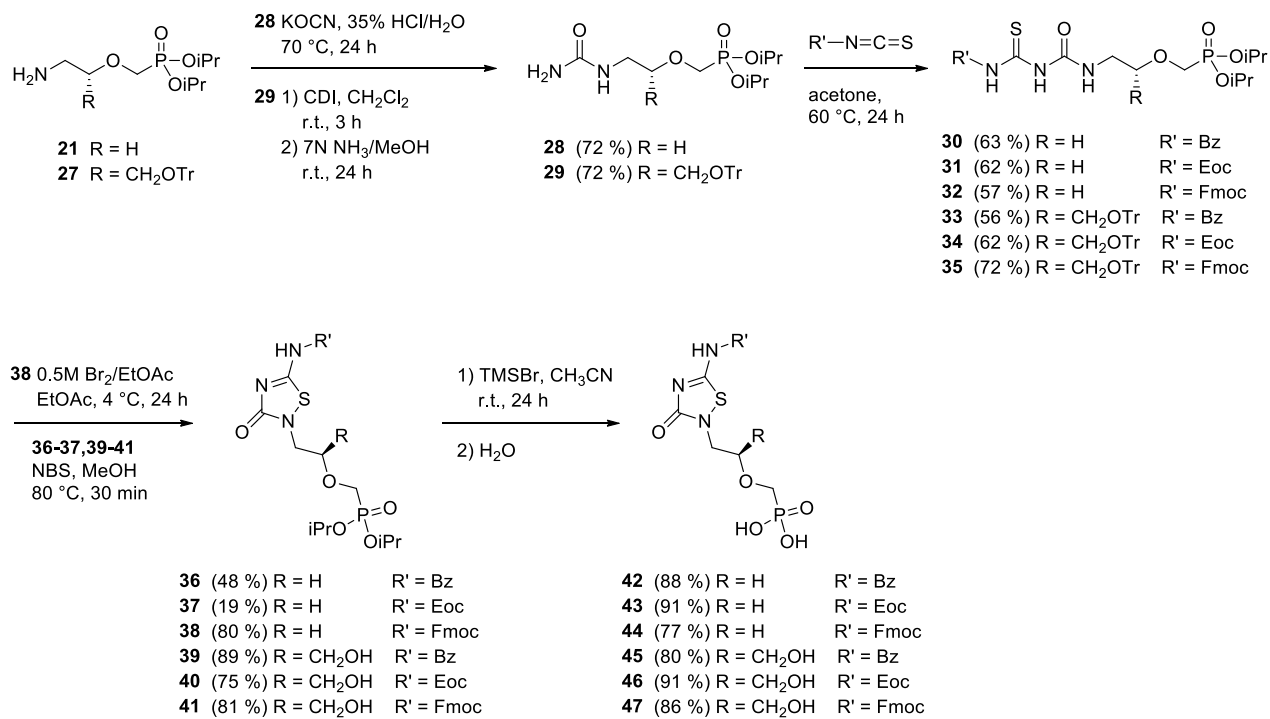


Scheme 3.8: Synthesis of protected (S)-HPMP-amine (27), a key intermediate for construction of thiadiazole derivatives of the HPMP series.

The obtained synthons, PME-amine (**21**) and (S)-HPMP-amine (**27**), were used for gradual assembly of the thiadiazole moiety (Scheme 3.9). In the first step, both amine synthons **21** and **27** were converted to the respective ureas. The PME-urea (**28**) was obtained from **21** by the action of potassium cyanate in aqueous hydrochloric acid [148]. However, this approach did not work for synthesis of the (S)-HPMP-urea **29** from **27**. Therefore, it was necessary to use 1,1'-carbonyldiimidazole (CDI) upon formation of the respective imidazole amide, which was subsequently cleaved by the action of methanolic ammonia [149] to obtain (S)-HPMP-urea (**29**). Both the ureas **28** and **29** were then reacted with various isothiocyanates bearing organic substituents – acetyl, benzoyl, ethoxycarbonyl (Eoc), and fluorenylmethyloxycarbonyl (Fmoc) – in acetone upon heating to 60 °C [111, 118]. While the reaction with acetyl isothiocyanate did not afford any products, all the other isothiocyanates yielded the respective *N*¹-protected *N*⁵-PME-substituted thiobiurets **30-32** and *N*¹-protected *N*⁵-HPMP-substituted thiobiurets **33-35** in good yields (57-72 %).

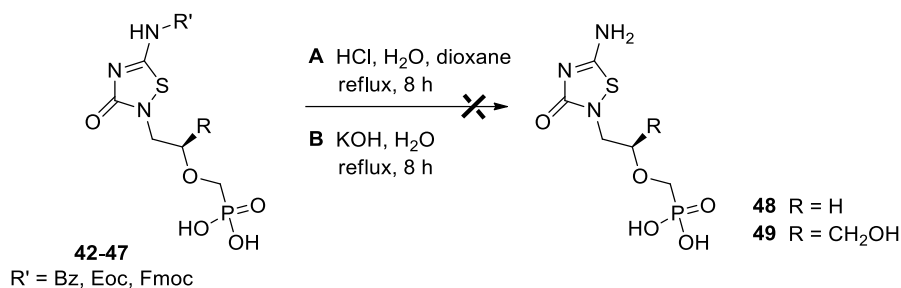
In the next step, cyclization of thiobiurets **30-35** was performed to obtain *N*⁵-protected *N*²-substituted thiadiazoles. Compound **32** was cyclized using cold bromine solution in ethyl acetate [144] to obtain thiadiazole **38**. All the other thiobiurets **30**, **31**, and **33-35** were subjected to cyclization by heating with NBS in methanol [144] to afford thiadiazoles **36**, **37**, and **39-41**. Important to note, deprotection of the trityl moiety in all the (S)-HPMP-derivatives took place during this cyclization step. Finally, the

cleavage of diisopropyl esters by bromotrimethylsilane, followed by hydrolysis [143] provided PME and (S)-HPMP derivatives as free phosphonic acids **42-47** in good to high yields.



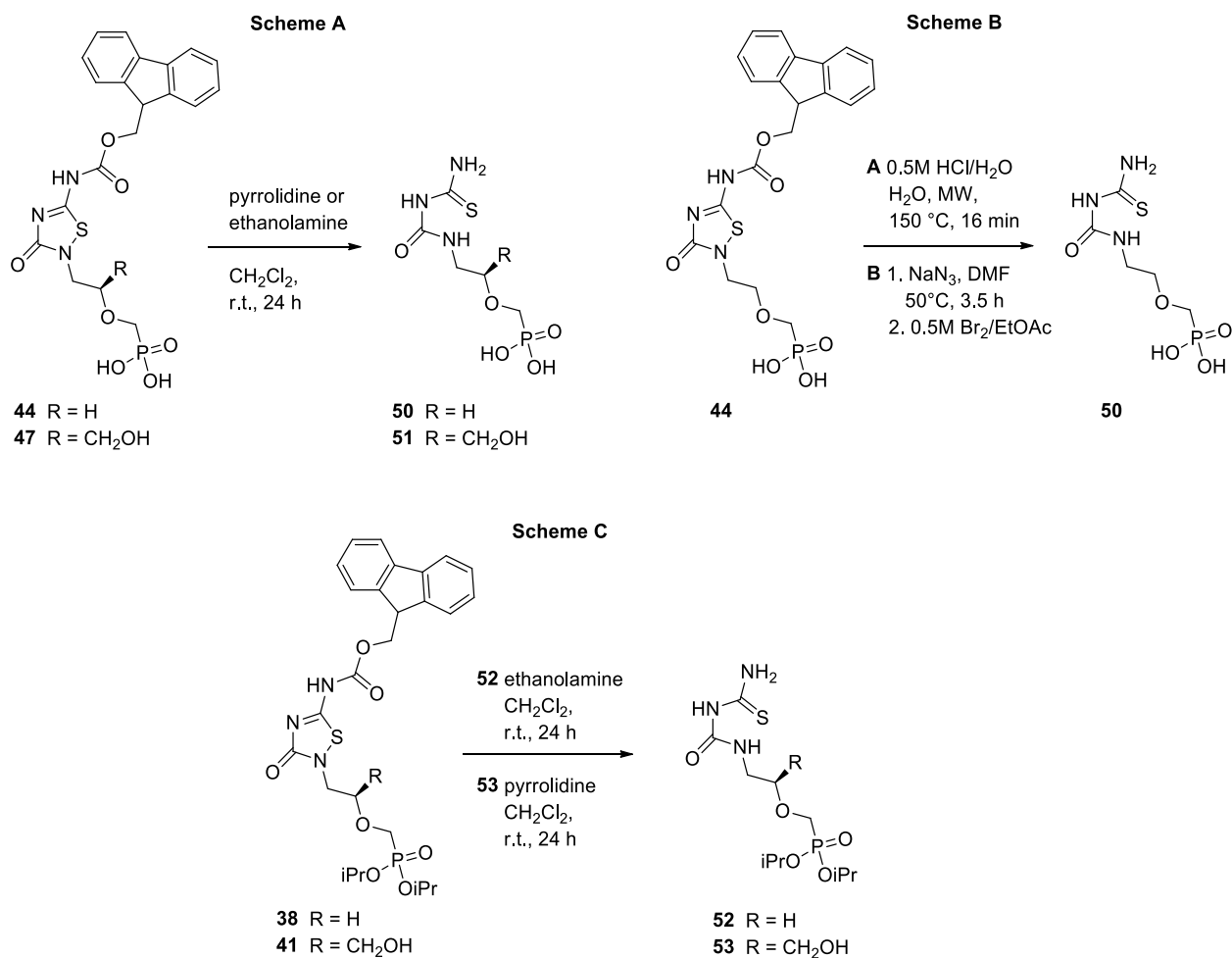
*Scheme 3.9: Synthesis of PME- and (S)-HPMP-derived N⁵-protected thiadiazoles **42-47** via gradual assembly of the thiadiazole ring.*

A special effort was paid to remove the protecting groups from the *N*⁵-position of compounds **42-47**. In this regard, acidic deprotection with HCl in H₂O/dioxane solution was attempted, as well as basic deprotection with aqueous KOH (Scheme 3.10). However, even under very severe conditions such as heating to reflux for 8 h under both the acidic and basic conditions, all the compounds **42-47** stayed unmodified.



*Scheme 3.10: Attempts to remove the protecting group from the N^5 -position of the thiadiazole ring in compounds **42-47**.*

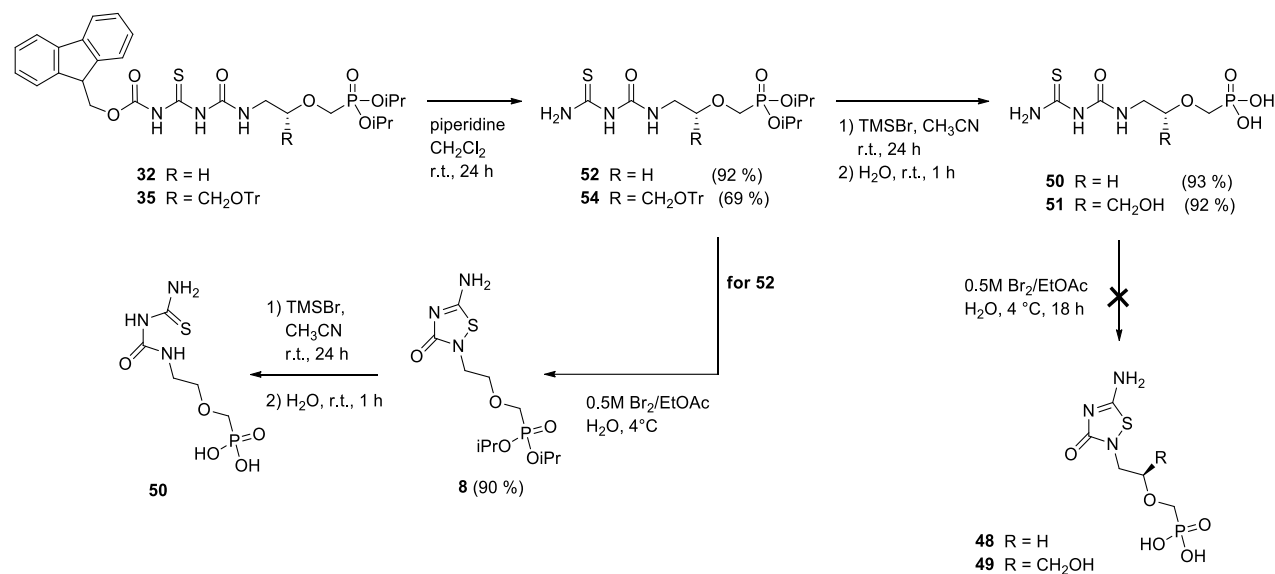
Regarding the Fmoc protecting group is generally known as easily removable, its deprotection by various reagents and methods was systematically studied (Scheme 3.11). First, free thiadiazole phosphonic acids **44** and **47** were treated with pyrrolidine or ethanolamine in dichloromethane [150] (Scheme 3.11A). However, removal of the Fmoc protecting group led to opening of the thiadiazole ring upon formation of the corresponding N^5 -substituted thiobiurets **50** and **51**. Likewise, when the Fmoc deprotection of compound **44** was attempted in aqueous HCl [151] using microwave (MW) irradiation (Scheme 3.11B, method A) or treated with NaN₃ as a base upon heating in DMF [152] (Scheme 3.11B, method B), only formation of the N^5 -substituted thiobiuret **50** was observed. The effort for its re-cyclization with Br₂ in ethyl acetate failed. Finally, when the Fmoc protected thiadiazole ANPs **38** and **41** (in the form of diisopropyl esters) were subjected to treatment with ethanolamine or pyrrolidine [150], respectively, the corresponding N^5 -substituted thiobiurets **52** and **53** were obtained, similarly as in previous cases (Scheme 3.11C).



*Scheme 3.11: Removal of the Fmoc protecting group from thiadiazole derivatives **38**, **41**, **44**, and **47** upon formation of the corresponding N⁵-substituted thiobiurets **50-53**.*

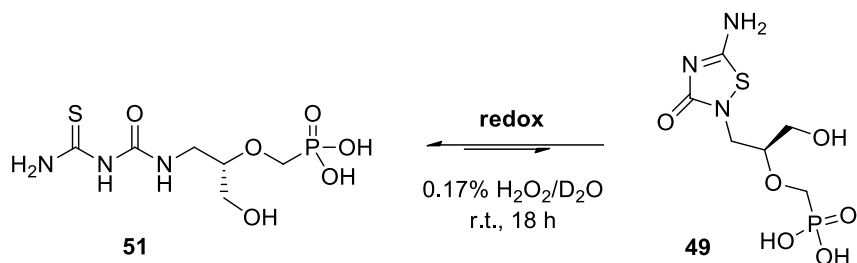
As the synthetic strategy based on deprotection of the NH₂ moiety of PME- and (S)-HPMP-alkylated thiadiazolones **42-47** did not lead to the desired outcome, cyclization of the unprotected N⁵-alkylated thiobiurets was attempted (Scheme 3.12). In the first step, removal of the Fmoc protecting group from N¹-position of thiobiurets **32** and **35** with piperidine in dichloromethane [150] provided thiobiurets **52** and **54** in very good yields. Cyclization of compound **52** with Br₂ solution in ethyl acetate [151] afforded the thiadiazole derivative **8** with a free NH₂ group in position 5, as expected. However, subsequent treatment of compound **8** with bromotrimethylsilane followed by hydrolysis to cleave diisopropyl esters [143] led to thiadiazole ring opening and formation of thiobiuret **50**. Therefore, it was necessary to perform cleavage of diisopropyl esters prior to thiobiuret cyclization. Compounds **52** and **54** were subjected to cleavage of esters with bromotrimethylsilane [143] to obtain free thiobiuret phosphonic acids **50** and **51** (yield 92-93 %). Cyclization of compounds **50** and **51** was attempted using Br₂ solution

in ethyl acetate at 4 °C [144] as in the case of successful cyclization of **52**. Nevertheless, no conversion was observed.



*Scheme 3.12: Synthetic efforts to prepare PME- and (S)-HPMP-thiadiazoles as free phosphonic acids via cyclization of N⁵-substituted thiobiurets **50** and **51** and cyclization of N⁵-substituted thiobiuret **52** as a diisopropyl ester.*

To elucidate the impossibility to cyclize compounds **50** and **51** upon thiadiazole ring formation, a study regarding redox character of the thiadiazole *S-N* bond was performed. For this purpose, cyclization of compound **51** was carried out in the NMR tube using 0.17% hydrogen peroxide in D₂O [153] (Scheme 3.13). Hydrogen peroxide was chosen as a cyclization agent as it is easily decomposed to water and does not disturb the measurement with by-product formation. The reaction was monitored at one-hour intervals over 8 hours by ³¹P NMR and showed gradual establishment of equilibrium between the cyclization product **49** and the linear compound **51**. The ³¹P spectra showed that the ratio of peaks for **51** and **49** was approximately 1:1. Addition of another equivalent of hydrogen peroxide after 8 hours did not influence the established equilibrium.



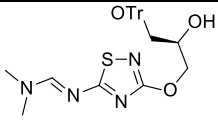
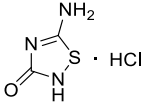
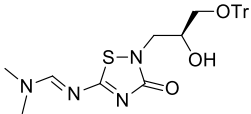
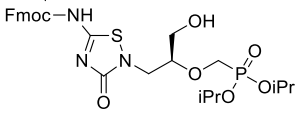
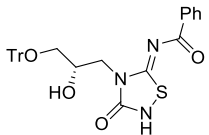
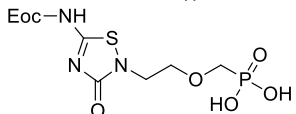
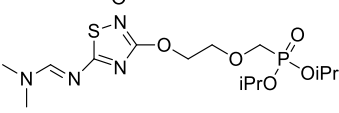
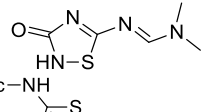
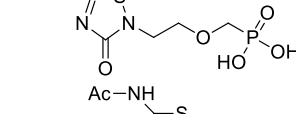
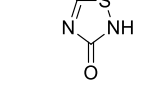
*Scheme 3.13: Cyclization of **51** by hydrogen peroxide; the experiment was performed in NMR tube in D₂O and monitored by ³¹P NMR spectroscopy.*

The existence of equilibrium between the thiobiuret **51** and the thiadiazole **49** is in accordance with the previously discussed thiadiazole ring opening during Fmoc deprotection of PME or (S)-HPMP thiadiazole derivatives **38**, **41**, **44**, and **47**. Moreover, it explains the impossibility to recycle the thiobiurets **50** and **51** in the free phosphonic acid form. The redox character of the thiadiazole *S-N* bond suggests that the *S-N* bond of *N*²-alkylated thiadiazole derivatives could be easily attacked by a nucleophile upon cleavage and formation of a substituted thiobiuret. This idea supports the use of the thiadiazole compounds prepared in this study as potential inhibitors of cysteine-dependent enzymes.

In summary, the target thiadiazoles **48** and **49** that would be *N*²-alkylated with PME and (S)-HPMP moiety and contain free NH₂ group in position 5 were not obtained in the form of free phosphonic acids by any of the reported synthetic strategies. Therefore, the NH₂ protected thiadiazoles in a free phosphonic acid form **42-47** were used as final compounds for biological activity testing, as well as most of their respective diisopropyl ester analogues (**36-37** and **39-41**).

3.3 Evaluation of biological activity

Twenty-one selected 5-amino-1,2,4-thiadiazol-3(2H)-one compounds including the parent molecule **1** were tested as potential inhibitors of human recombinant enzymes CatK and GSK-3 β (Table 3.1); their activity against GSK-3 β was compared to established GSK-3 β inhibitors tideglusib (**5**) and the molecule CHIR99021 (**55**). Both assays were performed *in vitro* at 10 μ M concentration of the compound and the inhibitory activity of the compound was expressed as residual activity of the enzyme (%). Moreover, cytotoxicity was determined on both tumor (CCRF-CEM, HL-60, HeLa S3, HepG2, and SH-SY5Y) and non-tumor (dental fibroblasts NHDF) cell lines at 100 μ M and 10 μ M and the data were expressed as cell viability in % (the ratio of viable cells and untreated control) (Table 3.2).

Compound	CatK residual activity (%)	GSK-3 β residual activity (%)
14a 	5.3 $\pm$ 1.5	72 $\pm$ 16.7
1 	16.9 $\pm$ 1.0	78 $\pm$ 7.3
14b 	28.3 $\pm$ 1.7	64 $\pm$ 14.1
41 	34.1 $\pm$ 2.4	76 $\pm$ 2.4
19 	34.2 $\pm$ 3.0	59 $\pm$ 13.6
43 	39.1 $\pm$ 1.0	87 $\pm$ 4.1
11 	52.9 $\pm$ 3.8	102 $\pm$ 8.8
10 	67.0 $\pm$ 5.8	97 $\pm$ 12.3
44 	67.0 $\pm$ 0.6	85 $\pm$ 4.8
18a 	76.6 $\pm$ 3.8	82 $\pm$ 9.7

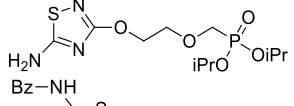
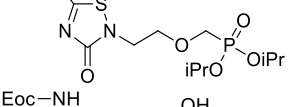
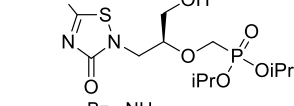
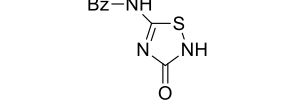
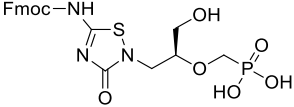
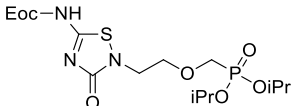
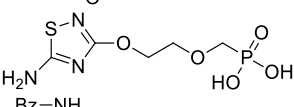
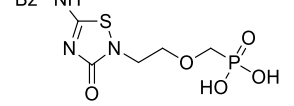
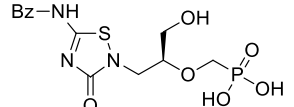
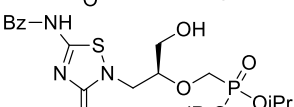
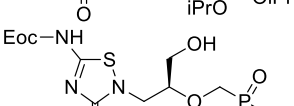
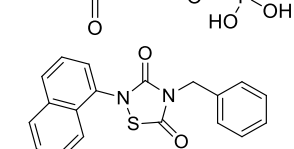
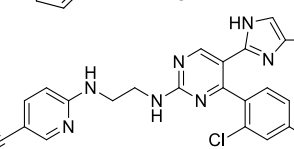
12		78.3 ± 2.6	113 ± 15.5
36		80.6 ± 5.0	87 ± 25.0
40		83.4 ± 8.6	110 ± 9.1
18b		85.0 ± 3.5	85 ± 18.5
47		85.5 ± 5.3	98 ± 12.5
37		86.8 ± 2.7	154 ± 0.5
13		90.0 ± 3.5	106 ± 6.2
42		90.3 ± 6.0	75 ± 7.4
45		92.3 ± 4.5	106 ± 12.3
39		92.9 ± 2.8	81 ± 3.5
46		97.5 ± 2.8	101 ± 10.0
5 (tideglusib)		N/A*	70 ± 7.2
55 (CHIR99021)		N/A	10 ± 5.2

Table 3.1: Inhibitory activity of human CatK and GSK-3 β by twenty-one 5-amino-1,2,4-thiadiazol-3(2H)-one derivatives at 10 μ M. The compound inhibitory effect is expressed as percentage residual enzyme activity relative to the uninhibited control (100%). The compounds are sorted in descending order of CatK inhibitory activity (*not applicable for CatK).

Cpd. No.	Cell viability [%]											
	CCRF-CEM		HL-60		HeLa S3		HepG2		SH-SY5Y		NHDF	
	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM
1	120	4	98	95	100	87	87	54	99	56	101	49
10	122	84	102	105	97	112	97	85	100	75	105	107
11	106	93	97	98	101	89	99	90	101	87	101	99
12	114	103	103	108	97	98	100	99	98	55	104	114
13	113	112	103	100	101	102	97	93	101	99	105	96
14a	101	2	86	1	91	2	92	1	99	2	97	1
14b	15	1	64	2	80	1	87	1	67	3	77	1
18a	104	102	105	108	97	102	103	88	102	99	99	105
18b	102	96	101	110	99	101	99	77	103	90	104	100
19	81	5	93	9	45	3	91	37	96	34	98	79
36	100	94	104	115	101	100	103	101	104	101	103	101
37	92	98	102	111	100	101	104	100	105	104	101	98
39	86	97	106	118	97	105	109	99	100	97	103	104
40	67	45	97	101	90	87	101	93	104	104	104	100
41	82	82	103	110	99	100	102	92	102	85	98	100
42	98	100	102	113	101	101	100	100	104	105	103	102
43	74	77	96	100	94	96	98	97	103	105	94	97
44	85	90	102	106	97	102	105	103	102	99	93	84
45	72	61	103	106	97	101	101	100	101	103	105	98
46	94	114	95	99	97	94	99	89	100	99	101	99
47	88	119	93	100	98	99	106	88	99	93	98	95
5 (tideglusib)	30	n.a.*	34	n.a.	69	n.a.	92	n.a.	80	n.a.	57	n.a.
55 (CHIR99021)	28	n.a.*	119	n.a.	67	n.a.	86	n.a.	77	n.a.	53	n.a.

Table 3.2: Cytotoxicity of the selected twenty-one 5-amino-1,2,4-thiadiazol-3(2H)-ones and reference molecules tideglusib (**5**) and CHIR99021 (**55**) towards both tumor (CCRF-CEM, HL-60, HeLa S3, HepG2, and SH-SY5Y) and non-tumor (NHDF) cell lines at 10 and 100 μM of the compound (*n.a. – not available). The cytotoxicity data are expressed as a percentage of viable cells and untreated control (100 %).

The inhibition of CatK (Table 3.1) was determined by the IOCB research group of Dr. Michael Mareš. Out of the set of tested thiadiazole derivatives, the six most active compounds exhibited inhibition above 50 % (residual enzyme activity lower than 50 %) and their IC₅₀ values ranged from 2.6 to 14.9 μM. These six compounds can be divided into three categories according to structural features: 1) the parent unsubstituted thiadiazole **1**; 2) the NH₂-protected derivatives carrying the 2-hydroxy-3-trityloxypopyl

moiety **14a**, **14b**, and **19**; and 3) the *N*²-alkylated PME or HPMP NH₂-protected derivatives, in the form of diisopropyl esters (**41**) or free phosphonic acids (**43**). Interestingly, the obtained data showed a few general trends regarding the structure-activity relationship (SAR). Some of the esterified phosphonates exhibited higher inhibitory activity than the corresponding free phosphonic acids (compare **12**, **36**, and **41** with **13**, **42**, and **47**, respectively). Moreover, in most cases, the PME-substituted compounds were more active than the HPMP-series (compare **36**, **42**, **43**, and **44** with **39**, **45**, **46**, and **47**, respectively). Finally, the Fmoc protected derivatives were more efficient inhibitors than their benzoyl protected analogues (compare **41** with **39**, **44** with **42**, and **47** with **45**).

The series of twenty-one thiadiazole derivatives was tested for GSK-3 β inhibition at 10 μ M concentration by the IOCB research group of Dr. Helena Mertlíková-Kaiserová. For reference, tideglusib (**5**) was used as a thiadiazole inhibitory molecule and CHIR99021 (**55**) [154] as a non-thiadiazole inhibitor of GSK-3 β . The results (Table 3.1) indicate that inhibition of GSK-3 β by the tested set of compounds including **5** was lower than previously reported [155], with IC₅₀ values exceeding 10 μ M. In contrast, IC₅₀ of the non-thiadiazole inhibitor **55** was 9.9 ± 0.65 nM. As a validated commercial GSK-3 β kit was used, the low inhibitory effect of all thiadiazole compounds might have been caused by unknown interferences of assay components with the thiadiazole moiety. Thus, compound **5** was chosen as a reference molecule for evaluation of the thiadiazole inhibitory activity. Generally, most compounds that exhibited similar or higher activity than compound **5** (the residual GSK-3 β activity around 70 % or lower) also belonged to the strongest CatK inhibitors – compounds **1**, **14a**, **14b**, **19**, and **41**. Therefore, these most active molecules probably follow a similar SAR pattern to that described for CatK inhibition.

The cytotoxicity testing (Table 3.2) revealed that the evaluated thiadiazole derivatives synthesized in this study are overall less toxic than tideglusib (**5**), which exhibited cytotoxicity at 10 μ M in two leukemic cell lines (HL-60 and CCRF-CEM) as well as in human fibroblasts (NHDF). Only the compounds containing trityl moiety – **14a**, **14b**, and **19** – and the parent unsubstituted 5-amino-1,2,4-thiadiazol-3(2*H*)-one molecule **1** were cytotoxic at 100 μ M; the rest of the tested compounds proved to be safe up to 100 μ M concentration. It is apparent that the presence of trityl moiety that increased inhibitory activity towards both CatK and GSK-3 β at the same time provided the compound with undesired cytotoxicity.

All new thiadiazole structures were also evaluated for their antiviral activity. The study was performed at the Rega Institute for Medical Research, Katholieke Universiteit Leuven in the team of Professor Graciela Andrei and Professor Robert Snoeck. The screening performed in cell cultures (human embryonic lung cells) included the following viruses: herpes simplex virus-1 (KOS strain), herpes simplex virus-2 (G strain), thymidine kinase deficient (acyclovir resistant) herpes simplex virus-1 (TK–KOS ACVr), vaccinia virus, human cytomegalovirus (AD-169 strain and Davis strains), varicella-zoster

virus, adenovirus 2 and human coronavirus 229E. Unfortunately, none of the compounds revealed *in vitro* antiviral activity.

4 Conclusions

1. In summary, six N^2 -substituted PME- and (*S*)-HPMP-thiadiazole derivatives with protected amino group (Bz, Eoc, or Fmoc) in position 5 (**42-47**) were obtained as chemically stable ANP congeners. These compounds were synthesized *via* gradual thiadiazole ring assembly that required the use of two newly prepared synthons, PME-amine **21** and (*S*)-HPMP-amine **27**. Moreover, O^3 -substituted PME-thiadiazole phosphonic acid **13** was afforded as a stable molecule *via* direct alkylation of the DMA-protected thiadiazole **10** with PME-Cl (**6**), followed by subsequent removal of the DMA-protecting group and cleavage of diisopropyl esters. Nevertheless, all attempts to prepare the intended NH_2 -unprotected N^2 -substituted PME- and (*S*)-HPMP-thiadiazole phosphonic acids **48** and **49** failed due to instability of the N^2 -substituted NH_2 -unprotected thiadiazole ring. Cyclization efforts of the unprotected N^5 -alkylated PME- and (*S*)-HPMP-thiobiuret phosphonic acids **50** and **51** in aqueous solution proved redox character of the thiadiazole *S-N* bond.

2. Biology testing of selected twenty newly prepared 5-amino-1,2,4-thiadiazol-3(2*H*)-one derivatives and the parent thiadiazole compound **1** revealed interesting inhibitory activities towards two cysteine-dependent enzymes – CatK and GSK-3 β . Six compounds – **1**, **14a**, **14b**, **19**, **41**, and **43** – exhibited CatK inhibition in low micromolar range, most of which – **1**, **14a**, **14b**, **19**, and **41** – also showed similar or higher activity against GSK-3 β than tideglusib, the well-established GSK-3 β inhibitor. Apparently, a similar SAR pattern applies for both CatK and GSK-3 β inhibition. Apart from compounds containing trityl moiety (**14a**, **14b**, and **19**) and the parent thiadiazole molecule **1**, all other tested thiadiazole derivatives proved to be non-toxic at concentrations up to 100 μM . No *in vitro* antiviral activity against selected viruses was reported for the tested set of compounds.

5 Experimental part

5.1 General information

All commercially available chemicals were obtained from the following sources: Sigma-Aldrich (Merck), PENTA, aber GmbH, and ACT Chemical. Thiobiuret (**4**) as a precursor was prepared by my colleague, Dr. Miroslav Otmar, and synthons PME-Cl (**6**), (*S*)-glycidyl trityl ether (**7**), diisopropyl (tosyloxy)methylphosphonate (**15**), and diisopropyl (bromomethyl)phosphonate (**25**) were prepared by other colleagues from the research group of Dr. Marcela Krečmerová.

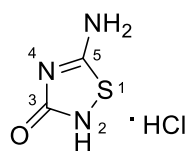
The column chromatography was performed on silica gel (Sigma S-0507, 40-63 μm), or on Dowex 50Wx8 H^+ /column. Thin-layer chromatography (TLC) analysis was performed on silica-gel plates with a fluorescent indicator (Silica gel 60 F₂₅₄-coated aluminum sheets, Merck, cat. no. 5554); the spots were visualized with UV lamp ENF-240C/FE (Spectroline) at 254/365 nm, or by spraying with ninhydrin (1% solution in ethanol) or 4-(*p*-nitrobenzyl)pyridine (1% solution in ethanol) – both followed by short heating to 300-400 °C. Sonication was performed with SONOREX Ultrasonic baths sonicator (BANDELIN). Synthesis employing MW irradiation was facilitated by the Microwave Synthesizer Discover (CEM Corporation).

The ^1H , ^{13}C , and ^{31}P NMR spectra were recorded on the Bruker Avance 400 instrument (^1H at 400 MHz, ^{13}C at 100.6 MHz, and ^{31}P at 162.0 MHz) and on the Bruker Avance 500 instrument (^1H at 500 MHz, ^{13}C at 125.7 MHz, and ^{31}P at 202.4 MHz), in CDCl_3 (referenced to TMS as an internal standard), in $\text{DMSO}-d_6$ (referenced to the solvent signal $\delta(^1\text{H}) = 2.50$ ppm and $\delta(^{13}\text{C}) = 39.70$ ppm, respectively), in $\text{MeOD}-d_4$ (referenced to the solvent signal $\delta(^1\text{H}) = 3.31$ ppm and $\delta(^{13}\text{C}) = 49.00$ ppm, respectively), or in D_2O (referenced to 1,4-dioxane as an internal standard). The complete assignment of signals was based on H,H-COSY , H,C-HSQC , and H,C-HMBC correlation experiments. Chemical shifts are given in δ -scale as parts per million (ppm); the coupling constants (J) are given in Hz.

The mass spectra were measured at the Mass Spectrometry Facility operated by IOCB. For compound characterization, ESI mass spectra were recorded on a Q-ToF micro (Waters) and the HR MS were taken on a LTQ Orbitrap XL (Thermo Fisher Scientific) spectrometer. The IR spectra were obtained on the Nicolett 6700 FT-IR spectrometer (Thermo Scientific) either in KBr pellets, in CCl_4 or in CHCl_3 solution. The melting points were determined by Kofler melting point apparatus with microscope KBT300 (SANTIAGO) and are uncorrected. Elemental analysis was performed on a PE 2400 Series II CHNS/O Analyzer (Perkin Elmer, USA).

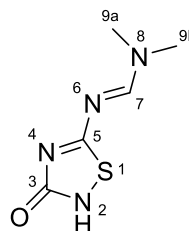
5.2 Synthetic part – reaction procedures and spectroscopic data

5-Amino-1,2,4-thiadiazol-3(2H)-one hydrochloride (1) [111].



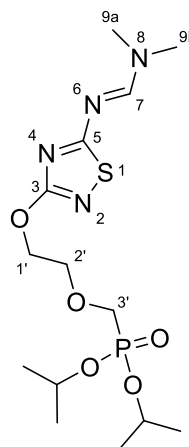
Thiobiuret (10.0 g, 83.9 mmol) was dissolved in 2M NaOH/H₂O (65 mL) and the solution was stirred in ice bath for 15 min. Hydrogen peroxide (30 %, 12 mL) was added dropwise and the reaction mixture was stirred for 3 h at 0 °C. Upon a successful conversion, the mixture was acidified to a pH 4.5 by adding 35% HCl. The resulting white precipitate was filtered and recrystallized from boiling water. White crystalline solid (6.185 g, yield 48 %). **Mp** = 219-220 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.90-9.75 (bs, 1H, NH), 8.05 (s, 2H, NH₂). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 176.25 (C-3), 168.31 (C-5). **R_f** (18/3/2/2 EtOAc/acetone/EtOH/H₂O) = 0.22. The spectra are in accordance with ref. [111].

(*E*)-*N,N*-Dimethyl-*N'*-(3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)formimidamide (10).



Compound **1** (5.450 g, 35.5 mmol) and DMF-DMA (12.4 mL, 93.3 mmol) were dissolved in acetonitrile (50 mL) and the reaction mixture was stirred overnight at r.t. An excess of diethylether was added and the resulting precipitate was filtered and washed with diethylether. White crystalline solid (4.706 g, yield 77 %). **Mp** = 197-198 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.56 (bs, 1H, NH), 8.45 (s, 1H, CH-7), 3.17 (s, 3H, 9b), 3.02 (s, 3H, 9a). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 187.01 (C-5), 168.21 (C-3), 157.97 (CH-7), 40.94 (9b), 35.03 (9a). **HRMS** (ESI) calcd. for C₅H₉ON₄S (M+H⁺): 173.0492, found: 173.0492. **R_f** (20/4/1 CHCl₃/MeOH/AcOH) = 0.56.

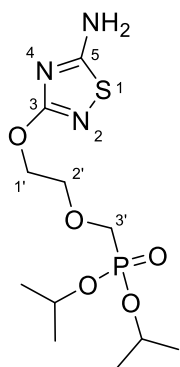
Diisopropyl (*E*)-((2-((5-(((dimethylamino)methylene)amino)-1,2,4-thiadiazol-3-yl)oxy)ethoxy)methyl)phosphonate (11); procedure according to ref. [142].



Compound **10** (4.59 g, 26.7 mmol), PME-Cl (**6**, 6.28 g, 24.3 mmol), and Cs₂CO₃ (11.89 g, 36.5 mmol) were dissolved in DMF (150 mL) and the reaction mixture was stirred at 95 °C for 5 h. After cooling to r.t., ethyl acetate (300 mL) was added and the mixture was washed with aqueous solution of NaHCO₃ (2x 250 mL) and with aqueous solution of NaCl (1x 250 mL). The organic phase was dried over MgSO₄, the solvent was evaporated and the residue was purified on silica gel column (0-5% MeOH in CHCl₃). Yellow oil (4.154 g, yield 39 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.40 (m, 1H, CH-7), 4.59 (dsept, 2H, *J*(H,P) = 7.8 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 4.37 (m, 2H, H-1'), 3.81 (m, 2H, H-2'), 3.80 (d, 2H, *J*(3',P) = 8.3 Hz, H-3'), 3.16 (d, 3H, *J*(9b,7) = 0.5 Hz, 9b), 3.02 (d, 3H, *J*(9a,7) = 0.8 Hz, 9a), 1.24 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.23 (d, 6H, *J*_{vic} = 6.1 Hz,

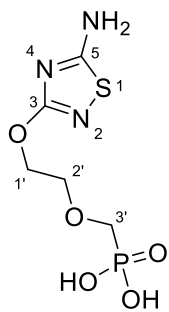
CH₃-*i*-Pr). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 191.16 (C-5), 168.05 (C-3), 157.93 (CH-7), 70.71 (d, *J*(2',P) = 12.0 Hz, C-2'), 70.35 (d, *J*(C,P) = 6.3 Hz, CH-*i*-Pr), 67.59 (C-1'), 65.08 (d, *J*(3',P) = 164.6 Hz, C-3'), 40.83 (9b), 34.99 (9a), 24.01 (d, *J*(C,P) = 3.8 Hz, CH₃-*i*-Pr), 23.87 (d, *J*(C,P) = 4.6 Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (162.0 MHz, DMSO-*d*₆): δ 20.40. **FTIR** (KBr, cm⁻¹) ν: 2980, 2881, 2820, 1705, 1624, 1468, 1375, 1253, 1193, 1107, 1060, 1010, 985, 950, 889. **HRMS** (ESI) calcd. for C₁₄H₂₇O₅N₄NaPS (M+Na⁺): 417.1332, found: 417.1337; calcd. for C₁₄H₂₈O₅N₄PS (M+H⁺): 395.1513, found: 395.1511. **R_f** (19/1 CHCl₃/MeOH) = 0.53.

Diisopropyl ((2-((5-amino-1,2,4-thiadiazol-3-yl)oxy)ethoxy)methyl)phosphonate (12).



To a compound **11** (4.10 g, 10.4 mmol) dissolved in methanol (15 ml), excess of hydrazine (8 mL, 35% in H₂O) was added and the mixture was stirred for 1 h at r.t. The solvent was removed *in vacuo* and the residue was purified on silica gel column (0-5% MeOH in CHCl₃). Colourless amorphous solid (2.657 g, yield 75 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.94 (s, 2H, NH₂), 4.59 (dsept, 2H, *J*(H,P) = 7.8 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 4.30 (m, 2H, H-1'), 3.79 (d, 2H, *J*(3',P) = 8.3 Hz, H-3'), 3.78 (m, 2H, H-2'), 1.24 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.23 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 182.76 (C-5), 167.37 (C-3), 70.72 (d, *J*(2',P) = 12.1 Hz, CH₂-2'), 70.38 (d, *J*(C,P) = 6.3 Hz, CH-*i*-Pr), 67.47 (CH₂-1'), 65.00 (d, *J*(3',P) = 164.4 Hz, CH₂-3'), 24.03 (d, *J*(C,P) = 3.8 Hz, CH₃-*i*-Pr), 23.89 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (162.0 MHz, DMSO-*d*₆): δ 20.38. **FTIR** (KBr, cm⁻¹) ν_{max}: 3298, 3162, 2980, 2935, 2882, 1631, 1548, 1507, 1465, 1454, 1409, 1387, 1375, 1337, 1237, 1222, 1178, 1142, 1105, 1013, 995, 891. **HRMS** (ESI) calcd. for C₁₁H₂₂O₅N₃PS (M+Na⁺): 362.0910, found: 362.0909. **R_f** (19/1 CHCl₃/MeOH) = 0.31.

((2-((5-Amino-1,2,4-thiadiazol-3-yl)oxy)ethoxy)methyl)phosphonic acid (13); procedure according to ref. [143].



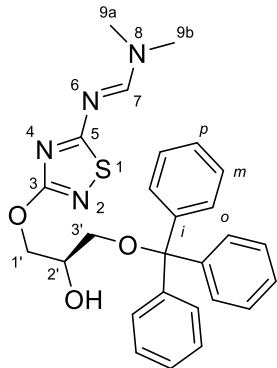
Compound **12** (2.78 g, 8.19 mmol) was dissolved in acetonitrile (50 mL), TMS-Br (21.6 mL, 0.164 mol) was added and the reaction mixture was stirred at r.t. overnight. The volatiles were removed *in vacuo* and the residue was co-evaporated with acetonitrile (3x) and water (1x). The residue was purified on a Dowex 50Wx8 H⁺/column with elution with 1 L of H₂O, followed by NH₃/H₂O gradient (0-2.5 %). The compound was crystallized from the mixture methanol/diethylether. White crystalline solid (1.515 g, yield 73 %). **Mp** = 161-163 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 8.00 (s, 2H, NH₂), 7.47 (vbs, NH₄⁺), 4.26 (m, 2H, H-1'), 3.71 (m, 2H, H-2'), 3.37 (d, 2H, *J*(3',P) = 8.7 Hz, H-3'). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 182.74 (C-5), 167.44 (C-3), 70.07 (d, *J*(2',P) = 10.0 Hz, CH₂-2'), 69.08 (d,

$J(3',P) = 155.0$ Hz, $\text{CH}_2\text{-}3'$), 67.88 ($\text{CH}_2\text{-}1'$). ^{31}P $\{^1\text{H}\}$ NMR (162.0 MHz, $\text{DMSO-}d_6$): δ 12.88. FTIR (KBr, cm^{-1}) ν_{max} : 3290, 3179, 2855, 1635, 1547, 1513, 1455, 1414, 1390, 1339, 1105, 1063, 975, 922. HRMS (ESI) calcd. for $\text{C}_5\text{H}_9\text{O}_5\text{N}_3\text{PS}$ (M-H^+): 254.0006, found: 254.0006. R_f (7/2/1 *i*-PrOH/ H_2O / NH_3) = 0.14.

(*R,E*)-*N'*-(3-(2-Hydroxy-3-(trityloxy)propoxy)-1,2,4-thiadiazol-5-yl)-*N,N*-dimethylformimidamide (14a) and (*S,E*)-*N'*-(2-(2-hydroxy-3-(trityloxy)propyl)-3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)-*N,N*-dimethylformimidamide (14b); procedure according to ref. [142].

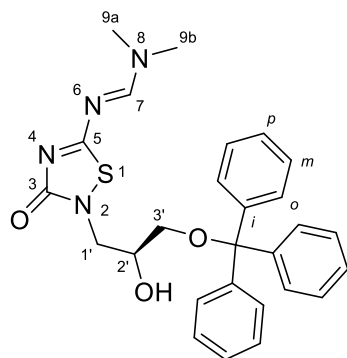
Compound **10** (2.50 g, 14.5 mmol), (*S*)-glycidyl trityl ether (**7**, 4.18 g, 13.2 mmol), and Cs_2CO_3 (0.860 g, 2.64 mmol) were dissolved in DMF (50 mL) and the reaction mixture was stirred at 95 °C for 4 h. diethylether (200 mL) was added after cooling to r.t. and the mixture was washed with aqueous solution of NaHCO_3 (2x 150 mL), followed by aqueous solution of NaCl (1x 150 mL). The organic phase was dried over MgSO_4 , the solvent was evaporated and the residue was purified on silica gel column (0-2% MeOH in CHCl_3); compound **14a** was eluted first, followed by compound **14b**.

(*R,E*)-*N'*-(3-(2-Hydroxy-3-(trityloxy)propoxy)-1,2,4-thiadiazol-5-yl)-*N,N*-dimethylformimidamide (14a).



White crystalline solid (2.505 g, yield 36 %). $\text{Mp} = 72\text{-}74$ °C. $[\alpha]_D -2.5$ (*c* 0.360 g/100 mL, MeOH). ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ 8.39 (m, 1H, H-7), 7.37-7.42 (m, 6H, H-*o*-Tr), 7.29-7.35 (m, 6H, H-*m*-Tr), 7.23-7.28 (m, 3H, H-*p*-Tr), 5.23 (d, 1H, $J(\text{OH},2') = 5.6$ Hz, OH), 4.33 (dd, 1H, $J_{\text{gem}} = 10.8$ Hz, $J(1'b,2') = 4.4$ Hz, H-1'b), 4.26 (dd, 1H, $J_{\text{gem}} = 10.8$ Hz, $J(1'a,2') = 5.8$ Hz, H-1'a), 3.97 (pentd, 1H, $J(2',1'a) = J(2',3'a) = J(2',3'b) = J(2',\text{OH}) = 5.7$ Hz, $J(2',1'b) = 4.4$ Hz, H-2'), 3.15 (bs, 3H, 9b), 3.04 (dd, 1H, $J_{\text{gem}} = 9.2$ Hz, $J(3'b,2') = 5.6$ Hz, H-3'b), 3.02 (d, 3H, $J(9a,7) = 0.8$ Hz, 9a), 3.00 (dd, 1H, $J_{\text{gem}} = 9.2$ Hz, $J(3'a,2') = 5.7$ Hz, H-3'a). ^{13}C NMR (100.6 MHz, $\text{DMSO-}d_6$): δ 191.07 (C-5, determined by HSQC and HMBC), 168.24 (C-3), 157.87 (CH-7), 143.98 (C-*i*-Tr), 128.47 (CH-*o*-Tr), 128.19 (CH-*m*-Tr), 127.19 (CH-*p*-Tr), 86.00 (C-Tr), 70.32 (C-1'), 67.91 (C-2'), 64.99 (C-3'), 40.83 (9b), 35.00 (9a). FTIR (KBr, cm^{-1}) ν_{max} : 3415, 3086, 3057, 3032, 3023, 3001, 2928, 2815, 1750, 1685, 1623, 1597, 1583, 1523, 1497, 1449, 1337, 1323, 1154, 1107, 1076, 1033, 1002, 946, 938, 765, 707, 700, 633, 316. HRMS (ESI) calcd. for $\text{C}_{27}\text{H}_{28}\text{O}_3\text{N}_4\text{NaS}$ ($\text{M}+\text{Na}^+$): 511.1774, found: 511.1773. R_f (20/4/1 CHCl_3 /*i*-PrOH/AcOH) = 0.82.

(*S,E*)-*N'*-(2-(2-Hydroxy-3-(trityloxy)propyl)-3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)-*N,N*-dimethylformimidamide (14b).

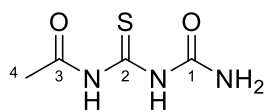


White crystalline solid (0.615 g, yield 9 %). **Mp** = 200-202 °C. $[\alpha]_D^{25}$ -3.4 (*c* 0.295 g/100 mL, MeOH). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 8.55 (s, 1H, H-7), 7.38-7.42 (m, 6H, H-*o*-Tr), 7.31-7.36 (m, 6H, H-*m*-Tr), 7.23-7.28 (m, 3H, H-*p*-Tr), 5.41 (d, 1H, *J*(OH,2') = 5.4 Hz, OH), 3.86 (dd, 1H, *J*_{gem} = 13.8 Hz, *J*(1'b,2') = 3.4 Hz, H-1'b), 3.81 (m, 1H, H-2'), 3.52 (dd, 1H, *J*_{gem} = 13.8 Hz, *J*(1'a,2') = 7.2 Hz, H-1'a), 3.20 (s, 3H, 9b), 3.03 (s, 3H, 9a), 2.97 (dd, 1H, *J*_{gem} = 9.3 Hz, *J*(3'b,2') = 5.2 Hz, H-3'b), 2.88 (dd, 1H, *J*_{gem} = 9.4 Hz, *J*(3'a,2') = 6.2 Hz, H-3'a). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 181.53 (C-5), 165.94 (C-3), 158.01 (CH-7), 143.88 (C-*i*-Tr), 128.48 (CH-*o*-Tr), 128.07 (CH-*m*-Tr), 127.20 (CH-*p*-Tr), 86.11 (C-Tr), 68.67 (C-2'), 65.34 (C-3'), 47.11 (C-1'), 41.12 (9b), 35.17 (9a). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3288, 3085, 3057, 3032, 3022, 3001, 2926, 2810, 1678, 1623, 1596, 1585, 1491, 1448, 1418, 1339, 1307, 1153, 1113, 1077, 1033, 1002, 950, 935, 762, 709, 699, 633, 617. **HRMS** (ESI) calcd. for C₂₇H₂₈O₃N₄Na₄S (M+Na⁺): 511.1774, found: 511.1773. **R_f** (20/4/1 CHCl₃/*i*-PrOH/AcOH) = 0.62.

The general procedure for synthesis of *N*¹-substituted thiobiurets (17) [118] and (3) [111]

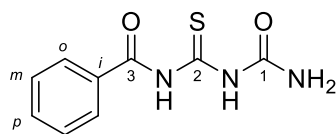
Potassium thiocyanate (48.0 g, 0.494 mol) was dissolved in acetone (400 mL) and acyl chloride (0.410 mmol) was added dropwise. The reaction mixture was stirred at 50 °C for 3.5 h. After cooling to r.t., KCl was removed by filtration over Celite and volume of the filtrate was reduced *in vacuo*. Urea (24.6 g, 0.410 mol) was added to the solution and the reaction mixture was stirred at 65 °C for 5 h. After cooling to r.t., the flask was cooled in ice bath to initiate crystallization and the solution was left at r.t. overnight. The crystals formed were collected by filtration and recrystallized from methanol.

***N*-(Carbamoylcarbamothioyl)acetamide (17) [118].**



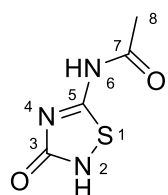
Reactants: acetyl chloride (29.2 mL, 0.410 mmol) and potassium thiocyanate (48.0 g, 0.494 mol). Yellowish crystalline solid (9.433 g, yield 14 %). **Mp** = 196-198 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 12.04 (bs, 1H, NH), 11.90 (bs, 1H, NH), 8.48 (bs, 1H, NH₂), 7.89 (bs, 1H, NH₂), 2.16 (s, 3H, H-4). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 179.62 (C-2), 172.73 (C-3), 152.81 (C-1), 24.33 (CH₃-4). **R_f** (9/1 CHCl₃/MeOH) = 0.59. The spectra are in accordance with ref. [118].

***N*-(Carbamoylcarbamothioyl)benzamide (3) [111].**



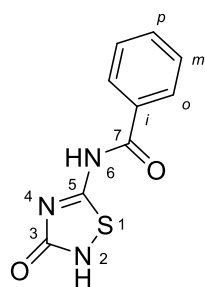
Reactants: benzoyl chloride (47.6 mL, 0.410 mmol) and potassium thiocyanate (48.0 g, 0.494 mol). Yellow crystalline solid (36.183 g, yield 40 %). **Mp** = 174-175 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 13.47 (bs, 1H, NH), 11.12 (bs, 1H, NH), 7.89 (m, 2H, H-*o*-Ph), 7.68 (m, 1H, H-*p*-Ph), 7.57 (m, 2H, H-*m*-Ph), 7.21 (vbs, 2H, NH₂). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 179.17 (C-2), 154.52 (C-1), 133.27 (CH-*p*-Ph), 132.96 (C-*i*-Ph), 128.93 (CH-*m*-Ph), 127.83 (CH-*o*-Ph); C-3 signal not detected. **R_f** (9/1 CHCl₃/MeOH) = 0.65. The spectra are in accordance with ref. [111, 118].

***N*-(3-Oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)acetamide (18a) [118]; procedure according to ref. [144].**



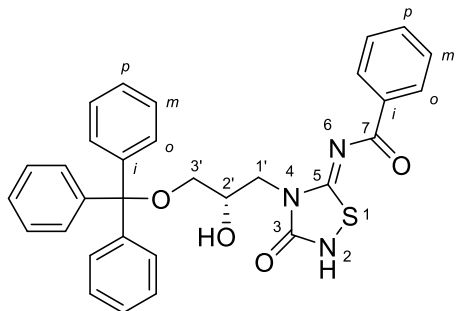
Compound **17** (5.00 g, 31.0 mmol) was dissolved in ethanol (40 mL), the solution was stirred at 35 °C and 1M Br₂ solution in CHCl₃ (43.8 mL, 43.8 mmol Br₂), was added dropwise. The reaction mixture was stirred at 35 °C for 20 min. After cooling to r.t., the crystals formed were filtered, washed with diethylether and recrystallized from water. White crystalline solid (2.952 g, yield 60 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 11.81 (vbs, 2H, NH-2, NH-6), 2.20 (s, 3H, H-8). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 172.97 (C-5), 171.98 (C-7), 166.53 (C-3), 22.65 (CH₃-8). **R_f** (9/1 CHCl₃/MeOH) = 0.26. The spectra are in accordance with ref. [118].

***N*-(3-Oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)benzamide (18b) [144].**



Compound **3** (7.46 g, 33.4 mmol) and NBS (7.43 g, 41.8 mmol) were dissolved in methanol (200 mL) and the reaction mixture was stirred at 70 °C for 6 h. After cooling to r.t., the crystals formed were filtered and washed with diethylether. White crystalline solid (4.154 g, yield 56 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 12.02 (vbs, 2H, NH-2, NH-6), 8.12 (m, 2H, H-*o*-Ph), 7.66 (m, 1H, H-*p*-Ph), 7.56 (m, 2H, H-*m*-Ph). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 173.5 (C-7), 133.23 (CH-*p*-Ph), 131.86 (C-*i*-Ph), 128.76 (CH-*m*-Ph), 128.61 (CH-*o*-Ph). **R_f** (9/1 CHCl₃/MeOH) = 0.30. The spectra are in accordance with ref. [144].

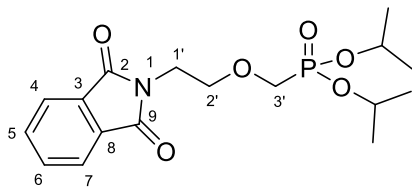
(*S,Z*)-*N*-(4-(2-Hydroxy-3-(trityloxy)propyl)-3-oxo-1,2,4-thiadiazolidin-5-ylidene)benzamide (19);
procedure according to ref. [142].



Compound **18b** (2.00 g, 9.04 mmol), (*S*)-glycidyl trityl ether (**7**, 2.62 g, 8.28 mmol), and Cs₂CO₃ (0.553 g, 1.70 mmol) were dissolved in DMF (70 mL) and the reaction mixture was stirred at 110 °C for 24 h. The solvent was removed *in vacuo* and the residue was purified on silica gel column (0-2% MeOH in CHCl₃) and recrystallized from ethyl acetate/hexane. White crystalline solid (1.016 g, yield 21 %). **Mp** = 176-177 °C. [α]_D -

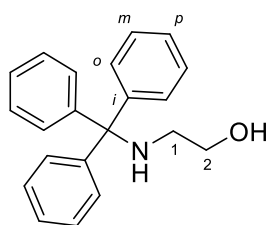
23.3 (*c* 0.313 g/100 mL, MeOH). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 10.24 (bs, 1H, NH, determined by HMBC), 8.16 (m, 2H, H-*o*-Ph), 7.63 (m, 1H, H-*p*-Ph), 7.46 (m, 2H, H-*m*-Ph), 7.35-7.41 (m, 6H, H-*o*-Tr), 7.24-7.30 (m, 6H, H-*m*-Tr), 7.19-7.24 (m, 3H, H-*p*-Tr), 5.30 (d, 1H, $J(\text{OH}, 2') = 5.8$ Hz, OH), 4.31 (bdpent, 1H, $J(2', 1'a) = 7.8$ Hz, $J(2', 1'b) = J(2', 3') = J(2', \text{OH}) = 5.7$ Hz, H-2'), 4.09 (dd, 1H, $J_{\text{gem}} = 13.2$ Hz, $J(1'b, 2') = 5.5$ Hz, H-1'b), 4.02 (dd, 1H, $J_{\text{gem}} = 13.2$ Hz, $J(1'a, 2') = 7.9$ Hz, H-1'a), 3.12 (dd, 1H, $J_{\text{gem}} = 9.5$ Hz, $J(3'b, 2') = 5.1$ Hz, H-3'b), 2.98 (dd, 1H, $J_{\text{gem}} = 9.5$ Hz, $J(3'a, 2') = 6.3$ Hz, H-3'a). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 176.97 (C-7), 171.52 (C-5), 153.51 (C-3), 143.91 (C-*i*-Tr), 133.97 (C-*i*-Ph), 133.23 (CH-*p*-Ph), 129.25 (CH-*o*-Ph), 128.80 (CH-*m*-Ph), 128.34 (CH-*o*-Tr), 128.02 (CH-*m*-Tr), 127.17 (CH-*p*-Tr), 86.27 (C-Tr), 66.70 (CH₂-3'), 66.33 (CH-2'), 48.34 (CH₂-1'). **HRMS** (ESI) calcd. for C₃₁H₂₇O₄N₃NaS (M+Na⁺): 560.1615, found: 560.1609. **R_f** (9/1 CHCl₃/MeOH) = 0.54.

Diisopropyl ((2-(*N*-phthalimido)ethoxy)methyl)phosphonate (20).



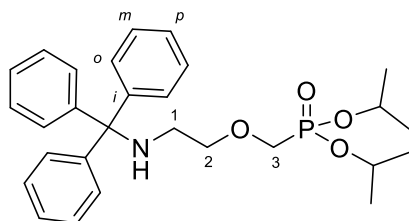
PME-Cl (**6**, 20.28 g, 78.4 mmol) and potassium phthalimide (14.52 g, 78.4 mmol) were dissolved in DMF (200 mL) and stirred at 120 °C for 3.5 h. The solvent was removed *in vacuo* and the residue was co-evaporated with toluene. The product was purified on silica gel column (0-5% MeOH in CHCl₃). White amorphous solid (24.668 g, yield 85 %). **¹H NMR** (400 MHz, CDCl₃): δ 7.77 (m, 2H, CH-4,7-Ar), 7.67 (m, 2H, CH-5,6-Ar), 4.63 (m, 2H, CH-*i*-Pr), 3.85 (m, 2H, H-1'), 3.80 (m, 2H, H-2'), 3.71 (d, 2H, $J(3', \text{P}) = 8.3$ Hz, H-3'), 1.19-1.22 (m, 12H, CH₃-*i*-Pr). **¹³C NMR** (100.6 MHz, CDCl₃): δ 167.95 (C-2, C-9), 133.85 (C_{Ar}-3,4), 131.93 (C_{Ar}-1,6), 123.09 (CH-4,7-Ar), 70.96 (d, $J(\text{C}, \text{P}) = 6.6$ Hz, CH-*i*-Pr), 69.55 (d, $J(2', \text{P}) = 10.8$ Hz, CH₂-2'), 65.28 (d, $J(3', \text{P}) = 167.2$ Hz, CH₂-3'), 36.90 (CH₂-1'), 23.87 (d, $J(\text{C}, \text{P}) = 3.8$ Hz, CH₃-*i*-Pr), 23.76 (d, $J(\text{C}, \text{P}) = 4.6$ Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (162.0 MHz, CDCl₃): δ 18.84. **FTIR** (CCl₄, cm⁻¹) ν_{max} : 3088, 3061, 2981, 2938, 2875, 1778, 1720, 1617, 1469, 1393, 1375, 1241, 1179, 1142, 1107, 1009, 990, 889, 718, 529. **HRMS** (ESI) calcd. for C₁₇H₂₄O₆NNaP (M+Na⁺): 392.1234, found: 392.1235. **R_f** (6/4/1 hexane/CHCl₃/MeOH) = 0.39.

2-(Tritylamino)ethan-1-ol (**22**) [146]; procedure according to ref. [145].



2-Aminoethan-1-ol (13.0 g, 0.212 mol) was dissolved in dichloromethane (250 mL) and TEA (60 mL, 0.431 mol) was added. Trityl chloride (59.1 g, 0.212 mol) was added to the solution while cooling in ice bath (exothermic reaction), then the mixture was stirred for 2 h at r.t. The solvent was removed *in vacuo*, the residue was dissolved in ethyl acetate (300 mL) and extracted with aqueous solution of NaHCO₃ (2x 250 mL) and aqueous solution of NaCl (1x 250 mL). The organic phase was dried over MgSO₄, the solvent was evaporated and the residue was crystallized from ethyl acetate/hexane. White crystalline solid (52.435 g, yield: 82 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.40 (m, 6H, H-*o*-Tr), 7.29 (m, 6H, H-*m*-Tr), 7.18 (m, 3H, H-*p*-Tr), 4.52 (t, 1H, *J*(OH,2) = 5.5 Hz, OH), 3.51 (q, 2H, *J*(2,1) = *J*(2,OH) = 5.8 Hz, H-2), 2.58 (t, 1H, *J*(NH,1) = 7.9 Hz, NH), 2.07 (dt, 2H, *J*(1,NH) = 7.8 Hz, *J*(1,2) = 6.1 Hz, H-1). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 146.39 (C-*i*-Tr), 128.59 (CH-*o*-Tr), 127.92 (CH-*m*-Tr), 126.28 (CH-*p*-Tr), 70.32 (C-Tr), 61.17 (CH₂-2), 46.23 (CH₂-1). *R_f* (9/1 CHCl₃/MeOH) = 0.71. The spectra are in accordance with ref. [146].

Diisopropyl ((2-(tritylamino)ethoxy)methyl)phosphonate (**23**); procedure according to ref. [145].



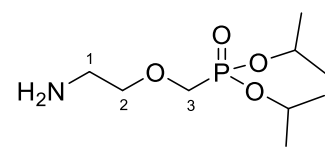
Compound **22** (5.0 g, 16.5 mmol), tosyloxymethanephosphonate diisopropyl ester (**15**, 6.94 g, 19.8 mmol), and magnesium di-*t*-butoxide (5.63 g, 33.0 mmol) were dissolved in NMP (30 mL) and stirred at 75-78 °C for 4.5 h. After cooling to r.t., water (30 mL) was added and the reaction mixture was extracted with diethylether (50 mL). The diethylether layer was washed with aqueous solution of NaHCO₃ (2x 50 mL) and aqueous solution of NaCl (1x 50 mL). The organic phase was dried over MgSO₄, the solvent was removed *in vacuo* and the residue was purified on silica gel column (CHCl₃/hexane, 1:9 to 1:1). Colourless oil (7.221 g, yield: 91 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.41 (m, 6H, H-*o*-Tr), 7.29 (m, 6H, H-*m*-Tr), 7.19 (m, 3H, H-*p*-Tr), 4.59 (dsept, 2H, *J*(H,P) = 7.8 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 3.68 (d, 2H, *J*(3,P) = 8.4 Hz, H-3), 3.62 (t, 2H, *J*(2,1) = 5.6 Hz, H-2), 2.59 (t, 1H, *J*(NH,1) = 7.9 Hz, NH), 2.16 (dt, 2H, *J*(1,NH) = 7.8 Hz, *J*(1,2) = 5.6 Hz, H-1), 1.23 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.21 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 146.09 (C-*i*-Tr), 128.47 (CH-*o*-Tr), 127.88 (CH-*m*-Tr), 126.29 (CH-*p*-Tr), 72.56 (d, *J*(2,P) = 12.1 Hz, CH₂-2), 70.27 (C-Tr), 70.22 (d, *J*(C,P) = 6.5 Hz, CH-*i*-Pr), 64.96 (d, *J*(3,P) = 165.0 Hz, CH₂-3), 43.09 (CH₂-1), 23.96 (d, *J*(C,P) = 3.9 Hz, CH₃-*i*-Pr), 23.88 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr). ³¹P {¹H} NMR (162.0 MHz, DMSO-*d*₆): δ 21.81. FTIR (CCl₄, cm⁻¹) ν_{max}: 3335, 3085, 3062, 3033, 2980, 2934, 2873, 1597, 1489, 1466, 1449, 1386, 1375, 1318, 1242, 1216, 1191, 1180, 1142, 1107, 1033, 1008, 990, 940, 902, 706, 698, 648, 625. HRMS (ESI) calcd. for C₂₈H₃₆O₄NNaP (M+Na⁺):

504.2274, found: 504.2273; calcd. for $C_{28}H_{37}O_4NP$ ($M+H^+$): 482.2455, found: 482.2455. R_f (6/4/1 hexane/ $CHCl_3$ /MeOH) = 0.55.

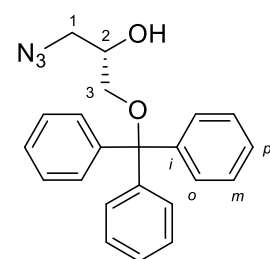
Diisopropyl ((2-aminoethoxy)methyl)phosphonate (21).

Method A - deprotection of phthalimide: Compound **20** (5.2 g, 14.1 mmol) was dissolved in ethanol (50 mL) and hydrazine (1M in THF, 140 mL, 0.141 mol) was added. The mixture was stirred for 2 h at r.t. Phthalhydrazide was removed by filtration, the filtrate was concentrated *in vacuo* and the residue was purified on silica gel column under basic conditions (2-10% 2.35M NH_3 /MeOH in $CHCl_3$). Colourless oil (3.065 g, yield 91 %).

Method B - deprotection of trityl protecting group: Compound **23** (7.0 g, 14.5 mmol) was dissolved in dioxane (40 mL), the solution was cooled in ice bath and HCl (35 %, 30 mL) was added. The reaction mixture was stirred for 1.5 h at r.t., then washed by diethylether to remove triphenylmethanol and the aqueous phase was concentrated *in vacuo*. The residue was purified on silica gel column under basic conditions (63/32/5 toluene/ $CHCl_3$ /TEA to 95/5 $CHCl_3$ /TEA). Colourless oil (2.260 g, yield 65 %).

 **^1H NMR** (400 MHz, $CDCl_3$): δ 4.73 (dsept, 2H, $J(H,P) = 7.7$ Hz, $J_{vic} = 6.2$ Hz, CH-*i*-Pr), 3.73 (d, 2H, $J(3,P) = 8.5$ Hz, H-3), 3.68 (t, 2H, $J(2,1) = 5.1$ Hz, H-2), 2.97 (t, 2H, $J(1,2) = 4.9$ Hz, H-1), 1.33 (d, 6H, $J_{vic} = 6.2$ Hz, CH_3 -*i*-Pr), 1.32 (d, 6H, $J_{vic} = 6.2$ Hz, CH_3 -*i*-Pr). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 75.34 (d, $J(C,P) = 11.2$ Hz, CH_2 -2), 70.89 (d, $J(C,P) = 6.7$ Hz, CH-*i*-Pr), 65.68 (d, $J(3,P) = 168.2$ Hz, CH_2 -3), 41.38 (CH_2 -1), 24.02 (d, $J(C,P) = 3.9$ Hz, CH_3 -*i*-Pr), 23.95 (d, $J(C,P) = 4.6$ Hz, CH_3 -*i*-Pr). **^{31}P { ^1H } NMR** (162.0 MHz, $CDCl_3$): δ 20.15. **FTIR** (CCl_4 , cm^{-1}) ν_{max} : 3334, 2981, 2934, 2876, 1467, 1387, 1376, 1241, 1179, 1142, 1106, 1010, 993, 889. **HRMS** (ESI) calcd. for $C_9H_{22}O_4NNaP$ ($M+Na^+$): 262.1179, found: 262.1179; calcd. for $C_9H_{23}O_4NP$ ($M+H^+$): 240.1359, found: 240.1360. R_f (12/5/4/1/1 $CHCl_3$ /*i*-PrOH/MeOH/ NH_3 / H_2O) = 0.52.

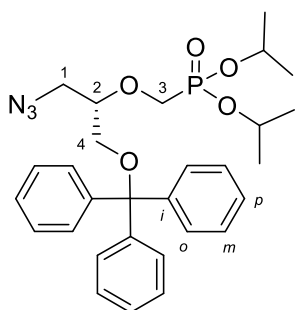
(S)-1-Azido-3-(trityloxy)propan-2-ol (24).



(S)-Glycidyl trityl ether (**7**, 10.0 g, 31.6 mmol) and sodium azide (41.1 g, 0.632 mol) were dissolved in DMF (500 mL) and the reaction mixture was stirred at 45°C for 7 days. Water (5.0 mL) was added and the reaction mixture was stirred for 10 min. The salts were filtered off by Büchner funnel and the solvent was removed *in vacuo*. The residue was extracted between water (350 mL) and ethyl acetate (2x 350 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residue was purified on silica gel column (17/3 hexane/ $CHCl_3$). Colourless oil

(7.525 g, yield 66 %). **[α]_D** -17.6 (*c* 0.250 g/100 mL, MeOH). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 7.36-7.42 (m, 6H, H-*o*-Tr), 7.30-7.38 (m, 6H, H-*m*-Tr), 7.23-7.30 (m, 3H, H-*p*-Tr), 5.35 (d, 1H, $J(\text{OH},2) = 5.3$ Hz, OH), 3.83 (ttd, 1H, $J(2,1b) = J(2,3b) = 6.5$ Hz, $J(2,\text{OH}) = J(2,3a) = 5.3$ Hz, $J(2,1a) = 3.6$ Hz, H-2), 3.36 (dd, 1H, $J_{\text{gem}} = 12.6$ Hz, $J(1a,2) = 3.6$ Hz, H-1a), 3.30 (dd, 1H, $J_{\text{gem}} = 12.6$ Hz, $J(1b,2) = 6.5$ Hz, H-1b), 3.01 (dd, 1H, $J_{\text{gem}} = 9.2$ Hz, $J(3a,2) = 5.3$ Hz, H-3a), 2.89 (dd, 1H, $J_{\text{gem}} = 9.2$ Hz, $J(3b,2) = 6.5$ Hz, H-3b). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 143.90 (C-*i*-Tr), 128.45 (CH-*o*-Tr), 128.08 (CH-*m*-Tr), 127.22 (CH-*p*-Tr), 86.11 (C-Tr), 69.14 (CH-2), 65.27 (CH₂-3), 53.80 (CH₂-1). **FTIR** (CCl₄, cm⁻¹) ν_{max} : 3600, 2928, 2858, 2105, 1737, 1467, 1393, 1087, 1077, 529. **HRMS** (ESI) calcd. for C₂₂H₂₁O₂N₃Na (M+Na⁺): 382.1526, found: 382.1523. **R_f** (6/4/1 hexane/CHCl₃/MeOH) = 0.61.

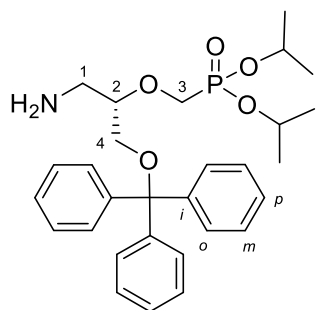
(Diisopropyl (S)-(((1-azido-3-(trityloxy)propan-2-yl)oxy)methyl)phosphonate (26); procedure according to ref. [46].



Compound **24** (7.500 g, 20.9 mmol) and NaH (60% suspension in mineral oil, 2.095 g, 52.3 mmol) were dissolved in DMF (300 mL) and stirred for 15 min at r.t. Diisopropyl (bromomethyl)phosphonate (**25**, 8.122 g, 31.4 mmol) was added and the mixture was stirred at 60 °C overnight. Water (30 mL) was added and the reaction mixture was stirred for 5 min. The solvent was removed *in vacuo* and the residue was extracted between water (300 mL) and ethyl acetate (2x 450 mL). The organic layers were collected and dried over

Na₂SO₄. The solvent was removed *in vacuo* and the residue was purified on silica gel column (3/2 hexane/CHCl₃ to 17/3 CHCl₃/MeOH). Colourless oil (7.850 g, yield 70 %). **[α]_D** -7.4 (*c* 0.298 g/100 mL, MeOH). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 7.36-7.43 (m, 6H, H-*o*-Tr), 7.31-7.38 (m, 6H, H-*m*-Tr), 7.25-7.29 (m, 3H, H-*p*-Tr), 4.53-4.64 (m, 2H, CH-*i*-Pr), 3.84 (dd, 1H, $J_{\text{gem}} = 13.9$ Hz, $J(3a,\text{P}) = 9.0$ Hz, H-3a), 3.81 (dd, 1H, $J_{\text{gem}} = 13.9$ Hz, $J(3b,\text{P}) = 9.0$ Hz, H-3b), 3.74 (dddd, 1H, $J(2,1b) = 5.7$ Hz, $J(2,4b) = 5.3$ Hz, $J(2,4a) = 4.9$ Hz, $J(2,1a) = 4.1$ Hz, H-2), 3.56 (dd, 1H, $J_{\text{gem}} = 13.1$ Hz, $J(1a,2) = 4.1$ Hz, H-1a), 3.45 (dd, 1H, $J_{\text{gem}} = 13.1$ Hz, $J(1b,2) = 5.7$ Hz, H-1b), 3.10 (dd, 1H, $J_{\text{gem}} = 10.2$ Hz, $J(4a,2) = 4.9$ Hz, H-4a), 3.08 (dd, 1H, $J_{\text{gem}} = 10.2$ Hz, $J(4b,2) = 5.3$ Hz, H-4b), 1.18-1.28 (m, 12H, CH₃-*i*-Pr). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 143.70 (C-*i*-Tr), 128.44 (CH-*o*-Tr), 128.12 (CH-*m*-Tr), 127.30 (CH-*p*-Tr), 86.36 (C-Tr), 79.35 (d, $J(2,\text{P}) = 12.2$ Hz, CH-2), 70.38 (d, $J(\text{C},\text{P}) = 6.3$ Hz, CH-*i*-Pr), 65.86 (CH₂-4), 64.07 (d, $J(3,\text{P}) = 165.2$ Hz, CH₂-3), 50.93 (CH₂-1), 23.72-24.06 (CH₃-*i*-Pr). **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 18.82. **FTIR** (CCl₄, cm⁻¹) ν_{max} : 3088, 3062, 3035, 3024, 2981, 2934, 2874, 2103, 1598, 1491, 1467, 1449, 1386, 1375, 1310, 1260, 1244, 1178, 1142, 1159, 1153, 1107, 1088, 1080, 1035, 1008, 991, 936, 889, 705, 697, 633, 555. **HRMS** (ESI) calcd. for C₂₉H₃₆O₅N₃NaP (M+Na⁺): 560.2285, found: 560.2284. **R_f** (15/10/1 hexane/CHCl₃/MeOH) = 0.46.

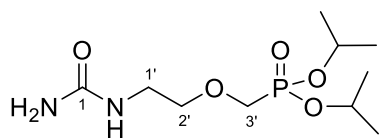
Diisopropyl (S)-(((1-amino-3-(trityloxy)propan-2-yl)oxy)methyl)phosphonate (27); procedure according to ref. [147].



Compound **26** (7.800 g, 14.5 mmol) was dissolved in THF (200 mL) and the solution was cooled in ice bath. Triphenylphosphine (4.185 g, 16.0 mmol) and water (2.0 mL) were added upon stirring and the reaction mixture was stirred overnight to reach r.t. The solvent was removed *in vacuo* and the residue was purified on silica gel column (0-5% MeOH in 1% TEA/CHCl₃). Colourless oil (4.819 g, yield 65 %). [α]_D -8.8 (*c* 0.656 g/100 mL, MeOH).

¹H NMR (500 MHz, DMSO-*d*₆): δ 7.38-7.42 (m, 6H, H-*o*-Tr), 7.31-7.36 (m, 6H, H-*m*-Tr), 7.23-7.28 (m, 3H, H-*p*-Tr), 4.55-4.65 (m, 2H, CH-*i*-Pr), 3.85 (dd, 1H, $J_{\text{gem}} = 13.3$ Hz, $J(3a,P) = 8.6$ Hz, H-3a), 3.81 (dd, 1H, $J_{\text{gem}} = 13.3$ Hz, $J(3b,P) = 8.6$ Hz, H-3b), 3.50 (dddd, 1H, $J(2,1b) = 6.1$ Hz, $J(2,4b) = 5.7$ Hz, $J(2,1a) = 5.2$ Hz, $J(2,4a) = 3.9$ Hz, H-2), 3.11 (dd, 1H, $J_{\text{gem}} = 10.0$ Hz, $J(4a,2) = 3.9$ Hz, H-4a), 3.04 (dd, 1H, $J_{\text{gem}} = 10.0$ Hz, $J(4b,2) = 5.7$ Hz, H-4b), 2.66 (dd, 1H, $J_{\text{gem}} = 13.2$ Hz, $J(1a,2) = 5.2$ Hz, H-1a), 2.62 (dd, 1H, $J_{\text{gem}} = 13.2$ Hz, $J(1b,2) = 6.1$ Hz, H-1b), 1.24 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.23 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.22 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.21 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 143.94 (C-*i*-Tr), 128.45 (CH-*o*-Tr), 128.06 (CH-*m*-Tr), 127.20 (CH-*p*-Tr), 86.15 (C-*Tr*), 82.78 (d, $J(2,P) = 11.8$ Hz, CH-2), 70.30 (d, $J(C,P) = 6.2$ Hz, CH-*i*-Pr), 70.26 (d, $J(C,P) = 6.2$ Hz, CH-*i*-Pr), 64.34 (d, $J(3,P) = 165.3$ Hz, CH₂-3), 63.83 (CH₂-4), 42.59 (CH₂-1), 24.02 (d, $J(C,P) = 3.6$ Hz, CH₃-*i*-Pr), 24.01 (d, $J(C,P) = 3.6$ Hz, CH₃-*i*-Pr), 23.93 (d, $J(C,P) = 4.5$ Hz, CH₃-*i*-Pr), 23.91 (d, $J(C,P) = 4.5$ Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 19.55. **FTIR** (CCl₄, cm⁻¹) ν_{max} : 3088, 3061, 3035, 3024, 2980, 2874, 1598, 1491, 1466, 1449, 1386, 1375, 1241, 1179, 1159, 1153, 1142, 1106, 1089, 1078, 992, 936, 890, 787, 764, 706, 633. **HRMS** (ESI) calcd. for C₂₉H₃₉O₅NP (M+H⁺): 512.2560, found: 512.2557. **R_f** (6/4/1 hexane/CHCl₃/MeOH) = 0.32.

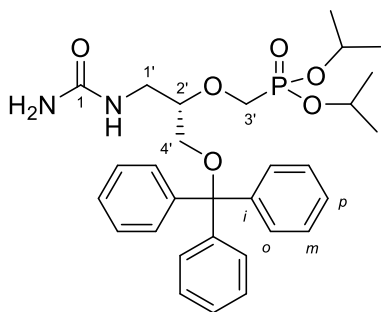
Diisopropyl ((2-ureidoethoxy)methyl)phosphonate (28); procedure according to ref. [148].



Compound **21** (5.12 g, 21.4 mmol) was dissolved in H₂O (50 mL) and the solution was cooled in ice bath to 10 °C upon stirring. HCl (35 %, 1.65 mL) was added dropwise, then potassium cyanate (3.47 g, 42.8 mmol) was added and the reaction mixture was stirred at 70 °C overnight. The mixture was extracted with chloroform (4x 70 mL), organic layer was dried over MgSO₄ and concentrated *in vacuo*. The residue was purified on silica gel column (0-5% MeOH in CHCl₃). Colourless oil (4.331 g, yield 72 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 5.95 (bt, 1H, $J(H,1') = 5.8$ Hz, NH), 5.48 (s, 2H, NH₂), 4.60 (dsept, 2H, $J(H,P) = 7.7$ Hz, $J_{\text{vic}} = 6.2$ Hz, CH-*i*-Pr), 3.72 (d, 2H, $J(3',P) = 8.2$ Hz, H-3'), 3.47 (t, 2H, $J(2',1') = 5.8$ Hz, H-2'), 3.12 (q, 2H, $J(1',2') = J(1',NH) = 5.8$ Hz, H-1'), 1.25 (d, 6H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.24 (d, 6H, $J_{\text{vic}} =$

6.2 Hz, CH₃-*i*-Pr). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 158.86 (C-1), 72.41 (d, *J*(2',P) = 11.2 Hz, CH₂-2'), 70.42 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 64.94 (d, *J*(3',P) = 164.4 Hz, CH₂-3'), 39.05 (CH₂-1'), 24.09 (d, *J*(C,P) = 3.7 Hz, CH₃-*i*-Pr), 23.98 (d, *J*(C,P) = 4.6 Hz, CH₃-*i*-Pr). ³¹P {¹H} NMR (162.0 MHz, DMSO-*d*₆): δ 19.67. FTIR (CCl₄, cm⁻¹) ν_{max}: 3362, 3210, 2982, 2935, 2876, 1690, 1665, 1612, 1555, 1467, 1387, 1376, 1240, 1179, 1142, 1105, 1014, 995, 890. HRMS (ESI) calcd. for C₁₀H₂₃O₅N₂NaP (M+Na⁺): 305.1237, found: 305.1237. R_f (12/5/4/1/1 CHCl₃/*i*-PrOH/MeOH/NH₃/H₂O) = 0.66.

Diisopropyl (S)-(((1-(trityloxy)-3-ureidopropan-2-yl)oxy)methyl)phosphonate (29); procedure according to ref. [149].



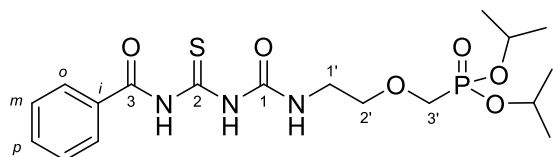
Compound **27** (4.750 g, 9.29 mmol) and 1,1'-carbonyldiimidazole (1.810 g, 11.2 mmol) were dissolved in dichloromethane (200 mL) and the reaction mixture was stirred at r.t. for 3 h. Afterwards, 7N NH₃/MeOH (50 mL) was added and the resulting mixture was stirred at r.t. overnight. The solvent was removed *in vacuo* and the residue was purified on silica gel column (0-5% MeOH in CHCl₃). Colourless oil (3.710 g, yield 72 %). [α]_D -3.7 (*c* 0.216 g/100 mL, MeOH). ¹H

NMR (500 MHz, CDCl₃): δ 7.43 (m, 6H, H-*o*-Tr), 7.30 (m, 6H, H-*m*-Tr), 7.24 (m, 3H, H-*p*-Tr), 6.26 (bs, 1H, NH), 4.77 (dsept, 1H, *J*(H,P) = 7.6 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 4.72 (dsept, 1H, *J*(H,P) = 7.6 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 4.52 (bs, 2H, NH₂), 4.13 (dd, 1H, *J*_{gem} = 14.1 Hz, *J*(3'a,P) = 6.4 Hz, H-3'a), 3.76 (dd, 1H, *J*_{gem} = 14.1 Hz, *J*(3'b,P) = 8.7 Hz, H-3'b), 3.62 (m, 1H, H-2'), 3.50 (ddd, 1H, *J*_{gem} = 13.8 Hz, *J*(1'a,2') = 7.8 Hz, *J*(1'a,NH) = 3.1 Hz, H-1'a), 3.17 (dd, 1H, *J*_{gem} = 10.2 Hz, *J*(4'a,2') = 6.1 Hz, H-4'a), 3.13 (dd, 1H, *J*_{gem} = 10.2 Hz, *J*(4'b,2') = 4.0 Hz, H-4'b), 3.01 (ddd, 1H, *J*_{gem} = 13.8 Hz, *J*(1'b,2') = 9.3 Hz, *J*(1'b,NH) = 3.0 Hz, H-1'b), 1.356 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.349 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.340 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.336 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). ¹³C NMR (125.7 MHz, CDCl₃): δ 159.02 (C-1), 143.65 (C-*i*-Tr), 128.61 (CH-*o*-Tr), 127.85 (CH-*m*-Tr), 127.10 (CH-*p*-Tr), 86.83 (C-Tr), 83.25 (d, *J*(2',P) = Hz, CH-2'), 71.82 (d, *J*(C,P) = 6.6 Hz, CH-*i*-Pr), 71.24 (d, *J*(C,P) = 7.1 Hz, CH-*i*-Pr), 66.99 (d, *J*(3',P) = 168.1 Hz, CH₂-3'), 64.42 (CH₂-4'), 42.16 (CH₂-1'), 24.15 (d, *J*(C,P) = 3.7 Hz, CH₃-*i*-Pr), 24.09 (d, *J*(C,P) = 4.6 Hz, CH₃-*i*-Pr), 24.02 (d, *J*(C,P) = 3.9 Hz, CH₃-*i*-Pr), 23.93 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr). ³¹P {¹H} NMR (202.4 MHz, CDCl₃): δ 21.09. FTIR (CHCl₃, cm⁻¹) ν_{max}: 3518, 3413, 3331, 3222, 3089, 3062, 3035, 2985, 2934, 2875, 1675, 1598, 1554, 1491, 1467, 1449, 1443, 1388, 1377, 1305, 1238, 1179, 1159, 1153, 1142, 1103, 1088, 1079, 1035, 1015, 1000, 890, 707, 701, 642, 633, 617. HRMS (ESI) calcd. for C₃₀H₃₉O₆N₂NaP (M+Na⁺): 577.2438, found: 577.2439; calcd. for C₃₀H₄₀O₆N₂P (M+H⁺): 555.2619, found: 555.2619. R_f (6/4/1 hexane/CHCl₃/MeOH) = 0.40.

The general procedure for synthesis of *N*¹-protected-*N*⁵-PME-substituted thiobiurets (30-32) and *N*¹-protected-*N*⁵-HPMP-substituted thiobiurets (33-35); procedure according to ref. [111, 118]

Compound **28-29** (3.00 mmol) was dissolved in acetone (30 mL) and an appropriate isothiocyanate (3.00 mmol) was added dropwise. The solution was stirred at 60 °C until quantitative conversion was observed (4-24 h). The solvent was removed *in vacuo* and the residue was purified on silica gel column (3/2 hexane/EtOAc to EtOAc).

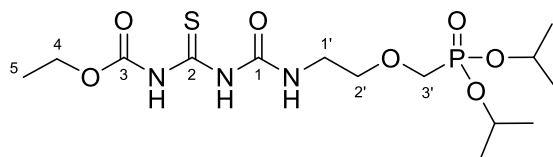
Diisopropyl (1,5-dioxo-1-phenyl-3-thioxo-9-oxa-2,4,6-triazadecan-10-yl)phosphonate (30).



Reactants: compound **28** (0.848 g, 3.00 mmol) and benzoyl isothiocyanate (**2**, 0.490 g, 3.00 mmol); reaction time 4 h. Yellowish oil (0.843 g, yield 63 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.90 (m, 2H, H-*o*-Ph), 7.68 (m,

1H, H-*p*-Ph), 7.58 (m, 2H, H-*m*-Ph), 4.60 (dsept, 2H, *J*(H,P) = 7.7 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 3.78 (d, 2H, *J*(3',P) = 8.5 Hz, H-3'), 3.61 (t, 2H, *J*(2',1') = 5.3 Hz, H-2'), 3.38 (m, 2H, H-1'), 1.24 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.23 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 178.96 (C-2), 168.08 (C-3), 153.60 (C-1, determined by HMBC), 133.56 (CH-*p*-Ph), 131.42 (C-*i*-Ph), 129.16 (CH-*m*-Ph), 128.09 (CH-*o*-Ph), 71.08 (d, *J*(2',P) = 11.4 Hz, CH₂-2'), 70.38 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 64.97 (d, *J*(3',P) = 164.5 Hz, CH₂-3'), 39.20 (CH₂-1'), 24.03 (d, *J*(C,P) = 3.8 Hz, CH₃-*i*-Pr), 23.92 (d, *J*(C,P) = 4.6 Hz, CH₃-*i*-Pr). ³¹P {¹H} NMR (162.0 MHz, DMSO-*d*₆): δ 19.34. FTIR (CCl₄, cm⁻¹) ν_{max}: 3497, 3423, 3330, 3237, 3068, 3035, 2981, 2937, 2875, 1723, 1689, 1675, 1603, 1582, 1540, 1502, 1491, 1467, 1451, 1386, 1375, 1316, 1238, 1179, 1143, 1106, 1070, 1013, 994, 890, 704, 686, 644. HRMS (ESI) calcd. for C₁₈H₂₈O₆N₃NaPS (M+Na⁺): 468.1329, found: 468.1328; calcd. for C₁₈H₂₉O₆N₃PS (M+H⁺): 446.1509, found: 446.1509. R_f (6/4/1 hexane/CHCl₃/MeOH) = 0.32.

Diisopropyl (6,10-dioxo-8-thioxo-2,11-dioxa-5,7,9-triazatridecyl)phosphonate (31).

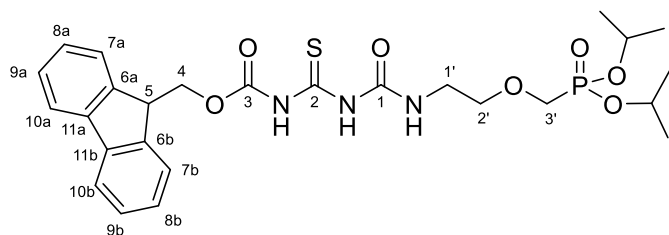


Reactants: compound **28** (0.848 g, 3.00 mmol) and ethoxycarbonyl isothiocyanate (0.394 g, 3.00 mmol); reaction time 4 h. Yellowish oil (0.782 g, yield 63 %).

¹H NMR (500 MHz, DMSO-*d*₆): δ 12.21 (bs, 1H, NH), 11.05 (bs, 1H, NH), 8.34 (bs, 1H, NH), 4.55-4.65 (m, 2H, CH-*i*-Pr), 4.18 (q, 2H, *J*(4,5) = 7.1 Hz, H-4), 3.76 (d, 2H, *J*(3',P) = 8.3 Hz, H-3'), 3.59 (bt, 2H, *J*(2',1') = 5.4 Hz, H-2'), 3.35 (bq, 2H, *J*(1',2') = 5.4 Hz, H-1'), 1.24 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.23 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.229 (t, 3H, *J*(5,4) = 7.1 Hz, H-5). ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 178.93 (C-2), 153.13 (C-1), 152.00 (C-3), 71.04 (d, *J*(2',P) = 12.1 Hz, CH₂-2'), 70.36 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 64.97 (d, *J*(3',P) = 164.4 Hz, CH₂-3'),

62.39 (CH₂-4), 39.22 (CH₂-1'), 24.00 (d, $J(\text{C},\text{P}) = 3.7$ Hz, CH₃-*i*-Pr), 23.90 (d, $J(\text{C},\text{P}) = 4.5$ Hz, CH₃-*i*-Pr), 14.18 (CH₃-5). **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 20.34. **FTIR** (CCl₄, cm⁻¹) ν_{max} : 3252, 3186, 2980, 2935, 2875, 1705, 1537, 1508, 1468, 1454, 1386, 1374, 1337, 1261, 1227, 1177, 1142, 1104, 1012, 993, 890, 794. **HRMS** (ESI) calcd. for C₁₄H₂₈O₇N₃NaPS (M+Na⁺): 436.1278, found: 436.1281; calcd. for C₁₄H₂₉O₇N₃PS (M+H⁺): 414.1458, found: 414.1451. **R_f** (6/4/1 hexane/CHCl₃/MeOH) = 0.41.

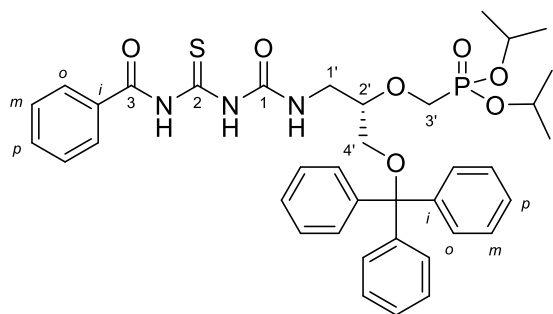
Diisopropyl (1-(9H-fluoren-9-yl)-3,7-dioxo-5-thioxo-2,11-dioxo-4,6,8-triazadodecan-12-yl)-phosphonate (32).



Reactants: compound **28** (0.848 g, 3.00 mmol) and 9-fluorenylmethoxycarbonyl isothiocyanate (1.013 g, 3.60 mmol); reaction time 6 h. White amorphous solid (0.912 g, yield 54 %). **¹H NMR** (500 MHz, DMSO-*d*₆):

δ 12.37 (bs, 1H, NH), 11.13 (bs, 1H, NH), 8.52 (bs, 1H, NH), 7.90 (dt, 2H, $J(10a,9a\&10b,9b) = 7.5$ Hz, $J(10a,8a\&10b,8b) = J(10a,7a\&10b,7b) = 1.1$ Hz, H-10a,b), 7.76 (bd, 2H, $J(7a,8a\&7b,8b) = 7.5$ Hz, H-7a,b), 7.43 (tdd, 2H, $J(9a,10a\&9b,10b) = J(9a,8a\&9b,8b) = 7.5$ Hz, $J(9a,7a\&9b,7b) = 1.2$ Hz, $J(9a,5\&9b,5) = 0.7$ Hz, H-9a,b), 7.35 (td, 2H, $J(8a,9a\&8b,9b) = J(8a,7a\&8b,7b) = 7.5$ Hz, $J(8a,10a\&8b,10b) = 1.1$ Hz, H-8a,b), 4.60 (dsep, 2H, $J(\text{H},\text{P}) = 7.7$ Hz, $J_{\text{vic}} = 6.2$ Hz, CH-*i*-Pr), 4.46 (d, 2H, $J(4,5) = 7.1$ Hz, H-4), 4.31 (bt, 1H, $J(5,4) = 7.1$ Hz, H-5), 3.77 (d, 2H, $J(3',\text{P}) = 8.2$ Hz, H-3'), 3.61 (t, 2H, $J(2',1') = 5.3$ Hz, H-2'), 3.37 (q, 2H, $J(1',2') = 5.3$ Hz, H-1'), 1.25 (d, 6H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr) 1.24 (d, 6H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 178.94 (C-2), 152.93 (C-1), 152.17 (C-3), 143.43 (C-6a,b), 140.94 (C-11a,b), 128.04 (CH-9a,b), 127.37 (CH-8a,b), 125.50 (CH-7a,b), 120.40 (CH-10a,b), 71.03 (d, $J(2',\text{P}) = 11.5$ Hz, CH₂-2'), 70.37 (d, $J(\text{C},\text{P}) = 6.4$ Hz, CH-*i*-Pr), 67.66 (CH₂-4), 64.98 (d, $J(3',\text{P}) = 164.3$ Hz, CH₂-3'), 46.22 (CH-5), 39.30 (CH₂-1'), 24.02 (d, $J(\text{C},\text{P}) = 3.7$ Hz, CH₃-*i*-Pr), 23.92 (d, $J(\text{C},\text{P}) = 4.5$ Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 20.32. **FTIR** (KBr, cm⁻¹) ν_{max} : 3322, 3243, 3186, 3066, 3040, 2979, 1721, 1705, 1609, 1538, 1509, 1466, 1451, 1386, 1360, 1338, 1323, 1224, 1209, 1172, 1142, 1105, 1084, 994, 840, 760, 741, 726, 621. **HRMS** (ESI) calcd. for C₂₆H₃₄O₇N₃NaPS (M+Na⁺): 586.1747, found: 586.1748. **R_f** (EtOAc) = 0.50.

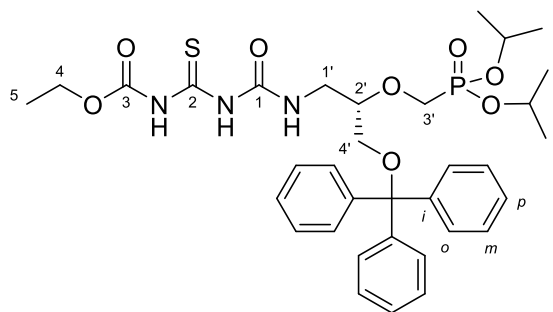
Diisopropyl (S)-(1,5-dioxo-1-phenyl-3-thioxo-8-((trityloxy)methyl)-9-oxa-2,4,6-triazadecan-10-yl)phosphonate (33).



Reactants: compound **29** (1.664 g, 3.00 mmol) and benzoyl isothiocyanate (0.490 g, 3.00 mmol); reaction time 24 h. Yellow amorphous solid (0.646 g, yield 30 %). $[\alpha]_D -3.4$ (*c* 0.205 g/100 mL, MeOH). $^1\text{H NMR}$ (500 MHz, DMSO-*d*₆): δ 7.86-7.89 (m, 2H, H-*o*-Ph), 7.67 (m, 1H, H-*p*-Ph), 7.54-7.58 (m, 2H, H-*m*-Ph), 7.38-7.41 (m, 6H, H-*o*-Tr), 7.30-7.35 (m, 6H, H-*m*-Tr), 7.25 (m, 3H,

H-*p*-Tr), 4.53-4.63 (m, 2H, CH-*i*-Pr), 3.84 (dd, 1H, $J_{\text{gem}} = 13.7$ Hz, $J(3'a,P) = 8.8$ Hz, H-3'a), 3.79 (dd, 1H, $J_{\text{gem}} = 13.7$ Hz, $J(3'b,P) = 9.3$ Hz, H-3'b), 3.75 (p, 1H, $J(2',1') = J(2',3') = 5.2$ Hz, H-2'), 3.42-3.47 (bm, 2H, H-1'), 3.09 (dd, 1H, $J_{\text{gem}} = 10.1$ Hz, $J(4'a,2') = 4.9$ Hz, H-4'a), 3.06 (dd, 1H, $J_{\text{gem}} = 10.1$ Hz, $J(4'b,2') = 5.3$ Hz, H-4'b), 1.22 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.21 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.20 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.19 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr). $^{13}\text{C NMR}$ (125.7 MHz, DMSO-*d*₆): δ 168.05 (C-3), 143.72 (C-*i*-Tr), 133.47 (CH-*p*-Ph), 133.10 (C-*i*-Ph), 129.09 (CH-*m*-Ph), 128.44 (CH-*o*-Tr), 128.07 (CH-*o*-Ph, CH-*m*-Tr), 127.26 (CH-*p*-Tr), 86.33 (C-Tr), 78.91 (d, $J(2',P) = 12.0$ Hz, CH-2'), 70.39 (d, $J(C,P) = 6.3$ Hz, CH-*i*-Pr), 70.38 (d, $J(C,P) = 6.3$ Hz, CH-*i*-Pr), 64.02 (d, $J(3',P) = 165.2$ Hz, CH₂-3'), 62.89 (CH₂-4'), 39.87 (CH₂-1'), 24.00 (d, $J(C,P) = 3.7$ Hz, CH₃-*i*-Pr), 23.90 (d, $J(C,P) = 4.4$ Hz, CH₃-*i*-Pr), 23.88 (d, $J(C,P) = 4.4$ Hz, CH₃-*i*-Pr); C-1 and C-2 not detected. $^{31}\text{P} \{^1\text{H}\}$ NMR (202.4 MHz, DMSO-*d*₆): δ 20.11. **FTIR** (CCl₄, cm⁻¹) ν_{max} : 3423, 3225, 3063, 3035, 2981, 2934, 1722, 1687, 1678, 1603, 1578, 1541, 1491, 1468, 1449, 1386, 1375, 1258, 1238, 1179, 1159, 1143, 1105, 1087, 1079, 1026, 1009, 993, 881, 706, 696, 648, 633. **HRMS** (ESI) calcd. for C₃₈H₄₄O₇N₃NaPS (M+Na⁺): 740.2530, found: 740.2524. **R_f** (6/4/1 hexane/CHCl₃/MeOH) = 0.45.

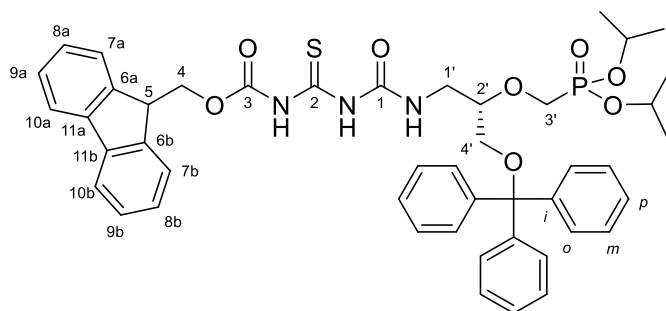
Diisopropyl (S)-(6,10-dioxo-8-thioxo-3-(trityloxymethyl)-2,11-dioxo-5,7,9-triazatridecyl)-phosphonate (34).



Reactants: compound **29** (1.664 g, 3.00 mmol) and ethoxycarbonyl isothiocyanate (0.592 g, 4.50 mmol); reaction time 4 h. Yellowish crystalline solid (1.275 g, yield 62 %). **Mp** = 61-62 °C. $[\alpha]_D -0.7$ (*c* 0.430 g/100 mL, MeOH). $^1\text{H NMR}$ (500 MHz, DMSO-*d*₆): δ 12.18 (bs, 1H, NH), 11.04 (bs, 1H, NH), 8.24 (bs, 1H, NH), 7.37-7.41 (m, 6H, H-*o*-Tr), 7.30-7.35 (m, 6H, H-*m*-Tr), 7.26 (m, 3H, H-*p*-Tr), 4.52-4.63 (m, 2H, CH-*i*-Pr), 4.18 (q, 2H, $J(4,5) = 7.1$ Hz, H-4), 3.83 (dd, 1H, $J_{\text{gem}} =$

13.7 Hz, $J(3'a,P) = 8.8$ Hz, H-3'a), 3.78 (dd, 1H, $J_{\text{gem}} = 13.7$ Hz, $J(3'b,P) = 9.3$ Hz, H-3'b), 3.72 (p, 1H, $J(2',1') = J(2',4') = 5.0$, H-2'), 3.35-3.48 (bm, 2H, H-1'), 3.08 (dd, 1H, $J_{\text{gem}} = 10.4$ Hz, $J(4'a,2') = 4.8$ Hz, H-4'a), 3.05 (dd, 1H, $J_{\text{gem}} = 10.4$ Hz, $J(4'b,2') = 5.3$ Hz, H-4'b), 1.16-1.26 (m, 15H, CH₃-*i*-Pr, H-5). ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 178.86 (C-2), 153.13 (C-1), 152.03 (C-3), 143.72 (C-*i*-Tr), 128.43 (CH-*o*-Tr), 128.08 (CH-*m*-Tr), 127.26 (CH-*p*-Tr), 86.33 (C-Tr), 78.84 (d, $J(2',P) = 12.2$ Hz, CH-2'), 70.38 (d, $J(C,P) = 6.3$ Hz, CH-*i*-Pr), 64.01 (d, $J(3',P) = 165.1$ Hz, CH₂-3'), 62.90 (CH₂-4'), 62.40 (CH₂-4), 39.70 (CH₂-1'), 24.00 (d, $J(C,P) = 3.7$ Hz, CH₃-*i*-Pr), 23.90 (d, $J(C,P) = 4.4$ Hz, CH₃-*i*-Pr), 23.87 (d, $J(C,P) = 4.4$ Hz, CH₃-*i*-Pr), 14.19 (CH₃-5). ³¹P {¹H} NMR (202.4 MHz, DMSO-*d*₆): δ 18.91. FTIR (CCl₄, cm⁻¹) ν_{max} : 3413, 3255, 3201, 3088, 3062, 3035, 3024, 2981, 2934, 2874, 1777, 1742, 1717, 1707, 1597, 1535, 1503, 1478, 1467, 1449, 1442, 1386, 1374, 1304, 1223, 1174, 1170, 1142, 1121, 1105, 1096, 1077, 1035, 1011, 993, 936, 890, 706, 696, 642, 633. HRMS (ESI) calcd. for C₃₄H₄₄O₈N₃NaPS (M+Na⁺): 708.2479, found: 708.2477. **R_f** (6/4/1 hexane/CHCl₃/MeOH) = 0.49.

Diisopropyl (S)-(1-(9H-fluoren-9-yl)-3,7-dioxo-5-thioxo-10-(trityloxymethyl)-2,11-dioxa-4,6,8-triazadodecan-12-yl)phosphonate (35).



Reactants: compound **29** (1.664 g, 3.00 mmol) and 9-fluorenylmethoxycarbonyl isothiocyanate (1.014 g, 3.60 mmol); reaction time 18 h. White crystalline solid (1.278 g, yield 51 %). **Mp** = 89-90 °C. [α]_D -2.8 (*c* 0.109 g/100 mL, MeOH). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.36 (bs, 1H, NH), 11.11

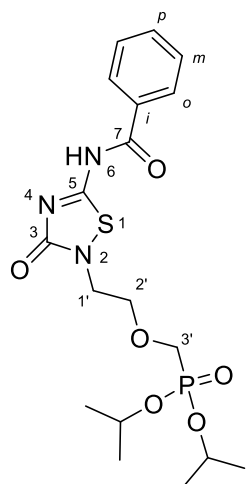
(bs, 1H, NH), 8.48 (bs, 1H, NH), 7.90 (dt, 2H, $J(10a,9a\&10b,9b) = 7.5$ Hz, $J(10a,8a\&10b,8b) = J(10a,7a\&10b,7b) = 0.9$ Hz, H-10a,b), 7.76 (bd, 2H, $J(7a,8a\&7b,8b) = 7.5$ Hz, H-7a,b), 7.43 (bt, 2H, $J(9a,10a\&9b,10b) = J(9a,8a\&9b,8b) = 7.5$ Hz, H-9a,b), 7.39 (m, 6H, H-*o*-Tr), 7.30-7.35 (m, 8H, H-8a,b, H-*m*-Tr), 7.25 (m, 3H, H-*p*-Tr), 4.53-4.63 (m, 2H, CH-*i*-Pr), 4.47 (d, 2H, $J(4,5) = 7.1$ Hz, H-4), 4.31 (t, 1H, $J(5,4) = 7.1$ Hz, H-5), 3.83 (dd, 1H, $J_{\text{gem}} = 13.7$ Hz, $J(3'a,P) = 8.8$ Hz, H-3'a), 3.79 (dd, 1H, $J_{\text{gem}} = 13.7$ Hz, $J(3'b,P) = 9.3$ Hz, H-3'b), 3.74 (p, 1H, $J(2',1') = J(2',3') = 5.2$ Hz, H-2'), 3.39-3.51 (bm, 2H, H-1'), 3.08 (dd, 1H, $J_{\text{gem}} = 10.8$ Hz, $J(4'a,2') = 5.2$ Hz, H-4'a), 3.06 (dd, 1H, $J_{\text{gem}} = 10.8$ Hz, $J(4'b,2') = 5.2$ Hz, H-4'b), 1.22 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.21 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.19 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr), 1.18 (d, 3H, $J_{\text{vic}} = 6.2$ Hz, CH₃-*i*-Pr). ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 178.90 (C-2), 152.94 (C-1), 152.18 (C-3), 143.74 (C-*i*-Tr), 143.43 (C-6a,b), 140.96 (C-11a,b), 128.46 (CH-*o*-Tr), 128.12 (CH-*m*-Tr), 128.08 (CH-9a,b), 127.39 (CH-8a,b), 127.29 (CH-*p*-Tr), 125.54 (CH-7a,b), 120.45 (CH-10a,b), 86.34 (C-Tr), 78.80 (d, $J(2',P) = 12.2$ Hz, CH-2'), 70.42 (d, $J(C,P) = 6.3$ Hz, CH-*i*-Pr), 67.70

(CH₂-4), 64.00 (d, $J(3',P) = 165.1$ Hz, CH₂-3'), 62.82 (CH₂-4'), 46.23 (CH-5), 39.70 (CH₂-1'), 24.05 (d, $J(C,P) = 3.7$ Hz, CH₃-*i*-Pr), 23.95 (d, $J(C,P) = 4.2$ Hz, CH₃-*i*-Pr), 23.92 (d, $J(C,P) = 4.2$ Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 20.11. **FTIR** (CHCl₃, cm⁻¹) $\nu_{\max}$: 3395, 3263, 3208, 3088, 3066, 2985, 1768, 1742, 1712, 1607, 1598, 1538, 1505, 1478, 1450, 1387, 1377, 1305, 1249, 1172, 1104, 1056, 999, 890, 707, 642, 545, 427. **HRMS** (ESI) calcd. for C₄₆H₅₀O₈N₃NaPS (M+Na⁺): 858.2948, found: 858.2949; calcd. for C₄₆H₅₁O₈N₃PS (M+H⁺): 836.3129, found: 836.3134. **R_f** (1/1 hexane/EtOAc) = 0.30.

The general procedure for synthesis of *N*⁵-protected-*N*²-PME-substituted thiadiazolones 36-37 and *N*⁵-protected-*N*²-HPMP-substituted thiadiazolones 39-41; procedure according to ref. [144]

Compound **36-37**, **39-41** (1.00 mmol) and NBS (0.223 g, 1.25 mmol) were dissolved in methanol (20 mL) and stirred at 80 °C for 30 min. After cooling to r.t., the solvent was removed *in vacuo* and the residue was purified on silica gel column (0-3% MeOH/CHCl₃).

Diisopropyl ((2-(5-benzamido-3-oxo-1,2,4-thiadiazol-2(3*H*)-yl)ethoxy)methyl)phosphonate (36).

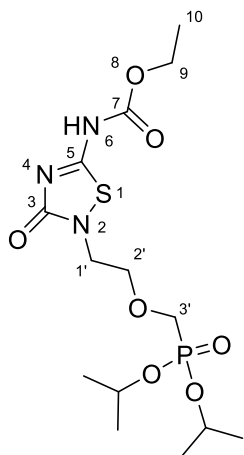


Reactants: compound **30** (0.445 g, 1.00 mmol) and NBS (0.223 g, 1.25 mmol).

White crystalline solid (0.213 g, yield 48 %). **Mp** = 83-84 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 13.42 (vbs, 1H, NH), 8.12 (m, 2H, H-*o*-Ph), 7.66 (m, 1H, H-*p*-Ph), 7.56 (m, 2H, H-*m*-Ph), 4.59 (dsept, 2H, $J(H,P) = 7.7$ Hz, $J_{\text{vic}} = 6.2$ Hz, CH-*i*-Pr), 3.80 (d, 2H, $J(3',P) = 8.2$ Hz, H-3'), 3.71-3.79 (m, 4H, H-1', H-2'), 1.23 (bt, 12H, $J_{\text{vic}} = 5.9$ Hz, CH₃-*i*-Pr). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 174.52 (C-7, determined by HMBC), 170.86 (C-5, determined by HMBC), 161.53 (C-3), 133.57 (CH-*p*-Ph), 130.56 (C-*i*-Ph), 129.04 (CH-*m*-Ph), 128.94 (CH-*o*-Ph), 70.74 (d, $J(2',P) = 11.5$ Hz, CH₂-2'), 70.40 (d, $J(C,P) = 6.4$ Hz, CH-*i*-Pr), 64.98 (d, $J(3',P) = 163.9$ Hz, CH₂-3'), 42.47 (CH₂-1'), 23.98 (d, $J(C,P) = 3.8$ Hz, CH₃-*i*-Pr), 23.88 (d, $J(C,P) = 4.5$ Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (162.0 MHz, DMSO-*d*₆): δ 19.01. **FTIR** (CCl₄, cm⁻¹) $\nu_{\max}$:

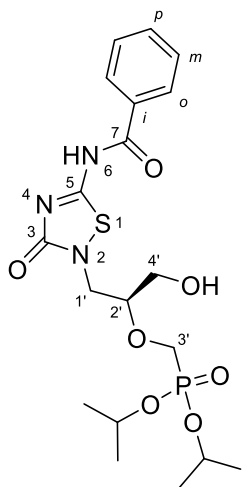
3503, 3430, 3123, 3068, 3032, 2981, 2930, 2877, 1714, 1706, 1684, 1676, 1653, 1601, 1570, 1559, 1488, 1468, 1449, 1387, 1375, 1336, 1302, 1231, 1177, 1143, 1106, 1069, 1017, 999, 890, 687, 639, 529. **HRMS** (ESI) calcd. for C₁₈H₂₆O₆N₃NaPS (M+Na⁺): 466.1172, found: 466.1171; calcd. for C₁₈H₂₇O₆N₃PS (M+H⁺): 444.1353, found: 444.1352. **R_f** (9/1 CHCl₃/MeOH) = 0.47.

Ethyl (2-(2-((diisopropoxyphosphoryl)methoxy)ethyl)-3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)carbamate (37).



Reactants: compound **31** (0.413 g, 1.00 mmol) and NBS (0.223 g, 1.25 mmol). White crystalline solid (0.078 g, yield 19 %). **Mp** = 84-86 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 12.79 (bs, 1H, NH), 4.59 (dsept, 2H, *J*(H,P) = 7.7 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 4.23 (q, 2H, *J*(9,10) = 7.1 Hz, CH₂-9), 3.77 (d, 2H, *J*(3',P) = 8.2 Hz, H-3'), 3.76 (m, 2H, H-1'), 3.69 (m, 2H, H-2'), 1.25 (t, 3H, *J*(10,9) = 7.1 Hz, H-10), 1.23 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.21 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 170.54 (C-7), 162.95 (C-3), 157.49 (C-5), 71.94 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 71.76 (d, *J*(2',P) = 11.2 Hz, CH₂-2'), 65.66 (d, *J*(3',P) = 164.7 Hz, CH₂-3'), 64.53 (CH₂-9), 43.70 (CH₂-1'), 24.85 (d, *J*(C,P) = 3.8 Hz, CH₃-*i*-Pr), 24.75 (d, *J*(C,P) = 4.6 Hz, CH₃-*i*-Pr), 15.23 (CH₃-10). **³¹P {¹H} NMR** (162.0 MHz, DMSO-*d*₆): δ 19.02. **FTIR** (CCl₄, cm⁻¹) *v*_{max}: 3426, 3145, 3076, 2982, 2933, 2876, 1716, 1689, 1635, 1576, 1467, 1386, 1375, 1320, 1306, 1252, 1233, 1178, 1144, 1106, 1014, 996, 890. **HRMS** (ESI) calcd. for C₁₄H₂₆O₇N₃NaPS (M+Na⁺): 434.1121, found: 434.1121; calcd. for C₁₄H₂₇O₇N₃PS (M+H⁺): 412.1302, found: 412.1302.

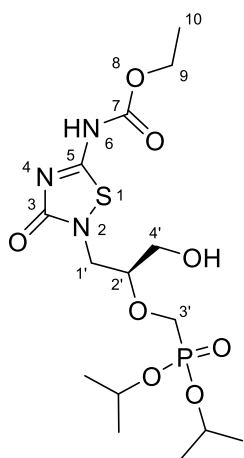
Diisopropyl (S)-(((1-(5-benzamido-3-oxo-1,2,4-thiadiazol-2(3H)-yl)-3-hydroxypropan-2-yl)oxy)-methyl)phosphonate (39).



Reactants: compound **33** (0.718 g, 1.00 mmol) and NBS (0.223 g, 1.25 mmol). White amorphous solid (0.420 g, yield 89 %). **[α]_D** -4.2 (*c* 0.169 g/100 mL, MeOH). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 13.40 (bs, 1H, NH), 8.10-8.14 (m, 2H, H-*o*-Ph), 7.66 (m, 1H, H-*p*-Ph), 7.54-7.58 (m, 2H, H-*m*-Ph), 4.86 (t, 1H, *J*(OH,4') = 5.3 Hz, OH), 4.54-4.65 (m, 2H, CH-*i*-Pr), 3.92 (dd, 1H, *J*_{gem} = 14.0 Hz, *J*(3'a,P) = 8.7 Hz, H-3'a), 3.84 (dd, 1H, *J*_{gem} = 14.0 Hz, *J*(3'b,P) = 8.7 Hz, H-3'b), 3.81 (dd, 1H, *J*_{gem} = 14.5 Hz, *J*(1'a,2') = 3.1 Hz, H-1'a), 3.67 (m, 1H, H-2'), 3.61 (dd, 1H, *J*_{gem} = 14.5 Hz, *J*(1'b,2') = 7.1 Hz, H-1'b), 3.45-3.54 (m, 2H, H-3'), 1.23 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.22 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.21 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.20 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 133.46 (CH-*p*-Ph), 129.01 (CH-*m*-Ph), 128.91 (CH-*o*-Ph), 80.53 (d, *J*(2',P) = 10.7 Hz, CH-2'), 70.41 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 70.34 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 63.86 (d, *J*(3',P) = 164.7 Hz, CH₂-3'), 60.22 (CH₂-4'), 43.75 (CH₂-1'), 23.97 (d, *J*(C,P) = 3.5 Hz, CH₃-*i*-Pr), 23.94 (d, *J*(C,P) = 3.5 Hz, CH₃-*i*-Pr), 23.88 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr), 23.85 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr); C-3, C-5, C-7, and C-*i*-Ph not detected. **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 20.38. **FTIR** (KBr, cm⁻¹) *v*_{max}: 3424,

3253, 2981, 1703, 1648, 1600, 1579, 1569, 1555, 1487, 1465, 1449, 1387, 1376, 1257, 1240, 1143, 1104, 1081, 1012, 1001, 994, 893, 690. **HRMS** (ESI) calcd. for $C_{19}H_{28}O_7N_3NaPS$ ($M+Na^+$): 496.1278, found: 496.1275; calcd. for $C_{19}H_{29}O_7N_3PS$ ($M+H^+$): 474.1458, found: 474.1458. **R_f** (9/1 $CHCl_3/MeOH$) = 0.43.

Ethyl (S)-(2-(2-((diisopropoxyphosphoryl)methoxy)-3-hydroxypropyl)-3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)carbamate (40).



Reactants: compound **34** (0.686 g, 1.00 mmol) and NBS (0.223 g, 1.25 mmol).

White amorphous solid (0.331 g, yield 75 %). **[α]_D** -5.6 (*c* 0.197 g/100 mL,

MeOH). **¹H NMR** (500 MHz, MeOD-*d*₄): δ 4.69-4.78 (m, 2H, CH-*i*-Pr), 4.33 (q,

2H, *J*(9,10) = 7.1 Hz, H-9), 4.02 (dd, 1H, *J*_{gem} = 13.9 Hz, *J*(3'a,P) = 9.0 Hz, H-3'a),

3.96 (dd, 1H, *J*_{gem} = 14.9 Hz, *J*(1'a,2') = 3.4 Hz, H-1'a), 3.93 (dd, 1H, *J*_{gem} = 13.9

Hz, *J*(3'b,P) = 9.3 Hz, H-3'b), 3.82 (dd, 1H, *J*_{gem} = 14.9 Hz, *J*(1'b,2') = 6.8 Hz, H-

1'b), 3.73 (m, 1H, H-2'), 3.68 (dd, 1H, *J*_{gem} = 12.1 Hz, *J*(4'a,2') = 4.7 Hz, H-4'a),

3.62 (dd, 1H, *J*_{gem} = 12.1 Hz, *J*(4'b,2') = 4.9 Hz, H-4'b), 1.31-1.37 (m, 15H, CH₃-*i*-

Pr, H-10). **¹³C NMR** (125.7 MHz, MeOD-*d*₄): δ 172.10 (C-5), 166.20 (C-3),

157.19 (C-7), 82.50 (d, *J*(2',P) = 11.6 Hz, CH-2'), 73.34 (d, *J*(C,P) = 6.8 Hz, CH-*i*-

Pr), 73.25 (d, *J*(C,P) = 6.8 Hz, CH-*i*-Pr), 65.28 (d, *J*(3',P) = 168.6 Hz, CH₂-3'), 64.84 (CH₂-9), 61.82

(CH₂-4'), 45.40 (CH₂-1'), 24.33 (d, *J*(C,P) = 3.6 Hz, CH₃-*i*-Pr), 24.32 (d, *J*(C,P) = 3.6 Hz, CH₃-*i*-Pr),

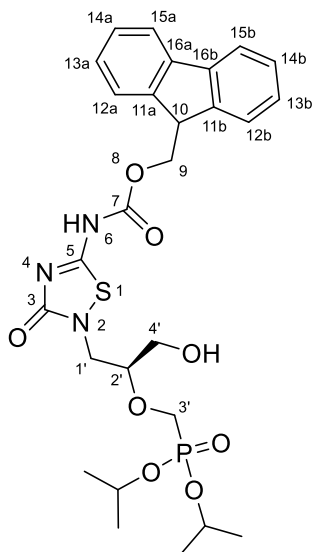
24.29 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr), 24.28 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr), 14.55 (CH₃-10). **³¹P {¹H}**

NMR (202.4 MHz, MeOD-*d*₄): δ 19.76. **FTIR** (CCl₄, cm⁻¹) ν_{max}: 3385, 2981, 2934, 2875, 2737, 1715,

1670, 1635, 1570, 1467, 1387, 1375, 1245, 1178, 1143, 1105, 1062, 1012, 996, 890, 690. **HRMS** (ESI)

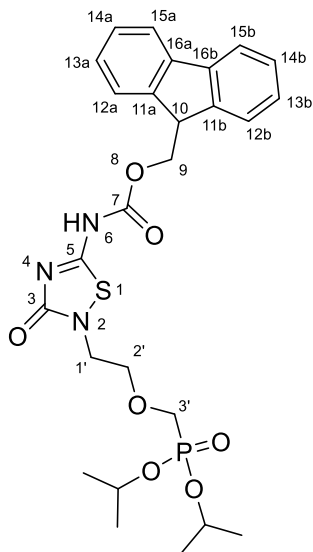
calcd. for $C_{15}H_{28}O_8N_3NaPS$ ($M+Na^+$): 464.1227, found: 464.1226. **R_f** (9/1 $CHCl_3/MeOH$) = 0.48.

(9*H*-Fluoren-9-yl)methyl (S)-2-(2-((diisopropoxyphosphoryl)methoxy)-3-hydroxypropyl)-3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)carbamate (41).



Reactants: compound **35** (0.835 g, 1.25 mmol) and NBS (0.223 g, 1.25 mmol). White crystalline solid (0.424 g, yield 72 %). **Mp** = 68-70 °C. $[\alpha]_D^{25}$ -2.8 (*c* 0.212 g/100 mL, MeOH). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 7.91 (ddd, 2H, *J*(15a,14a&15b,14b) = 7.5 Hz, *J*(15a,13a&15b,13b) = 1.2 Hz, *J*(15a,12a&15b,12b) = 0.7 Hz, H-15a,b), 7.71 (bd, 2H, *J*(12a,13a&12b,13b) = 7.5 Hz, H-12a,b), 7.43 (tdd, 2H, *J*(14a,13a&14b,13b) = *J*(14a,15a&14b,15b) = 7.5 Hz, *J*(14a,12a&14b,12b) = 1.2 Hz, *J*(14a,11a&14b,11b) = 0.7 Hz, H-14a,b), 7.34 (td, 2H, *J*(13a,12a&13b,12b) = *J*(13a,14a&13b,14b) = 7.5 Hz, *J*(13a,12a&13b,12b) = 1.2 Hz, H-13a,b), 4.84 (t, 1H, *J*(OH,4') = 5.4 Hz, OH), 4.52-4.63 (m, 4H, CH₂-9, CH-*i*-Pr), 4.34 (t, 1H, *J*(10,9) = 6.8 Hz, H-10), 3.89 (dd, 1H, *J*_{gem} = 14.0 Hz, *J*(3'a,P) = 8.8 Hz, H-3'a), 3.78-3.85 (m, 2H, H-1'a, H-3'b), 3.58-3.65 (m, 2H, H-2', H-1'b), 3.40-3.52 (m, 2H, H-4'), 1.219 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.214 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.208 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.199 (d, 3H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 143.41 (C-11a,b), 140.99 (C-16a,b), 128.03 (CH-14a,b), 127.37 (CH-13a,b), 125.34 (CH-12a,b), 120.44 (CH-15a,b), 80.64 (d, *J*(2',P) = 10.5 Hz, CH₂-2'), 70.41 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 70.34, (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 68.09 (CH₂-9), 63.85 (d, *J*(3',P) = 164.8 Hz, CH₂-3'), 60.16 (CH₂-4'), 46.36 (CH-10), 43.99 (CH₂-1'), 23.97 (d, *J*(C,P) = 3.5 Hz, CH₃-*i*-Pr), 23.94 (d, *J*(C,P) = 3.5 Hz, CH₃-*i*-Pr), 23.87 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr), 23.85 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr); C-3, C-5, and C-7 not detected. **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 20.34. **FTIR** (CCl₄, cm⁻¹) *v*_{max}: 3371, 3068, 3042, 3018, 2981, 2931, 2874, 2856, 2742, 1717, 1668, 1571, 1478, 1466, 1452, 1387, 1375, 1341, 1294, 1243, 1179, 1143, 1105, 1015, 997, 891, 729, 689, 621, 428. **HRMS** (ESI) calcd. for C₂₇H₃₃O₈N₃PS (M-H⁺): 590.1732, found: 590.1729. **R_f** (9/1 CHCl₃/MeOH) = 0.56.

(9H-Fluoren-9-yl)methyl (2-(2-((diisopropoxyphosphoryl)methoxy)ethyl)-3-oxo-2,3-dihydro-1,2,4-thiadiazol-5-yl)carbamate (38); procedure according to ref. [144].



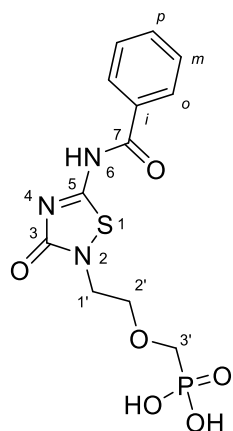
Compound **32** (2.540 g, 4.51 mmol) was dissolved in ethyl acetate (150 mL) and 0.5M Br₂ solution in ethyl acetate (19.0 mL, 9.5 mmol, 2.1 eq.) was added at 0 °C. The mixture was stirred overnight at 4 °C for 17 h. The solvent was removed *in vacuo* and the residue was purified on silica gel column (0-5% MeOH/CHCl₃). Colourless oil (2.030 g, yield 80 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.90 (bd, 2H, *J*(15a,14a&15b,14b) = 7.5 Hz, H-15a,b), 7.71 (bd, 2H, *J*(12a,13a&12b,13b) = 7.5 Hz, H-12a,b), 7.43 (bt, 2H, *J*(14a,13a&14b,13b) = *J*(14a,15a&14b,15b) = 7.5 Hz, H-14a,b), 7.34 (td, 2H, *J*(13a,12a&13b,12b) = *J*(13a,14a&13b,14b) = 7.5 Hz, H-13a,b), 4.57 (dsep, 2H, *J*(H,P) = 7.7 Hz, *J*_{vic} = 6.2 Hz, CH-*i*-Pr), 4.56 (d, 2H, *J*(9,10) = 6.8 Hz, H-9), 4.33 (bt, 1H, *J*(10,9) = 6.8 Hz, H-10), 3.77 (d, 2H, *J*(3',P) = 8.1 Hz, H-3'), 3.76 (bt, 2H, *J*(1',2') = 4.8 Hz, H-1'), 3.69 (bt, 2H, *J*(2',1') = 4.8

Hz, H-2'), 1.21 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr), 1.20 (d, 6H, *J*_{vic} = 6.2 Hz, CH₃-*i*-Pr). ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 169.90 (C-5), 160.40 (C-3), 157.55 (C-7), 143.39 (C-11a,b), 140.99 (C-16a,b), 128.03 (CH-14a,b), 127.37 (CH-13a,b), 125.34 (CH-12a,b), 120.44 (CH-15a,b), 70.84 (d, *J*(2',P) = 11.2 Hz, CH₂-2'), 70.39 (d, *J*(C,P) = 6.4 Hz, CH-*i*-Pr), 68.12 (CH-10), 64.94 (d, *J*(3',P) = 164.0 Hz, CH₂-3'), 46.35 (CH-10), 42.70 (CH₂-1'), 23.97 (d, *J*(C,P) = 3.7 Hz, CH₃-*i*-Pr), 23.86 (d, *J*(C,P) = 4.5 Hz, CH₃-*i*-Pr). ³¹P NMR {¹H} (202.4 MHz, DMSO-*d*₆): δ 20.04. FTIR (KBr, cm⁻¹) ν_{max}: 3435, 3250, 2980, 2927, 1716, 1636, 1568, 1478, 1465, 1451, 1232, 1254, 1180, 1145, 1105, 1003, 761, 741, 621. HRMS (ESI) calcd. for C₂₆H₃₂O₇N₃NaPS (M+Na⁺): 584.1591, found: 584.1592. *R*_f (19/1 CHCl₃/MeOH) = 0.20.

The general procedure for synthesis of *N*⁵-protected-*N*²-PME-substituted thiadiazolones (free phosphonic acids) 42-44; procedure according to ref. [143]

Compound **36-38** (0.50 mmol) was dissolved in acetonitrile (15 mL). TMS-Br (1.33 mL, 10 mmol) was added and the reaction mixture was stirred overnight at r.t. The volatiles were removed *in vacuo* and the residue was co-evaporated with acetonitrile (3x), then aqueous ammonia (25 %, 1x) and ethanol (3x). The product was recrystallized from ethanol.

((2-(5-Benzamido-3-oxo-1,2,4-thiadiazol-2(3*H*)-yl)ethoxy)methyl)phosphonic acid (42).

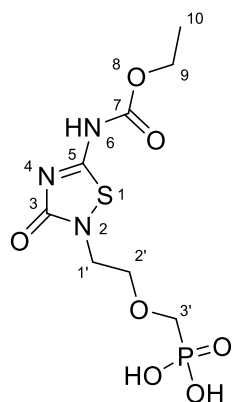


Reactants: compound **36** (0.221 g, 0.50 mmol) and TMS-Br (1.33 mL, 10 mmol).

White crystalline solid (0.172 g, yield 96 %). **Mp** = 219-221 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 8.09 (m, 2H, H-*o*-Ph), 7.40-7.48 (m, 3H, H-*m*-Ph, H-*p*-Ph), 6.97 (vbs, NH⁺), 3.68 (m, 2H, H-1'), 3.60 (m, 2H, H-2'), 3.36 (d, 2H, *J*(H,P) = 8.8 Hz, H-3'). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 176.89 (C-5), 175.28 (C-7), 166.16 (C-3), 136.88 (C-*i*-Ph), 130.96 (CH-*p*-Ph), 128.42 (CH-*o*-Ph), 128.22 (CH-*m*-Ph), 71.13 (d, *J*(C,P) = 10.7 Hz, CH₂-2'), 69.17 (d, *J*(C,P) = 154.4 Hz, CH₂-3'), 42.10 (CH₂-1'). **³¹P {¹H} NMR** (162.0 MHz, DMSO-*d*₆): δ 12.45. **FTIR** (KBr, cm⁻¹) *v*_{max}:

3127, 3010, 2930, 1669, 1655, 1617, 1603, 1582, 1564, 1536, 1496, 1448, 1399, 1334, 1302, 1145, 1113, 1070, 1015, 995, 952, 811, 772, 704, 687, 641, 529. **HRMS** (ESI) calcd. for C₁₂H₁₃O₆N₃PS (M-H⁺): 358.0268, found: 358.0268.

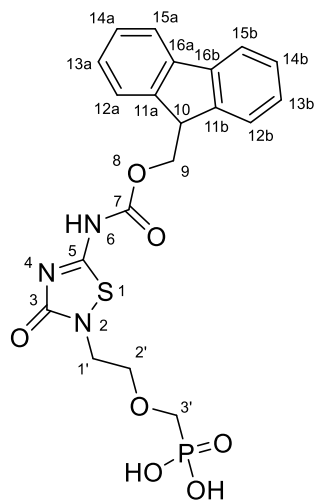
((2-(5-((Ethoxycarbonyl)amino)-3-oxo-1,2,4-thiadiazol-2(3*H*)-yl)ethoxy)methyl)phosphonic acid (43).



Reactants: compound **37** (0.206 g, 0.50 mmol) and TMS-Br (1.33 mL, 10 mmol). White crystalline solid (0.148 g, yield 91 %). **Mp** = 184-186 °C. **¹H NMR** (400 MHz, D₂O): δ 4.23 (q, 2H, *J*(9,10) = 7.1 Hz, H-9), 3.88 (m, 2H, H-1'), 3.82 (m, 2H, H-2'), 3.62 (d, 2H, *J*(3',P) = 8.5 Hz, H-3'), 1.29 (t, 3H, *J*(10,9) = 7.1 Hz, H-10). **¹³C NMR** (100.6 MHz, D₂O): δ 177.80 (C-5), 166.86 (C-3), 163.26 (C-7), 71.39 (d, *J*(2',P) = 10.4 Hz, CH₂-2'), 68.32 (d, *J*(3',P) = 154.2 Hz, CH₂-3'), 63.94 (CH₂-9), 43.70 (CH₂-1'), 14.41 (CH₃-10). **³¹P {¹H} NMR** (162.0 MHz, D₂O): δ 14.79. **FTIR** (KBr, cm⁻¹) *v*_{max}:

3138, 2978, 2934, 2884, 1706, 1665, 1500, 1483, 1403, 1339, 1312, 1218, 1107, 1027, 1011, 956, 937. **HRMS** (ESI) calcd. for C₈H₁₃O₇N₃PS (M-H⁺): 326.0217, found: 326.0213.

((2-(5-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-oxo-1,2,4-thiadiazol-2(3*H*)-yl)ethoxy)-methyl)phosphonic acid (44).

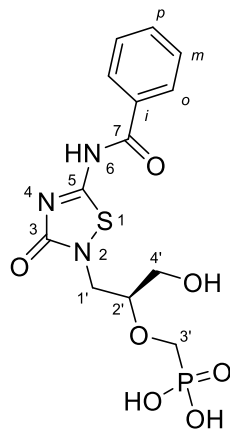


Reactants: compound **38** (0.281 g, 0.50 mmol) and TMS-Br (1.33 mL, 10 mmol). Yellowish amorphous solid (0.194 g, yield 82 %). ¹H NMR (500 MHz, MeOD-*d*₄): δ 7.78 (dt, 2H, *J*(15a,14a&15b,14b) = 7.5 Hz, *J*(15a,13a&15b,13b) = *J*(15a,12a&15b,12b) = 1.1 Hz, H-15a,b), 7.64 (dq, 2H, *J*(12a,13a&12b,13b) = 7.5 Hz, *J*(12a,14a&12b,14b) = *J*(12a,15a&12b,15b) = *J*(12a,10&12b,10) = 1.1 Hz, H-12a,b), 7.38 (tdd, 2H, *J*(14a,13a&14b,13b) = *J*(14a,15a&14b,15b) = 7.5 Hz, *J*(14a,12a&14b,12b) = 1.1 Hz, *J*(14a,11a&14b,11b) = 0.7 Hz, H-14a,b), 7.31 (td, 2H, *J*(13a,12a&13b,12b) = *J*(13a,14a&13b,14b) = 7.5 Hz, *J*(13a,15a&13b,15b) = 1.1 Hz, H-13a,b), 4.59 (d, 2H, *J*(9,10) = 6.5 Hz, H-9), 4.27 (t, 1H, *J*(10,9) = 6.5 Hz, H-10), 3.87 (m, 2H, H-1'), 3.79 (m, 2H, H-2'), 3.76 (d, 2H, *J*(3',P) = 8.3 Hz, H-3'). ¹³C NMR (125.7 MHz, MeOD-*d*₄): δ 171.87 (C-5), 165.70 (C-3), 156.99 (C-7), 144.47 (C-11a,b), 142.63 (C-16a,b), 129.04 (CH-14a,b), 128.29 (CH-13a,b), 126.04 (CH-12a,b), 121.08 (CH-15a,b), 72.34 (d, *J*(2',P) = 10.3 Hz, CH₂-2'), 69.91 (CH₂-9), 67.33 (d, *J*(3',P) = 162.9 Hz, CH₂-3'), 47.90 (CH-10), 44.44 (CH₂-1'). ³¹P NMR {¹H} (202.4 MHz, MeOD-*d*₄): δ 19.15. FTIR (KBr, cm⁻¹) ν_{max}: 3431, 3065, 3039, 3015, 2925, 2720, 2320, 1731, 1713, 1643, 1566, 1478, 1450, 1295, 1256, 1230, 1107, 1033, 1005, 942, 761, 741, 621, 428. HRMS (ESI) calcd. for C₂₀H₁₉O₇N₃PS (M-H⁺): 476.0684, found: 476.0684. R_f (7/2/1 *i*-PrOH/H₂O/NH₃) = 0.45.

The general procedure for synthesis of *N*⁵-protected-*N*²-HPMP-substituted thiadiazolones (free acids) 45-47; procedure according to ref. [143]

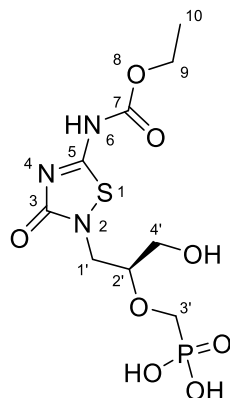
Compound **39-41** (0.50 mmol) was dissolved in acetonitrile (15 mL), TMS-Br (1.33 mL, 10 mmol) was added and the reaction mixture was stirred overnight at r.t. The volatiles were removed *in vacuo* and the residue was co-evaporated with acetonitrile (3x). MilliQ water (5.0 mL) was added and the mixture was stirred at r.t. for 1 h. Water was removed *in vacuo* and the residue was co-evaporated with ethanol (3x). The product was purified on reverse-phase column (0-60% acetonitrile/water). Fractions containing the product were concentrated by lyophilization.

(S)-(((1-(5-Benzamido-3-oxo-1,2,4-thiadiazol-2(3*H*)-yl)-3-hydroxypropan-2-yl)oxy)methyl)-phosphonic acid (45).



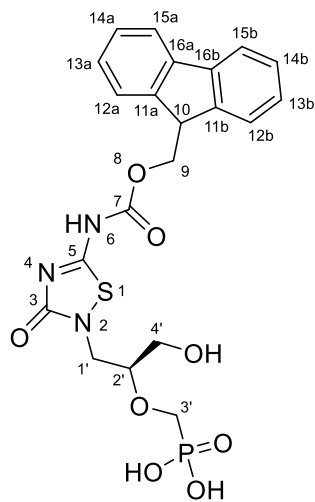
Reactants: compound **39** (0.237 g, 0.50 mmol) and TMS-Br (1.33 mL, 10 mmol). White lyophilizate (0.155 g, yield 80 %). $[\alpha]_D^{25}$ -1.2 (*c* 0.332 g/100 mL, H₂O). ¹H NMR (500 MHz, MeOD-*d*₄): δ 8.10-8.14 (m, 2H, H-*o*-Ph), 7.66 (m, 1H, H-*p*-Ph), 7.52-7.59 (m, 2H, H-*m*-Ph), 3.90 (dd, 1H, *J*_{gem} = 14.7 Hz, *J*(1'a,2') = 4.6 Hz, H-1'a), 3.78-3.86 (m, 2H, H-1'b, H-3'a), 3.76 (dd, 1H, *J*_{gem} = 12.2 Hz, *J*(4'a,2') = 3.6 Hz, H-4'a), 3.71 (m, 1H, H-2'), 3.67 (dd, 1H, *J*_{gem} = 12.8 Hz, *J*(3'b,P) = 9.9 Hz, H-3'b), 3.58 (dd, 1H, *J*_{gem} = 12.2 Hz, *J*(4'b,2') = 4.8 Hz, H-4'b). ¹³C NMR (125.7 MHz, MeOD-*d*₄): δ 173.57 (C-7), 171.68 (C-5), 162.56 (C-3), 134.74 (CH-*p*-Ph), 132.54 (C-*i*-Ph), 129.94 (CH-*m*-Ph), 129.82 (CH-*o*-Ph), 82.41 (d, *J*(2',P) = 10.4 Hz, CH-2'), 68.28 (d, *J*(3',P) = 157.6 Hz, CH₂-3'), 62.21 (CH₂-4'), 44.96 (CH₂-1'). ³¹P NMR {¹H} (202.4 MHz, MeOD-*d*₄): δ 16.50. FTIR (KBr, cm⁻¹) ν_{max}: 3390, 3200, 3136, 2893, 2516, 2270, 1753, 1705, 1687, 1652, 1600, 1589, 1547, 1487, 1472, 1449, 1303, 1261, 1185, 1157, 1107, 1057, 1027, 1013, 838, 709, 686, 632. HRMS (ESI) calcd. for C₁₃H₁₅O₇N₃PS (M-H⁺): 388.0374, found: 388.0370. *R*_f (1/1 MeOH/EtOAc) = 0.15.

(S)-(((1-(5-((Ethoxycarbonyl)amino)-3-oxo-1,2,4-thiadiazol-2(3*H*)-yl)-3-hydroxypropan-2-yl)-oxy)methyl)phosphonic acid (46).



Reactants: compound **40** (0.221 g, 0.50 mmol) and TMS-Br (1.33 mL, 10 mmol). White lyophilizate (0.130 g, yield 73 %). $[\alpha]_D^{25}$ -2.6 (*c* 0.272 g/100 mL, H₂O). ¹H NMR (500 MHz, MeOD-*d*₄): δ 4.33 (q, 2H, *J*(9,10) = 7.1 Hz, H-9), 3.95 (dd, 1H, *J*_{gem} = 14.8 Hz, *J*(1'a,2') = 3.9 Hz, H-1'a), 3.78-3.90 (m, 3H, H-1'b, 2H-4'), 3.68-3.74 (m, 2H, H-2', H-3'a), 3.60 (dd, 1H, *J*_{gem} = 13.5 Hz, *J*(3'b,2') = 6.3 Hz, H-3'b), 1.35 (t, 3H, *J*(10,9) = 7.1 Hz, H-10). ¹³C NMR (125.7 MHz, MeOD-*d*₄): δ 172.09 (C-5), 166.32 (C-3), 156.97 (C-7), 82.50 (d, *J*(2',P) = 10.7 Hz, CH-2'), 66.81 (d, *J*(3',P) = 162.1 Hz, CH₂-3'), 64.86 (CH₂-9), 62.02 (CH₂-4'), 45.14 (CH₂-1'), 14.53 (CH₃-10). ³¹P NMR {¹H} (202.4 MHz, MeOD-*d*₄): δ 19.45. FTIR (KBr, cm⁻¹) ν_{max}: 3259, 2281, 2137, 1758, 1714, 1689, 1563, 1513, 1370, 1363, 1323, 1276, 1257, 1231, 1034, 1010, 988, 942, 883, 701, 552. HRMS (ESI) calcd. for C₉H₁₅O₈N₃PS (M-H⁺): 356.0323, found: 356.0322. *R*_f (1/1 MeOH/EtOAc) = 0.15.

(S)-(((1-(5-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-oxo-1,2,4-thiadiazol-2(3H)-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonic acid (47).

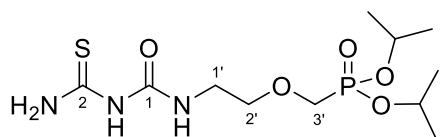


Reactants: compound **41** (0.296 g, 0.50 mmol) and TMS-Br (1.33 mL, 10 mmol). White lyophilizate (0.218 g, yield 86 %). $[\alpha]_D$ -1.8 (*c* 0.221 g/100 mL, H₂O). ¹H NMR (500 MHz, MeOD-*d*₄): δ 7.82 (ddd, 2H, *J*(15a,14a&15b,14b) = 7.6 Hz, *J*(15a,13a&15b,13b) = 1.3 Hz, *J*(15a,12a&15b,12b) = 0.7 Hz, H-15a,b), 7.67 (ddt, 2H, *J*(12a,13a&12b,13b) = 7.5 Hz, *J*(12a,14a&12b,14b) = 1.3 Hz, *J*(12a,13a&12b,13b) = *J*(12a,10&12b,10) = 0.7 Hz, H-12a,b), 7.41 (tdd, 2H, *J*(14a,13a&14b,13b) = *J*(14a,15a&14b,15b) = 7.5 Hz, *J*(14a,12a&14b,12b) = 1.3 Hz, *J*(14a,10&14b,10) = 0.7 Hz, H-14a,b), 7.33 (td, 2H, *J*(13a,12a&13b,12b) = *J*(13a,14a&13b,14b) = 7.5 Hz, *J*(13a,15a&13b,15b) = 1.3 Hz, H-13a,b), 4.65 (d, 2H, *J*(9,10) = 6.5 Hz, H-9), 4.32 (t, 1H, *J*(10,9) = 6.5 Hz, H-10), 3.94 (dd, 1H, *J*_{gem} = 14.9 Hz, *J*(1'a,2') = 3.8 Hz, H-1'a), 3.87 (dd, 1H, *J*_{gem} = 13.4 Hz, *J*(3'a,P) = 9.1 Hz, H-3'a), 3.83 (dd, 1H, *J*_{gem} = 13.4 Hz, *J*(3'b,P) = 9.5 Hz, H-3'b), 3.81 (dd, 1H, *J*_{gem} = 14.9 Hz, *J*(1'b,2') = 6.5 Hz, H-1'b), 3.65-3.72 (m, 2H, H-2', H-3'a), 3.60 (m, 1H, H-3'b). ¹³C NMR (125.7 MHz, MeOD-*d*₄): δ 172.11 (C-5), 166.11 (C-3), 157.00 (C-7), 144.53 (C-11a,b), 142.70 (C-16a,b), 129.07 (CH-14a,b), 128.32 (CH-13a,b), 126.07 (CH-12a,b), 121.10 (CH-15a,b), 82.52 (d, *J*(2',P) = 11.0 Hz, CH-2'), 69.93 (CH₂-9), 66.49 (d, *J*(3',P) = 163.0 Hz, CH₂-3'), 61.99 (CH₂-4'), 47.96 (CH-10), 45.16 (CH₂-1'). ³¹P NMR {¹H} (202.4 MHz, MeOD-*d*₄): δ 20.15. FTIR (KBr, cm⁻¹) ν_{max}: 3427, 3203, 3069, 3000, 1706, 1626, 1568, 1545, 1496, 1477, 1449, 1247, 1223, 1116, 1057, 1024, 1009, 621, 430. HRMS (ESI) calcd. for C₂₁H₂₀O₈N₃NaPS (M-2H⁺+Na⁺): 528.0612, found: 528.0608; calcd. for C₂₁H₂₁O₈N₃PS (M-H⁺): 506.0793, found: 506.0789. R_f (1/1 MeOH/EtOAc) = 0.14.

The general procedure for deprotection of the Fmoc-moiety upon synthesis of *N*⁵-PME-substituted thiobiuret diisopropyl esters 52-54; procedure according to ref. [150]

Compound **32**, **35**, or **41** (0.50 mmol) was dissolved in dichloromethane (20 mL) and piperidine (0.05 mL, 0.60 mmol) was added. The reaction mixture was stirred at r.t. overnight. The solvent was removed *in vacuo* and the residue was purified on silica gel column (0-3% MeOH/CHCl₃).

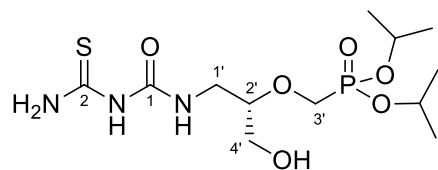
Diisopropyl ((2-(3-carbamothioylureido)ethoxy)methyl)phosphonate (52).



Reactants: compound **32** (0.282 g, 0.50 mmol) and piperidine (0.05 mL, 0.60 mmol). White crystalline solid (0.157 g, yield 92 %). **Mp** = 125-126 °C. ¹H NMR (500 MHz, CDCl₃): δ 9.75 (bs,

1H, NH), 7.22 (bs, 1H, NH), 7.12 (bs, 2H, NH₂), 4.76-4.89 (m, 2H, CH-*i*-Pr), 3.79 (d, 2H, $J(3',P) = 8.8$ Hz, H-3'), 3.66 (m, 2H, H-2'), 3.39 (m, 2H, H-1'), 1.35 (d, 6H, $J_{vic} = 6.3$ Hz, CH₃-*i*-Pr), 1.33 (d, 6H, $J_{vic} = 6.3$ Hz, CH₃-*i*-Pr). ¹³C NMR (125.7 MHz, CDCl₃): δ 182.43 (C-2), 154.35 (C-1), 72.39 (d, $J(2',P) = 12.1$ Hz, CH₂-2'), 71.99 (d, $J(C,P) = 6.8$ Hz, CH-*i*-Pr), 66.23 (d, $J(3',P) = 170.3$ Hz, CH₂-3'), 39.54 (CH₂-1'), 24.07 (d, $J(C,P) = 3.8$ Hz, CH₃-*i*-Pr), 24.00 (d, $J(C,P) = 4.7$ Hz, CH₃-*i*-Pr). ³¹P {¹H} NMR (202.4 MHz, CDCl₃): δ 19.98. FTIR (CHCl₃, cm⁻¹) ν_{max}: 3488, 3340, 3296, 3270, 3190, 2984, 2939, 2876, 1704, 1580, 1537, 1466, 1415, 1388, 1377, 1232, 1180, 1143, 1117, 1103, 1018, 1002, 891. HRMS (ESI) calcd. for C₁₁H₂₄O₅N₃NaPS (M+Na⁺): 364.1067, found: 364.1068; calcd. for C₁₁H₂₅O₅N₃PS (M+H⁺): 342.1247, found: 342.1248. R_f (9/1 CHCl₃/MeOH) = 0.47.

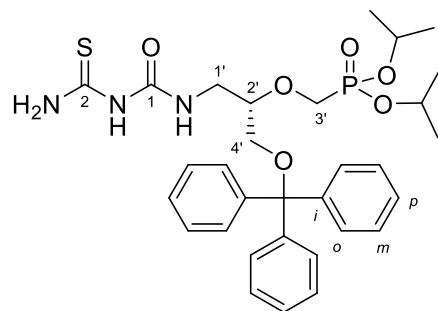
Diisopropyl (S)-(((1-(3-carbamothioylureido)-3-hydroxypropan-2-yl)oxy)methyl)phosphonate (53).



Reactants: compound **41** (0.296 g, 0.50 mmol) and piperidine (0.05 mL, 0.60 mmol). White crystalline solid (0.124 g, yield 67 %). ¹H NMR (400 MHz, CDCl₃): δ 4.68-4.81 (m, 2H, CH-*i*-Pr), 3.83 (m, 1H, H-3'a), 3.78 (m, 1H, H-3'b), 3.50-3.65 (m, 2H, H-1'a,

H-2'), 3.16-3.11 (m, 2H, H-4'), 3.03 (m, 1H, H-1'b), 1.35 (d, 6H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr), 1.32 (d, 6H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr). ³¹P {¹H} NMR (162.0 MHz, CDCl₃): δ 20.34. LRMS (ESI) calcd. for C₁₂H₂₆O₆N₃NaPS (M+Na⁺): 394.1, found: 394.1. R_f (6/4/1 hexane/CHCl₃/MeOH) = 0.16.

Diisopropyl (S)-(((1-(3-carbamothioylureido)-3-(trityloxy)propan-2-yl)oxy)methyl)phosphonate (54).



Reactants: compound **35** (0.417 g, 0.50 mmol) and piperidine (0.05 mL, 0.60 mmol). White crystalline solid (0.212 g, yield 69 %). Mp = 77-78 °C. [α]_D -4.6 (c 0.240 g/100 mL, MeOH). ¹H NMR (500 MHz, DMSO-*d*₆): δ 9.75 (d, 1H, ⁴*J* = 1.6 Hz, CSNH), 9.36 (d, 1H, $J_{gem} = 3.3$ Hz, NH₂), 8.95 (d, 1H, $J_{gem} = 3.3$ Hz, NH₂), 7.37-7.41 (m, 6H, H-*o*-Tr), 7.31-7.36 (m, 6H, H-*m*-Tr), 7.26 (m, 3H, H-*p*-Tr), 6.82 (bt, 1H, $J(NH,1') = 5.3$ Hz, CONH),

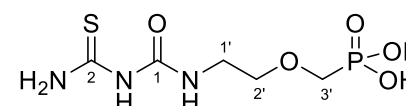
4.53-4.63 (m, 2H, CH-*i*-Pr), 3.81 (dd, 1H, $J_{gem} = 13.7$ Hz, $J(3'a,P) = 8.7$ Hz, H-3'a), 3.76 (dd, 1H, $J_{gem} = 13.7$ Hz, $J(3'b,P) = 9.3$ Hz, H-3'b), 3.65 (p, 1H, $J(2',1') = J(2',4') = 5.1$ Hz, H-2'), 3.23-3.35 (m, 2H, H-1'), 3.05 (dd, 1H, $J_{gem} = 10.4$ Hz, $J(4'a,2') = 4.6$ Hz, H-4'a), 3.02 (dd, 1H, $J_{gem} = 10.4$ Hz, $J(4'b,2') = 5.4$ Hz, H-4'b), 1.23 (d, 6H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr), 1.22 (d, 6H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr), 1.20 (d, 3H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr), 1.19 (d, 3H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr). ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 181.51 (C-2), 154.31 (C-1), 143.77 (C-*i*-Tr), 128.44 (CH-*o*-Tr), 128.09 (CH-*m*-Tr), 127.28 (CH-*p*-Tr), 86.32 (C-Tr),

79.17 (d, $J(2',P) = 12.0$ Hz, CH-2'), 70.42 (d, $J(C,P) = 6.3$ Hz, CH-*i*-Pr), 70.39 (d, $J(C,P) = 6.3$ Hz, CH-*i*-Pr), 64.00 (d, $J(3',P) = 165.1$ Hz, CH₂-3'), 63.01 (CH₂-4'), 39.48 (CH₂-1'), 24.02 (d, $J(C,P) = 3.6$ Hz, CH₃-*i*-Pr), 23.91 (d, $J(C,P) = 4.4$ Hz, CH₃-*i*-Pr), 23.87 (d, $J(C,P) = 4.4$ Hz, CH₃-*i*-Pr). **³¹P {¹H} NMR** (202.4 MHz, DMSO-*d*₆): δ 18.99. **FTIR** (CHCl₃, cm⁻¹) $\nu_{\max}$: 3488, 3390, 3340, 3295, 3264, 3088, 3063, 3035, 2984, 2935, 2875, 1705, 1599, 1578, 1519, 1491, 1466, 1449, 1442, 1412, 1388, 1377, 1228, 1180, 1159, 1152, 1143, 1117, 1101, 1090, 1080, 1036, 1018, 1002, 891, 707, 700, 643, 633, 617. **HRMS** (ESI) calcd. for C₃₁H₄₀O₆N₃NaPS (M+Na⁺): 636.2268, found: 636.2269. **R_f** (9/1 CHCl₃/MeOH) = 0.55.

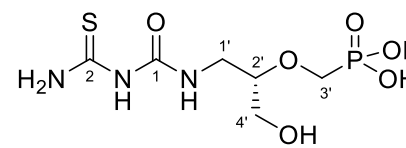
The general procedure for synthesis of *N*⁵-PME-substituted thiobiurets (free phosphonic acids) **50-51; procedure according to ref. [143]**

Compound (**52** or **54**, 0.50 mmol) was dissolved in acetonitrile (10 mL) and TMS-Br (1.00 mL, 7.5 mmol) was added. The reaction mixture was stirred at r.t. overnight. The volatiles were removed *in vacuo* and the residue was co-evaporated with acetonitrile (1x). Water was added and the solution was stirred at r.t. for 1 h. Water was removed *in vacuo* and the residue was codistilled with water (1x). For compound **51**, the residue was then extracted between diethylether and water to remove trityl alcohol; water phase was collected and concentrated *in vacuo*.

((2-(3-Carbamothioylureido)ethoxy)methyl)phosphonic acid (50**).**

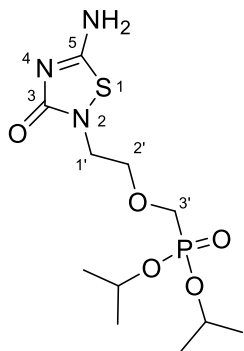
 Reactants: compound **52** (0.171 g, 0.50 mmol) and TMS-Br (1.00 mL, 7.5 mmol). Yellowish crystalline solid (0.120 g, yield 93 %). **Mp** = 139-140 °C. **¹H NMR** (500 MHz, MeOD-*d*₄): δ 3.76 (d, 2H, $J(3',P) = 9.0$ Hz, H-3'), 3.64-3.67 (m, 2H, H-2'), 3.36-3.39 (m, 2H, H-1'). **¹³C NMR** (125.7 MHz, MeOD-*d*₄): δ 183.67 (C-2), 155.73 (C-1), 72.84 (d, $J(2',P) = 11.9$ Hz, CH₂-2'), 67.08 (d, $J(3',P) = 163.7$ Hz, CH₂-3'), 40.16 (CH₂-1'). **³¹P {¹H} NMR** (202.4 MHz, MeOD-*d*₄): δ 19.57. **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3336, 3247, 3199, 3139, 2675, 2258, 1723, 1714, 1626, 1604, 1563, 1535, 1223, 1120, 1102. **HRMS** (ESI) calcd. for C₅H₁₁O₅N₃PS (M-H⁺): 256.0163, found: 256.0161. **R_f** (7/2/1 *i*-PrOH/H₂O/NH₃) = 0.12.

(*S*)-(((1-(3-Carbamothioylureido)-3-hydroxypropan-2-yl)oxy)methyl)phosphonic acid (51**).**

 Reactants: compound **54** (0.307 g, 0.50 mmol) and TMS-Br (1.00 mL, 7.5 mmol). Colourless oil (0.132 g, yield 92 %). **[α]_D** -11.7 (*c* 0.214 g/100 mL, MeOH). **¹H NMR** (500 MHz, MeOD-*d*₄): δ 3.85-3.91 (m, 2H, CH₂-3'), 3.65 (dd, 1H, $J_{\text{gem}} = 11.8$ Hz, $J(4'a,2') = 4.5$ Hz, H-4'a), 3.59 (dd, 1H, $J_{\text{gem}} = 11.8$ Hz, $J(4'b,2') = 5.3$ Hz, H-4'b), 3.55 (m, 1H, H-2'), 3.46 (dd, 1H, $J_{\text{gem}} = 14.2$ Hz, $J(1'a,2') = 4.5$ Hz, H-1'a), 3.27 (dd, 1H, $J_{\text{gem}} = 14.2$ Hz, $J(1'b,2') = 6.1$ Hz, H-1'b). **¹³C NMR** (125.7 MHz, MeOD-*d*₄): δ 183.74 (C-

2), 155.89 (C-1), 82.72 (d, $J(2',P) = 11.5$ Hz, CH-2'), 66.04 (d, $J(3',P) = 164.2$ Hz, CH₂-3'), 62.51 (CH₂-4'), 40.64 (CH₂-1'). ³¹P {¹H} NMR (202.4 MHz, MeOD-*d*₄): δ 21.13. FTIR (KBr, cm⁻¹) ν_{max}: 3375, 3280, 2945, 2306, 1730, 1711, 1643, 1599, 1546, 1437, 1222, 1120, 1040, 1002, 943. HRMS (ESI) calcd. for C₆H₁₃O₆N₃PS (M-H⁺): 286.0268, found: 286.0267. R_f (7/2/1 *i*-PrOH/H₂O/NH₃) = 0.08.

Diisopropyl ((2-(5-amino-3-oxo-1,2,4-thiadiazol-2(3*H*)-yl)ethoxy)methyl)phosphonate (8);
procedure according to ref. [144].



Compound **52** (0.201 g, 0.589 mmol) was dissolved in ethyl acetate (10 mL) and 0.5M bromine solution in ethyl acetate (2.5 mL, 12.5 mmol) was added at 4 °C. The mixture was stirred overnight at 4 °C for 16.5 h. The solvent was removed *in vacuo* and the residue was purified by preparative TLC (5% MeOH/CHCl₃). Colourless oil (0.180 g, yield 90 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 8.33 (bs, 2H, NH₂), 4.59 (dsep, 2H, $J(H,P) = 7.7$ Hz, $J_{vic} = 6.2$ Hz, CH-*i*-Pr), 3.76 (d, 2H, $J(3',P) = 8.2$ Hz, H-3'), 3.66 (m, 2H, H-1'), 3.64 (m, 2H, H-2'), 1.24 (dd, 6H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr), 1.23 (dd, 6H, $J_{vic} = 6.2$ Hz, CH₃-*i*-Pr). ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 172.14 (C-5), 164.86 (C-3), 71.22 (d, $J(2',P) = 11.3$ Hz, CH₂-2'), 70.46 (d, $J(C,P) = 6.4$ Hz, CH-*i*-Pr), 64.95 (d, $J(3',P) = 164.1$ Hz, CH₂-3'), 43.16 (CH₂-1'), 24.07 (d, $J(C,P) = 3.6$ Hz, CH₃-*i*-Pr), 23.95 (d, $J(C,P) = 4.5$ Hz, CH₃-*i*-Pr). ³¹P NMR {¹H} (202.4 MHz, MeOD-*d*₄): δ 19.08. FTIR (CHCl₃, cm⁻¹) ν_{max}: 3464, 3398, 3274, 3126, 2983, 2877, 1668, 1630, 1608, 1544, 1388, 1377, 1237, 1179, 1143, 1103, 1000. HRMS (ESI) calcd. for C₁₁H₂₂O₅N₃NaPS (M+Na⁺): 362.0910, found: 362.0910. R_f (19/1 CHCl₃/MeOH) = 0.24.

6 References

1. Holý, A.: Principy bioorganické chemie ve vývoji antivirotik a cytostatik. *Univerzita Palackého v Olomouci*, 1st Edition, Olomouc, Czech Republic **2004**, ISBN: 80-244-0855-4.
2. De Clercq, E.; Holý, A.; Rosenberg, I.; Sakuma, T.; Balzarini, J.; and Maudgal, P. C.: A novel selective broad-spectrum anti-DNA virus agent. *Nature* **1986**, *323*, 464-467.
3. Naesens, L.; Snoeck, R.; Andrei, G.; Balzarini, J.; Neyts, J.; and De Clercq, E.: HPMPC (cidofovir), PMEA (adefovir) and related acyclic nucleoside phosphonate analogues: a review of their pharmacology and clinical potential in the treatment of viral infections. *Antivir. Chem. Chemother.* **1997**, *8*, 1-23.
4. De Clercq, E.; and Holý, A.: Acyclic nucleoside phosphonates: a key class of antiviral drugs. *Nature* **2005**, *4*, 928-940.
5. Balzarini, J.; Verstuyf, A.; Hatse, S.; Goebels, J.; Sobis, H.; Vandeputte, M.; and De Clercq, E.: The human immunodeficiency virus(HIV) inhibitor 9-(2-phosphonylmethoxyethyl)adenine (pmea) is a strong inducer of differentiation of several tumor cell lines. *Int. J. Cancer* **1995**, *61*, 130-137.
6. Hatse, S.; Naesens, L.; De Clercq, E.; and Balzarini, J.: Potent differentiation-inducing properties of the antiretroviral agent 9-(2-phosphonylmethoxyethyl)adenine (PMEA) in the rat choriocarcinoma (RCHO) tumor cell model. *Biochem. Pharmacol.* **1998**, *56*, 851-859.
7. Hatse, S.; Naesens, L.; Degrève, B.; Segers, C.; Vandeputte, M.; Waer, M.; De Clercq, E.; and Balzarini, J.: Potent antitumor activity of the acyclic nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine in choriocarcinoma-bearing rats. *Int. J. Cancer* **1998**, *76*, 595-600.
8. Neyts, J.; Sadler, R.; De Clercq, E.; Raab-Traub, N.; and Pagano, J. S.: The Antiviral Agent Cidofovir [(S)-1-(3-Hydroxy-2-phosphonyl-methoxypropyl) Cytosine] Has Pronounced Activity against Nasopharyngeal Carcinoma Grown in Nude Mice. *Cancer Res.* **1998**, *58*, 384-388.
9. Murono, S.; Raab-Traub, N.; and Pagano, J. S.: Prevention and Inhibition of Nasopharyngeal Carcinoma Growth by Antiviral Phosphonated Nucleoside Analogs. *Cancer Res.* **2001**, *61*, 7875-7877.
10. Andrei, G.; Snoeck, R.; Piette, J.; Delvenne, P.; and De Clercq, E.: Inhibiting Effects of Cidofovir (HPMPC) on the Growth of the Human Cervical Carcinoma (SiHa) Xenographs in Athymic Nude Mice. *Oncol. Res.* **1998**, *10*, 533-539.

11. Liekens, S.; Andrei, G.; Vandeputte, M.; De Clercq, E.; and Neyts, J.: Potent Inhibition of Hemangioma Formation in Rats by the Acyclic Nucleoside Phosphonate Analogue Cidofovir. *Cancer Res.* **1998**, *58*, 2562-2567.
12. Liekens, S.; Verbeken, E.; De Clercq, E.; and Neyts, J.: Potent Inhibition of Hemangiosarcoma Development in Mice by Cidofovir. *Int. J. Cancer* **2001**, *92*, 161-167.
13. Liekens, S.; Neyts, J.; De Clercq, E.; Verbeken, E.; Ribatti, D.; and Presta, M.: Inhibition of Fibroblast Growth Factor-2-induced Vascular Tumor Formation by the Acyclic Nucleoside Phosphonate Cidofovir. *Cancer Res.* **2001**, *61*, 5057-5064.
14. Andrei, G.; Snoeck, R.; Schols, D.; and De Clercq, E.: Induction of Apoptosis by Cidofovir in Human Papillomavirus (HPV)-Positive Cells. *Oncol. Res.* **2001**, *12*, 397-408.
15. Abdulkarim, B.; Sabri, S.; Deutsch, E.; Chagraoui, H.; Maggiorella, L.; Thierry, J.; Eschwege, F.; Vainchenker, W.; Chouaïb, S.; and Bourhis, J.: Antiviral agent Cidofovir restores p53 function and enhances the radiosensitivity in HPV-associated cancers. *Oncogene* **2002**, *21*, 2334-2346.
16. Shen, Y.; Zhukovskaya, N. L.; Zimmer, M. I.; Soelaiman, S.; Bergson, P.; Wang, C.-R.; Gibbs, C. S.; and Tang, W.-J.: Selective inhibition of anthrax edema factor by adefovir, a drug for chronic hepatitis B virus infection. *PNAS* **2004**, *101*, 3242-3247.
17. Keough, D. T.; Hocková, D.; Rejman, D.; Špaček, P.; Vrbková, S.; Krečmerová, M.; Eng, W. S.; Jans, H.; West, N. P.; Naesens, L. M. J.; De Jersey, J.; and Guddat, L. W.: Inhibition of the *Escherichia coli* 6-Oxopurine Phosphoribosyltransferases by Nucleoside Phosphonates: Potential for New Antibacterial Agents. *J. Med. Chem.* **2013**, *56*, 6967-6984.
18. Hocková, D.; Keough, D. T.; Špaček, P.; Janeba, Z.; Edstein, M. D.; Chavchich, M.; Wang, T.-H.; Eng, W. S.; West, N. P.; De Jersey, J.; and Guddat, L. W.: Acyclic nucleoside bisphosphonates as inhibitors of 6-oxopurine phosphoribosyltransferases: potential antimalarial and antibacterial agents. *Collection Symposium Series* **2014**, *14*, 107-110.
19. Smeijsters, L. J. J. W.; Franssen, F. F. J.; Naesens, L.; De Vries, E.; Holý, A.; Balzarini, J.; De Clercq, E.; and Overdulve, J. P.: Inhibition of the in vitro growth of *Plasmodium falciparum* by acyclic nucleoside phosphonates. *Int. J. Antimicrob. Agents* **1999**, *12*, 53-61.
20. Keough, D. T.; Hocková, D.; Holý, A.; Naesens, L. M. J.; Skinner-Adams, T. S.; De Jersey, J.; and Guddat, L. W.: Inhibition of Hypoxanthine-Guanine Phosphoribosyltransferase by Acyclic Nucleoside Phosphonates: A New Class of Antimalarial Therapeutics. *J. Med. Chem.* **2009**, *52*, 4391-4399.
21. Holý, A.: Acyklické nukleosidfosfonáty: představy a skutečnost. Habilitation procedure, Appendix 2, Copy of cited parts I (ref. 30-69), Palacký University Olomouc, **2003**.

22. Holý, A.: Phosphonomethoxyalkyl Analogs of Nucleotides. *Curr. Pharm. Des.* **2003**, 9, 2567-2592.
23. De Clercq, E.: Tenofovir alafenamide (TAF) as the successor of tenofovir disoproxil fumarate (TDF). *Biochem. Pharmacol.* **2016**, 119, 1-7.
24. Cundy, K. C.; Shaw, J.-P.; and Lee, W. A.: Oral, Subcutaneous, and Intramuscular Bioavailabilities of the Antiviral Nucleotide Analog 9-(2-Phosphonylmethoxyethyl) Adenine in Cynomolgus Monkeys. *Antimicrob. Agents Chemother.* **1994**, 38, 365-368.
25. Hatse, S.; Balzarini, J.; and De Clercq, E.: Induction of Erythroid Differentiation of Human Leukemia K562 Cells by the Acyclic Nucleoside Phosphonate 9-(2-Phosphonylmethoxyethyl)adenine (PMEA). *Nucleos. Nucleot.* **1995**, 14, 649-652.
26. Hatse, S.; De Clercq, E.; and Balzarini, J.: Evidence for distinction of the differentiation-inducing activities and cytostatic properties of 9-(2-phosphonylmethoxyethyl)adenine and a variety of differentiation-inducing agents in human erythroleukemia K562 cells. *Mol. Pharmacol.* **1996**, 50, 1231-1242.
27. Otová, B.; Sladká, M.; Votruba, I.; Holý, A.; and Křen, V.: Cytostatic effect of 9-(2-phosphonomethoxyethyl) adenine (PMEA). I. Lymphatic leukemia KHP-Lw-I in Lewis rats. *Folia Biol.* **1993**, 39, 136-141.
28. Coutinho, B.; and Prasad, R.: Emtricitabine/Tenofovir (Truvada) for HIV Prophylaxis. *Am. Fam. Physician* **2013**, 88, 535-540.
29. Elliott, W.; and Chan, J.: Tenofovir Alafenamide Tablets (Vemlidy). *Intern. Med. Alert* **2017**, 39, 3.
30. Elliott, W.; and Chan, J.: Tenofovir Alafenamide vs. Tenofovir Disoproxil Fumarate. *Infect. Dis. Alert* **2016**, 35, 8.
31. Lalezari, J. P.; Stagg, R. J.; Kuppermann, B. D.; Holland, G. N.; Kramer, F.; Ives, D. V.; Youle, M.; Robinson, M. R.; Drew, W. L.; and Jaffe, H. S.: Intravenous Cidofovir for Peripheral Cytomegalovirus Retinitis in Patients with AIDS. *Ann. Intern. Med.* **1997**, 126, 257-263.
32. Tjon Pian Gi, R. E. A.; Dietz, A.; Djukic, V.; Eckel, H. E.; Friedrich, G.; Golusinski, W.; Hantzakos, A.; Lawson, G.; Remacle, M.; Rihkanen, H.; and Dikkers, F. G.: Treatment of recurrent respiratory papillomatosis and adverse reactions following off-label use of cidofovir (Vistide®). *Eur. Arch. Otorhinolaryngol.* **2012**, 269, 361-362.
33. Field, H. J.; and De Clercq, E.: Antiviral Chemistry & Chemotherapy's current antiviral agents FactFile (2nd edition): DNA viruses. *Antivir. Chem. Chemother.* **2008**, 19, 51-62.
34. Izzedine, H.; Launay-Vacher, V.; and Deray, G.: Antiviral Drug-Induced Nephrotoxicity. *Am. J. Kidney Dis.* **2005**, 45, 804-817.

35. Krečmerová, M.; Dračinský, M.; Snoeck, R.; Balzarini, J.; Pomeisl, K.; and Andrei, G.: New prodrugs of pyrimidine acyclic nucleoside phosphonates: Synthesis and antiviral activity. *Bioorg. Med. Chem.* **2017**, *25*, 4637-4648.
36. De Clercq, E.: Antiviral drug discovery: ten more compounds, and ten more stories (part B). *Med. Res. Rev.* **2009**, *29*, 571-610.
37. Balzarini, J.; Holý, A.; Jindřich, J.; Dvořáková, H.; Hao, Z.; Snoeck, R.; Herdewijn, P.; Johns, D. G.; and De Clercq, E.: 9-[(2RS)-3-fluoro-2-phosphonylmethoxypropyl] derivatives of purines: a class of highly selective antiretroviral agents in vitro and in vivo. *PNAS* **1991**, *88*, 4961-4965.
38. Dvořáková, H.; and Holý, A.: Synthesis and Biological Effects of N-(2-Phosphonomethoxyethyl) Derivatives of Deazapurine Bases. *Collect. Czech. Chem. Commun.* **1993**, *58*, 1419-1429.
39. Holý, A.: Synthetic approaches to “opened-ring” acyclic nucleoside phosphonates – novel type of antivirals. *Collection Symposium Series* **2002**, *5*, 27-35.
40. Holý, A.; Votruba, I.; Masojídková, M.; Andrei, G.; Snoeck, R.; Naesens, L.; De Clercq, E.; and Balzarini, J.: 6-[2-(Phosphonomethoxy)alkoxy]pyrimidines with Antiviral Activity. *J. Med. Chem.* **2002**, *45*, 1918-1929.
41. Balzarini, J.; Pannecouque, C.; De Clercq, E.; Aquaro, S.; Perno, C.-F.; Egberink, H.; and Holý, A.: Antiretrovirus Activity of a Novel Class of Acyclic Pyrimidine Nucleoside Phosphonates. *Antimicrob. Agents Chemother.* **2002**, *46*, 2185-2193.
42. Hocková, D.; Holý, A.; Masojídková, M.; Andrei, G.; Snoeck, R.; De Clercq, E.; and Balzarini, J.: 5-Substituted-2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidines – Acyclic Nucleoside Phosphonate Analogues with Antiviral Activity. *J. Med. Chem.* **2003**, *46*, 5064-5073.
43. Krejčová, R.; Horská, K.; Votruba, I.; and Holý, A.: Phosphorylation of Purine (Phosphonomethoxy)alkyl Derivatives by Mitochondrial AMP Kinase (AK2 Type) from L1210 Cells. *Collect. Czech. Chem. Commun.* **2000**, *65*, 1653-1668.
44. Kramata, P.; Votruba, I.; Otová, B.; and Holý, A.: Different Inhibitory Potencies of Acyclic Phosphonomethoxyalkyl Nucleotide Analogs toward DNA Polymerases alpha, delta and epsilon. *Mol. Pharmacol.* **1996**, *49*, 1005-1011.
45. De Clercq, E.: Potential of acyclic nucleoside phosphonates in the treatment of DNA virus and retrovirus infections. *Expert Rev. Anti-infect. Ther.* **2003**, *1*, 21-43.
46. Holý, A.; and Rosenberg, I.: Synthesis of 9-(2-phosphonylmethoxyethyl)adenine and related compounds. *Collect. Czech. Chem. Commun.* **1987**, *52*, 2801-2809.
47. Holý, A.: Synthesis of Acyclic Nucleoside Phosphonates. *Curr. Protoc. Nucleic Acid Chem.* **2005**, *22*, 14.2.1-14.2.38.

48. Krečmerová, M.; and Otmar, M.: 5-azacytosine compounds in medicinal chemistry: current stage and future perspectives. *Future Med. Chem.* **2012**, *4*, 991-1005.
49. Krečmerová, M.; Pohl, R.; Masojídková, M.; Balzarini, J.; Snoeck, R., and Andrei, G.: *N*⁴-Acyl derivatives as lipophilic prodrugs of cidofovir and its 5-azacytosine analogue, (S)-HPMP-5-azaC: Chemistry and antiviral activity. *Bioorg. Med. Chem.* **2014**, *22*, 2896-2906.
50. Borin, A. C.; Mai, S.; Marquetand, P.; and González, L.: *Ab initio* molecular dynamics relaxation and intersystem crossing mechanisms of 5-azacytosine. *Phys. Chem. Chem. Phys.* **2017**, *19*, 5888-5894.
51. Pískala, A.; and Šorm, F.: Nucleic acids components and their analogues. LI. Synthesis of 1-glycosyl derivatives of 5-azauracil and 5-azacytosine. *Collect. Czech. Chem. Commun.* **1964**, *29*, 2060-2076.
52. Šorm, F.; Pískala, A.; Čihák, A.; and Veselý, J.: 5-Azacytidine, a new, highly effective cancerostatic. *Experientia* **1964**, *20*, 202-203.
53. Hanka, L. J.; Evans, J. S.; Mason, D. J.; and Dietz, A.: Microbiological production of 5-azacytidine. I. Production and biological activity. *Antimicrob. Agents Chemother.* **1966**, *6*, 619-624.
54. Pliml, J.; and Šorm, F.: Synthesis of a 2'-deoxy-D-ribofuranosyl-5-azacytosine. *Collect. Czech. Chem. Commun.* **1964**, *29*, 2576-2578.
55. Šorm, F.; and Veselý, J.: Effect of 5-aza-2'-deoxycytidine against leukemic and hemopoietic tissues in AKR mice. *Neoplasma* **1968**, *15*, 339-343.
56. Seidel, C.; Florean, C.; Schnekenburger, M.; Dicato, M.; and Diederich, M.: Chromatin-modifying agents in anti-cancer therapy. *Biochimie* **2012**, *94*, 2264-2279.
57. Hollenbach, P. W.; Nguyen, A. N.; Brady, H.; Williams, M.; Ning, Y.; Richard, N.; Krushel, L.; Aukerman, S. L.; Heise, C.; and MacBeth, K. J.: A Comparison of Azacitidine and Decitabine Activities in Acute Myeloid Leukemia Cell Lines. *PLoS One* **2010**, *5*, e9001.
58. Szmigielska-Kaplon, A.; and Robak, T.: Hypomethylating Agents in the Treatment of Myelodysplastic Syndromes and Myeloid Leukemia. *Curr. Cancer Drug Targets* **2011**, *11*, 837-848.
59. Costa, R.; Abdulhaq, H.; Haq, B.; Shadduck, R. K.; Latsko, J.; Zenati, M.; Atem, F. D.; Rossetti, J. M.; Sahovic, E. A.; and Lister, J.: Activity of Azacitidine in Chronic Myelomonocytic Leukemia. *Cancer* **2011**, *117*, 2690-2696.
60. Hagemann, S.; Heil, O.; Lyko, F.; and Brueckner, B.: Azacytidine and Decitabine Induce Gene-Specific and Non-Random DNA Demethylation in Human Cancer Cell Lines. *PLoS One* **2011**, *6*, e17388.

61. Li, L. H.; Olin, E. J.; Buskirk, H. H.; and Reineke, L. M.: Cytotoxicity and Mode of Action of 5-Azacytidine on L1210 Leukemia. *Cancer Res.* **1970**, *30*, 2760-2769.
62. Christman, J. K.: 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* **2002**, *21*, 5483-5495.
63. Veselý, J.; Čihák, A.; and Šorm, F.: Biochemical mechanisms of drug resistance. IX. Metabolic alterations in leukemic mouse cells following 5-aza-2'-deoxycytidine. *Collec. Czech. Chem. Commun.* **1969**, *34*, 901-909.
64. Veselý, J.; and Čihák, A.: 5-Azacytidine: Mechanism of action and biological effects in mammalian cells. *Pharm. Ther.* **1978**, *2*, 813-840.
65. Lu, L.-J. W.; and Randerath, K.: Mechanism of 5-Azacytidine-induced Transfer RNA Cytosine-5-methyltransferase Deficiency. *Cancer Res.* **1980**, *40*, 2701-2705.
66. Lee, T. T.; and Karon, M. R.: Inhibition of protein synthesis in 5-azacytidine-treated HeLa cells. *Biochem. Pharmacol.* **1976**, *25*, 1737-1742.
67. Jones, P. A.; and Taylor, S. M.: Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **1980**, *20*, 85-93.
68. Yang, X.; Lay, F.; Han, H.; and Jones, P. A.: Targeting DNA methylation for epigenetic therapy. *Trends Pharmacol. Sci.* **2010**, *31*, 536-546.
69. Ren, J.; Singh, B. N.; Huang, Q.; Li, Z.; Gao, Y.; Mishra, P.; Hwa, Y. L.; Li, J.; Dowdy, S. C.; and Jiang, S.-W.: DNA hypermethylation as a chemotherapy target. *Cell. Signal.* **2011**, *23*, 1082-1093.
70. Nie, J.; Liu, L.; Li, X.; and Han, W.: Decitabine, a new star in epigenetic therapy: the clinical application and biological mechanism in solid tumors. *Cancer Lett.* **2014**, *354*, 12-20.
71. Cowan, L. A.; Talwar, S.; and Yang, A. S.: Will DNA methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors. *Epigenomics* **2010**, *2*, 71-86.
72. Estey, E. H.: Epigenetics in clinical practice: the examples of azacitidine and decitabine in myelodysplasia and acute myeloid leukemia. *Leukemia* **2013**, *27*, 1803-1812.
73. Gailhouste, L.; Liew, L. C.; Hatada, I.; Nakagama, H.; Ochiya, T.: Epigenetic reprogramming using 5-azacytidine promotes an anti-cancer response in pancreatic adenocarcinoma cells. *Cell Death Dis.* **2018**, *9*, 468-479.
74. Dračinský, M.; Krečmerová, M.; and Holý, A.: Study of chemical stability of antivirally active 5-azacytosine acyclic nucleoside phosphonates using NMR spectroscopy. *Bioorg. Med. Chem.* **2008**, *16*, 6778-6782.

75. Matoušová, M.; Votruba, I.; Otmar, M.; Tloušťová, E.; Günterová, J.; and Mertlíková-Kaiserová, H.: 2'-deoxy-5,6-dihydro-5-azacytidine – a less toxic alternative of 2'-deoxy-5-azacytidine. A comparative study of hypomethylating potential. *Epigenetics* **2011**, *6*, 769-776.
76. Issa, J.-P. J.; Garcia-Manero, G.; Giles, F. J.; Mannari, R.; Thomas, D.; Faderl, S.; Bayar, E.; Lyons, J.; Rosenfeld, C. S.; Cortes, J.; and Kantarjian, H. M.: Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood* **2004**, *103*, 1635-1640.
77. Stresemann, C.; and Lyko, F.: Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int. J. Cancer* **2008**, *123*, 8-13.
78. Beisler, J. A.; Abbasi, M. M.; Kelley, J. A.; and Driscoll, J. S.: Synthesis and Antitumor Activity of Dihydro-5-azacytidine, a Hydrolytically Stable Analogue of 5-Azacytidine. *J. Med. Chem.* **1977**, *20*, 806-812.
79. Glazer, R. I.; Peale, A. L.; Beisler, J. A.; and Abbasi, M. M.: The effect of 5-azacytidine and dihydro-5-azacytidine on nuclear ribosomal RNA and poly(A) RNA synthesis in L1210 cells in vitro. *Mol. Pharmacol.* **1980**, *17*, 111-117.
80. Traganos, F.; Staiano-Coico, L.; Darzynkiewicz, Z.; and Melamed, M. R.: Effects of Dihydro-5-azacytidine on Cell Survival and Cell Cycle Progression of Cultured mammalian Cells. *Cancer Res.* **1981**, *41*, 780-789.
81. Yogelzang, N. J.; Herndon, J. E.; Cirrincione, C.; Harmon, D. C.; Antman, K. H.; Corson, J. M.; Suzuki, Y.; Citron, M. L.; and Green, M. R.: Dihydro-5-Azacytidine in Malignant Mesothelioma. *Cancer* **1997**, *79*, 2237-2242.
82. Pískala, A.; Česneková, B.; and Veselý, J.: Preparation and biological activity of 5,6-dihydro-5-azapyrimidine nucleosides. *Nucleic Acids Symp. Ser.* **1987**, *18*, 57-60.
83. Murakami, E.; Basavapathruni, A.; Bradley, W. D.; and Anderson, K. S.: Mechanism of action of a novel viral mutagenic covert nucleotide: molecular interactions with HIV-1 reverse transcriptase and host cell DNA polymerases. *Antiviral Res.* **2005**, *67*, 10-17.
84. Krečmerová, M.; Holý, A.; Pískala, A.; Masojídková, M.; Andrei, G.; Naesens, L.; Neyts, J.; Balzarini, J.; De Clercq, E.; and Snoeck, R.: Antiviral Activity of Triazine Analogues of 1-(S)-[3-Hydroxy-2-(phosphonomethoxy)propyl]cytosine (Cidofovir) and Related Compounds. *J. Med. Chem.* **2007**, *50*, 1069-1077.
85. Krečmerová, M.; Holý, A.; Pohl, R.; Masojídková, M.; Andrei, G.; Naesens, L.; Neyts, J.; Balzarini, J.; De Clercq, E.; and Snoeck, R.: Ester Prodrugs of Cyclic 1-(S)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine: Synthesis and Antiviral Activity. *J. Med. Chem.* **2007**, *50*, 5765-5772.

86. Krečmerová, M.; Masojídková, M.; and Holý, A.: Acyclic nucleoside phosphonates with 5-azacytosine base moiety substituted in C-6 position. *Bioorg. Med. Chem.* **2010**, *18*, 387-395.
87. Dawood, K. M.; and Farghaly, T. A.: Thiadiazole inhibitors: a patent review. *Expert Opin. Ther. Pat.* **2017**, *27*, 477-505.
88. Kumar, K. A.; Kumar, G. V.; and Renuka, N.: Thiadiazoles: Molecules Of Diverse Applications - A Review. *Int. J. Pharmtech Res.* **2013**, *5*, 239-248.
89. Surov, A. O.; Bui, C. T.; Proshin, A. N.; Roussel, P.; Idrissi, A.; and Perlovich, G. L.: Novel 1,2,4-Thiadiazole Derivatives: Crystal Structure, Conformational Analysis, Hydrogen Bond Networks, Calculations, and Thermodynamic Characteristics of Crystal Lattices. *J. Phys. Chem. B* **2013**, *117*, 10414-10429.
90. Unangst, P. C.; Shrum, G. P.; Connor, D. T.; Dyer, R. D.; and Schrier, D. J.: Novel 1,2,4-Oxazolines and 1,2,4-Thiadiazoles as Dual 5-Lipoxygenase and Cyclooxygenase Inhibitors. *J. Med. Chem.* **1992**, *35*, 3691-3698.
91. Song, Y.; Connor, D. T.; Sercel, A. D.; Sorenson, R. J.; Doubleday, R.; Unangst, P. C.; Roth, B. D.; Beylin, V. G.; Gilbertsen, R. B.; Chan, K.; Schrier, D. J.; Guglietta, A.; Bornemeier, D. A.; and Dyer, R. D.: Synthesis, Structure-Activity Relationships, and in Vivo Evaluations of Substituted Di-*tert*-butylphenols as a Novel Class of Potent, Selective, and Orally Active Cyclooxygenase-2 Inhibitors. 2. 1,3,4- and 1,2,4-Thiadiazole Series. *J. Med. Chem.* **1999**, *42*, 1161-1169.
92. Kohara, Y.; Kubo, K.; Imamiya, E.; Wada, T.; Inada, Y.; and Naka, T.: Synthesis and Angiotensin II Receptor Antagonistic Activities of Benzimidazole Derivatives Bearing Acidic Heterocycles as Novel Tetrazole Bioisosteres. *J. Med. Chem.* **1996**, *39*, 5228-5235.
93. Hara, R.; Sakamoto, K.; Hisamichi, H.; and Nagano, N.: Structure-activity Relationships of Cephalosporins Having a (Dimethylisoxazolidinio)vinyl Moiety at Their 3-Position. *J. Antibiot.* **1996**, *49*, 1162-1171.
94. Ishikawa, T.; Iizawa, Y.; Okonogi, K.; and Miyake, A.: Studies on Anti-MRSA Parenteral Cephalosporins. I. Synthesis and Antibacterial Activity of 7 β -[2-(5-Amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-hydroxyiminoacetamido]-3-(substituted imidazo[1,2-*b*]-pyridazinium-1-yl)methyl-3-cephem-4-carboxylates and Related Compounds. *J. Antibiot.* **2000**, *53*, 1053-1070.
95. Gupta, A.; Mishra, P.; Pandeya, S. N.; Kashaw, S. K.; Kashaw, V.; and Stables, J. P.: Synthesis and anticonvulsant activity of some substituted 1,2,4-thiadiazoles. *Eur. J. Med. Chem.* **2009**, *44*, 1100-1105.

96. Castro, A.; Castaño, T.; Encinas, A.; Porcal, W.; and Gil, C.: Advances in the synthesis and recent therapeutic applications of 1,2,4-thiadiazole heterocycles. *Bioorg. Med. Chem.* **2006**, *14*, 1644-1652.
97. Fawzi, A. B.; MacDonald, D.; Benbow, L. L.; Smith-Torhan, A.; Zhang, H.; Weig, B. C.; Ho, G.; Tulshian, D.; Linder, M. E.; and Graziano, M. P.: SCH-202676: An Allosteric Modulator of Both Agonist and Antagonist Binding to G Protein-Coupled Receptors. *Mol. Pharmacol.* **2001**, *59*, 30-37.
98. Perlovich, G. L.; Proshin, A. N.; Volkova, T. V.; Petrova, L. N.; and Bachurin, S. O.: Novel 1,2,4-Thiadiazole Derivatives as Potent Neuroprotectors: Approach to Creation of Bioavailable Drugs. *Mol. Pharm.* **2012**, *9*, 2156-2167.
99. Martinez, A.; Alonso, M.; Castro, A.; Pérez, C.; and Moreno, F. J.: First Non-ATP Competitive Glycogen Synthase Kinase 3 β (GSK-3 β) Inhibitors: Thiadiazolidinones (TDZD) as potential Drugs for the Treatment of Alzheimer's Disease. *J. Med. Chem.* **2002**, *45*, 1292-1299.
100. Lovestone, S.; Boada, M.; Dubois, B.; Hüll, M.; Rinne, J. O.; Huppertz, H.-J.; Calero, M.; Andrés, M. V.; Gómez-Carrillo, B.; León, T.; and Del Ser, T.: A Phase II Trial of Tideglusib in Alzheimer's Disease. *J. Alzheimers Dis.* **2015**, *45*, 75-88.
101. Iizawa, Y.; Okonogi, K.; Hayashi, R.; Iwahi, T.; Yamazaki, T.; and Imada, A.: Therapeutic Effect of Cefozopran (SCE-2787), a New Parenteral Cephalosporin, against Experimental Infections in Mice. *Antimicrob. Agents Chemother.* **1993**, *37*, 100-105.
102. Pham, C.-D.; Weber, H.; Hartmann, R.; Wray, V.; Lin, W.; Lai, D.; and Proksch, P.: New Cytotoxic 1,2,4-Thiadiazole Alkaloids from the Ascidian *Polycarpa aurata*. *Org. Lett.* **2013**, *15*, 2230-2233.
103. Chen, M.-H.; Lin, S.; Wang, Y.-N.; Zhu, C.-G.; Li, Y.-H.; Jiang, J.-D.; and Shi, J.-G.: Antiviral stereoisomers of 3,5-bis(2-hydroxybutyl-3-en-1-yl)-1,2,4-thiadiazole from the roots of *Isatis indigotica*. *Chin. Chem. Lett.* **2016**, *27*, 643-648.
104. Yang, Z.; Huang, N.; Xu, B.; Huang, W.; Xie, T.; Cheng, F.; and Zou, K.: Cytotoxic 1,3-Thiazole and 1,2,4-Thiadiazole Alkaloids from *Penicillium oxalicum*: Structural Elucidation and Total Synthesis. *Molecules* **2016**, *21*, 232-243.
105. Heitz, S.; Durgeat, M.; Guyot, M.; Brassy, C.; and Bachet, B.: Nouveau derive indolique du thiadiazole-1,2,4, isole d'un tunicier (*Dendrodoa grossularia*). *Tetrahedron Lett.* **1980**, *21*, 1457-1458.
106. Yang, T.; Zhang, T.; Guan, X.-N.; Dong, Z.; Lan, L.; Yang, S.; and Yang, C.-G.: Tideglusib and Its Analogues As Inhibitors of *Staphylococcus aureus* SrtA. *J. Med. Chem.* **2020**, *63*, 8442-8457.

107. Marrano, C.; De Macédo, P.; Gagnon, P.; Lapierre, D.; Gravel, C.; Keillor, J. W.: Synthesis and evaluation of novel dipeptide-bound 1,2,4-Thiadiazoles as irreversible inhibitors of guinea pig liver transglutaminase. *Bioorg. Med. Chem.* **2001**, *9*, 3231-3241.
108. Leung-Toung, R.; Wodzinska, J.; Li, W.; Lowrie, J.; Kukreja, R.; Desilets, D.; Karimian, K.; and Fat Tam, T.: 1,2,4-Thiadiazole: A Novel Cathepsin B Inhibitor. *Bioorg. Med. Chem.* **2003**, *11*, 5529-5537.
109. Storer, A. C.; and Ménard, R.: Recent insights into cysteine protease specificity: Lessons for drug design. *Perspect. Drug Discov. Des.* **1996**, *6*, 33-46.
110. Barrett, A.; Rawlings, N.; and Woessner, J.: Handbook of Proteolytic Enzymes, pp 545. *Academic Press*, 3rd Edition, London **1998**, ISBN: 9780123822208.
111. Párkányi, C.; Yuan, H. L.; Cho, N. S.; Jaw, J.-H. J.; Woodhouse, T. E.; and Aung, T. L.: Synthesis of 2-(2',3'-Dihydroxypropyl)-5-amino-2*H*-1,2,4-thiadiazol-3-one and 3-(2',3'-Dihydroxypropyl)-5-amino-3*H*-1,3,4-thiadiazol-3-one. *J. Heterocyclic Chem.* **1989**, *26*, 1331-1334.
112. Pískala, A.; Vachálková, A.; Masojídková, M.; Horváthová, K.; Ovesná, Z.; Pačes, V.; and Novotný, L.: Improved synthesis, antibacterial activity and potential carcinogenicity of 5-amino-1,2,4-thiadiazol-3(2*H*)-one. *Pharmazie* **2004**, *59*, 756-762.
113. Abraham, D. J.: Burger's Medicinal Chemistry and Drug Discovery, pp 690. *Wiley Online Library* **2003**, Online ISBN: 9780471266945.
114. Li, Y.; Geng, J.; Liu, Y.; Yu, S.; and Zhao, G.: Thiadiazole – a Promising Structure in Medicinal Chemistry. *ChemMedChem* **2013**, *8*, 27-41.
115. Revankar, G. R.; and Robins, R. K.: Synthesis of certain 1,2,4-Thiadiazole nucleosides. *J. Heterocycl. Chem.* **1976**, *13*, 169-170.
116. Revankar, G. R.; and Robins, R. K.: 1,2,4-Thiadiazolidine-3,5-dione. ICN Pharmaceuticals, Patent US4093624A (06.06.1978).
117. Ling Chwang, T.; Němec, J.; and Welch, A. D.: Synthesis and characterization of 5-amino-2-(beta-D-ribofuranosyl)-1,2,4-thiadiazoline-3-one, a sulfur-containing analog of cytidine. *J. Carbohydr. Nucleosides Nucleotides* **1980**, *7*, 159-166.
118. Ra, D. Y.; Cho, N. S.; Moon, J. H.; and Kang, S. K.: Synthetic and Spectroscopic Study of 5-Amino-2-acyl-1,2,4-thiadiazolin-3-ones. *J. Heterocyclic Chem.* **1998**, *35*, 1435-1439.
119. Cho, N. S.; Park, Y. C.; Ra, D. Y.; and Kang, S. K.: Tautomerism and Alkylation of 5-Amino-2*H*-1,2,4-thiadiazoline-3-one. *J. Korean Chem. Soc.* **1995**, *39*, 564-571.
120. Fonović, M.; and Turk, B.: Cysteine cathepsins and their potential in clinical therapy and biomarker discovery. *Proteomics Clin. Appl.* **2014**, *8*, 416-426.

121. Wijkman, J.; and Gossen, J.: Inhibitors of cathepsin K: a patent review (2004 – 2010). *Expert Opin. Ther. Patents* **2011**, *21*, 1611-1629.
122. Turk, V.; Stoka, V.; Vasiljeva, O.; Renko, M.; Sun, T.; Turk, B.; and Turk, D.: Cysteine cathepsins: From structure, function and regulation to new frontiers. *Biochim. Biophys. Acta* **2012**, *1824*, 68-88.
123. Zhao, Q.; Jia, Y.; and Xiao, Y.: Cathepsin K: A therapeutic target for bone diseases. *Biochem. Biophys. Res. Commun.* **2009**, *380*, 721-723.
124. Stoch, S. A.; and Wagner, J. A.: Cathepsin K Inhibitors: A Novel Target for Osteoporosis Therapy. *Clin. Pharmacol. Ther.* **2008**, *83*, 172-176.
125. Xiao, Y.; Junfeng, H.; Tianhong, L.; Lu, W.; Shulin, C.; Yu, Z.; Xiaohua, L.; Weixia, J.; Sheng, Z.; Yanyun, G.; Guo, L.; and Min, L.: Cathepsin K in Adipocyte Differentiation and Its Potential Role in the Pathogenesis of Obesity. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 4520-4527.
126. Yang, M.; Sun, J.; Zhang, T.; Liu, J.; Zhang, J.; Shi, M. A.; Darakhshan, F.; Guerre-Millo, M.; Clement, K.; Gelb, B. D.; Dolgiov, G.; and Shi, G.-P.: Deficiency and Inhibition of Cathepsin K Reduce Body Weight Gain and Increase Glucose Metabolism in Mice. *Arterioscler. Thromb. Vasc. Biol.* **2008**, *28*, 2202-2208.
127. Lu, J.; Wang, M.; Wang, Z.; Fu, Z.; Lu, A.; and Zhang, G.: Advances in the discovery of cathepsin K inhibitors on bone resorption. *J. Enzyme Inhib. Med. Chem.* **2018**, *33*, 890-904.
128. Drake, M. T.; Clarke, B. L.; Oursler, M. J.; and Khosla, S.: Cathepsin K Inhibitors for Osteoporosis: Biology, Potential Clinical Utility, and Lessons Learned. *Endocr. Rev.* **2017**, *38*, 325-350.
129. Walz, A.; Ugolkov, A.; Chandra, S.; Kozikowski, A.; Carneiro, B. A.; O'Halloran, T. V.; Giles, F. J.; Billadeau, D. D.; and Mazar, A. P.: Molecular Pathways: Revisiting Glycogen Synthase Kinase-3 β as a Target for the Treatment of Cancer. *Clin. Cancer Res.* **2017**, *23*, 1891-1897.
130. Embi, N.; Rylatt, D. B.; and Cohen, P.: Glycogen Synthase Kinase-3 from Rabbit Skeletal Muscle. Separation from Cyclic-AMP-Dependent Protein Kinase and Phosphorylase Kinase. *Eur. J. Biochem.* **1980**, *107*, 519-527.
131. Forde, J. E.; and Dale, T. C.: Glycogen synthase kinase 3: A key regulator of cellular fate. *Cell. Mol. Life Sci.* **2007**, *64*, 1930-1944.
132. Kaidanovich-Beilin, O.; and Woodgett, J. R.: GSK-3: Functional Insights from Cell Biology and Animal Models. *Front. Mol. Neurosci.* **2011**, *4*, 40.
133. Hoeflich, K. P.; Luo, J.; Rubie, E. A.; Tsao, M.-S.; Jin, O.; and Woodgett, J. R.: Requirement for glycogen synthase kinase-3 β in cell survival and NF-kappaB activation. *Nature* **2000**, *406*, 86-90.

134. Pandey, M. K.; and DeGrado, T. R.: Glycogen Synthase Kinase-3 (GSK-3)-Targeted Therapy and Imaging. *Theranostics* **2016**, *6*, 571-593.
135. Hernández, F.; and Avila, J.: The role of glycogen synthase kinase 3 in the early stages of Alzheimer's disease. *FEBS Lett.* **2008**, *582*, 3848-3854.
136. Mathuram, T. L.; Ravikumar, V.; Reece, L. M.; Karthik, S.; Sasikumar, C. S.; and Cherian, K. M.: Tideglusib induces apoptosis in human neuroblastoma IMR32 cells, provoking sub-G₀/G₁ accumulation and ROS generation. *Environ. Toxicol. Pharmacol.* **2016**, *46*, 194-205.
137. Zhou, A.; Lin, K.; Zhang, S.; Chen, Y.; Zhang, N.; Xue, J.; Wang, Z.; Aldape, K. D.; Xie, K.; Woodgett, J. R.; and Huang, S.: Nuclear GSK3 β promotes tumorigenesis by phosphorylating KDM1A and inducing its deubiquitination by USP22. *Nat. Cell. Biol.* **2016**, *18*, 954-966.
138. Horrigan, J.; Gomes, T. B.; Snape, M.; Nikolenko, N.; McMorn, A.; Evans, S.; Yaroshinsky, A.; Della Pasqua, O.; Oosterholt, S.; and Lochmüller, H.: A Phase 2 Study of AMO-02 (Tideglusib) in Congenital and Childhood-Onset Myotonic Dystrophy Type 1 (DM1). *Pediatr. Neurol.* **2020**, *112*, 84-93.
139. Shinji, M.; Hiroshige, F.; and Hajime, T.: Efficacy and Safety of Glycogen Synthase Kinase 3 Inhibitors for Alzheimer's Disease: A Systematic Review and Meta-Analysis. *J. Alzheimers Dis.* **2019**, *69*, 1031-1039.
140. Neves, V. C. M.; Babb, R.; Chandrasekaran, D.; and Sharpe, P. T.: Promotion of natural tooth repair by small molecule GSK3 antagonists. *Sci. Rep.* **2017**, *7*, 39654.
141. Wang, H.; Huang, S.; Yan, K.; Fang, X.; Abussaud, A.; Martinez, A.; Sun, H.-S.; and Feng, Z.-P.: Tideglusib, a chemical inhibitor of GSK3 β , attenuates hypoxic-ischemic brain injury in neonatal mice. *Biochim. Biophys. Acta* **2016**, *1860*, 2076-2085.
142. Česnek, M.; Skácel, J.; Jansa, P.; Dračinský, M.; Šmídková, M.; Mertlíková-Kaiserová, H.; Soto-Velasquez, M. P.; Watts, V. J.; and Janeba, Z.: Nucleobase Modified Adefovir (PMEA) Analogues as Potent and Selective Inhibitors of Adenylate Cyclases from *Bordetella pertussis* and *Bacillus anthracis*. *ChemMedChem* **2018**, *13*, 1779-1796.
143. Holý, A.; Rosenberg, I.; and Dvořáková, H.: Synthesis of N-(2-phosphonylmethoxyethyl) derivatives of heterocyclic bases. *Collect. Czech Chem. Commun.* **1989**, *54*, 2190-2210.
144. Castro, A.; Encinas, A.; Gil, C.; Bräse, S.; Porcal, W.; Pérez, C.; Moreno, F. J.; and Martínez, A.: Non-ATP competitive glycogen synthase kinase 3 β (GSK-3 β) inhibitors: Study of structural requirements for thiadiazolidinone derivatives. *Bioorg. Med. Chem.* **2008**, *16*, 495-510.
145. Cai, Z. R.; Jabri, S. Y.; Jin, H.; Kim, C. U.; Metobo, S. E.; Mish, M. R.; and Pastor, R. M.: Antiviral phosphonate conjugates for inhibition of HIV. Patent WO/2007/14352 (01.02.2007).

146. Xie, A.; Yan, J.; Yue, L.; Feng, F.; Mir, F.; Abdel-Halim, H.; Chebib, M.; Le Breton, G. C.; Standaert, R. F.; Qian, H.; and Pepperberg, D. R.: 2-Aminoethyl Methylphosphonate, a Potent and Rapidly Acting Antagonist of GABA_A-p1 Receptors. *Mol. Pharmacol.* **2011**, *80*, 965-978.
147. Sleczkowski, M. L.; Meijer, E. W.; and Palmans, A. R. A.: Cooperative Folding of Linear Poly(dimethyl siloxane)s via Supramolecular Interactions. *Macromol. Rapid Commun.* **2017**, *38*, 1700566.
148. Tiwari, L.; Kumar, V.; Kumar, B.; and Mahajan, D.: A practically simple, catalyst free and scalable synthesis of *N*-substituted ureas in water. *RSC Adv.* **2018**, *8*, 21585-21595.
149. Panicker, B.; Lim, D. S.; Mishra, R. K.; Oehlen, L. J. W. M.; Tarrant, J. G.; Zhu, X.; and Jung, D.: Cytochrome P450 inhibitors and uses thereof. Patent US2018/148438 A1 (31.05.2018).
150. Chang, C.-D.; Waki, M.; Ahmad, M.; Meienhofer, J.; Lundell, E. O.; and Haug, J. D.: Preparation and properties of *N*^α-9-fluorenylmethyloxycarbonylamino acids bearing *tert*-butyl side chain protection. *Int. J. Peptide Protein Res.* **1980**, *15*, 59-66.
151. Zhang, Z.; Feng, L.; Liu, S.; Wang, C.; Li, J.; Feng, J.: Synthesis method of 5-aminolevulinic acid hydrochloride. Patent CN109608421 (12.04.2019).
152. Chen, C.-C.; Rajagopal, B.; Liu, X. Y.; Chen, K. L.; Tyan, Y.-C.; Lin, F.; and Lin, P.-C.: A mild removal of Fmoc group using sodium azide. *Amino Acids* **2014**, *46*, 367-374.
153. Kurzer, F.; and Taylor, S. A.: Thiadiazoles. Part VI. 5-Amino-3-hydroxy-1 : 2 : 4-thiadiazole Derivatives. *J. Chem. Soc.* **1958**, *0*, 379-386.
154. Cohen, P.; and Goedert, M.: GSK3 Inhibitors: Development and Therapeutic Potential. *Nat. Rev. Drug Discov.* **2004**, *3*, 479-487.
155. Domínguez, J. M.; Fuertes, A.; Orozco, L.; del Monte-Millán, M.; Delgado, E.; and Medina, M.: Evidence for Irreversible Inhibition of Glycogen Synthase Kinase-3β by Tideglusib. *J. Biol. Chem.* **2012**, *287*, 893-904.

Part II

Development of small molecules targeting *c-MYC* oncogene

Part of the results described and discussed in this section of the thesis was published in the following article:

Pomeislová, A.; Vrzal, L.; Kozák, J.; Dobiaš, J.; Hubálek, M.; Dvořáková, H.; Reyes-Gutiérrez, P. E.; Teplý, F.; and Veverka, V.: Kinetic Target-Guided Synthesis of Small-Molecule G-Quadruplex Stabilizers. *ChemistryOpen* **2020**, *9*, 1236-1250.

The results summarized in Chapter 9.1.2.3 (Structural basis of compound binding by chemical-shift perturbation NMR) were obtained by Dr. Lukáš Vrzal and presented in his Ph.D. thesis (ref. [10]), which was properly cited in the text.

7 Introduction

7.1 Methods in drug discovery

Drug development methods have been experiencing boom as there is a growing need of innovative medicines for various diseases, such as many types of cancer and infections, neurodegenerative diseases, and diabetes. In spite of increasing investment into research and development, the number of molecules approved by the USA Food and Drug Administration per year remains constant at 20-30 compounds. Novel strategies in drug development efforts, which would enhance the efficiency of this process, are therefore necessary [1]. In the following overview, drug discovery methods will be presented with emphasis on fragment-based lead discovery FBLD [2] and target-guided synthesis (TGS) [3, 4] that were employed in our research, both taking advantage of click chemistry.

7.1.1 Fragment-based lead discovery

The FBLD technique identifies small biologically active molecules, so-called "hits", that are capable of binding to a specific target. After their evaluation for target binding by biophysical methods, these compounds serve as starting points for further elaboration in the drug discovery process. FBLD is an alternative method to the more traditional high-throughput screening (HTS), which is based on screening of large chemical libraries – usually numbering hundreds of thousands of molecules – against a biological target. Over the last two decades, FBLD has become an established technique both in industry and academia and its impact has been still growing [2, 5-9].

Generally, FBLD starts with screening of a small library of low molecular weight compounds (in the M_r range ~ 150 -300), so-called "fragments", for their binding ability to a particular target – usually a protein. Contrary to HTS, the FBLD fragment libraries are usually small – they typically comprise few hundred to few thousand of compounds. The main advantage of the use of fragments is their low degree of complexity: they are big enough to efficiently interact with the target, while they are sufficiently small to bind to regions difficult to access (e.g. allosteric sites or essential small binding pockets) and to avoid unfavourable interactions, such as steric clashes. As a result, fragments that are identified as hits usually contain ideal binding motifs and thus constitute desirable starting points for further optimization. The binding affinity of such fragment hits is weak, usually in a μM -mM range, because they possess fewer heavy atoms (usually 8-20) able to form multiple attractive interactions with the target compared to larger molecules in HTS (M_r range ~ 250 -600). Enhancing hit potency after their identification can be achieved by fragment linking, fragment growing, or fragment merging strategies, which represent the key approaches of FBLD [8-9]. The first approved drug that evolved from FBLD hit is Vemurafenib that

targets BRAF kinase for metastatic melanoma treatment [10, 11]. Another example of FBLD hit-originated molecule is Venetoclax used for treatment of chronic lymphocytic leukemia targeting the BCL2 protein [10, 12].

7.1.1.1 Fragment library screening methods for hit identification

The aim of fragment hit identification is to select only those fragments that specifically bind to the target. Initially, a primary screen is performed with direct binding techniques, such as X-ray and NMR based methods, surface plasmon resonance, thermal shift assay methods, and microscale thermophoresis. Due to the low affinity of fragments, high concentrations (up to 1 or 2 mM) of screened compounds are used for the primary selection. Consequently, a number of fragments are identified as target binding molecules, including non-specific binders and false-positive hits. It is therefore necessary to use at least one secondary screening method for hit confirmation that would be based on different measurement principles. For this purpose, the most convenient are biophysical or biochemical techniques such as Förster resonance energy transfer (FRET) and polymerase extension assay (PEA) [9]. Both FRET and PEA will be further discussed in Chapter 7.2.3 in regard of ligand interaction with G4.

7.1.1.2 Fragment-to-lead optimization

Once the best fragment hit is identified, it is further elaborated by chemical synthesis to improve its affinity towards the target. There are three basic medicinal chemistry approaches to achieve this: fragment growing, fragment linking, and fragment merging (Figure 7.1) [7, 9, 13].

In the fragment growing approach, additional moieties that increase the affinity towards the target are attached upon increasing the size of the molecule. To achieve this, it is necessary to identify essential positions of the fragment scaffold, so-called "growth vectors", to which additional atoms, functional groups, or other binding scaffolds can be attached to increase its potency or selectivity. On the other hand, functionalities essential for target binding should be preserved. A successful lead candidate is obtained when the fragment is grown in the right direction and the size of substituents as well as attachment flexibility is appropriate. This method can be successfully combined with the other two approaches [9, 13].

Fragment linking constitutes another concept: two or more separate fragment hits that bind to different but adjacent sites of the target are linked together using a suitable linker moiety. Ideally, combination of several fragments with relatively low affinity results in larger molecule with significantly increased affinity towards the target. The main challenge is to retain the favourable orientation of each fragment as the resulting molecule becomes more rigid due to linking. Therefore, the binding mode of all fragments must be identified prior any linking. Optimal linker must not disrupt any functionality that is

critical for target binding, must not take any unfavourable interactions with the protein, and should be in a relaxed, unstrained state. For that reason, fragment linking approach has resulted in fewer clinical candidates than other methods [9, 13, 14].

The fragment merging approach is applied when two or more fragments bind to overlapping regions of the target. In such case, motifs of all fragments are merged into one compound that desirably exhibits higher affinity towards the target than original fragments. To perform merging of two or more fragments successfully, it is necessary to know the geometry and binding patterns of all fragments. Moreover, merging is easier when there are obvious structural similarities of the ligands. When no such similarities are apparent, structural data of ligand-target complexes are necessary to guide this approach [9, 13].

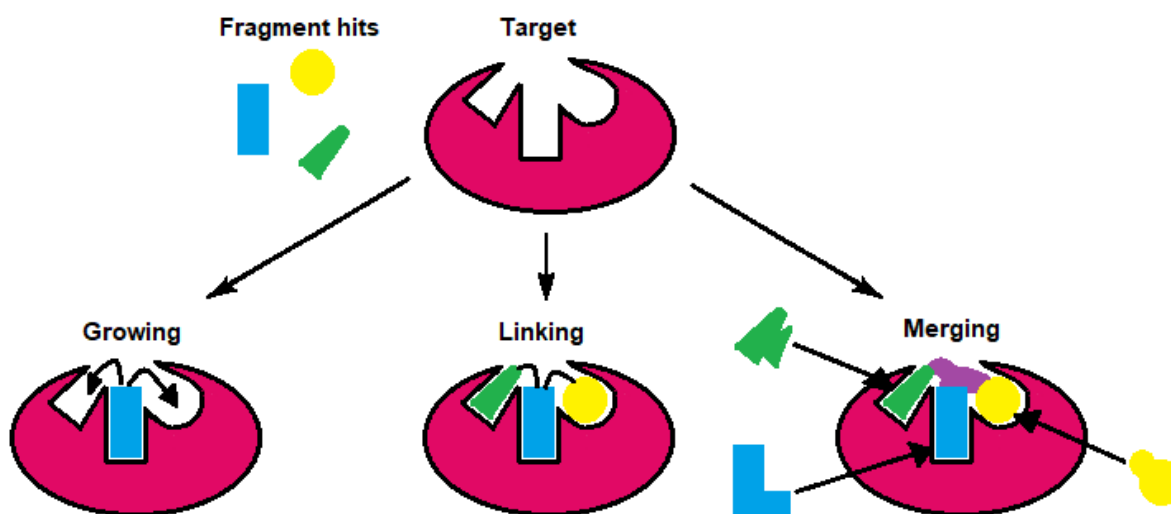


Figure 7.1: Hit-to-lead optimization approaches: fragment growing, fragment linking, and fragment merging (drawn according to ref.[13] with the consent of Prof. R. E. Hubbard).

After hit-to-lead optimization, the new derivatives can be evaluated with the previously used secondary (and sometimes also primary) hit identification techniques. Most importantly, these compounds should be compared with the original fragment hit regarding the affinity to the target. It is also desirable to determine their binding mode towards the target [9].

7.1.2 Target-guided synthesis and click chemistry

Contrary to FBLD, TGS is a dynamic template-assisted strategy to discover ligands that are assembled with the aid of the target. The ligands are formed *in situ* out of a pool of fragments bearing complementary reactive functional groups. Specific interactions between fragments and the biological target bring the two complementary molecules into a close proximity with a suitable orientation. As it is

the active site of the biological target that directs the choice of best binding partners, the most efficient ligand is assembled. This method addresses the problem encountered by FBLD that regards identification of low-affinity fragments and biologically efficient linkages [2-4, 15-17].

TGS has been used for over the last 20 years and bridges the gap between chemical synthesis and screening assays [3, 15]. The first discovery achieved through TGS was that of potent inhibitors of acetylcholine esterase, which constitutes a target for Alzheimer's disease [3, 16]. Further TGS applications led to invention of ligands targeting various enzymes, e.g., HIV-1 protease [3, 17], or NAD kinase from *L. monocytogenes* and *S. aureus* [3, 18].

There are two basic classes of TGS: dynamic combinatorial chemistry and kinetic target-guided synthesis (KTGS); the principle of both classes is depicted in Figure 7.2. In dynamic combinatorial chemistry, two libraries of complementary reacting fragments assemble in a reversible fashion and provide all possible combinations of assembled products in a "dynamic fragment library". When the target is added, the equilibrium of such assembled products is shifted towards compounds exhibiting the highest affinity towards the target. In order to determine the best hit combinations, composition of dynamic fragment libraries in the presence and absence of the target should be compared [3, 4].

On the other hand, KTGS employs only reactive fragments that assemble in an irreversible manner to form a larger molecule. Therefore, hit identification is based on the fact whether a given combination of reactive fragments can be readily detected. Provided that the product of KTGS is structurally similar to the transition state, its affinity towards the target is significantly improved compared to the affinity of each reagent [3, 4]. As KTGS was applied in our research, further text will be dedicated to this method.

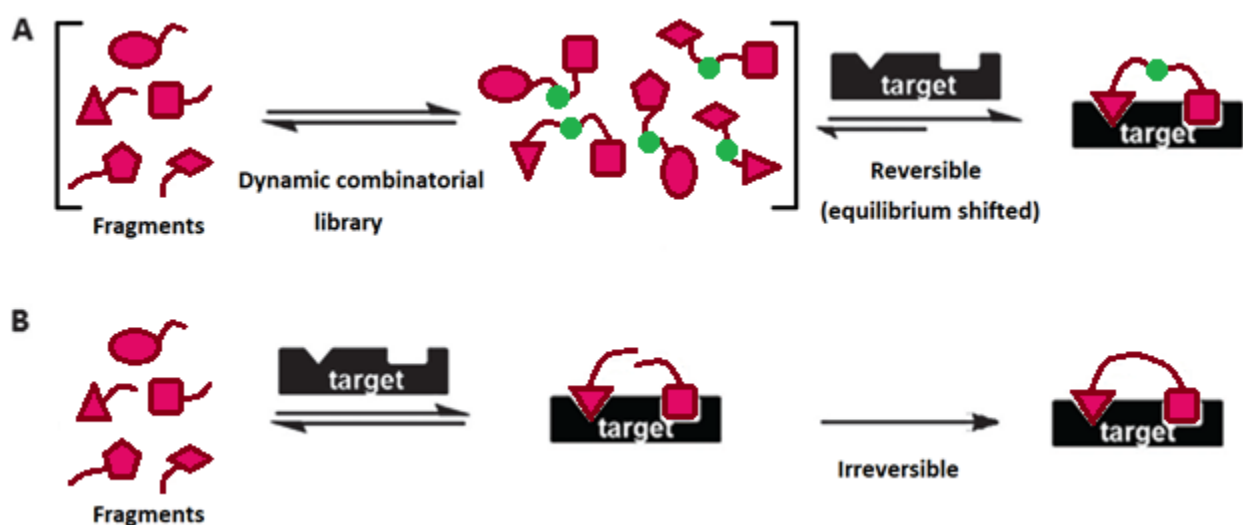
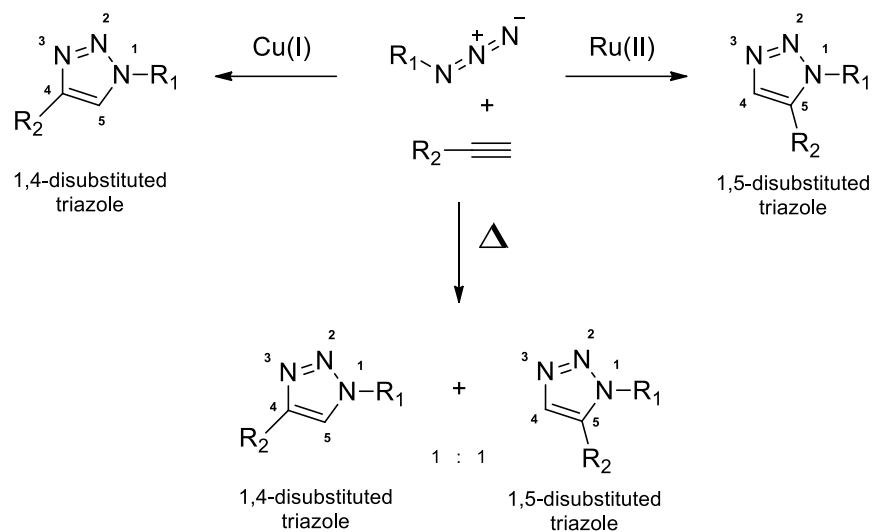


Figure 7.2: Basic principle of TGS: A – dynamic combinatorial chemistry approach, B – KTGS approach (adapted according to ref. [3, 19] with the consent of Prof. P.-Y. Renard).

As outlined previously, the TGS combinatory product is assembled from two fragments possessing complementary reactive groups [2]. To achieve this, KTGS employs irreversible chemical reaction, referred to as "click reaction", which joins two fragments with a heteroatom linker (C-X-C) to obtain a "click product". The concept of click chemistry was first introduced by K. B. Sharpless in 2001 [20] and since then has become widely adopted for the use in drug discovery – KTGS in particular [2]. A typical click reaction is modular, wide in scope, gives very high yields, generates only inoffensive byproducts that can be removed by nonchromatographic methods (such as crystallization or distillation), and is stereospecific, but not necessarily enantioselective. The criteria of a click reaction are as follows: simple and mild reaction conditions (ideally insensitive to water and oxygen), readily available starting materials and reagents, no solvent or a solvent that is benign (e.g., water), and simple product isolation. Moreover, the click product must be stable under physiological conditions [20]. The reaction types that possess these characteristics and have been so far used in KTGS are the Huisgen 1,3-dipolar cycloaddition [3, 19, 21-24], the thiol-ene reaction [3, 25-29], the sulfo-click reaction [3, 30-33], epoxide ring opening by thiols or amines [3, 34, 35], alkylation of thiols or amines with alkyl halides [3, 36, 37], and amidation reaction between acids and amines [3, 18]. In the following text, the Huisgen 1,3-dipolar cycloaddition will be introduced.

7.1.2.1 The Huisgen 1,3-dipolar cycloaddition

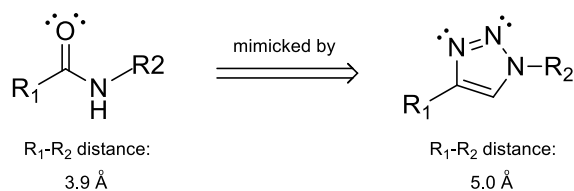
The most common click reaction used in KTGS is the Huisgen 1,3-dipolar cycloaddition – a reaction between an azide and an alkyne that results in 1,2,3-triazole formation. When uncatalyzed, it proceeds under harsh thermal conditions and provides a mixture of two regioisomers [19, 21]. Nevertheless, if an appropriate catalyst – Ru(II) or Cu(I) – is used, we can obtain each of these regioisomers selectively (Scheme 7.1) [22].



Scheme 7.1: The Huisgen 1,3-dipolar cycloaddition. Uncatalyzed reaction of a terminal alkyne with an azide affords 1,4- and 1,5-disubstituted triazoles approximately in the ratio 1:1. Under copper(I) catalysis we obtain 1,4-regioisomer, while the ruthenium(II) catalysis affords 1,5-regioisomer only (drawn according to ref. [19]).

The Huisgen 1,3-dipolar cycloaddition reaction was first reported by A. Michael more than 100 years ago [19, 23], but its mechanism was elucidated in 1960s by R. Huisgen *et al.* [21, 24] and its potential as a bioorthogonal reaction for KTGS was disclosed in 2001 by W. L. Mock *et al.* with cucubituril, a synthetic receptor, catalyzing the cycloaddition [19, 38]. Generally, the rate of the non-catalyzed reaction between an azide and a terminal alkyne is rather low due to necessity of the correct orbital alignment of reactants. In KTGS, specific interactions between fragments and the biological target bring the two molecules into a close proximity with a suitable orientation, which decreases the negative $\Delta S^\ddagger$ values of 1,3-dipolar cycloaddition, thereby overcoming the otherwise low reaction rate under non-catalyzed conditions [16, 39].

1,2,3-Triazoles possess an interesting biological activity: they behave as rigid linking units that can mimic the atom placement and electronic properties of a peptide bond without susceptibility to hydrolytic cleavage. Nevertheless, an increase in R_1 - R_2 distance of 1.1 Å over the typical amide bond was calculated due to the extra atom in the triazole backbone (Scheme 7.2) [40]. The main drawback of the Huisgen 1,3-dipolar cycloaddition reaction is azide handling, which is associated with specific hazards due to azide reactivity and sensitivity towards shock and friction. Therefore, adequate safety precautions must be taken during azide synthesis and isolation [3]. Nevertheless, it is an excellent tool for TGS [3] as there have been countless KTGS and dynamic combinatorial chemistry applications reported. For instance, we can mention discovery of acetylcholinesterase inhibitors [16, 41-44], HIV-1 protease inhibitors [17], or carbonic anhydrase II inhibitors [45].



Scheme 7.2: Topological and electronic similarities of amides and 1,4-disubstituted 1,2,3-triazoles. R_1 - R_2 distance is depicted for each structure (drawn according to ref. [40]).

In principle, the alkyne moiety stands for a dipolarophile (LUMO orbital) that interacts with the azide as a 1,3-dipole (HOMO orbital). Under non-catalyzed thermal conditions, the reaction proceeds through concerted mechanism and affords 1,4- and 1,5-disubstituted regioisomers in an approximate ratio of 1:1, provided that a terminal alkyne is used. This lack of regioselectivity is due to similar magnitude differences in HOMO-LUMO energy levels for both azides and alkynes; hence, the reaction is controlled by both dipole-HOMO and dipole-LUMO interactions simultaneously [40, 46, 47]. The advantage of the non-catalyzed Huisgen reaction producing both 1,4- and 1,5-disubstituted triazoles lies in enhancing the chemical diversity of potential ligands without expanding the number of reagents, nor the quantity of the target required [3].

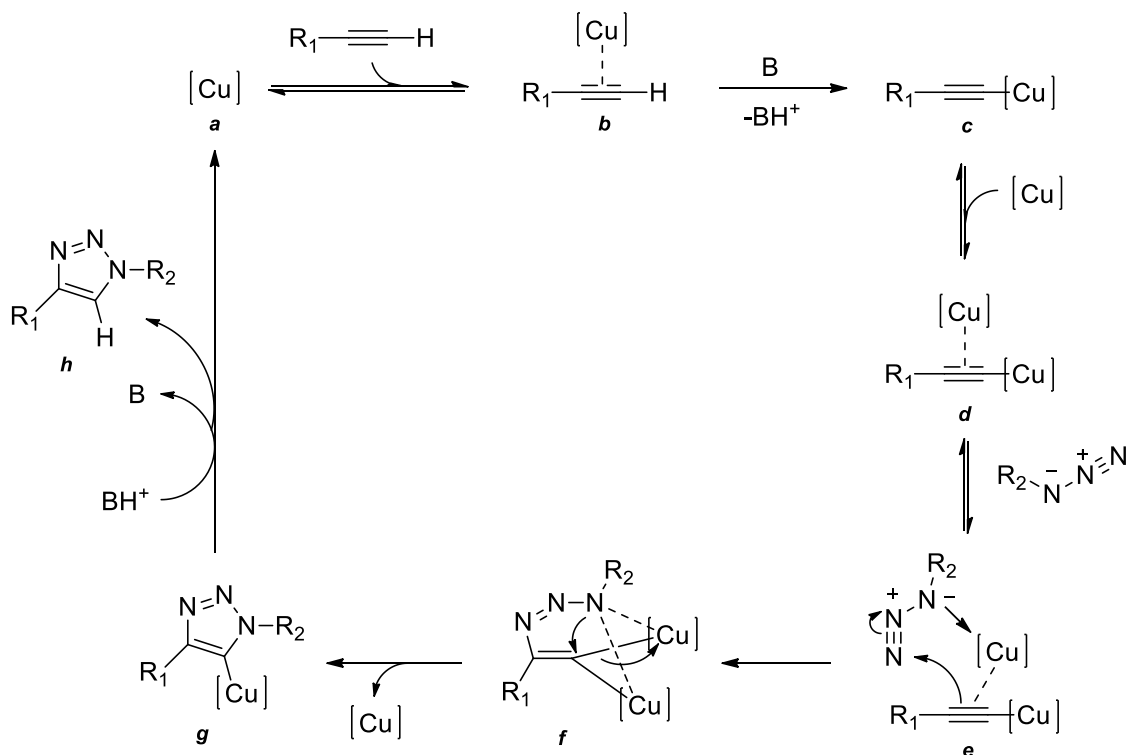
To obtain a particular regioisomer as the only product, there are two different catalytic approaches that complement each other in the sense of regioselectivity: the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) and ruthenium(II)-catalyzed azide-alkyne cycloaddition (RuAAC) [2, 22]. CuAAC affords 1,4-regioisomer of 1,2,3-triazoles selectively and is applicable only for terminal alkynes [2, 22]. On the contrary, RuAAC provides only the 1,5-regioisomer and works with both terminal and internal alkynes [2, 22]. However, only CuAAC has been considered as a click reaction; therefore, RuAAC will not be further discussed.

7.1.2.1.1 Copper(I)-catalyzed azide-alkyne cycloaddition

CuAAC is by far the most popular and utilized click reaction. It was discovered in 2002 simultaneously and independently by K. B. Sharpless [48] and M. Meldal [49]. The reaction can be performed with Cu(I) salts, such as CuBr or CuI, but it proceeds more efficiently when Cu(II) (e.g., Cu(OAc)₂ or CuSO₄) is used in a mixture with a reducing agent as sodium ascorbate to produce Cu(I) *in situ*. The reducing agent not only reduces Cu(II) to active Cu(I) for the reaction, but also reduces any dioxygen present and thus decreases the amount of oxidative byproducts. As a solvent, THF, DMSO, or alcohols (e.g., methanol, ethanol, *tert*-butanol) can be used, often in a mixture with water to dissolve all reaction components including Cu salts [2, 47]. The rate of CuAAC is more than 10⁷ times higher than of the non-catalyzed reaction; therefore it proceeds efficiently at r.t. [2, 40]. Moreover, this reaction tolerates

a variety of functional groups and provides the click product in a high yield with minimal work-up and purification [40].

In contrast to the non-catalyzed reaction, CuAAC no longer proceeds in concerted fashion, but rather in a sequence of discreet steps leading to triazole formation [47]. This fact was based on density functional theory calculations [40]. However, it is difficult to establish a precise mechanism as there are involved multiple equilibria between several reaction intermediates [2, 50]. Here, the mechanism comprising two copper atoms that was proposed by V. V. Fokin and his colleagues in 2013 [50] is presented (Scheme 7.3).



Scheme 7.3: Mechanism of CuAAC proposed by V. V. Fokin and his group in 2013 (drawn according to ref. [50]).

Initially, the Cu(I) species **a** generated *in situ* forms a π -complex **b** with the triple bond of the terminal alkyne. Then, the terminal hydrogen of the triple bond is deprotonated by a base and σ -bound copper-acetylide intermediate **c** is formed. Involvement of another copper species leads to intermediate **d** in which the second copper atom forms a weak π -complex with the triple bond. This catalytically active complex then coordinates an organic azide upon complex **e** formation. Afterwards, nucleophilic attack at the terminal nitrogen atom of azide by the β -carbon atom of acetylide forms the first covalent C-N bond, producing intermediate **f**. A fast ligand exchange (not displayed) leads to ring closure and a triazolide **g**

containing a copper atom in position 5 is formed. Finally, the 1,4-disubstituted 1,2,3-triazole as product ***h*** is dissociated upon protonation and catalyst species ***a*** regeneration [40, 50].

The main reason for a wide use of CuAAC lies in suitable properties of azides and alkynes: they tolerate oxygen, water, common organic synthetic conditions, biological molecules, a large number of solvents, a broad range of pH, and reaction conditions in living systems. However, the Cu(I) toxicity limits the CuAAC use for *in vivo* applications [2]. Nevertheless, CuAAC was successfully applied e.g., in TGS upon discovery of macrolide antibiotics targeting the bacterial ribosome [51], in oligonucleotide labelling [52], in polymer synthesis [53, 54], in receptor-ligand binding studies [55], or in designing molecular probes for biomolecule detection *in vivo* [56, 57].

7.2 The *c-MYC* protooncogene G-quadruplex in the role of target

7.2.1 G-quadruplexes

Apart from the standard right-handed double stranded B-form, DNA can adopt a variety of alternative conformations based on particular sequence motifs and interactions with various proteins. These non-canonical secondary structures include G-quadruplexes (G4s) [58] that were discovered in 1988 by D. Sen *et al* [59]. G4 structures consist of two or more G-tetrads that are stacked on the top of each other, forming a helical shape (Figure 7.3, Panel B). Each G-tetrad is composed of four guanines that are held in a planar arrangement through Hoogsteen hydrogen-bonding with stabilization provided by a monovalent cation such as K^+ or Na^+ , coordinated to the O^6 -lone pairs of each guanine (Figure 7.3, Panel A) [58-61]. While the stacked G-tetrads form the G4 structure, the intervening sequences are extruded as single-strand loops that vary in size and sequence [58].

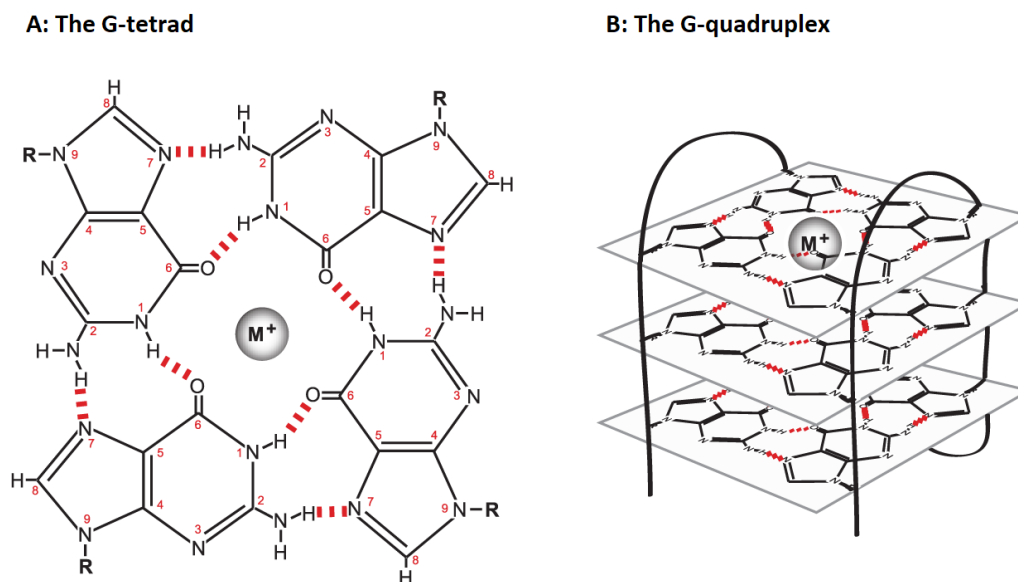


Figure 7.3: Panel A – the G-tetrad composed of four guanines bound by Hoogsteen base pairing; the metal cation in the cavity stabilizes the structure. Panel B – schematic representation of the G4 structure (taken from ref. [61] with the consent of Dr. D. K. Orren).

The high thermodynamic G4 stability under near-physiological conditions allows that these structures are formed in genomic DNA *in vivo*. Indeed, the G4 structures are found in guanine-rich regions of DNA that are present in telomeric regions, in both mitotic and meiotic double-strand break sites, and transcriptional start sites close to promoter regions. These findings suggest involvement of G4s in multiple roles such as replication, recombination, and transcription. Furthermore, it was found that failure to form the G4 structures leads to genomic instability [58, 60, 62-65].

This work concerns the G4 motifs near promoter regions that participate on gene regulation. Especially the promoters of human oncogenes and regulatory genes, such as *c-MYC*, *c-KIT*, *VEGF*, *BCL-2*, and *KRAS*, contain G4 motifs, while they are under-represented in tumor suppressor genes [58, 66, 67]. Provided that the G4 motif is encoded by the template DNA strand, the folded G4 secondary structure inhibits transcription as it prevents transcription factors from binding to transcription factor recognition sites [58, 67, 68, 69]. Furthermore, the G4 motif can be targeted by a small-molecule ligand that could stabilize the G4 structure and thus reinforce the transcription inhibition (Figure 7.4) [70, 71].

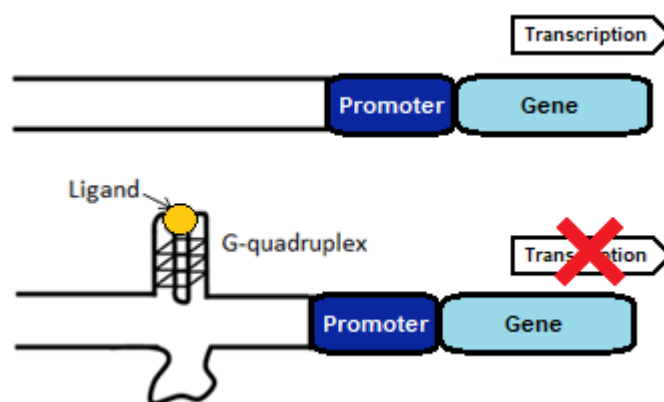


Figure 7.4: The impact of G4 formation near the promoter region on transcriptional activity. In the absence of G4 (top), transcription can proceed, while the presence of G4 (bottom) close to the promoter prevents transcription, especially with the aid of a ligand (adapted according to ref [70] with the consent of the publisher).

There is a general consensus that G4 binders stabilizing this secondary structure can represent an efficient tool in anti-cancer treatment. The known G4 binders are usually planar aromatic molecules that interact with the G4 structure mostly *via* external π - π stacking. Moreover, electrostatic interactions among positively charged ligands and negatively charged G4-DNA scaffolds also substantially participate on G4 stabilization. These positively charged ligands can be either cationic or *in situ* protonated. Nevertheless, there are also metallo-organic ligands that interact with G4 tetrad by positioning its metal centre over the cation channel of G4. These metallo-organic ligands target mostly telomeric G4s, although there are examples of such promoter-G4 binding molecules. [71]. Several examples of ligand types regarding the near-promoter G4s are shown in Figure 7.5.

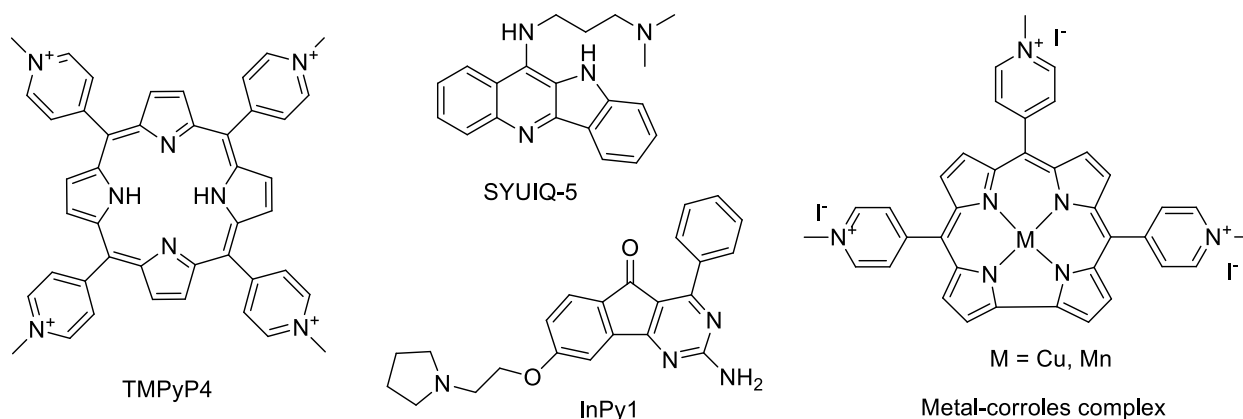


Figure 7.5: Examples of ligands targeting near-promoter G4s: TMPyP4 as a cationic ligand (drawn according to ref. [71]), SYUIQ-5 (drawn according to ref. [72]) and InPy1 (drawn according to ref. [73]) as ligands protonated *in situ*, and metal-corroles complex as a metallo-organic ligand (drawn according to ref. [74]).

Furthermore, there is growing evidence that the nature of the G4 loop has a significant impact on ligand selectivity for particular quadruplex fold [75]. It was shown that the flexibility of loops containing at least three nucleotides may be a determining factor in the selectivity of ligand binding, although the role of loops in ligand recognition is not yet well understood [68, 76]. As there are selective interactions between ligands and the G4 loops, there will be a growing trend to design ligands that would target the G4 secondary structure regarding its loop rather than the general G4 tetrad [68].

7.2.2 G-quadruplex of the protooncogene *c-MYC*

The human *c-MYC* is an oncoprotein transcription factor that is involved in regulation of cell growth, proliferation, differentiation, and apoptosis [77-81]. The gene that encodes this protein is tightly regulated in normal cells. However, its aberrant overexpression is associated with many cancer types [77], such as lung carcinoma [82], breast carcinoma [83, 84], and rare cases of colon carcinoma [85]. Downstream of the *c-MYC* promoter, there is the nuclease hypersensitive element III₁ (NHE III₁) that controls over 80 % of the *c-MYC* transcriptional activity. The NHE III₁ contains a sequence for a G4 motif, which represses transcription upon *in vivo* formation [58, 65] and is thus the key regulatory factor of the *c-MYC* gene [86].

There are several biologically relevant sequences derived from the human NHE III₁ that can give rise to the G4 motif [77]. The 27-nucleotide purine-rich strand denoted as Pu27, which constitutes the core sequence of the wild-type *c-MYC*, contains six guanine tracts that can form multiple G4s [65, 67, 77, 87, 88]. Furthermore, the G4 structure that involves the four central Pu27 guanine tracts, so-called 'propeller-type' G4, was also found to be biologically relevant as its stabilization by various ligands

decreased the *c-MYC* transcriptional level [65, 77, 87-90]. Moreover, the 24-nucleotide strand Pu24 that contains five guanine tracts is another Pu27-derived sequence that can form a single G4 fold. Pu24 represents a unique intramolecular parallel-stranded foldback G4 in which a guanine from the 3' tail is plugged back into the core through guanine tetrad formation (Figure 7.6) [77].

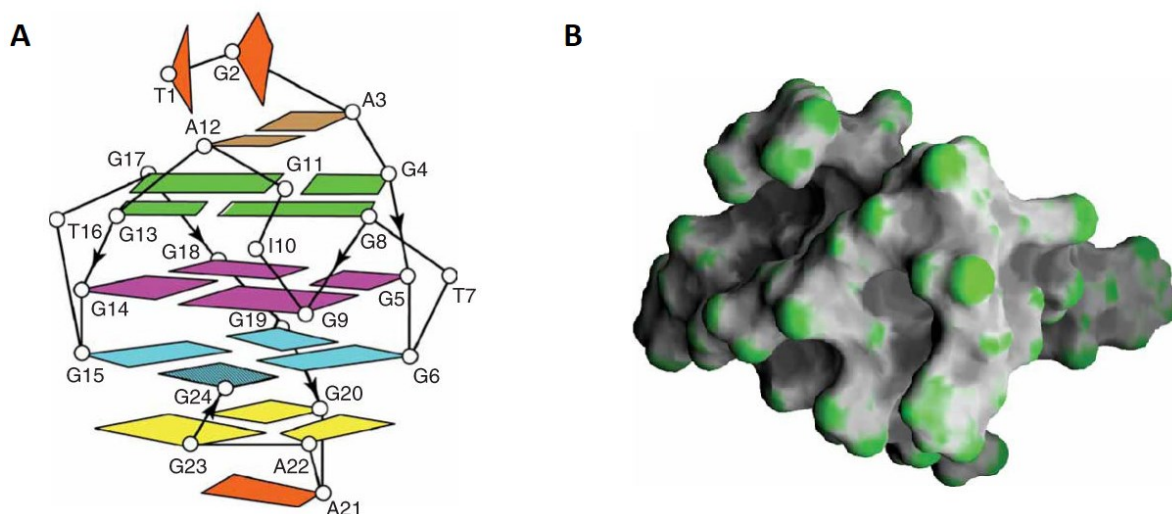


Figure 7.6: Structure of the Pu24 G4. A: a scheme displaying the spatial arrangement of nucleotides within the Pu24 sequence; B: a surface view of the Pu24 G4. Taken from the ref. [77] with the consent of the publisher.

There have been numerous efforts to discover or synthesize efficient G4 ligands that would inhibit *c-MYC* transcription [70, 91-95]. One of the first compounds that were found to stabilize the *c-MYC* G4 was the cationic porphyrin TMPyP4 (Figure 7.5) [65], which was originally established as a telomeric G4 ligand [96]. The TMPyP4 molecule serves as a standard binder in biochemical assays for evaluation of ligand stabilization efficiency [97]. A different example of a *c-MYC* binding ligand is the synthetic dye Hoechst 33258, which is widely used for cell imaging as it binds to the minor groove of the duplex DNA [98]. Hoechst 3325 was confirmed to convert Pu27 to G4s and to interact with the AAGT loop of Pu27 G4s [99, 100]. Other molecules that were identified as *c-MYC* ligands included e.g., quindoline [92-93] and berberine [94] derivatives, carbazoles [101], a perylene derivative PIPER [102], and a dihydroxyanthracene-9,10-dione derivative MYRA-A [103]. Nevertheless, the only drug candidate that has so far entered clinical trials was Quarfloxin, a fluoroquinolone derivative that proved to interact with G4s *in vivo*. The structural basis for Quarfloxin was laid in 2001 by L. H. Hurley's group, but the final structure was developed by Cyclene Pharmaceuticals [70, 104]. Quarfloxin has recently completed Phase II trials as a candidate therapeutic agent against several tumors, including neuroendocrine tumors, carcinoid tumors, and lymphoma [105, 106]. However, the Phase III trials for Quarfloxin are not

proceeding due to high albumin binding [105, 107]. The overview of selected *c-MYC* ligands is presented in Figure 7.7.

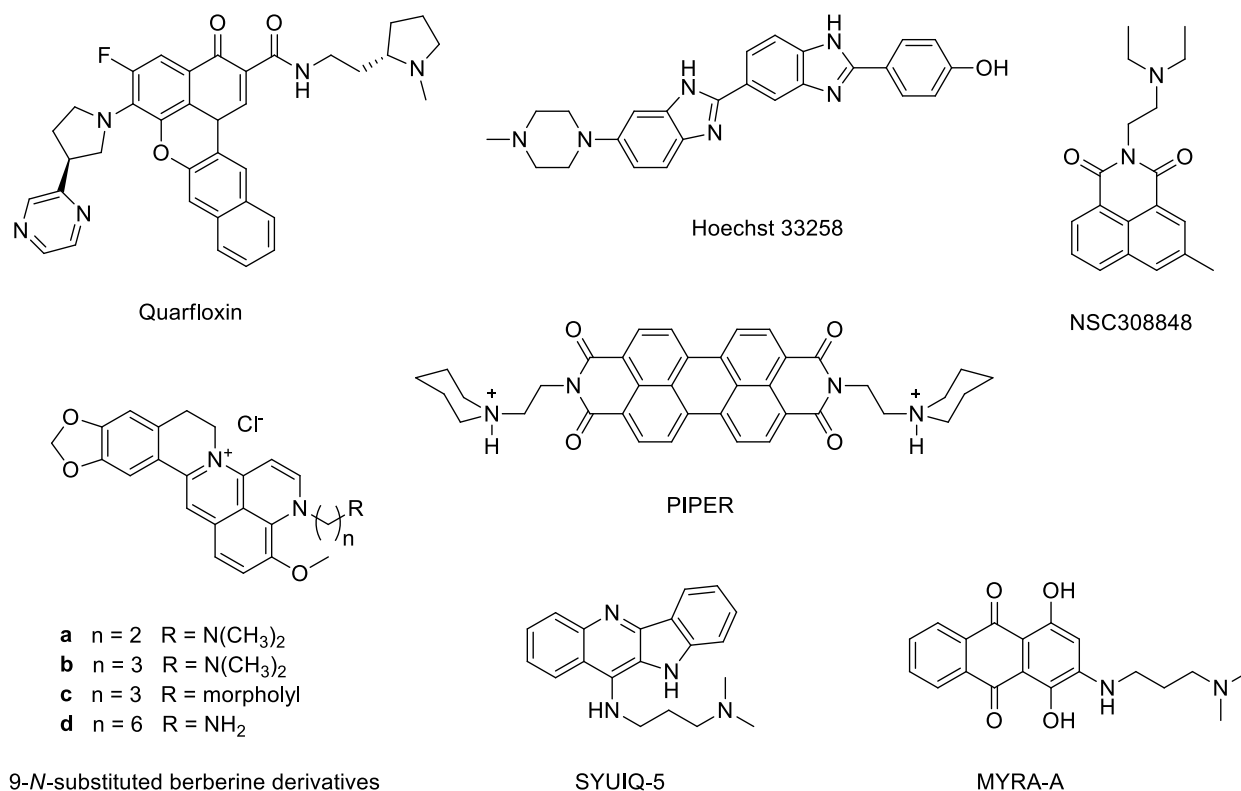


Figure 7.7: Structure of selected *c-MYC* ligands: *Quarfloxin* [108], *Hoechst 33258* [99], compound *NSC308848* [99], *PIPER* [102], 9-*N*-substituted berberine derivatives [109], *SYUIQ-5* [93], and *MYRA-A* [99].

7.2.3 Ligand-G4 interaction study methods

There are a number of experimental and computational approaches that have been successfully employed to identify new ligands and to investigate their binding mode towards G4s. These include virtual screening, isothermal titration calorimetry, mass spectrometry (MS), nuclear magnetic resonance (NMR), circular dichroism, various methods based on fluorescence spectroscopy, and biochemical assays [110, 111]. To obtain an overall knowledge of a particular G4-ligand system, it is necessary to use several complementary techniques since each technique provides a certain type of information (e.g., stoichiometries, type and strength of the binding, number of binding sites, kinetics of the binding reaction) [111]. In this study, FRET melting assay as a fluorescence-based method, PEA as a biochemical assay, and chemical-shift perturbation NMR were used to evaluate newly synthesized ligands. Therefore, these three methods will be discussed in the following text.

7.2.3.1 Förster resonance energy transfer melting assay

FRET melting assay is a technique based on non-radiative energy transfer from a donor fluorophore to an acceptor molecule (fluorophore or chromophore) through dipole-dipole coupling, which occurs through space when the donor and the acceptor are in a close proximity (1-10 nm). In practice, a short DNA sequence that represents the G4 structure is labelled at opposite ends with donor and acceptor molecules. For a successful energy transfer, it is necessary that the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. Usually, 6-carboxyfluorescein (6-FAM) serves as a donor and 6-carboxy-tetramethylrhodamine (6-TAMRA) is used as an acceptor fluorophore (Figure 7.8). In the folded G4, the two fluorophores (or fluorophore and chromophore) are close enough to each other and FRET is observed. As the efficiency of the energy transfer is inversely proportional to the sixth power of the distance, FRET can be significantly affected by a slight distance change between the donor and acceptor molecule. Therefore, the change in the distance of these two labelled sites indicates their separation as a result of disintegration of the G4 secondary structure [111-114].

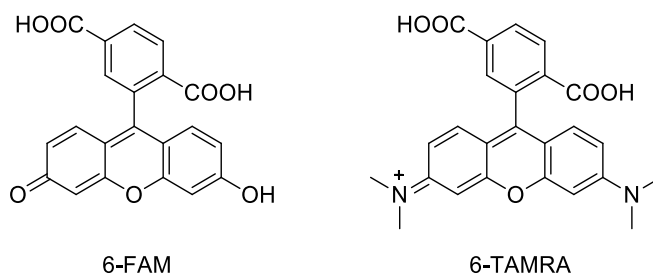


Figure 7.8: The structure of fluorophores commonly used in FRET: 6-carboxyfluorescein (6-FAM) [115], and 6-carboxy-tetramethylrhodamine (6-TAMRA) [116].

There are two types of the FRET experimental layout. In the first layout type (Figure 7.9A) the G4 sequence is labelled at both ends with a fluorescent dye (fluorophore). When the G4 sequence is folded, both dyes are close to each other and the energy may be transferred from the excited dye to the other dye, which then re-emits at a longer wavelength. The amount of direct emission versus FRET emission indicates how close the dyes are. In the second type (Figure 7.9B), the G4 template is labelled at one end with a fluorescent dye (fluorophore) and at the other end with a quencher (chromophore) that absorbs energy but does not re-emit it. As we observe fluorescence only in the unfolded form, this layout can be used to monitor G4 folding [112, 113, 117].

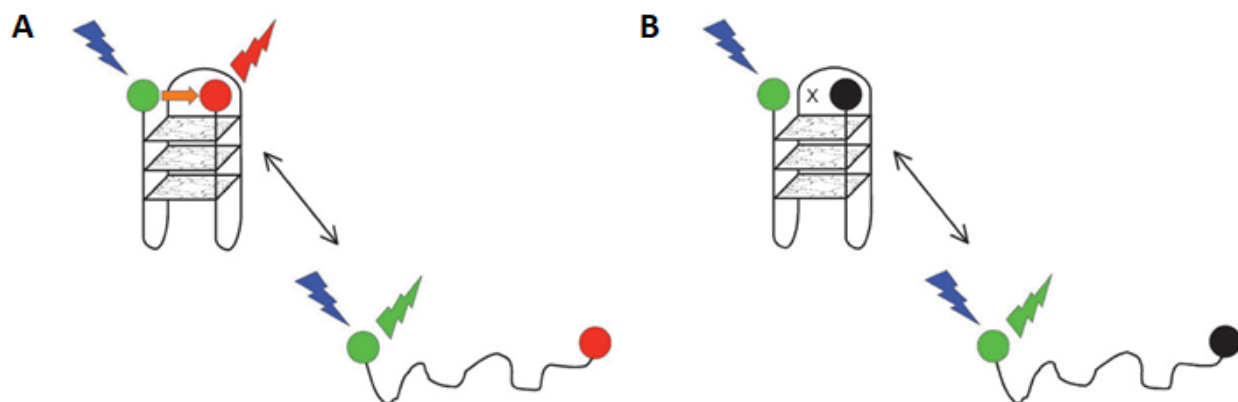


Figure 7.9: FRET experimental layout variants: A – G4 is labelled on both ends with a fluorescent dye; B – G4 is labelled on one end with a fluorescent dye and on the other with a quencher (adapted according to ref. [113] with the consent of Dr. J. L. Huppert).

During the FRET melting assay experiment, the temperature of the system is gradually increased and we simultaneously observe decrease of fluorescence intensity caused by denaturation of the G4 secondary structure. The G4 melting point (T_m) is defined as a temperature where 50 % of the original G4 amount is unfolded. If a suitable ligand capable of stabilizing the G4 secondary structure is present, the T_m of such system will increase. The difference in T_m between the G4 itself and G4 in the presence of a defined concentration of the tested ligand can therefore determine stabilizing efficiency of such ligand. Melting curves for both the G4 itself and ligand-G4 solution (Figure 7.10) are obtained as a change in emission of a fluorescent label involved in FRET pair during temperature increase. T_m is determined by the inflexion point of each melting curve and the T_m increase (ΔT_m) is assessed from the distance of inflexion points of both melting curves [112, 113, 117, 118].

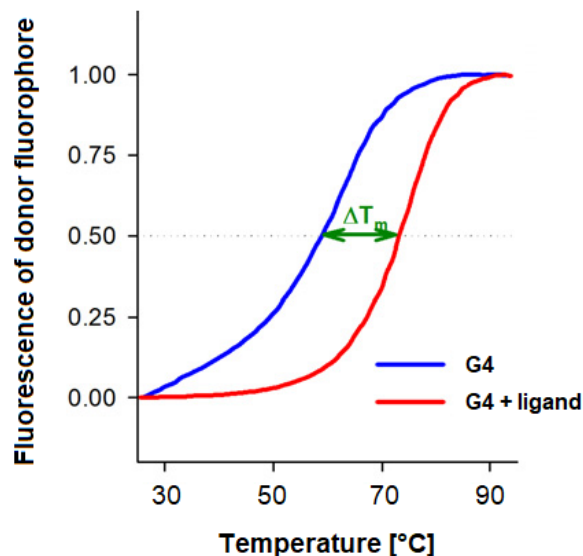


Figure 7.10: FRET melting assay determination of the T_m increase (ΔT_m) from melting curves of G4 itself (blue curve) and G4 in the presence of a ligand (red curve) (adapted according to ref. [118] with the consent of Dr. J.-L. Mergny).

7.2.3.2 Polymerase extension assay

The principle of this method consists in extension of a radiolabelled primer with thermostable *Taq* DNA polymerase [119] alongside the template strand that contains a sequence for intramolecular G4 formation (Figure 7.11). If the G4 secondary structure on the template oligonucleotide is unfolded, polymeration can proceed and a full-length product is formed. Stabilization of G4 by a suitable ligand induces pausing of primer extension by *Taq* polymerase and we observe formation of a stop product that is shorter than the full-length product. The PEA products are separated based on their size by gel electrophoresis and visualized by autoradiography. The stronger is the stabilizing efficiency of a ligand, the stronger stop product band is obtained relative to the full-length-product band (Figure 7.12). Thus, PEA serves for evaluation of ligand interaction with the intramolecular G4 structure [117, 120-122].

The main advantage of PEA is stabilization efficiency determination of a given ligand by other means than the change of intensity of either emitted or absorbed light/radiation, which can also be influenced by the ligand. However, some ligands can inhibit *Taq* polymerase, which manifests itself as enhanced intensity in the primer region. Moreover, manipulation with a radiolabelled primer requires increased security measures [117, 120-122].

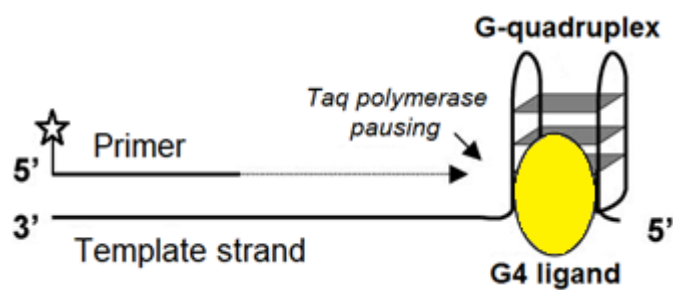


Figure 7.11: The principle of PEA. Elongation of radiolabelled primer alongside the template strand by Taq polymerase is paused in the presence of a ligand stabilizing the G4 structure on the template strand (adapted according to ref. [121] with the consent of Dr. J.-L. Mergny).

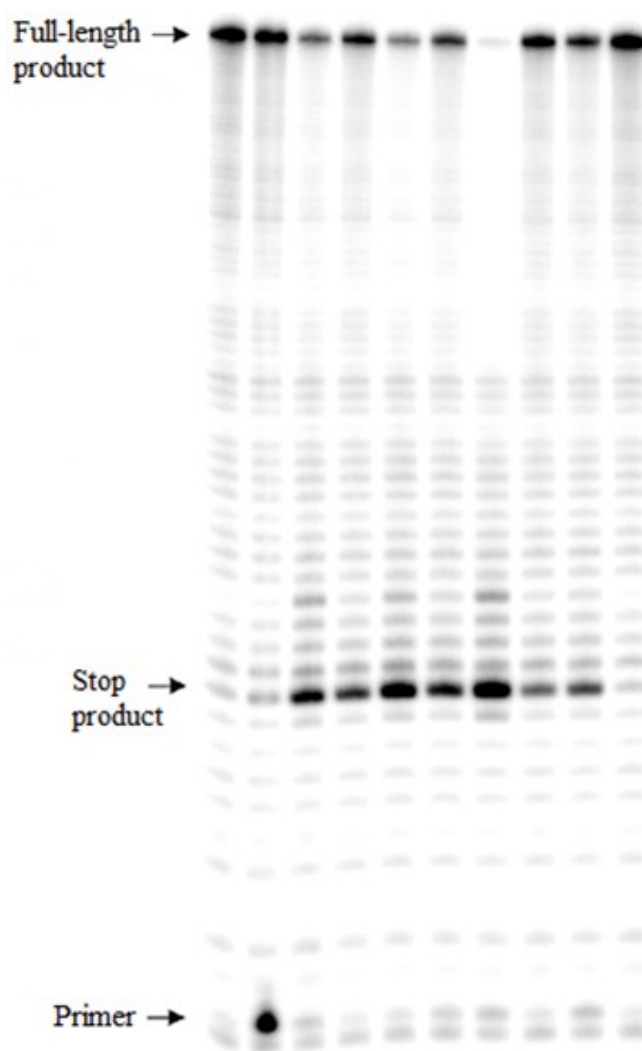


Figure 7.12: Autoradiogram of a PEA gel. The position of full-length product, stop product, and primer are indicated by arrows (kindly provided by Dr. Kozák from IOCB).

7.2.3.3 Chemical-shift perturbation NMR

Chemical-shift perturbation NMR (Figure 7.13) is a target-observing method that regards the chemical-shift perturbation of target NMR signals, which is caused by covalent or non-covalent interactions between ligand and target. This way we can determine the binding mode of the ligand towards the target. For the purpose of this method, it is usually necessary to use a ^{15}N - and/or ^{13}C -labelled target and unlabelled ligand. A ^{15}N or ^{13}C NMR spectrum (usually HSQC or TROSY) of the labelled target in complex with the ligand is compared with the spectrum of the labelled target only [9, 10, 123, 124]. Important to mention, ^1H NMR spectra can be also applied for the chemical-shift perturbation method. However, the chemical shift range for ^1H NMR is smaller than for ^{15}N or ^{13}C NMR experiments, which requires the calculations of ^1H chemical shifts to be more accurate [124].

Any chemical shift change is very sensitive to structural changes and can be measured very accurately, which means that almost every genuine binding interaction will produce chemical-shift perturbation. If a ligand does not bind to the target, no chemical shift change will be observed. Furthermore, we can compare which signals of the target change upon addition of various ligands and thus determine whether or not these ligands bind to the target in the same manner. The main drawback of this method is the necessity of isotope labelling in ^{15}N or ^{13}C NMR experiments [9, 10, 123, 124].



Figure 7.13: Chemical-shift perturbation ^1H NMR spectra for various small molecules targeting G4 of c-MYC, represented by the oligonucleotide Pu24 [10]. Amidic regions of Pu24 are observed and the respective signals are marked with a coloured vertical line and numbered. “Ref.” is a reference Pu24 spectrum in the absence of a ligand (kindly provided by Dr. Veverka from IOCB).

7.3 Chemistry of fragments used for library construction

The basis for construction of the fragment library presented in this work was provided by two distinct compounds: 4-(4-methylpiperazin-1-yl)aniline as a piperazine derivative and pyridostatin. The chemistry and biological activity of these compounds will be discussed in the following text.

7.3.1 Piperazine and 4-(4-methylpiperazin-1-yl)aniline

Piperazine (**1**, Figure 7.14) is a privileged structure found in biologically active compounds with therapeutic uses as antifungals, antidepressants, antivirals, and serotonin 5-HT receptor antagonists/agonists [125]. As a result, piperazine derived building blocks have been used for construction of screening libraries [126]. One such fragment library member derived from piperazine is 4-(4-methylpiperazin-1-yl)aniline [127] (**2**, Figure 7.14) that was recognized in 2014 by Nasiri *et al* (the Balasubramanian research group) as a hit when targeting G4 of the human *c-MYC* promoter, using an intercalator-displacement assay for the library screen. The binding affinity of **2** towards the *c-MYC* G4 was further evaluated and confirmed by molecular modelling and NMR docking studies. This compound (**2**) contains an amino functionality, which may aid in the electrostatic binding to the G4 DNA [128]. Interestingly, the 4-(4-methylpiperazin-1-yl)aniline moiety is incorporated in the fluorescent dye Hoechst 33258 (**3**, Figure 7.14), an established *c-MYC* G4 ligand [98-100] that has already been introduced in Chapter 7.2.2.

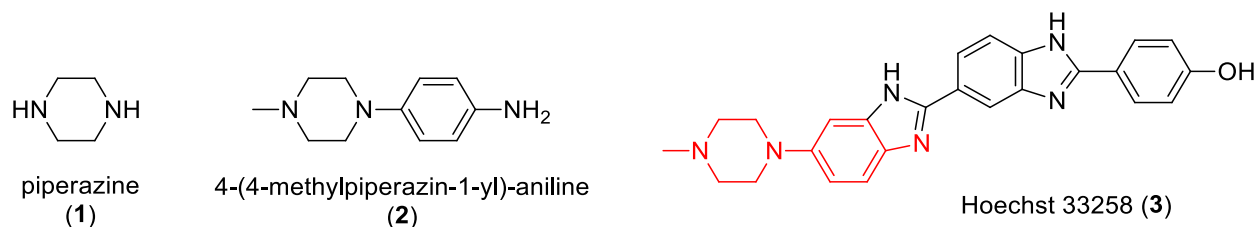


Figure 7.14: Structure of piperazine (**1**), its derivative 4-(4-methylpiperazin-1-yl)-aniline (**2**), and the dye Hoechst 33258 (**3**), which contains 4-(4-methylpiperazin-1-yl)-aniline moiety (highlighted in red).

7.3.2 Pyridostatin

Pyridostatin (**4**, Figure 7.15A) is an efficient stabilizer of telomeric G4s that is based on *N,N'*-bis(quinolinyl) pyridine-2,6-dicarboxamide. The pyridostatin molecule, introduced by Balasubramanian research group in 2008 [129], was designed after an intensive research on the biology of G4 nucleic acids. Its design was initially based on certain structural features (Figure 7.15B) that are typical for known G4 binders, such as electron-rich aromatic surface, potential for a flat conformation, and ability to participate

in hydrogen bonding. As a result, pyridostatin possesses an optimal electronic density of the aromatic surface that enables π - π interactions with the G-tetrad tuned by substituents. The ability to adopt a flat but flexible conformation is facilitated by an internal hydrogen bonding network, prone to adapt to the dynamic and polymorphic nature of diverse G4 structures. Finally, the free nitrogen lone pairs can coordinate with a water molecule or, alternatively, sequester a monovalent potassium cation in the centre, thus locking the flat surface of the molecule and facilitating the interaction with G-tetrads. These key features distinguish pyridostatin from other structurally related G4 interacting molecules [129, 130].

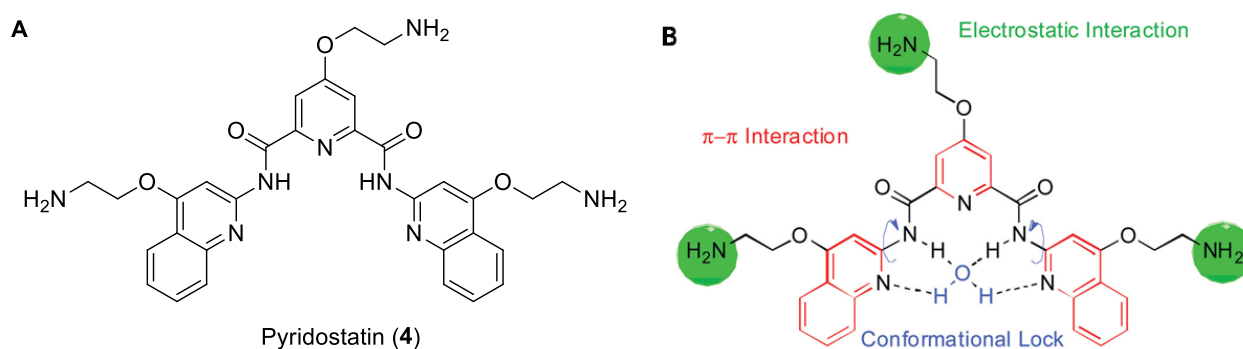


Figure 7.15: A – the pyridostatin (4) structure (drawn according to ref. [129]). B – the design rationale highlighting key features for G4 stabilization: electrostatic interactions, π - π interactions, and conformational lock (taken from the ref. [130] with the consent of Prof. S. Balasubramanian).

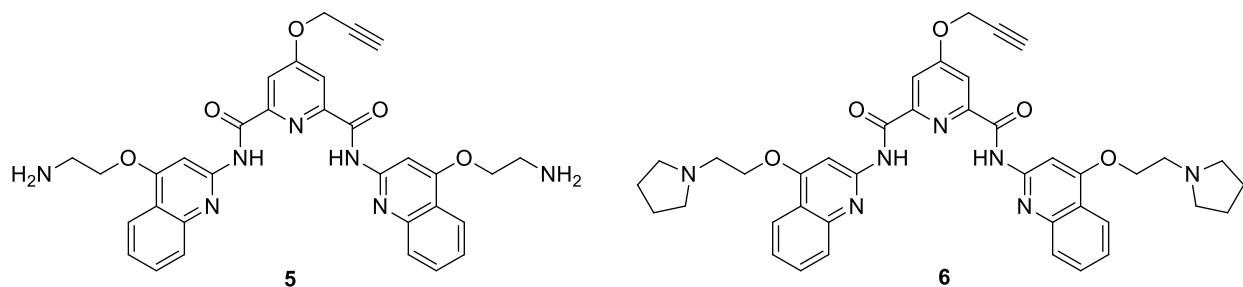
FRET melting experiments performed on the human telomeric G4 (H-Telo, a 21-mer oligonucleotide sequence) revealed a maximum ΔT_m of 35 K at 0.18 μ M concentration of pyridostatin when using 60 mM K^+ , and 44 K at 0.34 μ M concentration of pyridostatin with 100 mM Na^+ . In contrast, the ligand-induced double-stranded DNA stabilization was negligible. These findings suggest the highest induced shifts in T_m for the telomeric G4 so far, accompanied by a high level of selectivity over duplex DNA. Moreover, pyridostatin was shown to induce telomere disfunction by competing for binding with telomere-associated proteins such as human POT1 [129, 130]. However, FRET melting assay assessment of pyridostatin binding affinity towards various G4s including both H-Telo and *c-MYC* G4 did not show strong selectivity for any given G4 target. The interaction of pyridostatin with *c-MYC* G4 and H-Telo was comparable. Therefore, further research regarding pyridostatin modifications is required to obtain an ultimate specific G4 ligand [131].

There have been several efforts to modify or functionalize the pyridostatin molecule in search for novel pyridostatin derivatives with enhanced affinity or selectivity towards various, but mainly telomeric G4s [39, 130, 132-134]. To my knowledge, a study concerning pyridostatin derivatives for targeting *c-MYC* G4 has not been published so far. In 2012, Müller *et al* (the Balasubramanian research group) [130]

performed various pyridostatin modifications on its alkylamine side chains to gain further insight into structure-activity relationship (SAR). Evaluation of the binding affinity of such derivatives towards H-Telo using FRET melting assay confirmed necessity of amino moieties for interaction with G4, as their removal on both the pyridine and quinolone cores led to reduction of the G4 stabilization potential. Moreover, the nature of the amine in the side chains bound to quinolone moieties had a small influence on the stabilization potential of the analogues following the general trend: $\text{NMe}_2 \geq \text{NH}_2 \geq \text{pyrrolidine}$ [130].

The most significant work that laid the basis for our study regarding pyridostatin derivatives was presented by Di Antonio *et al* (the Balasubramanian research group) in 2012 [39]. In the concerned study, the research group explored the ability of H-Telo to act as the reaction vessel for 1,3-dipolar cycloaddition to generate potent telomere-targeting small molecules. For this purpose, the pyridine moiety was modified in position 4 to contain an alkynyl instead of the alkylamine chain in order to obtain a pyridostatin-alkyne derivative **5** (Figure 7.16) amenable for *in situ* click chemistry. An analogous pyridostatin-alkyne compound **6** (Figure 7.16) was prepared by substitution of the NH_2 groups on the quinolone side chains with pyrrolidine moieties for comparison regarding the stabilization potential. The pyridostatin-alkynes **5** and **6** were subjected to *in situ* click reaction with a series of small-molecule azides **7-12** (Figure 7.16). These azides comprised both positively and negatively charged atoms, aromatic substrates, and a neutral sugar to cover several types of interaction modes including π -stacking as well as electrostatic and hydrogen bonding, with the aim to maximize the chances of identifying a strong H-Telo interacting ligand [39].

A: Pyridostatin derived alkynes



B: Azides

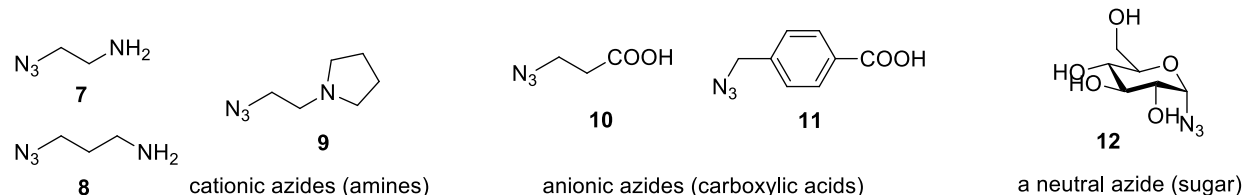


Figure 7.16: A - Structure of pyridostatin-alkynes **5** and **6**. B – Structure of azides **7-12** used for *in situ* click experiment (drawn according to ref. [39]).

In situ click experiments with alkynes **5** and **6** and azides **7-12** were performed with and without H-Telo DNA, both in the presence and in the absence of a copper catalyst, and composition of the reaction mixture was monitored by LC-MS. The presence of a copper catalyst showed to be essential for the click reaction to reach completion because copper-free experiments with and without H-Telo suffered from poor kinetics. The experiment performed with H-Telo and a stoichiometric amount of a copper catalyst showed a shift of product distribution towards sugar-containing click products (with azide **12**) at the expense of other click products identified in the presence of only the copper catalyst (without H-Telo). This indicates that the G4 DNA overrides to some extent the lack of specificity imposed by the kinetically favoured copper-catalyzed reaction [39].

Furthermore, all possible click products were prepared *via* chemical synthesis and their stabilization efficiency towards the H-Telo G4 was determined by FRET melting assay at 1 μ M and compared with both pyridostatin-alkynes and the original pyridostatin molecule. There was a general trend in which adducts derived from **5** exhibited slightly better stabilizing properties than the analogues derived from **6** [39]. This finding was in accordance with the results previously published by Müller *et al* [130] and discussed above, showing that the H-Telo stabilization potential in FRET was higher for compounds with NH₂ moiety in the side chains bound to quinolone moieties than for their pyrrolidine analogues. Additionally, the stabilization properties of each adduct followed the general trend of neutral (sugar) > basic moiety (amines) > acidic moiety (carboxylic acids), with ΔT_m value ranging from 30 K for sugars to 20 K for amines and 6 K for carboxylic acids. Generally, click products were more potent H-Telo stabilizers than the starting materials **5** and **6**, with the exception of adducts containing a carboxylic acid moiety that were even weaker stabilizers. However, neither any click product, nor the pyridostatin-alkynes exhibited higher increase of ΔT_m of H-Telo than the pyridostatin molecule with the ΔT_m value of 35.4 ± 1.2 at 1 μ M [39].

8 Aims of the work

1. Design and synthesis of new *c-MYC* G4 ligands based on the 4-(4-methylpiperazin-1-yl)aniline (**2**) structure that was previously reported as a *c-MYC* G4 binder by Nasiri *et al* [128]:
 - Modification of the 4-(4-methylpiperazin-1-yl)aniline molecule (**2**) and determination of the impact of different chemical moieties and their respective positions on the *c-MYC* G4 stabilization.
 - Functionalization of the 4-(4-methylpiperazin-1-yl)aniline template for click chemistry with selected azides (inspired by Di Antonio *et al* [39]) and construction of a focused fragment library of the respective click products.
 - KTGS employing CuAAC to observe click product formation *in situ*, thus to evaluate the best fragment combination directed by Pu24 as the target.
 - Evaluation of *c-MYC* G4 binding affinity of the resulting click products as well as the original clickable fragments based on results from the FRET metling assay, PEA, and chemical-shift perturbation NMR provided by the biochemical department.
2. Design and synthesis of new pyridostatin derived ligands:
 - Functionalization of pyridostatin (**4**) with an alkynyl or azidoalkyl moiety for click chemistry with selected small-molecule azides (inspired by Di Antonio *et al* [39]), azides derived from 4-(4-methylpiperazin-1-yl)aniline, and alkynes derived from 4-(4-methylpiperazin-1-yl)aniline to form a focused fragment library of the respective click products.
 - KTGS employing CuAAC to observe pyridostatin click product formation *in situ*, thus to evaluate the best fragment combination directed by Pu24 as the target.
 - Evaluation of *c-MYC* G4 binding affinity of the resulting click products as well as the original clickable fragments based on results from the FRET metling assay provided by the biochemical department. As long as the click products exhibit activity in FRET and their sufficient formation ratio is observed during *in situ* click experiments, PEA and chemical-shift perturbation NMR will be performed as well.

9 Results and discussion

9.1 The focused fragment library based on 4-(4-methylpiperazin-1-yl)aniline

9.1.1 Modification of the 4-(4-methylpiperazin-1-yl)aniline molecule

Before functionalizing 4-(4-methylpiperazin-1-yl)aniline (**2**) for click chemistry, it was necessary to ascertain how different chemical moieties, attached to the compound **2** structure, and their respective position contribute to *c-MYC* G4 stabilization. For this purpose, a set of commercially available derivatives of compound **2** that comprised modifications at the piperazine nitrogen and/or at the aniline moiety were provided (Figure 9.1, compounds **13-24**). Additionally, compounds **25** and **26** (Figure 9.1), both containing a tertiary alkylamine chain bound to the aniline NH₂ moiety, were synthesized and included into the set of the 4-(4-methylpiperazin-1-yl)aniline derivatives to determine the necessity of this amino moiety for interaction with *c-MYC*.

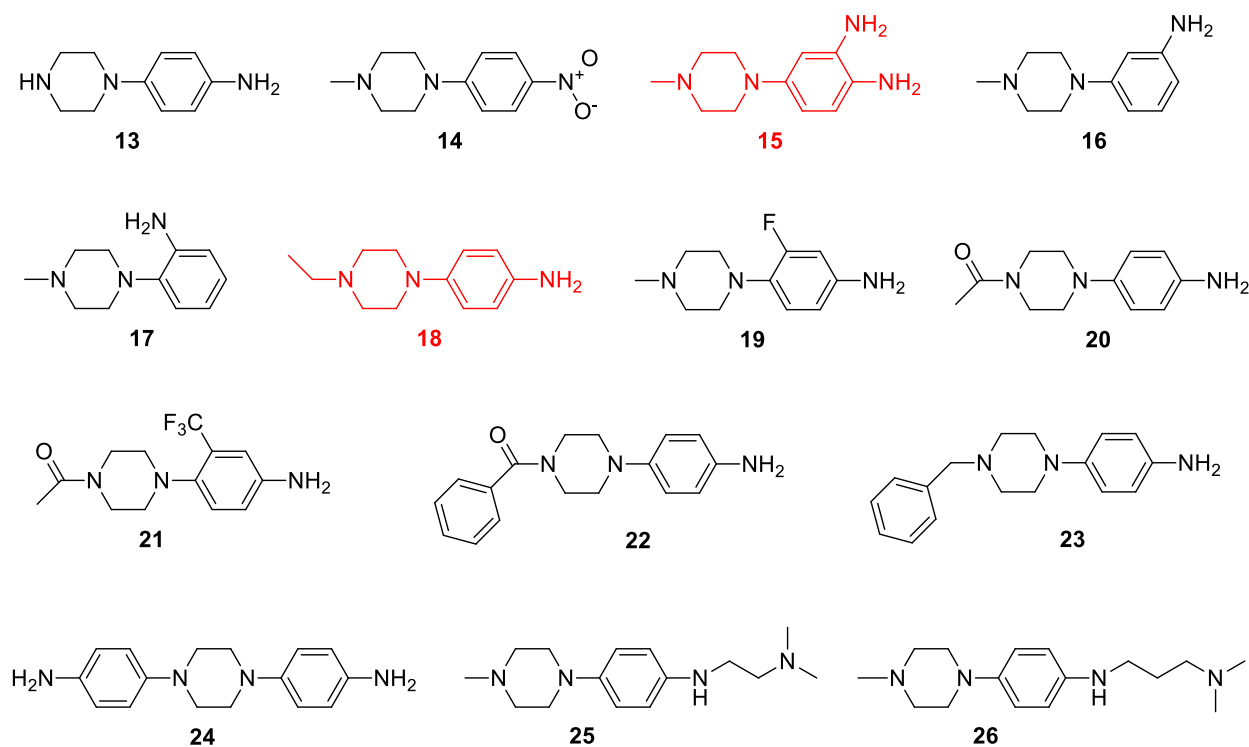
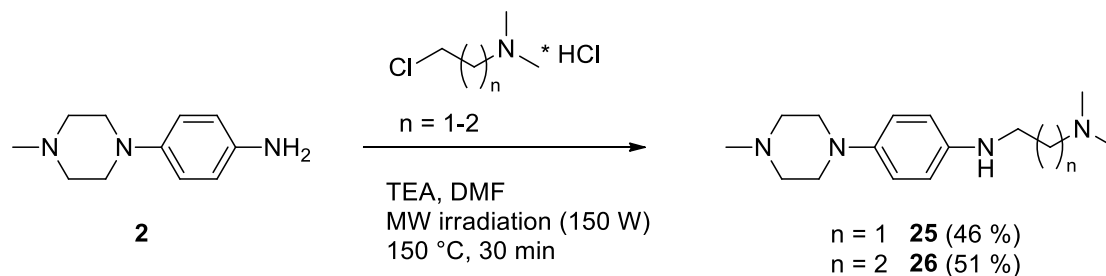


Figure 9.1: A set of 4-(4-methylpiperazin-1-yl)aniline derivatives containing various chemical moieties. Compounds **13-24** were purchased, compounds **25** and **26** were synthesized. Compounds **15** and **18**, which exhibited restored activity in FRET and/or PEA compared to the reference active molecule **2**, are highlighted with red colour.

Synthesis of compounds **25** and **26** was performed by alkylation of compound **2** on the NH₂ group using 2-chloro-*N,N*-dimethylethanamine and 3-chloro-*N,N*-dimethylpropan-1-amine as an alkylating agent, respectively. The reaction was carried out according to ref. [135] under basic conditions in DMF, MW irradiation, and high temperature (150 °C) to obtain both compounds **25** and **26** in moderate yield (Scheme 9.1).



Scheme 9.1: Alkylation of 2 on the aniline NH₂ group with two tertiary alkylamines to obtain compounds 25 and 26.

The obtained set of compounds (**13-26**) were subsequently tested at 100 μM in FRET melting assay using dual-labelled (5'-(6-FAM) and 3'-(6-TAMRA) 25-mer oligonucleotide Fc-myc25T, and at 100 μM in PEA using a template strand containing the Pu27 sequence. The results (Table 9.1) were compared to the template compound **2** that induced a ΔT_m increase of 10.4 ± 0.6 °C at a 100 μM in FRET. Compound **14**, the nitro-analogue of compound **2**, produced no stop band in PEA and therefore served as a negative control for other compounds tested to their *c-MYC* binding ability in PEA.

The highest activity in both biochemical assays was exhibited by the compound **15** that possesses an additional NH₂ group in position 2 of the aromatic ring. In FRET, the ΔT_m induced by the presence of **15** exceeded 35 °C, representing a more than 3-fold ΔT_m increase compared to the original molecule **2**. Moreover, the PEA results showed fully restored activity of **15** when compared to **2**. Other tested compounds did not exhibit any or only negligible activity in both assays apart from compounds **18** and **14**. Compound **18** showed partially restored activity in PEA, but a negligible ΔT_m in FRET (1.7 °C) compared to compound **2**. Although compound **14** did not produce any stop band in PEA, its FRET activity was partially restored when compared to compound **2**.

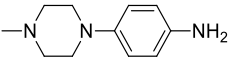
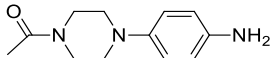
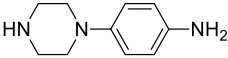
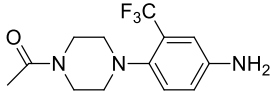
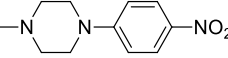
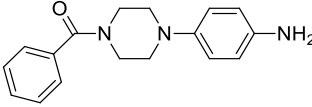
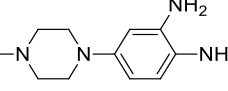
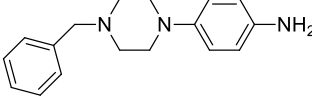
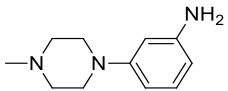
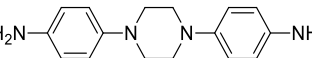
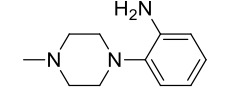
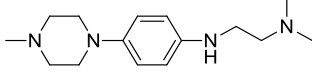
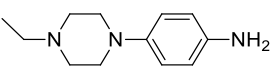
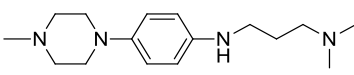
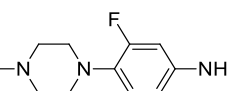
Cpd. No	Structure	PEA at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 100 μ M	Cpd. No	Structure	PEA at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 100 μ M
2		1.0	10.4 $\pm$ 0.6	20		0.3	1.8
13		0.2	1.6	21		0.1	0.9
14		0.2	3.7 $\pm$ 0.5	22		0.2	1.2
15		1.1	> 35	23		0.2	1.1
16		0.2	0.0	24		0.2	1.1
17		-	-0.3	25		-	-1.5
18		0.6	1.7	26		-	1.0
19		0.2	1.1				

Table 9.1: SAR data summary of the FRET and PEA results at 100 μ M for compound 2 and its derivatives. Data for PEA were determined as a quotient of stop band density of the respective compound divided by the stop band density of the parent compound 2.

Amino groups are a common feature of well-established G4 ligands as they bind to phosphate groups of the G4 backbone *via* electrostatic interactions mediated by bridging water molecules [68]. Correspondingly, our results showed inactivity of compounds **25** and **26** in both assays and inactivity of the nitro-compound **14** in PEA. Moreover, compounds **16** and **17** possessing the amino group in position 2 or 3 of the aromatic ring and thus isomeric to the parent compound **2** were inactive in both assays. All these findings underline the necessity of the unsubstituted NH₂ group at the position 1 of the aromatic ring for interaction of the ligand with *c-MYC*. Moreover, the presence of additional amino group in position 2 of the aromatic ring significantly increased the affinity of the fragment **15** towards *c-MYC*. Furthermore, inactivity of compound **13**, containing free piperazine nitrogen, in both assays and partially restored PEA activity of **18**, homologous to compound **2**, indicate necessity of an alkyl chain bound to the piperazine nitrogen.

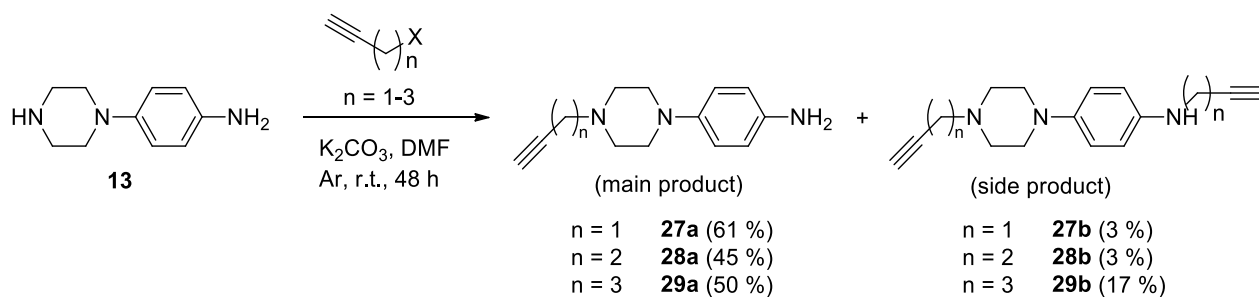
9.1.2 Construction of the fragment library based on the 4-(4-methylpiperazin-1-yl)aniline structure

Based on the FRET and PEA results regarding modifications of compound **2**, it was decided to synthesize two series of 4-(4-methylpiperazin-1-yl)aniline-derived compounds: a mono-amino series that was directly inspired by compound **2**, and the bis-amino series that contained an additional amino group in position 2 of the aromatic ring. These two series were evaluated for their *c-MYC* binding ability in FRET and PEA biochemical assays separately. Important to mention, results from the SAR study regarding the mono-amino series were applied for construction of the bis-amino series.

9.1.2.1 The mono-amino series

9.1.2.1.1 Design and synthesis of the mono-amino series

Evaluation of various derivatives of the compound **2** using FRET melting assay and PEA showed that the aniline NH₂ moiety cannot be used for further derivatization, while alkylation on the piperazine nitrogen was necessary for restoration of its binding ability towards *c-MYC* G4. Therefore, it was decided to modify the 4-(4-methylpiperazin-1-yl)aniline scaffold for click chemistry on its piperazine nitrogen in position 4. Compound **13** was used as a starting point for alkylation with terminal alkynyl halides containing 1-3 methylene units in the chain to obtain a series of homologous piperazine *N*⁴-alkynylated derivatives. The reaction was performed in DMF using 10-fold excess of K₂CO₃ as a base [136], and both the product of mono-alkylation (**27a** [137], **28a**, and **29a**) and bis-alkylation (**27b**, **28b**, and **29b**) were obtained (Scheme 9.2). For each reaction, the main product, alkylated on the piperazine nitrogen only, was afforded in moderate to good yield (45-61 %). On the contrary, the yield of the side product, which was alkylated on both the piperazine and the aniline nitrogens, was negligible (3 %) for compounds **27b** and **28b** and poor for compound **29b** (17 %).



*Scheme 9.2: N-alkylation of 4-(piperazin-1-yl)aniline with alkynyl halides of various methylene units in the alkynyl chain. As a result, the mono-alkylated main products (**27a**, **28a**, **29a**) as well as the bis-alkylated side products (**27b**, **28b**, **29b**) were obtained.*

For functionalization in the following click reaction, only the mono-alkylated homology series was chosen due to the previously showed necessity of unsubstituted NH₂ group for *c-MYC* G4 binding.

The choice of azides suitable for click chemistry was inspired by the study performed by Di Antonio *et al* [39], who employed the following compounds (Table 9.2): 2-azidoethanamine (**7**), 3-azidopropanamine (**8**), and 1-(2-azidoethyl)pyrrolidine (**9**) as cationic azides (amines); 3-azidopropanoic acid (**10**) and 4-(azidomethyl)benzoic acid (**11**) as anionic azides (carboxylic acids); and 1-azido-1-deoxy- β -D-glukopyranoside (**12**) as a neutral azide (sugar).

To determine the most suitable azide type for further growing of the mono-alkynylated fragments in click chemistry, initial *in situ* click experiments were performed by my colleague, Dr. Harish R. Talele. For each of the two homologous alkynes **28a** and **29a** added to the mixture of six azides (**7-12**), a set of four *in situ* click experiments was performed as a combination of the following criteria: either in the presence or in the absence of Cu^I as a catalyst, and with either Pu24 or Pu27 as an oligonucleotide. The reaction conditions were performed according to the protocol presented by Di Antonio *et al* [39]. After quenching the reaction with TFA, composition of each reaction mixture was analyzed by LC-MS; the results are summarized in Table 9.2.

No product formation was observed in experiments that were performed in the absence of Cu^I (data not shown). Apparently, the presence of Cu^I was essential for click product formation as this catalysis significantly improved reaction kinetics, which has also been reported previously by Di Antonio *et al* [39]. Interestingly, in this study regarding *c-MYC*, the respective click products were formed almost exclusively with cationic azides **7-9**. On the contrary, click products with the neutral sugar moiety were preferentially formed in the presence of H-Telo, which was reported Di Antonio *et al* [39].

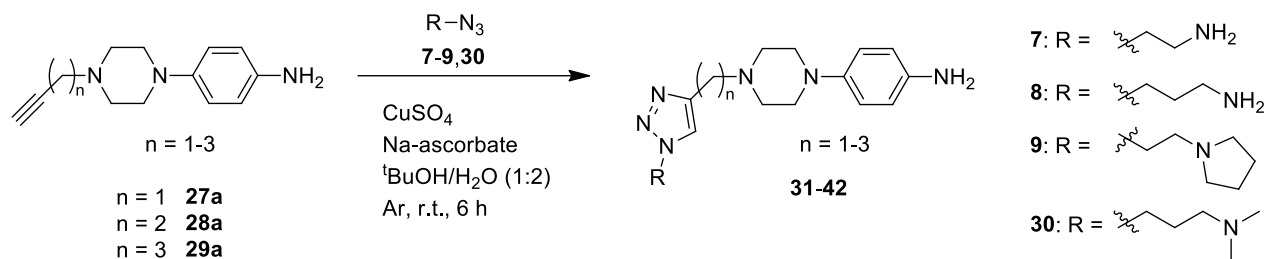
As the individual click products differ in their ionizability, we cannot compare them regarding their integral intensity in LC-MS, but we can compare their formation in the presence of different oligonucleotides (Pu24 vs. Pu27). Notably, higher formation rate was observed in the presence of Pu27 compared to Pu24, which is attributed to more G4 folding options of Pu27. It is most likely that one of the Pu27 G4 folds, which is different from the only G4 fold in Pu24, is either better stabilized by the respective ligands or is generally more stable and thus catalyzes the *in situ* click reaction more efficiently. Additionally, there was a difference in the order of tens of the integral intensity among click products derived from alkynes **28a** and **29a** in the favour of **28a** derivatives. It was supposed that their increased formation yield was rather due to increased *c-MYC* binding ability than due to different ionizability of each click product family.

Alkyne		28a	29a
Azide			
7		Pu24: 765 Pu27: 1,53·10 ³	Pu24: – Pu27: 150
8		Pu24: 1,40·10 ³ Pu27: 2,08·10 ³	Pu24: 84 Pu27: 436
9		Pu24: 3,86·10 ³ Pu27: 8,20·10 ³	Pu24: 181 Pu27: 567
10		Pu24: – Pu27: –	Pu24: – Pu27: –
11		Pu24: – Pu27: –	Pu24: – Pu27: –
12		Pu24: – Pu27: 132	Pu24: – Pu27: –

Table 9.2: LC-MS results of Cu^I catalyzed *in situ* click experiments of alkyne **28a** and **29a** with azides A-F, either in the presence of Pu24 or Pu27.

Based on the *in situ* click experiments performed, only the cationic azides **7-9** possessing either a pyrrolidine or a primary amine moiety were selected for growing of the mono-alkylated series. Additionally, we decided to include 3-azido-*N,N*-dimethylpropan-1-amine (**30**, Table 9.3) to our selection, as this azide resembles the quinolone substituent containing the -NMe₂ moiety that led to slight increase of H-Telo stabilization potential in the respective pyridostatin derivatives [130]. Furthermore, compound **30** represents a tertiary-amino-analogue of the azide **8**.

Chemical synthesis of click products as a combination of the alkynes **27a-29a** and azides **7-9** and **30** was performed under Cu^I catalysis to obtain the respective 1,4-disubstituted triazoles as the only product (Scheme 9.3). The CuAAC reaction was carried out in the mixture of *tert*-butanol/H₂O (1:2) under argon atmosphere and with CuSO₄ as a Cu catalyst, which was reduced from the oxidation state II to I by sodium ascorbate [39]. The 1,4-products of the chemical click (**31-42**) were obtained in moderate to very good yield (46-85 %); their structure as a combination of the respective alkyne and azide is summarized in Table 9.3.



Scheme 9.3: CuAAC of 4-(4-methylpiperazin-1-yl)aniline-derived alkynes (**27a**, **28a**, and **29a**) and azides (**7-9**, **30**) to form click products of the mono-amino series (**31-42**).

Alkyne	(27a)	(28a)	(29a)
Azide			
(7)	(31)	(32)	(33)
(8)	(34)	(35)	(36)
(9)	(37)	(38)	(39)
(30)	(40)	(41)	(42)

Table 9.3: Structure of click products (**31-42**) as a combination of the corresponding alkynes and azides.

9.1.2.1.2 The structure-activity relationship regarding the mono-amino series

All compounds synthesized as derivatives of 4-(4-methylpiperazin-1-yl)aniline were subjected to biological evaluation in FRET melting assay at 100 μ M using dual-labelled (5'-(6-FAM) and 3'-(6-TAMRA)) 25-mer oligonucleotide Fc-myc25T, and in PEA at 100 μ M using a template strand containing the Pu27 sequence. Their activity in both assays was compared to the template molecule **2**. Moreover, compound **14** was included in this series as a negative control for the PEA biology assay. The results from both FRET and PEA are summarized in Table 9.4.

According to the data obtained, no 4-(4-methylpiperazin-1-yl)aniline derivative of the mono-amino series exhibited *c-MYC* G4 affinity comparable to the compound **2** in FRET. All the alkynes **27a**, **28a**, and **29a** exhibited only small ΔT_m increase ranging from 2.4 ± 0.9 to 3.3 ± 0.4 $^{\circ}$ C, which pointed to negligible biological activity of these clickable fragments. Additionally, negative ΔT_m values were measured for click products **31-36** derived from azides **7** and **8**, suggesting destabilization of the Fc-myc25T G4. On the other hand, click products **37-42** derived from azides **9** and **30** exhibited positive, although low ΔT_m values. Generally, click products **37-39** derived from azide **9** exhibited higher activity than click products **40-42** originated from azide **30**. Moreover, there was a trend of increasing ΔT_m with the number of methylene units in the linker that connects the piperazine and the triazole ring, making compounds **39** and **42** derived from alkyne **29a** the best ligands. Among these two compounds, fragment **39** with ΔT_m increase of 7.6 ± 0.4 $^{\circ}$ C was far the most active.

In PEA, the clickable alkynes **27a** and **28a** displayed negligible activity. The alkyne **29a** and vast majority of click products exhibited only partially restored activity compared to the parent compound **2**. Generally, click products **37-42** derived from azides **9** and **30** bearing a tertiary amino moiety exhibited slightly higher activity than click products **31-36** that originated from azides **7** and **8** with a primary amino moiety. Also, among each homology series of click products or alkynes, the activity was usually highest for the compound with three methylene units at the alkynyl linker. A full restoration of activity in PEA when compared to compound **2** was observed only for fragments **39** and **41**. The activity of compound **39** that resulted from click reaction between alkyne **29a** with three methylene units at the alkynyl chain and azide **9** was slightly higher than the activity of compound **41** originating from alkyne **28a** with two methylene units and azide **30**. The highest activity for compound **39** in PEA is in accordance with the FRET results discussed previously.

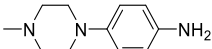
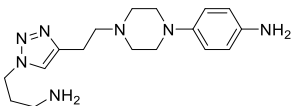
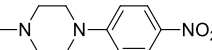
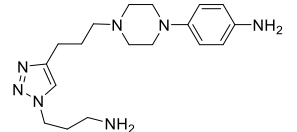
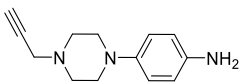
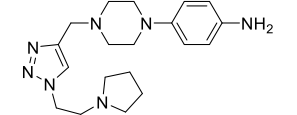
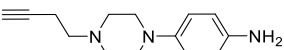
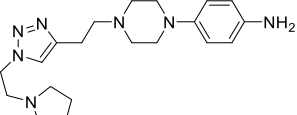
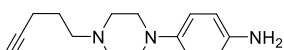
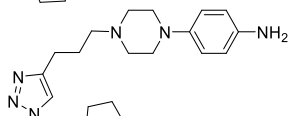
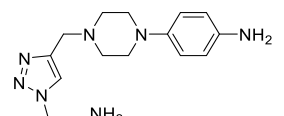
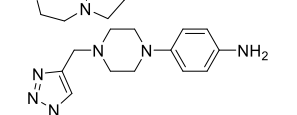
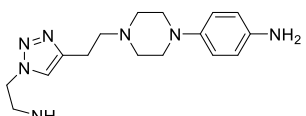
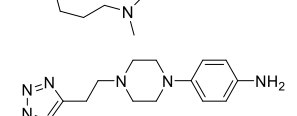
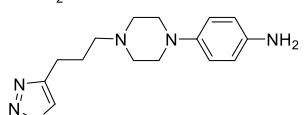
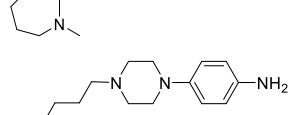
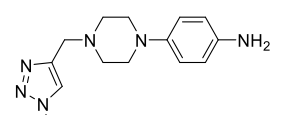
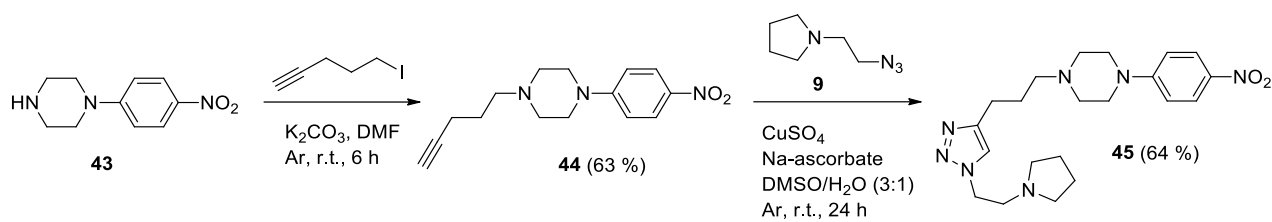
Cpd. No	Structure	PEA at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 100 μ M	Cpd. No	Structure	PEA at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 100 μ M
2		1.0	10.4 $\pm$ 0.6	35		0.5	-2.5 $\pm$ 0.4
14		0.2	3.7 $\pm$ 0.5	36		0.7	-2.3 $\pm$ 0.3
27a		0.1	3.3 $\pm$ 0.4	37		0.5	3.0 $\pm$ 0.8
28a		0.1	2.4 $\pm$ 0.9	38		0.6	3.9 $\pm$ 0.4
29a		0.5	2.4 $\pm$ 0.4	39		1.2	7.6 $\pm$ 0.4
31		0.4	-2.3 $\pm$ 0.7	40		0.7	3.6 $\pm$ 0.5
32		0.5	-1.9 $\pm$ 0.3	41		0.9	3.6 $\pm$ 0.8
33		0.5	-2.7 $\pm$ 0.2	42		0.8	4.2 $\pm$ 0.8
34		0.6	-1.4 $\pm$ 0.3				

Table 9.4: SAR data summary of the mono-amino series showing both the FRET and PEA results at 100 μ M. Data for PEA were determined as a quotient of stop band density of the respective compound divided by the stop band density of the parent compound 2.

To confirm the impact of the free NH₂ group on *c-MYC* binding, a nitro analogue of the most active mono-amino click product **39** was synthesized as a negative control. The reaction procedure (Scheme 9.4) followed the same principles as synthesis of the mono-amino series: the commercially obtained compound **43** was alkylated at the piperazine N^H position with 5-iodopent-1-yne under basic conditions [136] to obtain the alkyne **44** with three methylene units at the alkynyl linker (yield 63 %). Compound **44** was then subjected to CuAAC [39] with azide **9** to obtain the nitro click product **45** in good yield (64 %).



*Scheme 9.4: Synthesis of compound **45** that serves as a nitro analogue of compound **39**.*

As expected, the nitro derivative **45** did not exhibit any biological activity in PEA at 100 μ M – no stop product was observed. On the contrary, the FRET melting assay at 100 μ M showed partially restored activity (ΔT_m of 3.9 ± 0.1 °C) comparable to the mono-amino click product **38** that is also derived from azide **9** and that contains two methylene units at the piperazine-triazole linker. The ΔT_m increase induced by **45** was most likely due to the presence of the pyrrolidine moiety, which proved to have G4 stabilizing effect for all azide **9** derivatives. FRET data at 100 μ M for all compounds of the mono-amino series are displayed in Chart 9.1.

In conclusion, the click products **37-42**, which originated from azides **9** and **30** with a tertiary amine moiety, were the best *c-MYC* G4 ligands according to both FRET and PEA biology assays. Moreover, the FRET activity of these compounds positively correlated with the number of methylene units at the linker connecting the piperazine and the triazole ring, and was generally higher for click products derived from azide **9** with the pyrrolidine moiety. Nevertheless, no compound of the mono-amino series exhibited fully restored or increased activity in FRET melting assay when compared to the original molecule **2**. In PEA, only the click products **39** and **41** exhibited fully restored activity compared to compound **2**. Compound **39**, which is derived from alkyne **29a** and azide **9**, proved to be the most active in both assays. Finally, comparison of **39** with its nitro analogue **45** proved the necessity of amino moiety in position 1 of the aromatic ring for *c-MYC* G4 affinity.

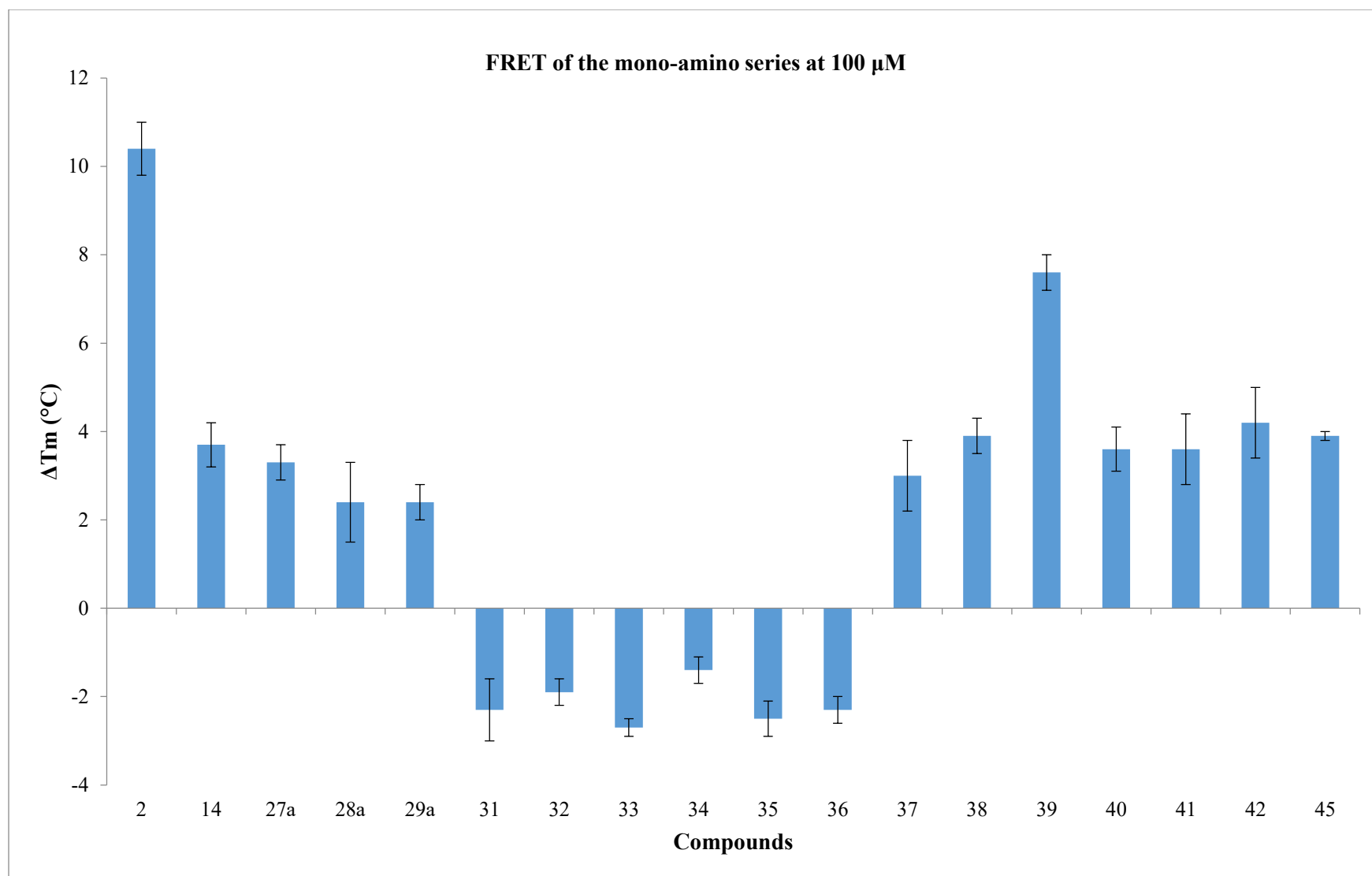


Chart 9.1: FRET analysis of all compounds of the mono-amino series (alkynes **27a**, **28a**, **29a**, and click products **31-42**) at 100 μ M compared to the template molecule **2** and its nitro analogue **14**.

9.1.2.1.3 KTGS of the mono-amino click products

KTGS employing the azide-alkyne cycloaddition *in situ* was performed in order to evaluate the co-catalytic activity of the *c-MYC* on formation of the respective mono-amino click products (**31-42**). In a standard *in situ* click experiment, detection of a click product in the case of a templated reaction directly suggests the templating effect. However, in non-catalyzed reactions, amounts of the formed triazole are usually small and hard to detect and quantify in complex reaction mixtures [39]. The lack of product formation was also observed during initial copper-free *in situ* click experiments that were performed to choose the best clickable azides (Chapter 9.1.2.1.1). Therefore, all *in situ* click experiments contained catalytic Cu^I species to increase triazole adduct formation and to facilitate the subsequent analysis [39]. For the purpose of establishing the *c-MYC* co-catalytic activity, only Pu24 was chosen as an oligonucleotide catalyst because it was used in all NMR experiments as well. Moreover, as Pu24 gives rise to only one G4 fold type, the KTGS data obtained would be more easily interpretable than upon the use of Pu27, which can form multiple G4 folds.

Initially, two series of *in situ* click experiments, both combining alkynes (**27a**, **28a**, and **29a**) and a different set of two azides, was performed. As some of the click products **31-42** possess the same molecular weight, it was necessary for the subsequent analysis by LC-MS to choose such combination of azides for a given experiment so that the molecular weight of each possible click product in the reaction mixture was unique. Therefore, alkynes **27a**, **28a**, and **29a** were reacted in experiment X-1 with azides **7** and **30**, and in experiment Y-1 with azides **8** and **9**. Within each experiment X-1 and Y-1, three different *in situ* click sub-experiments A-C were performed simultaneously: in the presence of the Pu24 oligonucleotide (A), in the absence of Pu24 (B), and a control reaction (C), where Pu24 was added just 1 min. before quenching the reaction with TFA. All these sub-experiments were carried out in triplicates to obtain statistically relevant results.

As outlined previously, after 2 hours of incubation, the *in situ* click reaction was quenched with TFA and composition of each reaction mixture was analyzed by LC-MS. However, the yield of individual click products cannot be directly derived from the MS intensities because of their different ionization states. Therefore, the substantial increase in their formation rate as the Pu24 co-catalytic effect was expressed as an A/B ratio of MS integrals in experiment A (with Pu24) and B (without Pu24). Experiment C and the related A/C ratio were introduced to assess the influence of Pu24 to the LC-MS analysis, as possible binding of the emerging click products on the Pu24 G4 may decrease its concentration in the sample for the LC-MS analysis.

The experiment X-1 provided click products **31-33** and **40-42**, while the experiment Y-1 produced click products **34-36** and **37-39** (Table 9.5). Our data revealed that click products **40-42** from experiment X-1 and click products **37-39** from experiment Y-1 exhibited higher A/B and A/C ratios than

the other compounds **31-33** and **34-36**, respectively. This finding was in accordance with the SAR study performed previously, where all the click products **37-42** prepared by chemical synthesis from azides **9** and **30** exhibited higher overall FRET activity than click products **31-36** that originated from azides **7** and **8**. Therefore, an additional *in situ* click experiment Z-1 combining alkynes **27a**, **28a**, and **29a** with these two azide molecules (**9** and **30**) was performed to compare the click product **37-39** and **40-42** formation in one reaction mixture.

The average A/B and A/C ratios for all 12 products of the *in situ* click experiments X-1 to Z-1 are presented in Table 9.5. The reaction mixture composition of experiments X-1 to Z-1 and the sub-experiments A-C, as well as the average formation yield of the *in situ* click reaction for each product, are summarized in Experimental part, Chapter 11.3.2.1 (for mixture composition, see Tables 11.2-11.4; for *in situ* formation yields, see Tables 11.5-11.7).

To confirm that the co-catalytic activity of the Pu24 oligonucleotide on ligand assembly is facilitated by the G4 secondary structure, control *in situ* click experiments were carried out. For this purpose, the G4 non-forming 'Q sequence' referred to as “ds26” that forms a double-stranded hairpin [138] was used. The control *in situ* click experiments X-2 to Z-2 were performed in the same manner as the standard *in situ* click experiments co-catalyzed with Pu24, as summarized in Experimental part, Chapter 11.3.2.1 (for mixtures composition, see Tables 11.2-11.4; for *in situ* formation yields, see Tables 11.8-11.10). The average A/B and A/C ratios for all 12 products of the *in situ* click experiments X-2 to Z-2 are presented in Experimental part, Chapter 11.3.2.1, Table 11.11.

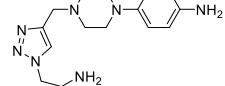
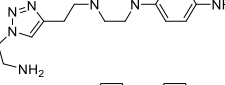
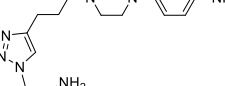
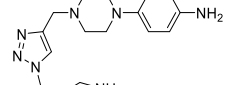
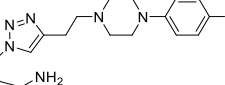
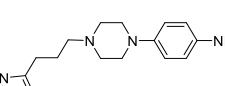
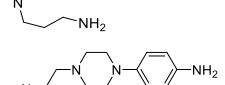
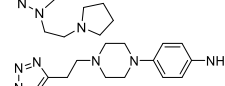
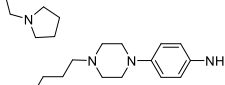
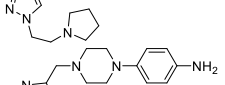
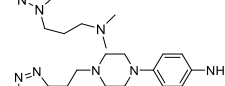
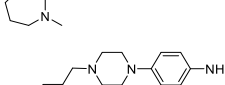
Compound	CH ₂ units	Experiment X-1		Experiment Y-1		Experiment Z-1	
		A/B	A/C	A/B	A/C	A/B	A/C
31 	1	1.8	1.3	-	-	-	-
32 	2	1.7	1.3	-	-	-	-
33 	3	4.4	3.0	-	-	-	-
34 	1	-	-	1.4	1.2	-	-
35 	2	-	-	1.5	1.2	-	-
36 	3	-	-	2.5	1.9	-	-
37 	1	-	-	4.8	3.8	5.4	4.5
38 	2	-	-	3.4	2.5	3.7	2.6
39 	3	-	-	9.8	5.5	16.9	10.5
40 	1	7.1	4.7	-	-	6.6	5.1
41 	2	5.2	3.1	-	-	7.4	4.4
42 	3	22.7	10.8	-	-	55.0	42.7

Table 9.5: The relative formation yields of compounds **31-42** given by reaction ratios. X-1, Y-1, and Z-1 are the combination reactions of three alkynes with the corresponding azides. A, B, and C are *in situ* click reactions in the presence of Pu24, without Pu24 and a control reaction, respectively. For click products with the highest A/B and A/C ratios, the respective values are marked in bold.

According to the data obtained, all the compounds **31-42** were prepared with increased yields in the presence of Pu24, which suggested a possible co-catalytic activity of G4 during click product

formation (Table 9.5, A/B and A/C ratios). Notably, the presence of three methylene units at the alkynyl chain attached to the N^4 position of the piperazine moiety led to a significantly increased yield formation of the respective click reaction products **33**, **36**, **39**, and **42**, while there was no substantial difference observed among compounds with one and two methylene units (Table 9.5). Additionally, A/B and A/C ratios of *in situ* click products derived from azides **9** and **30** were higher when compared to those of click products derived from azides **7** and **8**. In this regard, click products **40-42** derived from azide **30** had the highest A/B and A/C ratios at all, which was also confirmed in experiment Z-1 that directly compared formation of click products from azides **9** and **30**. The highest A/B and A/C ratios of *in situ* click experiments were obtained for compound **42**, followed by **39**, both of which originated from compound **29a** with the longest alkynyl linker (three methylene units). All these results were in agreement with the SAR study performed previously (Table 9.4) with the exception of compound **39** that showed better activity than **42** in both FRET and PEA at 100 μ M. Important to mention, the control reaction C showed small but significant increase in reaction yields when compared to B, indicating a rapid click product formation due to addition of Pu24 oligonucleotide just 1 min. before quenching the reaction. For that reason, it is more accurate to consider here the A/B ratio as the corresponding relative formation yield of a particular compound than the A/C ratio.

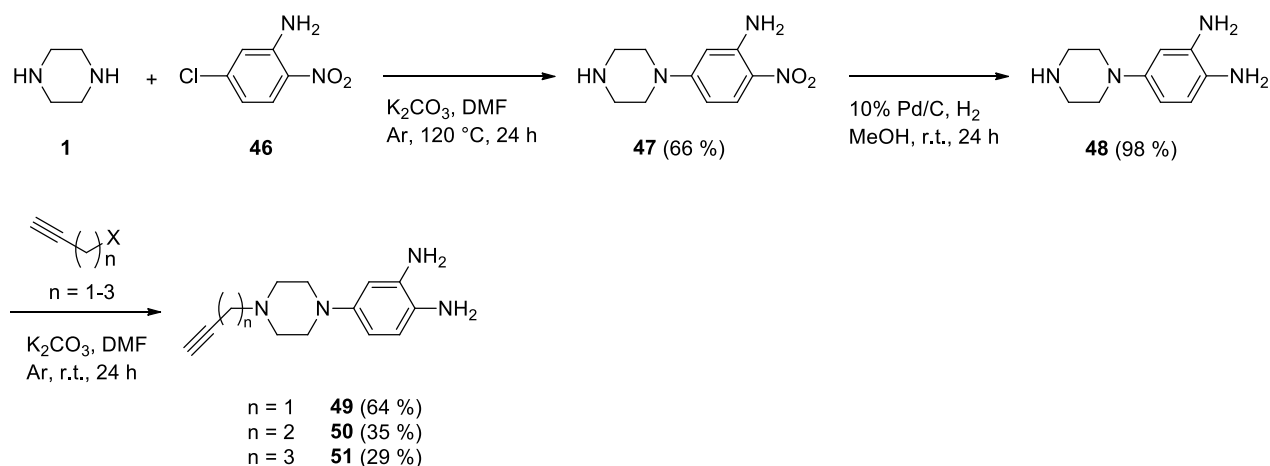
In contrast, the results concerning the control *in situ* click experiments showed no remarkable product formation increase in experiments A with ds26 over experiments B without ds26 and control experiments C. The respective A/B and A/C ratios were in most cases close to 1.0 (Experimental part, Chapter 11.3.2.1, Table 11.11). However, a considerable decrease of the A/B and A/C ratios was observed for compounds **31**, **32**, and **34**, which were derived from azides A and B with a primary amino moiety. This suggests binding of the respective click products to the double-stranded ds26 sequence due to the primary amino group bound to the aliphatic chain. Nevertheless, based on the control *in situ* click experiments, we can suppose that the click product formation yield increase in the presence of Pu24 oligonucleotide is due to the G4 co-catalytic activity.

To sum up, the presence of Pu24 in the reaction mixture led to increased click product formation, which suggested a possible co-catalytic activity of this oligonucleotide. The Pu24 co-catalysis led to significantly increased formation of click products from azides **9** and **30** containing a tertiary amino moiety, and click products that originated from azide **29a** with three methylene units in the alkynyl linker. On the other hand, control *in situ* click experiments with G4 non-forming ds26 sequence did not show any substantial increase of click product formation. Therefore, we can assume that the increase of click product formation in the presence of Pu24 is due to the co-catalytic activity of the *c-MYC* G4.

9.1.2.2 The bis-amino series

9.1.2.2.1 Design and synthesis of the bis-amino series

The bis-amino series was based on the compound **15** analogous to the original mono-amino molecule **2**, and its construction followed the same principles as in the case of the mono-amino series. In the first stage of the process, a homology series of alkynes suitable for click chemistry was prepared. The synthesis started by nucleophilic aromatic substitution of 5-chloro-2-nitroaniline (**46**) with piperazine (**1**) to obtain the compound **47** [139] with an *o*-nitroaniline moiety in fair yield (66 %). Compound **47** was subsequently reduced by Pd/C under hydrogen atmosphere to afford its *o*-aminoaniline derivative **48** [139] in quantitative yield (98 %). The compound **48** was then *N*-alkylated under basic conditions [136] with three alkynyl halides of different linker length to form the bis-amino homology series of compounds **49-51** with 1, 2, or 3 methylene units at the alkynyl chain in low to fair yield (29-64 %, Scheme 9.5).

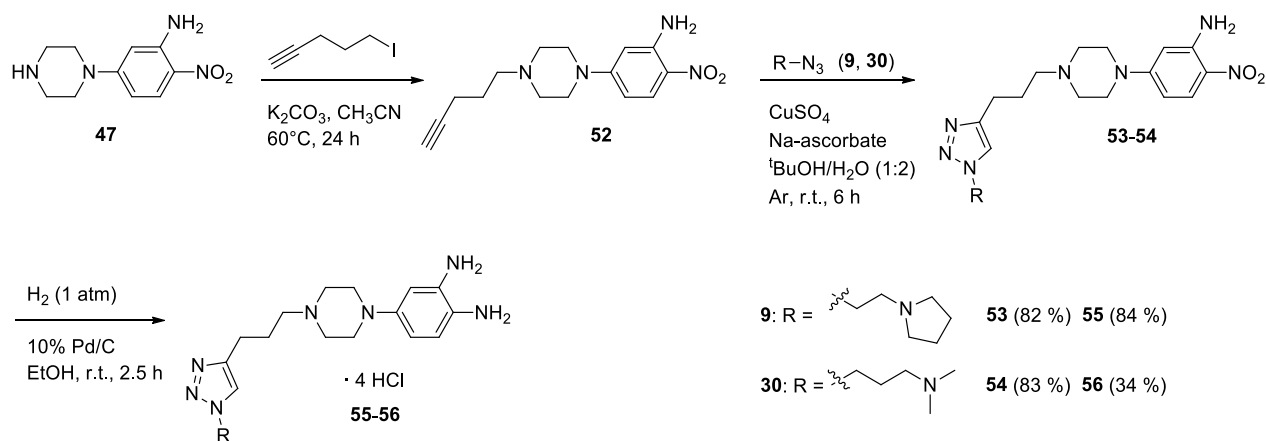


*Scheme 9.5: Synthesis of the homology series of the bis-amino clickable alkynes **49-51** with 1, 2, or 3 methylene units at the alkynyl chain.*

In the course of handling small samples of the bis-amino alkynes **49-51** at r.t. and in the presence of air, obvious decomposition was observed. After performing chromatography on a silica gel column and evaporating the mobile phase, the colour of these compounds changed within several minutes from beige amorphous solid to dark brown oil. Oxidation of the *o*-aminoaniline moiety, which was previously described for aromatic bis(*o*-aminoanils) [140], was eventually confirmed for compounds **49-51** by MS spectroscopy (data not shown). Therefore, these compounds required careful handling under argon atmosphere or vacuum and prompt freezing after solvent evaporation to avoid their decomposition.

Within the bis-amino series, only click products derived from an alkyne **51** with 3 methylene units were prepared because the previously performed SAR study regarding the mono-amino series (Table 9.4, FRET results) and *in situ* click experiments (Table 9.5) showed that the *c-MYC* stabilizing

activity of click products positively correlated with the length of the linker connecting the piperazine and the triazole ring. In particular, compound **29a** containing the *N*-alkynyl chain with three methylene units produced mono-amino click products with the best *c-MYC* G4 stabilizing activity. Furthermore, only azides **9** and **30** were chosen for the respective CuAAC, as they provided more active mono-amino click products when compared with azides **7** and **8**. However, the analogous direct click reaction of the bis-amino alkyne derivative **51** with azides **9** and **30** was not performed due to low stability of the *o*-aminoaniline moiety. Therefore, reaction steps leading to the respective bis-amino click products (Scheme 9.6) had to be carried out in a different order. First, the *o*-nitroaniline compound **47** was alkylated with 5-iodopent-1-yne [136] to form alkyne **52** in quantitative yield (97 %), which was then reacted in CuAAC [39] with azides **9** and **30** resulting in *o*-nitroaniline click products **53-54**, respectively (yield 82-83 %). These *o*-nitroaniline compounds were subsequently reduced by Pd/C under hydrogen atmosphere to their *o*-aminoaniline counterparts, **55-56**, and immediately converted to HCl salts to stabilize them. According to elemental analysis, the tetrahydrochloride salt of each compound was obtained (yield 34-84 %).



Scheme 9.6: Synthesis of o-aminoaniline click products 55-56 as derivatives of alkyne 51 with three methylene units in the alkynyl chain.

Easy decomposition of the *o*-aminoaniline moiety was the reason why KTGS (*in situ* click experiment) regarding *o*-aminoaniline click products **55-56** was not carried out. It was assumed that performing a direct click reaction of **51** with azides **9** and **30** in aqueous solution, in the presence of air, and under prolonged (2-hour) shaking at r.t. would lead to quick oxidation of the *o*-aminoaniline alkyne **51**.

Finally, the compound **57** (Figure 9.2) as the *o*-nitroaniline analogue of compound **15** was provided by my colleague, Dr. Paul E. Reyes-Gutiérrez, who synthesized this compound by nucleophilic aromatic substitution of 5-chloro-2-nitroaniline (**46**) with 1-methylpiperazine under the same conditions

as for compound **47** (Scheme 9.5). Both the compounds **57** and **15** were included in the bis-amino series and tested for *c-MYC* binding ability.

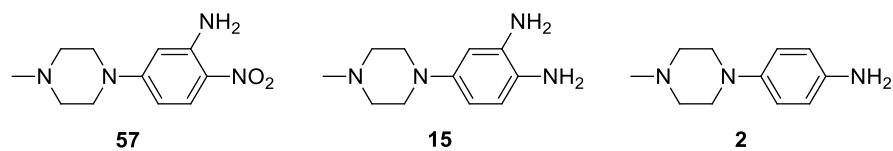


Figure 9.2: Structure of compound 57 as an o-nitroaniline analogue of 2 and 15.

9.1.2.2.2 The structure-activity relationship regarding the bis-amino series

All the compounds belonging to the bis-amino series were evaluated in FRET melting assay at 100 μM using dual-labelled (5'-(6-FAM) and 3'-(6-TAMRA)) 25-mer oligonucleotide Fc-myc25T, and in PEA at 100 μM using a template strand containing the Pu27 sequence. The results were then compared with the template **2** molecule of the mono-amino series and its nitro derivative **14**, which was used as a negative control for PEA. As all the *o*-aminoaniline derivatives exhibited significant ΔT_m increase in FRET at 100 μM when compared to compound **2**, additional FRET experiments at 50 μM and 25 μM were performed for all compounds of the bis-amino series, including the *o*-nitroaniline derivatives. The results for *o*-aminoaniline (**15**, and **48-51**) and *o*-nitroaniline (**47**, **52**, and **57**) derivatives are summarized in Table 9.6. Similarly, the results for *o*-nitroaniline (**53-54**) and *o*-aminoaniline (**55-56**) click products are shown in Table 9.7. Chart 9.2 displays the FRET activity of all respective compounds of the bis-amino series at 25 μM , 50 μM , and 100 μM .

According to the results obtained, all the *o*-aminoaniline derivatives, including the alkynes **49-51** and the click products **55-56**, exhibited ΔT_m increase at 25 μM , 50 μM , and 100 μM in FRET when compared to compound **2** at the respective concentrations. Moreover, the PEA results for the *o*-aminoaniline derivatives showed either partial restoration (**49-50**) or increase (**15**, **48**, **51**, **55**, and **56**) of activity when compared to compound **2**. These findings confirmed that the presence of additional amino group in position 2 enhances the activity towards the *c-MYC*, as was previously shown in biology assays performed on commercially available derivatives of 4-(4-methylpiperazin-1-yl)aniline (**2**) (Figure 9.1). The highest increase in FRET was observed for the *o*-aminoaniline derivatives **15** and **48** and click products **55-56**, which also appeared to be the most active compounds in PEA. Additionally, both the FRET and PEA activity of the *o*-aminoaniline alkynes **49-51** positively correlated with the length of the alkynyl linker, outlining the compound **51** with three methylene units as the most efficient alkynyl ligand.

In contrast, the activity of all *o*-nitroaniline derivatives (**47**, **52**, and **57**) was similar to negative control (**14**) in PEA and lower than compound **2** (and similar to compound **14**) in FRET at 50 μM and 100 μM . At 25 μM , the FRET activity of *o*-nitroaniline compounds was comparable to both **2** and **14**. Therefore, these *o*-nitroaniline derivatives served as negative controls for their respective *o*-aminoaniline analogues in FRET, as illustrated by Chart 9.2. Notably, partially restored FRET activity at 100 μM regarding the template molecule **2** was observed for the *o*-nitroaniline alkyne **52** and the *o*-nitroaniline click product **56** derived from azide **30**. Based on these results, we can conclude that the presence of alkynyl chain bound to the N^4 piperazine nitrogen of **52** and its subsequent growth in click reaction enhances the activity towards *c-MYC* when compared to its N^4 -methyl analogue **57** or compound **47** with an unsubstituted N^4 position of piperazine.

As both the *o*-nitroaniline and *o*-aminoaniline and click products **54** and **56** derived from azide **30** exhibited slightly better activity in FRET (compound **56** at all concentrations, compound **54** at 100 μ M) than their respective azide **9** analogues **53** and **55**, it is assumed that the presence of the *N,N*-dimethyl-3-aminopropyl moiety on the triazole ring provides the click product with better biological activity than the *N*-ethylpyrrolidine moiety. This finding is in accordance with the results from *in situ* click experiments presented for the mono-amino series that outlined the click product **42** derived from azide **30** and alkyne **29a** as the most active. On the other hand, it contradicts PEA and FRET results at 100 μ M that depicted the mono-amino click product **39** containing the *N*-ethylpyrrolidine moiety as more active than its *N,N*-dimethyl-3-aminopropyl derivative **42**.

To determine the G4-binding specificity over duplex DNA, all the bis-amino compounds as well as compounds **2** and **14** of the mono-amino series were tested in control FRET experiment with a G4 non-forming template at 25 μ M and 50 μ M. For this purpose, a dual-labelled FdsT oligonucleotide containing ds26 sequence that forms a double-stranded hairpin [138] was used. The results of these control experiments together with Fc-myc25T data are illustrated by Chart 9.3; the FRET data for control measurements are available in Experimental part, Chapter 11.3.1, Table 11.1. Obviously, the control FRET experiments with FdsT showed in most cases negligible stabilization potential at both concentrations. Although a slight ΔT_m increase was observed for compound **48** at 50 μ M, the value was negligible when compared to the ΔT_m increase induced for Fc-myc25T stabilization at 50 μ M for the same compound. Therefore, we can conclude that the *o*-aminoaniline compounds of the bis-amino series selectively interact with the *c-MYC* G4 motif in Fc-myc25T relative to the double-stranded secondary structure formed by FdsT.

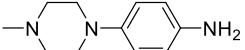
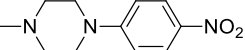
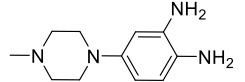
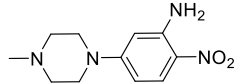
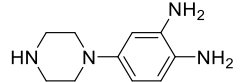
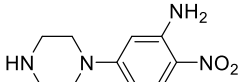
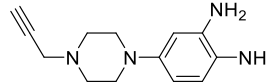
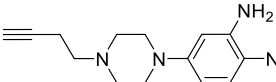
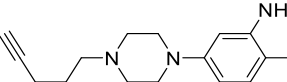
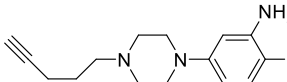
Cpd. No	Structure	PEA at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 50 μ M	ΔT_m ($^{\circ}$ C) FRET at 25 μ M
2		1.0	10.4 ± 0.6	2.0 ± 0.5	-0.5 ± 1.2
14		0.2	3.7 ± 0.5	-0.1 ± 0.8	0.0 ± 1.5
15		1.6	> 35	26.2 ± 0.4	18.6 ± 0.5
57		0.1	1.0 ± 1.0	-2.1 ± 0.5	-0.5 ± 1.5
48		1.6	> 35	27.8 ± 1.3	18.7 ± 0.3
47		0.3	4.1 ± 1.1	1.4 ± 0.6	0.1 ± 1.4
49		0.5	13.0 ± 0.9	7.7 ± 0.7	1.9 ± 0.4
50		0.5	15.9 ± 1.0	9.4 ± 0.5	4.2 ± 0.6
51		1.6	29.5 ± 0.3	25.0 ± 0.7	17.9 ± 1.8
52		0.1	5.6 ± 0.4	1.3 ± 0.7	-1.7 ± 0.9

Table 9.6: SAR data summary of the *o*-aminoaniline alkynes and other **15** derivatives in comparison to compounds **2** and **14** of the mono-amino series. The table displays PEA results at 100 μ M, and FRET results at 100 μ M, 50 μ M, and 25 μ M. Data for PEA were determined as a quotient of stop band density of the respective compound divided by the stop band density of the parent compound **2**.

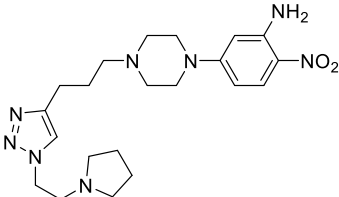
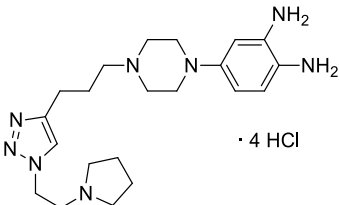
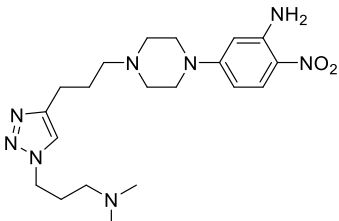
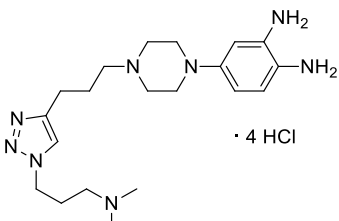
Cpd. No	Structure	PEA at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 100 μ M	ΔT_m ($^{\circ}$ C) FRET at 50 μ M	ΔT_m ($^{\circ}$ C) FRET at 25 μ M
53		0.5	3.5 ± 0.4	-0.5 ± 1.1	-1.2 ± 0.3
55	 • 4 HCl	4.5	33.9 ± 0.2	27.0 ± 2.3	6.4 ± 1.5
54		0.7	5.1 ± 0.7	0.5 ± 2.3	-2.9 ± 2.2
56	 • 4 HCl	4.5	> 35	28.5 ± 0.4	9.2 ± 1.1

Table 9.7: SAR data summary of *o*-nitroaniline and *o*-aminoaniline click products showing PEA results at 100 μ M and FRET results at 100 μ M, 50 μ M, and 25 μ M. Data for PEA were determined as a quotient of stop band density of the respective compound divided by the stop band density of the parent compound **2**.

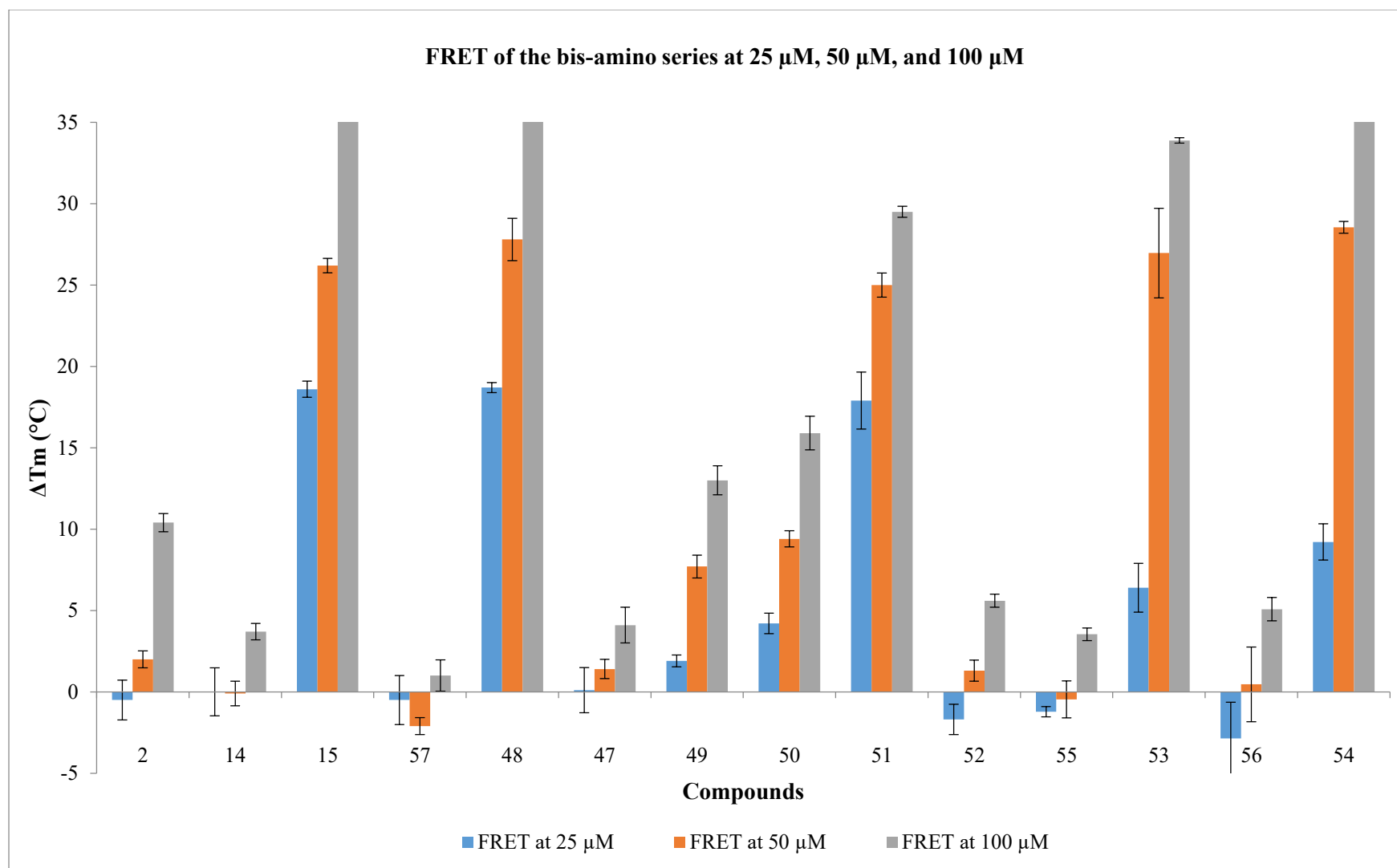


Chart 9.2: FRET analysis of the bis-amino series at 25 μ M, 50 μ M, and 100 μ M in comparison with compounds **2** and **14**. Juxtapositioning each *o*-aminoaniline compound with its *o*-nitroaniline analogue showed necessity of the additional amino moiety for *c*-MYC interaction.

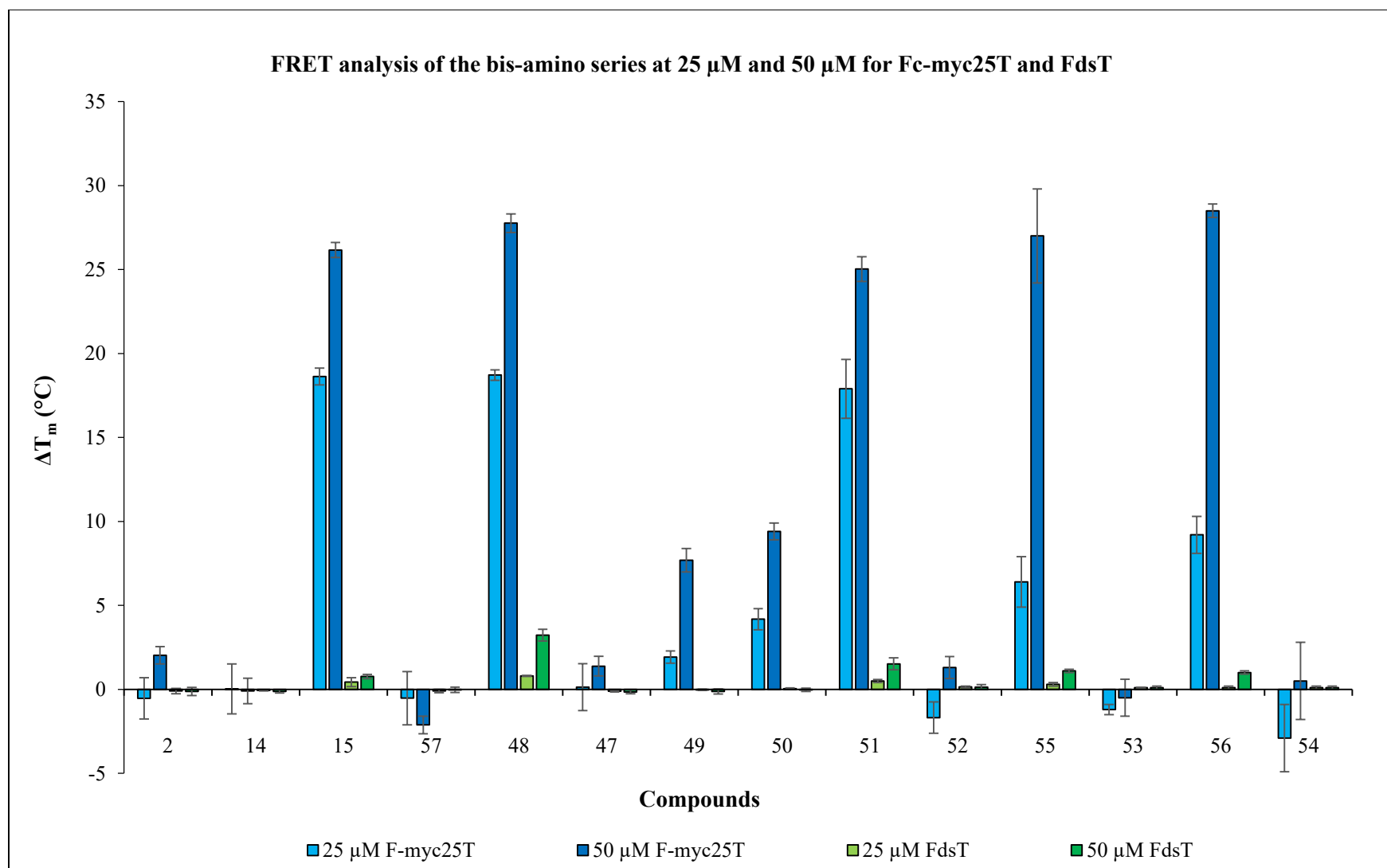


Chart 9.3: FRET analysis of the bis-amino series and compounds **2** and **14** at 25 μ M and 50 μ M using Fc-myc25T and FdsT. Juxtapositioning Fc-myc25T results with FdsT values for each compound showed selectivity of interaction of the *o*-aminoaniline compounds with the *c*-MYC motif.

Generally, all *o*-aminoaniline derivatives of the bis-amino series exhibited notably higher affinity towards the *c*-MYC G4 in FRET and PEA when compared to the mono-amino series (Table 9.4). Both the *o*-aminoaniline click products **55** and **56** showed a significant increase of activity in FRET experiments at 100 μ M that was comparable to **15** and **51** and even 3-fold higher than the activity measured for **2**. Moreover, based on PEA measurements, **55** and **56** appeared to be the strongest ligands prepared in this study with 4.5-fold activity increase compared to the parent molecule **2**. Among the azides **49-51**, an increase of FRET activity was observed with growing number of methylene units in the alkynyl linker, which was in accordance with the results regarding the mono-amino series. The inactivity of the analogous *o*-nitroaniline compounds (**47**, **52-54**, and **57**) underlines the importance of the second amino moiety in position 2 of the aromatic ring for the *c*-MYC G4 stabilization potency. Finally, FRET control experiments performed with the G4 non-forming template FdsT at 25 μ M and 50 μ M showed zero or only negligible ΔT_m increase and thus confirmed validity and relevance of our Fc-myc25T measurements.

9.1.2.3 Structural basis of compound binding by chemical-shift perturbation NMR

Apart from testing in FRET and PEA biology assays, all compounds from both the mono- and the bis-amino series were evaluated by chemical-shift perturbation NMR to determine their binding mode towards the *c-MYC* G4. For this purpose, the oligonucleotide Pu24 was used and chemical shift changes in guanine imino-region of ^1H NMR spectra upon ligand addition were observed. The ^1H NMR spectroscopy was performed and the data obtained were analyzed by Dr. Lukáš Vrzal, and the results were presented in his Ph.D. thesis [10]. With the consent of Dr. Lukáš Vrzal, a brief overview regarding the chemical-shift perturbation NMR is provided to obtain full and accurate picture of the *c-MYC* binding ability of each compound.

The G4 core of Pu24 is formed by three guanine tetrads, depicted in Figure 9.3A as a 5' G-tetrad (green), a central G-tetrad (purple), and a 3' G-tetrad (blue) [77]. All compounds were classified based on perturbations or disappearing of individual assigned ^1H NMR guanine imino-signals of Pu24 either as Pu24 binders and further sorted into three distinct classes I-III according to the binding mode (Figure 9.3B), or as non-binders [10].

Compounds **15**, **48**, **55**, and **56**, outlined as the most potent class I binders, induced 5' G-tetrad destabilization that manifested itself by disappearance of the imino signals of G4, G13, G8 and G17. The residues from the central G-tetrad (G18, G5, G14) and from the 3' G-tetrad (G15, G6, G19) were significantly shifted, which indicated ligand stacking on the top of the central G-tetrad or intercalation between the central and the 3' G-tetrad [10]. Notably, all the class I binders **15**, **48**, **55**, and **56** contained *o*-aminoaniline moiety and exhibited high stabilizing potency towards Pu24 in both PEA and FRET biology assays. Moreover, compounds **55** and **56** were the most active click products prepared in this study.

The class II of binders comprised the homology series of the *o*-aminoaniline alkynes **49-51** and also the *o*-nitroaniline click products **53** and **54**. These compounds induced perturbations within the 5' G-tetrad (G4, G13, G8 and G17) and central G-tetrad (G5, G14), indicating selective binding of the ligand only to the 5' G-tetrad; this mode of interaction did not disrupt the G4 structure [10].

All compounds of the class III binders carried a nitro group: **52** and **57** were *o*-nitroaniline derivatives, while **45** was a click product of the mono-amino series possessing a nitro group on the aromatic ring. The interaction mode of these ligands was supposed to be the weakest as they induced perturbations of only two guanine NH signals from the 5' G-tetrad (G4, G17), suggesting a specific interaction that has only minimal effect on the overall Pu24 conformation. Additionally, chemical shift changes were only moderate when compared to class I and II compounds [10]. These observations were in accordance with the SAR study performed previously, which showed that activity of **45**, **52**, and **57** was similar to negative control in PEA and lower than **2** in FRET.

The compounds classified as non-binders induced no significant effects in the imino regions of ^1H NMR spectra. This category comprised all the mono-amino click products **31-42**, the alkynes **27a**, **28a**, and **29a**, non-alkylated *o*-nitroaniline derivative **47**, compound **14** as a nitro-analog of **2**, and the compound **2** itself [10]. For compounds **14**, **27a**, **28a**, and **29a**, and **47** and for most mono-amino click products, the ^1H NMR measurements were consistent with zero or only partially restored activity FRET when compared to **2** and activity similar to negative control in PEA, both of which suggested low binding affinity towards *c-MYC*. However, the non-binder class also included the template molecule **2** as well as the mono-amino click products **39** and **42** that exhibited some stabilizing activity in FRET and their formation was promoted in the presence of Pu24 during *in situ* click experiment. In this case, alternative binding mechanism that does not perturb imino groups in the core G4 tetrads is suggested.

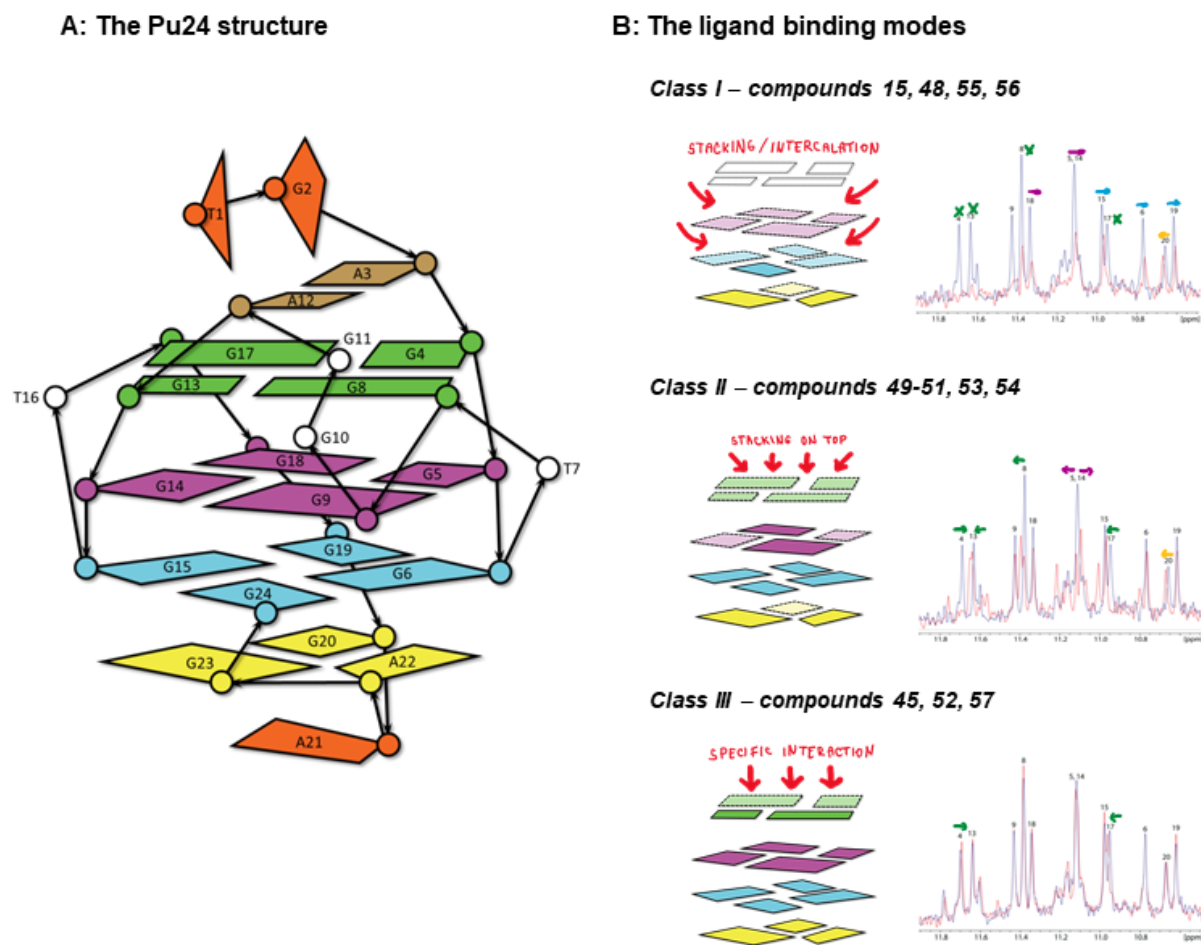
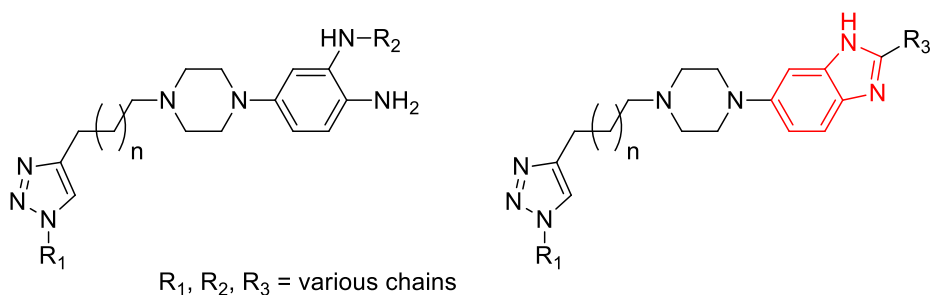


Figure 9.3: (A) Schematic representation of the Pu24 oligonucleotide [77]. (B) Three classes (I-III) of Pu24 binders determined by changes of peaks of individual guanine NH groups within the amidic region of ^1H NMR spectra [7, 10].

9.1.3 Outlook

Based on all SAR results including FRET and PEA biology assays and chemical-shift perturbation ^1H NMR, it is clear that *o*-aminoaniline compounds of the bis-amino series are substantially stronger *c-MYC* ligands than the mono-amino derivatives of 4-(4-methylpiperazin-1-yl)aniline (**2**). Further chemical elaboration of the *o*-aminoaniline compounds and especially the click products might lead to development of effective tool for cancer treatment. Nevertheless, the issue of instability of the *o*-aminoaniline moiety should be solved first, most probably *via* substitution of the additional NH_2 moiety in position 2 of the aromatic ring. Additionally, modification of both amino groups to obtain 1*H*-benzo[*d*]imidazole moiety, which is a recurring motif present in the structure of an established G4 binder Hoechst 33258 (**3**), should be achieved. Finally, further extension of the alkynyl linker length should be performed, as it may provide the respective click product with increased flexibility and therefore facilitate more efficient interaction with the *c-MYC* G4. Possible modifications of the bis-amino click products and structure of the 1*H*-benzo[*d*]imidazole template are shown in Figure 9.4.

A: Possible modifications of the bis-amino derivatives



B: Hoechst 33258 as a 1*H*-benzo[*d*]imidazole structural template

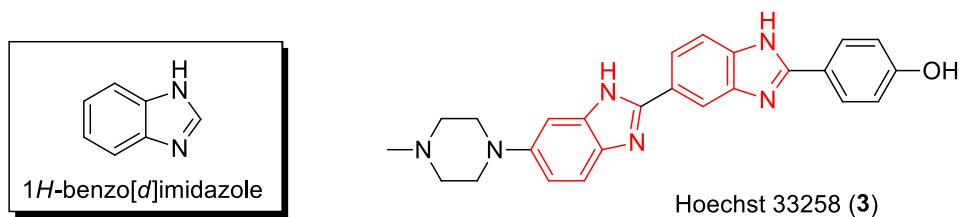


Figure 9.4: A – possible modifications of the *o*-aminoaniline click products with regard to their *o*-aminoaniline moiety and the piperazine-triazole linker length. B – 1*H*-benzo[*d*]imidazole as a structural basis for the dye Hoechst 33258 (**3**), which represents an established G4 binder. In both panels, the 1*H*-benzo[*d*]imidazole moiety incorporated in the possible bis-amino derivative and Hoechst 33258 is marked in red.

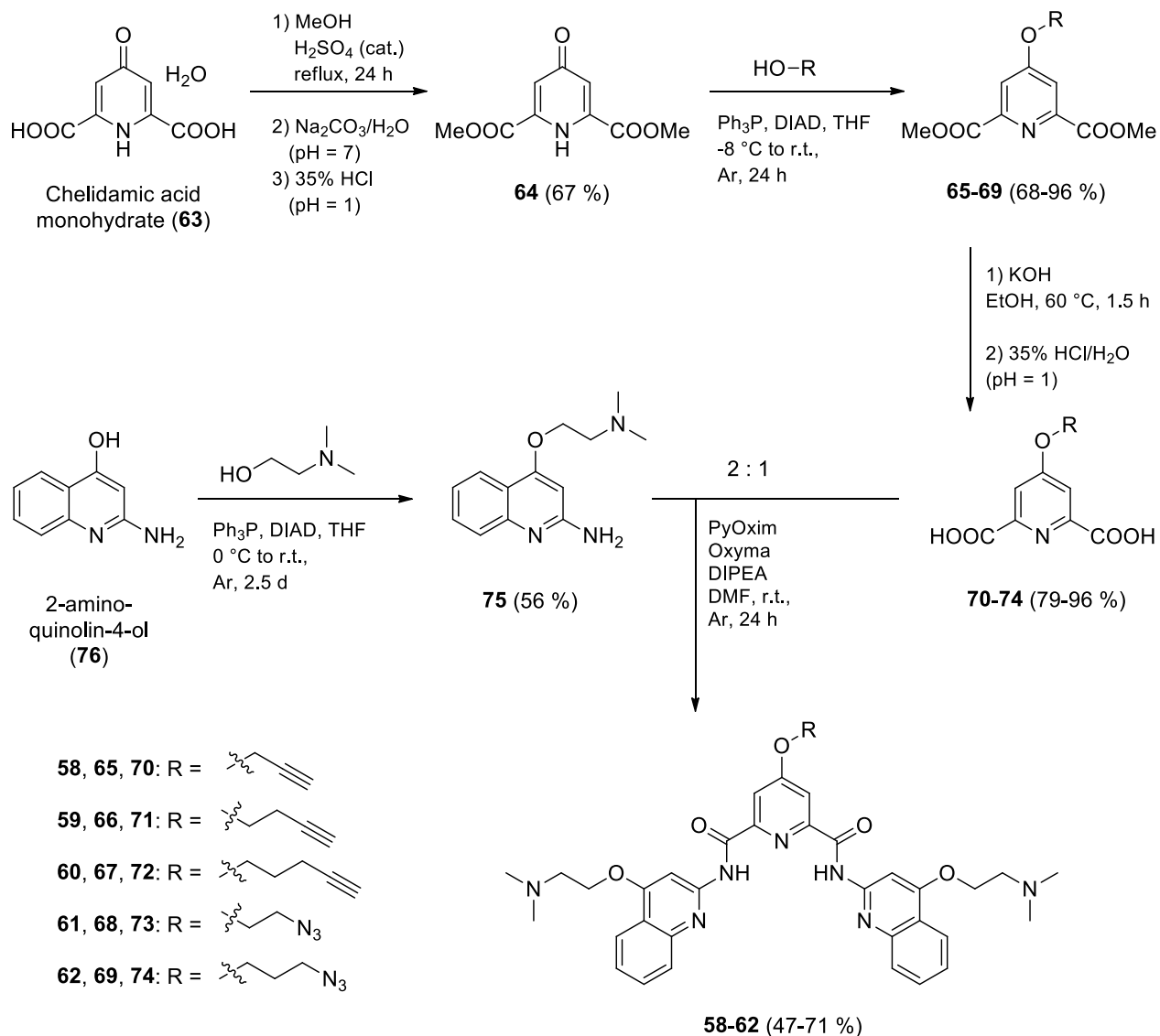
9.2 The focused fragment library based on pyridostatin

9.2.1 Design and synthesis of the pyridostatin derived series

Construction of the pyridostatin fragment library was directly inspired by the study performed by Di Antonio *et al.* In his work regarding H-Telo ligands, two pyridostatin derived alkynes were prepared and employed in click chemistry with azides **7-12** both *in situ* and *via* chemical synthesis [39] (Introduction, Chapter 7.3.2). Here, I intended to prepare a homology series of pyridostatin derivatives that would contain either alkyne or azide functional group suitable for subsequent modification in click chemistry. Moreover, according to the study presented by Müller *et al.*, pyridostatin derivatives containing the -NMe₂ functionality in the quinolone side chains exhibited slightly higher stabilization potential towards H-Telo than the original pyridostatin molecule (**4**) with NH₂ group and other respective pyridostatin analogues [130] (Introduction, Chapter 7.3.2). Therefore, the whole pyridostatin based library presented in this work was modified accordingly.

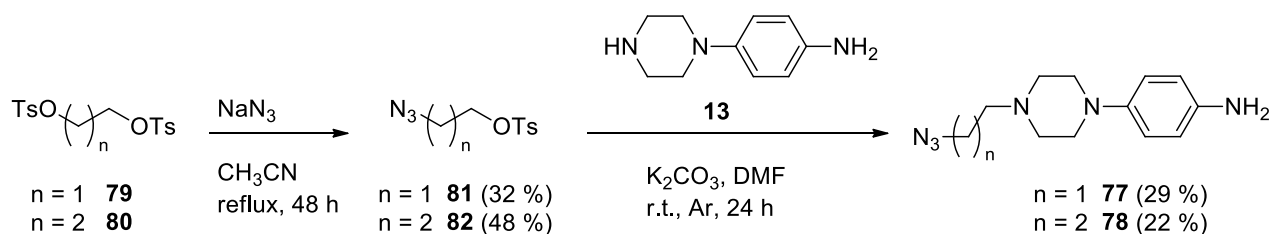
As a starting point for click chemistry, two pyridostatin derived homology series were synthesized: a series of pyridostatins containing an alkynyl chain with 1-3 methylene units (**58-60**), and a series of pyridostatins containing an azidoalkyl chain with 2-3 methylene units (**61-62**). Both homology series were synthesized in the same fashion (Scheme 9.7). First, chelidamic acid monohydrate (**63**) was converted to dimethyl ester **64** [129, 130]. Instead of transforming carboxylic functionalities to acyl chlorides by SOCl₂ and their subsequent esterification with methanol as described in the original protocol [39, 129, 130], direct esterification with methanol using catalytic amount of H₂SO₄ followed by precipitation of the ester by adjusting the pH [141] was performed to obtain **64** in moderate yield (67 %). This methodology was chosen for its simplicity over the two-step reaction commonly used in pyridostatin synthesis. In the next stage, the alkynyl- or azidoalkyl- moiety was introduced to the hydroxyl group of **64** through the Mitsunobu reaction using an appropriate alcohol, Ph₃P, and DIAD to get the respective alkyne (**65** [39] and **66-67**) or azide (**68-69**) product in good to excellent yield (68-96 %). The subsequent cleavage of methyl esters under basic conditions followed by product precipitation by pH adjustment afforded synthons **70** [39] and **71-74** with free carboxylic groups in very good to excellent yields (79-96 %). To prepare the aminoquinoline-derived synthon **75** [130], 2-aminoquinolin-4-ol (**76**) was *O*-alkylated with 2-(dimethylamino)ethan-1-ol through the Mitsunobu reaction [39, 129, 130] using Ph₃P and DIAD to obtain the desired compound in moderate yield (56 %). Finally, each of the chelidamic acid derived synthons **70** [39] and **71-74** were condensed with two equivalents of the aminoquinoline synthon **75**. The peptide bond formation using Ph₃P, which is described for pyridostatin synthesis [39, 129, 130], provided the desired product in a very low yield. Therefore, PyOxim was used as a coupling reagent and DIPEA was used as a base [142]. Moreover, the reaction was performed with the aid of Oxyma (ethyl (hydroxyimino)cyanoacetate)), which was reported in the literature as an accelerating agent that increased

the reaction yield [143]. The resulting pyridostatin derivatives **58-62** were obtained in moderate yields (47-71 %). This methodology of amide bond formation was new regarding the pyridostatin synthesis and provided substantially higher yields of modified pyridostatins than the original procedure described in the literature [129, 130].



*Scheme 9.7: Synthesis of pyridostatin derived homology series of alkynes **58-60** and azides **61-62**.*

The pyridostatin derived azides **61** and **62** were intended to be coupled with the mono-amino homology series of alkynes **27a**, **28a**, and **29a** that were synthesized as derivatives of compound **2** (Chapter 9.1.2.1.1, Scheme 9.2). As in the case of the focused fragment library based on the 4-(4-methylpiperazin-1-yl)aniline structure, the choice of azides for the click reaction with the pyridostatin derived alkynes **58-60** was determined by the results from the initial *in situ* click experiment (Chapter 9.1.2.1.1, Table 9.2) performed according to ref. [39]. The cationic azides **7-9** with the amino moiety were chosen, complemented by azide **30** for its similarity with the quinolone substituent containing the -NMe₂ moiety that led to slight increase of H-Telo stabilization potential in the respective pyridostatin derivatives [130]. Apart from that, a homology series of two azides (**77-78**) as compound **2** derivatives was prepared for the purpose of click chemistry with pyridostatin derived alkynes **58-60** (Scheme 9.8). Initially, S_N2 reaction of an appropriate bis(p-toluenesulfonate) **79-80** with one equivalent of sodium azide afforded alkylating agents **81** [144] and **82** [145] bearing an azido moiety in low to moderate yield (32-48 %). The compounds **81-82** were then used for N⁴-alkylation of compound **13** under basic conditions in another S_N2 reaction [136] to obtain the azides **77** and **78**. The low reaction yield of 22-29 % is attributed to difficult purification on a silica gel column, as several compounds with an R_f value similar to the desired product were present in each reaction mixture.

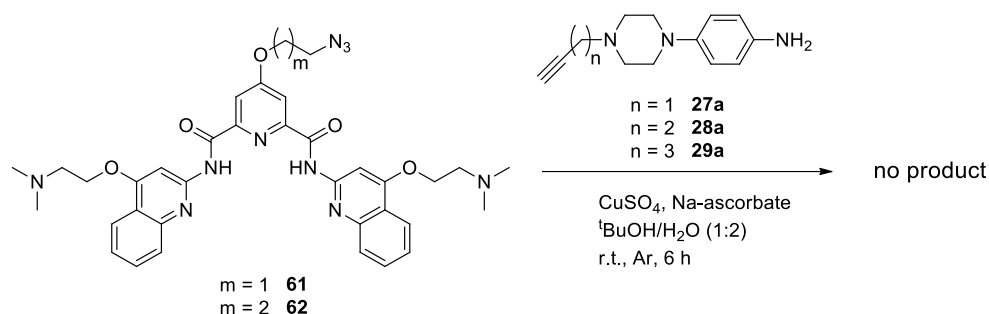


Scheme 9.8: Synthesis of homology series of azides **77-78** as derivatives of compound **2**.

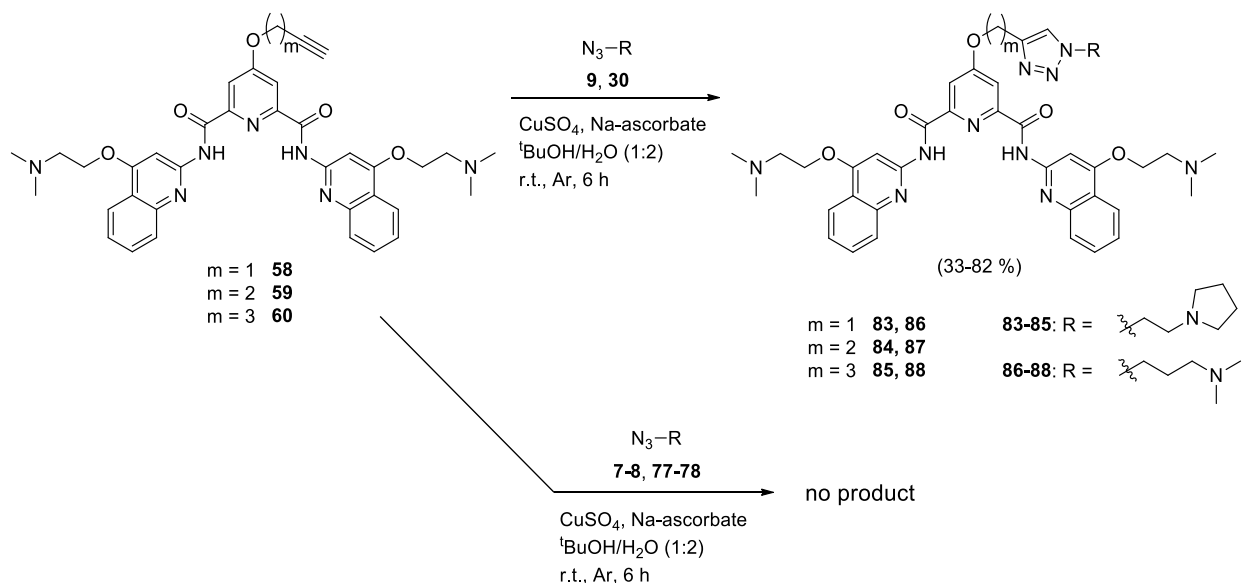
As proposed, the pyridostatin derived azides **61-62** were allowed to couple with 4-(4-methylpiperazin-1-yl)aniline-derived alkynes **27a**, **28a**, and **29a** in CuAAC using CuSO₄ as a catalyst and sodium ascorbate as a reducing agent. The reaction was performed in the *tert*-butanol/H₂O (1:2) mixture under argon atmosphere according to the ref. [39] (Scheme 9.9). Likewise, the copper-catalyzed chemical click was attempted between the pyridostatin derived alkynes **58-60** and azides **7-9**, **30**, and **77-78** (Scheme 9.10). However, the click reaction products were obtained only for the CuAAC reaction of alkynes **58-60** with azides **9** and **30** containing a tertiary amino moiety. The respective click products **83-88** were afforded in low to good yield (33-82 %).

In order to obtain the remaining pyridostatin derived click products, there were several efforts to modify reaction conditions, e.g., increasing concentration of CuSO₄ and sodium ascorbate, or using

DMSO/water mixture in various ratios as a solvent, but without any effect. It is notable that the presence of the primary amino moiety during CuAAC regarding both the mono-amino series of compound **2** derivatives did not cause any problem (Chapter 9.1.2.1.1, Scheme 9.3). Therefore, we anticipated that the reason why the CuAAC with alkynes **27a**, **28a**, **29a**, and **7-8** and azides **77-78** containing a primary amino group did not provide pyridostatin click products was attributed to interaction of the free NH₂ functionality with the pyridostatin core. Interestingly, no such difficulty was reported by Di Antonio *et al* who successfully performed the CuAAC of pyridostatin derived alkynes **5-6** (for structures, see Figure 7.16A in Introduction, Chapter 7.3.2) with azides **7-8** by synthetic means [39].

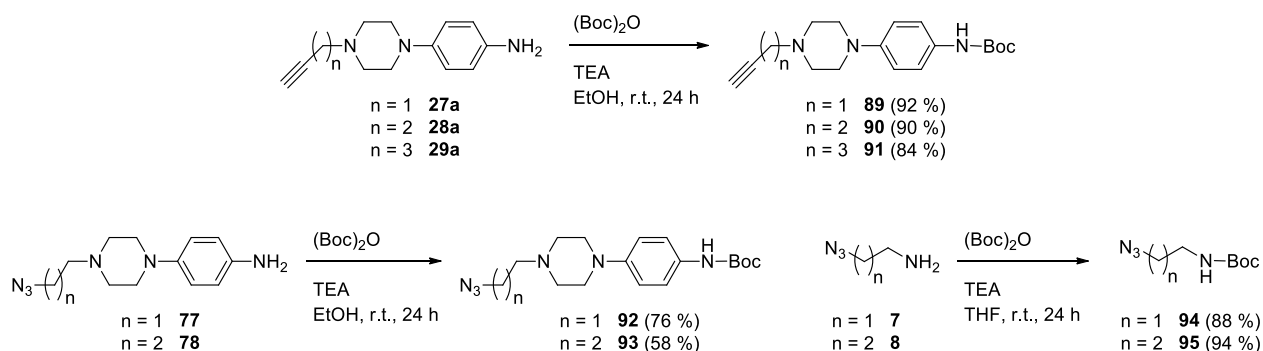


Scheme 9.9: The CuAAC reaction between pyridostatin derived azides **61-62** and 4-(4-methylpiperazin-1-yl)aniline-derived alkynes **27a**, **28a**, and **29a**. No reaction product was observed.



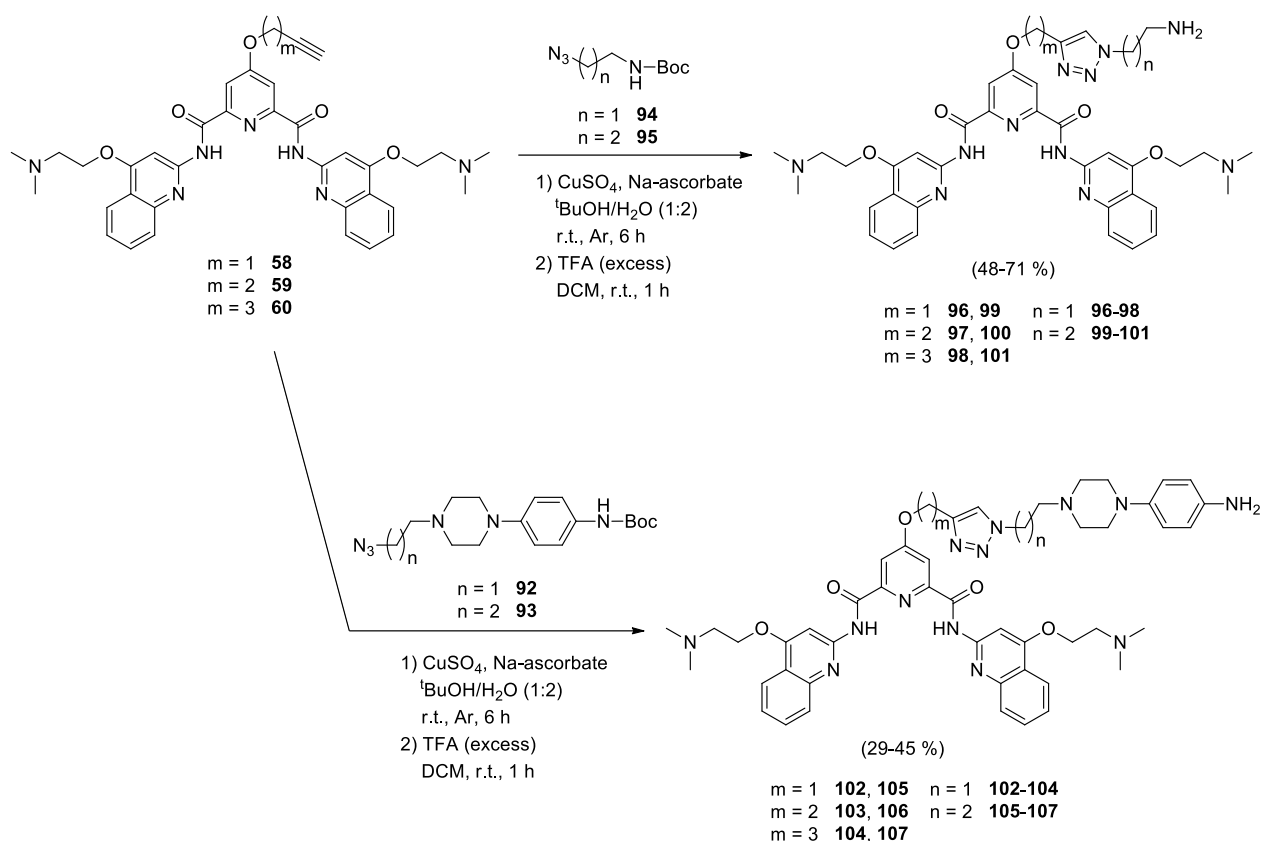
Scheme 9.10: The CuAAC reaction between pyridostatin derived alkynes **58-60** and azides **7-9**, **30**, and **27a**, **28a**, and **29a**. The click products **83-88** were afforded when using azides **9** and **30** with the tertiary amino moiety, while no reaction product was observed for azides **7-8** and **27a**, **28a**, and **29a** containing the primary amino group.

To obtain the desired pyridostatin derived click products, it was necessary to protect the NH_2 moiety of alkynes **27a**, **28a**, and **29a** and azides **7-8** and **77-78** with the Boc protecting group. This was performed by stirring the respective compound with Boc-anhydride and excess of TEA as a base (Scheme 9.11). For Boc-protection of the 4-(4-methylpiperazin-1-yl)aniline derived alkynes **27a**, **28a**, and **29a** and azides **77-78**, 1.5 eq. of the Boc-anhydride was used to obtain the relevant products in considerable amount, and the reaction was performed in ethanol [146]. Indeed, the Boc-protected alkynes **89-91** and azides **92-93** were obtained in moderate to very good yields (58-92 %). On the other hand, as azides **7-8** and their Boc-protected counterparts **94-95** are easily volatilized, only 1.1 eq. of the Boc-anhydride was used for **7-8** protection to ease the subsequent product purification, which consisted in evaporation of the solvent, the *tert*-butanol formed in the course of the reaction, and the excess of the Boc-anhydride. For the same reason, the reaction was carried out in THF [147] due to its lower boiling point compared to ethanol. The azides **94** [148] and **95** [149] were obtained in a very good yield (88-94 %).

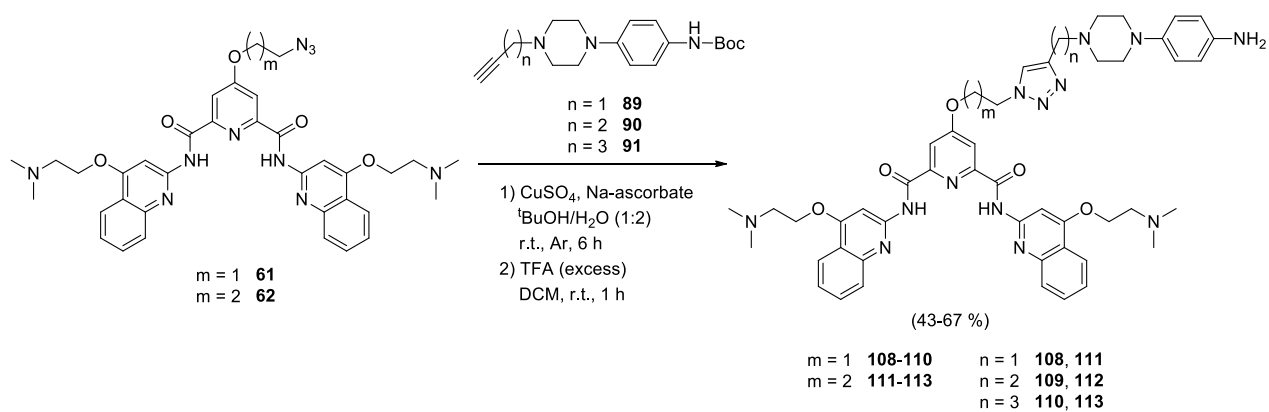


*Scheme 9.11: Boc-protection of the NH_2 moiety of alkynes **27a**, **28a**, and **29a** and azides **77-78** and **7-8** to obtain a homology series of alkynes **89-91** and azides **92-93** and **94-95**.*

The CuAAC of pyridostatin derived alkynes **58-60** with Boc-protected azides **92-93** and **94-95** (Scheme 9.12) was performed with CuSO_4 as a catalyst, which was immediately reduced from the oxidation state II to I by sodium ascorbate, and the reaction was carried out in the *tert*-butanol/ H_2O (1:2) mixture under argone atmosphere [39]. The subsequent deprotection of the Boc moiety with TFA provided 1,4-disubstituted click products **96-101** and **102-107** in low to good yields (29-71 %). The reaction of pyridostatin derived azides **61-62** with alkynes **89-91** (Scheme 9.13) was performed in the same manner [39] and afforded compounds **108-113** in moderate yields (43-67 %).



*Scheme 9.12: The CuAAC reaction between pyridostatin derived alkynes **58-60** and Boc-protected azides **94-95** and **92-93** to afford the click products **96-101** and **102-107**, respectively.*

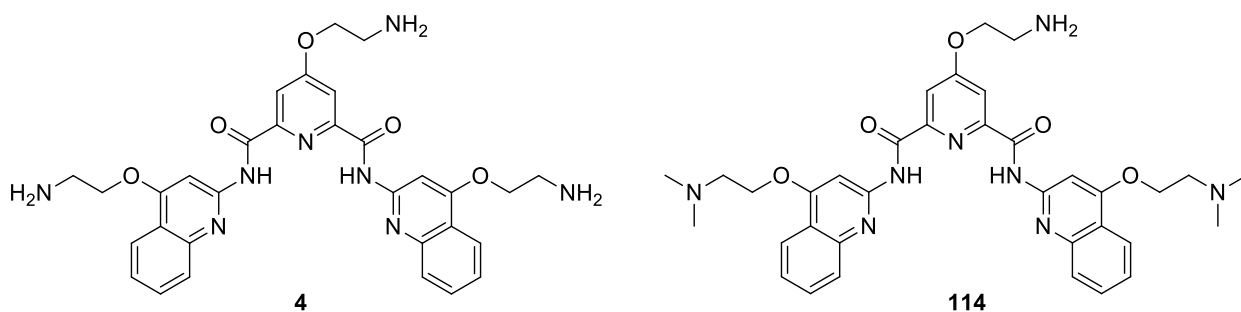


*Scheme 9.13: The CuAAC reaction between pyridostatin derived azides **61-62** and Boc-protected alkynes **89-91** to afford the click products **108-113**.*

9.2.2 The structure-activity relationship regarding the pyridostatin fragment library

The pyridostatin derived alkynes **58-60** and azides **61-62** as well as their respective click products **83-88** and **96-113** were subjected to biological evaluation in FRET melting assay at 2 μ M using dual-labelled (5'-(6-FAM) and 3'-(6-TAMRA)) 25-mer oligonucleotide Fc-myc25T. Their FRET activity was compared to activity of the original pyridostatin molecule **4**, which was purchased from Sigma-Aldrich®, and to the activity of the pyridostatin derivative **114** containing the -NMe₂ functionality in the quinolone side chains. Compound **114** was synthesized by Dr. Paul E. Reyes-Gutiérrez according to the protocol for synthesis of pyridostatin-alkynes and azides described in this thesis (Scheme 9.7). Both compounds **4** and **114** (Figure 9.5A) served as reference molecules for evaluation of other pyridostatin derivatives for their *c-MYC* binding ability in both biochemical assays and KTGS. Structure of the pyridostatin residue and its -NMe₂ derivative present in pyridostatin click products are defined in Figure 9.5B for the purpose of clarification in the following tables regarding FRET (Table 9.8 and 9.9) and *in situ* click (Table 9.10 and 9.11) results.

A: Structure of the pyridostatin template molecules



B: Structure of the pyridostatin residues

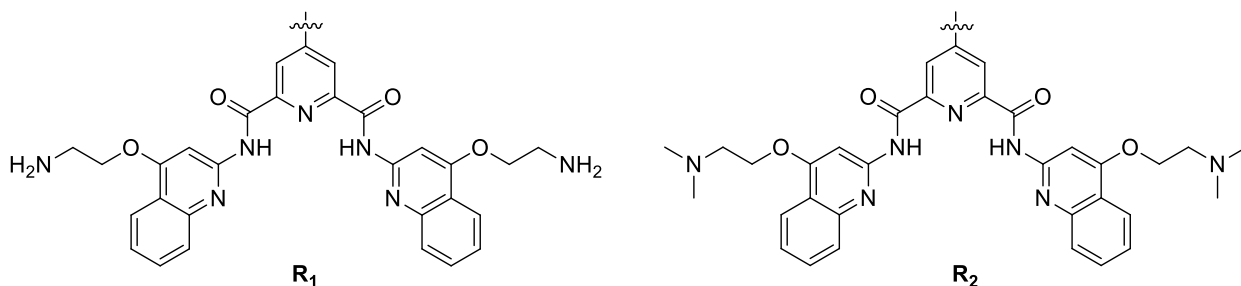


Figure 9.5: A – Structure of the pyridostatin molecule **4** and its derivative **114** that bears the -NMe₂ functionality in the quinolone side chains, both used as standard compounds for the FRET melting assay evaluation. B – Structure of the pyridostatin residues for the clarification purpose in Tables 9.8-9.11 concerning the FRET and *in situ* click experiment results.

The results concerning the pyridostatin derived alkynes **58-60** and their respective click products **83-88** and **96-107** did not show any significant increase of activity in FRET (Table 9.8) when compared to the template pyridostatin molecules **4** and **114**. The FRET activity of the alkynes **58-60**, click products **83-88** derived from azides **9** and **30** bearing a tertiary amino moiety, and click products **102-107** derived from azides **77** and **78** bearing the 4-(piperazin-1-yl)aniline moiety was only partially restored, with the ΔT_m value ranging from 2.5 ± 0.4 °C to 9.6 ± 0.7 °C. For click products **96-101** derived from azides **7** and **8** with the primary NH₂ group, the ΔT_m values were comparable to both the pyridostatin molecules **4** and **114**. The only exception was represented by compound **99**, which showed slightly higher ΔT_m increase (17.5 ± 0.1 °C). The FRET ΔT_m increase of the discussed compounds at 2 μ M is illustrated by Chart 9.4.

Based on the FRET melting assay, we can conclude that the pyridostatin based molecule interacts with *c-MYC* preferentially *via* its core composed of the pyridine and quinolone moieties. Therefore, the linker bearing the NH₂ group, reinforcing G4 binding, should be more flexible to reach to the *c-MYC* G4 structure. This idea is supported by the fact that the lowest FRET activity was exhibited by click products **102-104** bearing the structure of azide **77** that contains the relatively rigid 4-(piperazin-1-yl)aniline moiety and possesses only two methylene units in the azidoalkyl linker. On the other hand, the presence of the primary amino group on a flexible linker bound to the oxygen attached to position 4 of the pyridine moiety in compounds **96-101** facilitates interaction of the NH₂ group with *c-MYC*.

Similar to this study, the FRET measurement at 1 μ M evaluating stabilization potential of H-Telo, which was performed by Di Antonio *et al* [39], showed that the pyridostatin-alkynes **5** and **6** (Introduction, Chapter 7.3.2, Figure 7.16A) as well as their respective click products with azides **7-12** did not exhibit higher ΔT_m values than the original pyridostatin molecule **4**. Though, all pyridostatin derivatives prepared by Di Antonio *et al* showed overall higher H-Telo stabilization potential (ΔT_m values ranging from 5.9 ± 0.3 °C to 30.0 ± 0.1 °C at 1 μ M) than similar compounds prepared in this study regarding *c-MYC* (ΔT_m from 2.5 ± 0.4 °C to 9.6 ± 0.7 °C at 2 μ M). It is therefore assumed that pyridostatin derived compounds exhibit higher binding affinity towards H-Telo than towards *c-MYC*.

Cpd. No	Structure	ΔT_m (°C) FRET at 2 μ M	Cpd. No	Structure	ΔT_m (°C) FRET at 2 μ M
4		11.9 $\pm$ 0.7	97		12.7 $\pm$ 0.1
114		10.4 $\pm$ 0.5	98		12.3 $\pm$ 0.2
58		7.8 $\pm$ 0.4	99		17.5 $\pm$ 0.1
59		8.9 $\pm$ 0.0	100		11.7 $\pm$ 0.1
60		5.5 $\pm$ 0.1	101		14.2 $\pm$ 0.3
83		7.1 $\pm$ 1.4	102		2.5 $\pm$ 0.4
84		8.2 $\pm$ 0.6	103		5.7 $\pm$ 0.0
85		8.0 $\pm$ 0.3	104		3.9 $\pm$ 0.7
86		9.6 $\pm$ 0.7	105		6.2 $\pm$ 0.6
87		8.8 $\pm$ 0.7	106		6.2 $\pm$ 0.2
88		8.1 $\pm$ 0.7	107		7.5 $\pm$ 0.1
96		11.5 $\pm$ 0.2			

Table 9.8: FRET results at 2 μ M concerning the pyridostatin based fragment library of alkynes **58-60** and the respective click products **83-88** and **96-107** compared to the original pyridostatin molecule **4** and its derivative **114**.

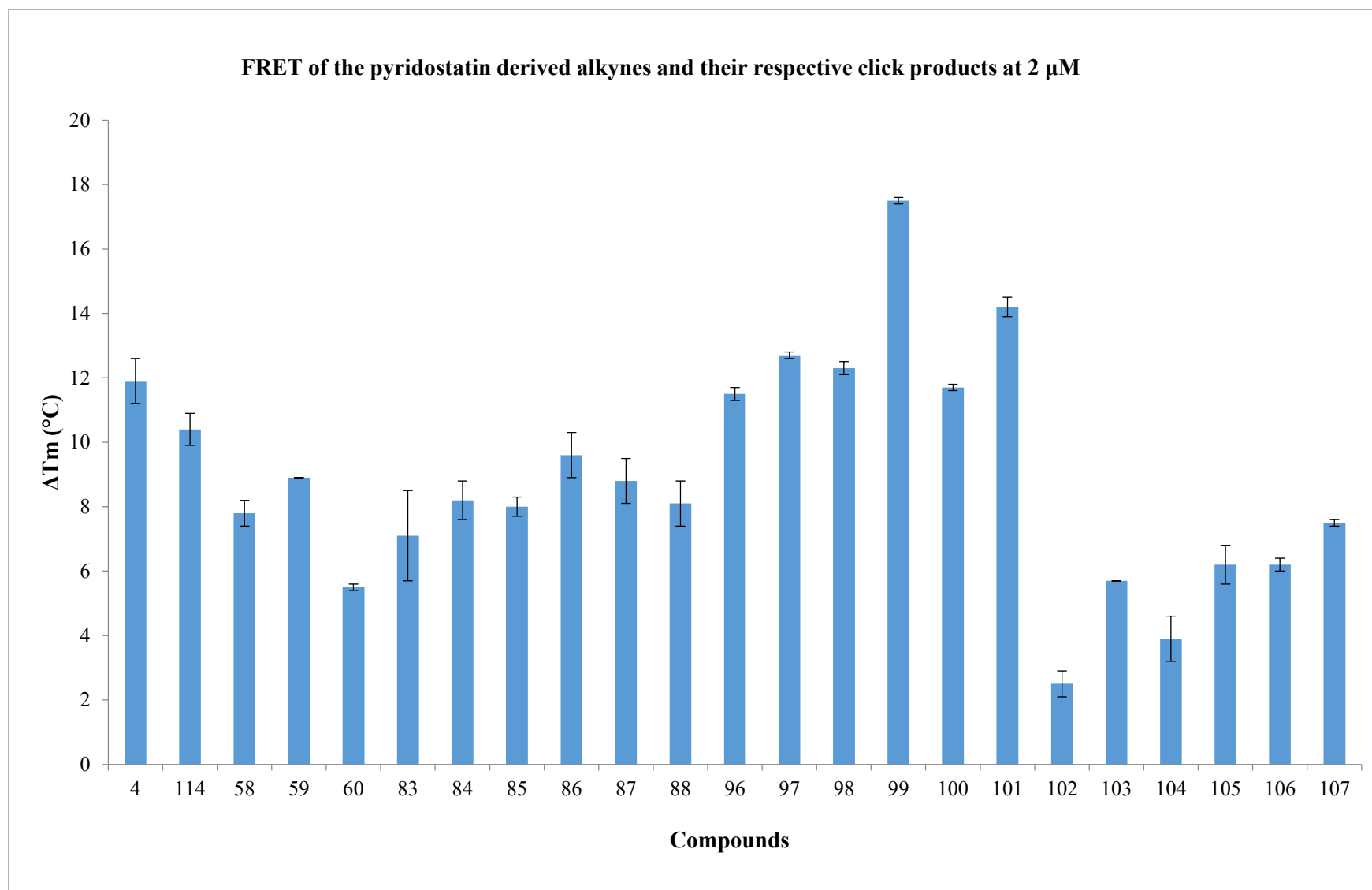


Chart 9.4: FRET results at 2 μ M concerning the pyridostatin based fragment library of alkynes **58-60** and the respective click products **83-88** and **96-107** compared to the original pyridostatin molecule **4** and its derivative **114**.

Both the pyridostatin derived azides **61-62** and their respective click products **108-113**, which were derived from alkynes **27a**, **28a**, and **29a** bearing the 4-(piperazin-1-yl)aniline moiety, displayed only partially restored activity in FRET at 2 μ M when compared to pyridostatin compounds **4** and **114** (Table 9.9, Chart 9.5). They induced ΔT_m increase ranging from 3.7 ± 0.2 $^{\circ}$ C to 7.2 ± 0.3 $^{\circ}$ C, comparable to the ΔT_m values exhibited by pyridostatin-alkyne click products **102-107** also containing the 4-(piperazin-1-yl)aniline moiety. Apparently, there is no synergy between the pyridostatin and the 4-(piperazin-1-yl)aniline moiety in *c-MYC* G4 binding, as FRET activity decrease compared to **4** and **114** was observed for compounds **102-107** and **108-113**. Most likely, these two moieties bind to the G4 in the same fashion and thus compete for the same binding area. As pyridostatin is a significantly stronger binder, it prevents the 4-(piperazin-1-yl)aniline moiety, and thus the aniline NH₂ group, from interaction with *c-MYC* G4. This leads to activity decrease of such click product in comparison to the parent pyridostatin compounds **4** and **114**.

Cpd. No	Structure	ΔT_m ($^{\circ}$ C) FRET at 2 μ M
4		11.9 ± 0.7
114		10.4 ± 0.5
61		7.2 ± 0.3
62		3.7 ± 0.2
108		6.7 ± 0.0
109		7.0 ± 0.3
110		6.7 ± 0.8
111		5.9 ± 0.1
112		6.2 ± 0.6
113		6.2 ± 0.1

Table 9.9: FRET results at 2 μ M concerning the pyridostatin based fragment library of azides **61-62** and the respective click products **108-113** compared to the original pyridostatin molecule **4** and its derivative **114**.

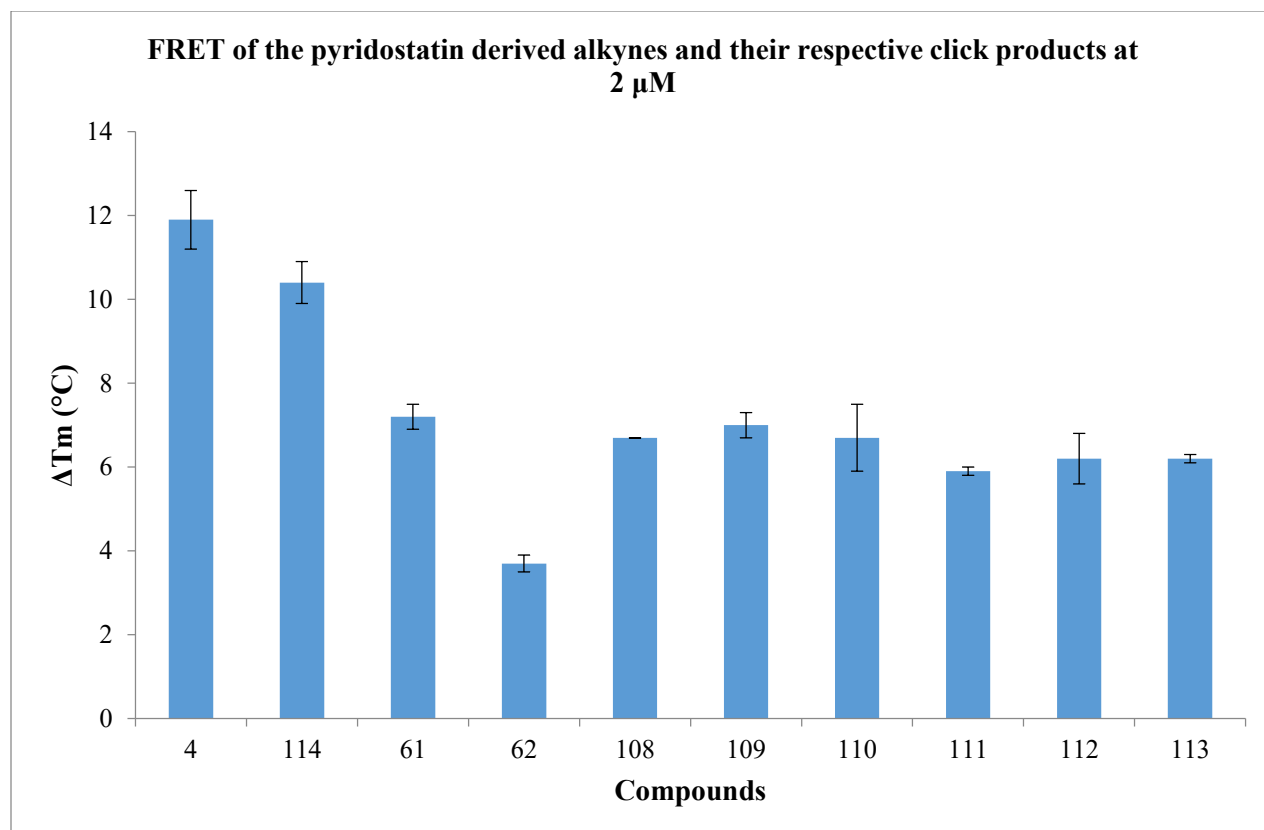


Chart 9.5: FRET results at 2 μ M concerning the pyridostatin based fragment library of azides **61-62** and the respective click products **108-113** compared to the original pyridostatin molecule **4** and its derivative **114**.

Overall, there was no general trend regarding the influence of the number of methylene units in either the alkynyl or the azidoalkyl linker on the ΔT_m value. Within each set of three click products derived from the same azide and a series of alkynes differing in the number of methylene units (1-3) in the alkynyl linker, the FRET activity was comparable (Table 9.8, compound sets **83-85**, **86-88**, **96-98**, **99-101**, **102-104**, **105-107**; Table 9.9, compound sets **108-110**, and **111-113**). This conclusion can be also made for most compound groups originated from the same alkyne and homologous azides differing in the number of methylene units (1-2) in the azidoalkyl linker (Table 9.8, compound sets **96** and **99**, **97** and **100**, and **98** and **101**). Nevertheless, as discussed above, there was a slightly higher ΔT_m observed for click products **105-107** with three methylene units in the azidoalkyl linker than for compounds **102-104** possessing only two methylene units. It is therefore believed that longer linkers facilitate to some extent interaction of the NH_2 group bound to the rigid 4-(piperazin-1-yl)aniline moiety with the *c-MYC* G4 structure.

Apart from discussing activity of the pyridostatin derived alkynes, azides, and their corresponding click products, it is also important to compare the FRET activity of the newly synthesized pyridostatin

derivative **114** (ΔT_m of 10.4 ± 0.5 °C) containing the -NMe₂ substituted linkers bound to the quinolone moieties with the template pyridostatin molecule **4** (ΔT_m of 11.9 ± 0.7 °C). It is obvious that substitution of the primary amino group with the -NMe₂ functionality did not increase the *c-MYC* binding ability of compound **114**. This finding does not correspond with the FRET measurements investigating the binding ability towards H-Telo, in which the presence of the -NMe₂ moiety generally induced a slight ΔT_m increase for various pyridostatin derivatives [130]. Apart from that, the FRET stabilization potential of **4** towards the *c-MYC* G4 represented by the oligonucleotide Fc-myc25T (ΔT_m of 11.9 ± 0.7 °C at 2 μ M) was considerably lower than its stabilization potential towards the telomeric H-Telo 21-mer oligonucleotide (ΔT_m of 35.4 ± 1.2 °C at 1 μ M) [39]. Therefore, the pyridostatin molecule **4** acts as a better ligand for H-Telo than for the *c-MYC* G4, although not being entirely selective. This evidence is in agreement with a study performed by De Rache *et al* [131] who reported no distinctive selectivity of pyridostatin binding to H-Telo in FRET and proved interaction of pyridostatin with *c-MYC* G4 comparable but lower than towards H-Telo.

To conclude, a fully restored FRET activity compared to the template pyridostatin molecules **4** and **114** was displayed only by click products **96-101** that possessed a primary amino moiety bound to a relatively flexible linker, which enabled interaction of this amino moiety with the *c-MYC* G4 structure. The importance of the NH₂ group for *c-MYC* binding was also supported by FRET ΔT_m decrease exhibited by pyridostatin alkyne **59** and pyridostatin azide **61** when compared to their amino analogues **4** and **114** with the same number of methylene units (2) in the linker bearing the concerned functionality. No synergic effect was observed between the pyridostatin and the 4-(piperazin-1-yl)aniline moiety in *c-MYC* G4 binding, as both of these two moieties stack to the G4 binding area in the same fashion.

As no significant FRET activity at 2 μ M was observed for the discussed pyridostatin derived fragment library when compared to the original pyridostatin molecule **4** and its derivative **114**, it was unnecessary to perform FRET at lower concentrations (e.g., 1 μ M or 0.5 μ M). Moreover, it was decided by the team not to perform the PEA biochemical assay or the chemical-shift perturbation ¹H NMR.

9.2.3 KTGS of the pyridostatin derived click products

All pyridostatin derived click products **83-88** and **96-113** were allowed to form through CuAAC *in situ*. As in the case of the mono-amino series of 4-(piperazin-1-yl)aniline-derived click products (Chapter 9.1.2.1.3, Table 9.5), KTGS was performed to evaluate the co-catalytic activity of the *c-MYC* G4 on pyridostatin click product formation, since the click product yield directly suggests the templating effect of *c-MYC*. Likewise, the *in situ* click experiments performed were catalyzed by a Cu^I species to increase triazole adduct formation [39] and Pu24 was used as an oligonucleotide catalyst.

To obtain all desired click product combinations, four *in situ* click experiments D-G were performed. Both the experiments D and E combined the homology series of pyridostatin derived alkynes **58-60** and a different set of three azides: experiment D employed azides **8**, **9**, and **78**; experiment E involved azides **7**, **30**, and **77**. Furthermore, each of the experiments F and G combined three homologous 4-(piperazin-1-yl)aniline-derived alkynes **27a**, **28a**, and **29a** with a different pyridostatin-azide: compound **61** was used in experiment F, while azide **62** was employed in experiment G. The combination of alkynes and azides in each experiment was selected so that the molecular weight of each possible click product in the reaction mixture was unique. This was particularly important in order to ascertain integral intensity of a particular click product in a subsequent LC-MS analysis.

Within the scope of each experiment D-G, three different *in situ* click sub-experiments A-C were performed simultaneously: in the presence of the Pu24 oligonucleotide (A), in the absence of Pu24 (B), and a control sub-experiment (C), in which Pu24 was added just 1 min. before quenching the reaction with TFA. All these sub-experiments were carried out in triplicates to obtain statistically relevant results. As the yield of individual click products cannot be directly derived from the MS intensities because of their different ionization states, the substantial increase in click product formation rate was expressed as an A/B ratio of MS integrals in experiment A (with Pu24) and B (without Pu24). Experiment C and the related A/C ratio were introduced to assess the influence of Pu24 to the LC-MS analysis.

The experiment D yielded click products **83-85**, **99-101**, and **105-107**, while the experiment E provided click products **86-88**, **96-98**, and **102-104**. The data concerning the A/B and A/C formation ratios in experiments D and E are summarized in Table 9.10. Analogously, the A/B and A/C formation ratios of click products **108-110** formed in experiment F and click products **111-113** formed in experiment G are presented in Table 9.11. The reaction mixture composition of experiments D-G and the sub-experiments A-C, as well as the average yield of the *in situ* click reaction for each product, are summarized in Experimental part, Chapter 11.3.2.2 (for mixtures composition, see Tables 11.12-11.17; for *in situ* formation yields, see Tables 11.18-11.21).

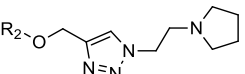
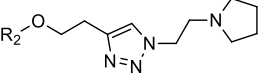
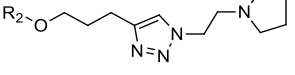
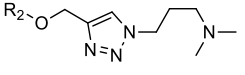
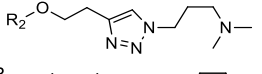
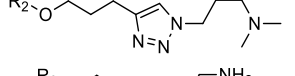
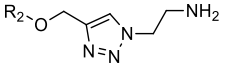
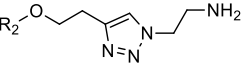
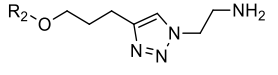
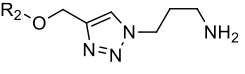
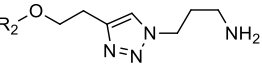
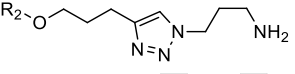
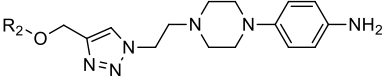
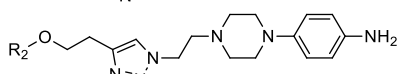
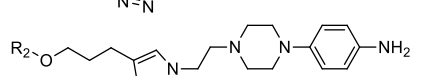
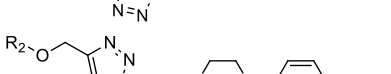
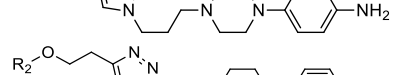
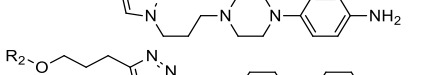
Compound	CH ₂ units	Experiment D		Experiment E	
		A/B	A/C	A/B	A/C
83 	1	1.2	1.2	-	-
84 	2	0.9	0.9	-	-
85 	3	1.0	1.0	-	-
86 	1	-	-	1.2	1.0
87 	2	-	-	1.9	1.0
88 	3	-	-	1.5	1.2
96 	1	-	-	0.6	0.5
97 	2	-	-	0.8	0.5
98 	3	-	-	0.9	0.5
99 	1	1.0	1.0	-	-
100 	2	0.7	0.7	-	-
101 	3	0.8	0.8	-	-
102 	1	-	-	0.6	0.6
103 	2	-	-	0.7	0.6
104 	3	-	-	0.6	0.6
105 	1	0.8	0.9	-	-
106 	2	0.7	0.7	-	-
107 	3	0.9	0.9	-	-

Table 9.10: The relative formation yields of compounds **83-88** and **96-113** expressed as A/B and A/C reaction ratios in experiments D and E – each combining pyridostatin-alkynes **58-60** with a different set of three azides (experiment D – azides **8**, **9**, **78**; experiment E – azides **7**, **30**, **77**). For click products with the highest A/B and A/C ratios, the respective values are marked in bold.

Compound		CH ₂ units	Experiment F A/B A/C		Experiment G A/B A/C	
108		1	0.6	0.7	-	-
109		2	0.8	1.0	-	-
110		3	1.4	1.7	-	-
111		1	-	-	1.0	1.0
112		2	-	-	0.9	1.0
113		3	-	-	1.5	1.9

Table 9.11: The relative formation yields of compounds **108-113** expressed as A/B and A/C reaction ratios in experiments F and G – each combining three homologous 4-(piperazin-1-yl)aniline-derived alkynes **27a**, **28a**, and **29a** with a different pyridostatin-azide (experiment F – azide **61**; experiment G – azide **62**). The highest values of A/B and A/C ratios are marked in bold.

When first seen, the data obtained suggest no substantial increase of click product formation in the presence of Pu24, as the A/B ratio ranges from 0.6 to 1.9. However, these numerical values can be biased by stacking of the emerging click product to the G4 formed by Pu24 and thus depleting the ligand from the mixture for the subsequent LC-MS measurement. Since most of the A/B ratios are lower than 1.0, the click product binding to Pu24 is most probable, and this assumption is supported by the fact that pyridostatins are generally good G4 ligands. Nevertheless, the integral intensities measured by LC-MS for each click product were very low in general (Experimental part, Chapter 11.3.2.2, Tables 11.18-11.21), while there was still a significant amount of reactants present in the samples analyzed (data not shown). This fact can be explained by poor reactivity of alkynes **27a**, **28a**, and **29a** and azides **7**, **8**, **77**, and **78** bearing the primary amino moiety with pyridostatin derived alkynes and azides, respectively, which was previously reported for the click reaction performed by chemical means (Scheme 9.9 and 9.10). Though, low integral intensities were found even for click products **83-88** originated from azides **9** and **30** containing a tertiary amino moiety, for which the chemical CuAAC ran smoothly. Moreover, the A/C ratio, which was introduced to eliminate the effect of possible ligand binding to Pu24 and thus decreasing its amount in the analyzed sample, was in most cases equal or comparable to the A/B ratio.

The observed low co-catalytic reaction rate may be explained by easy and quick stacking of all pyridostatin derived alkynes or azides on the surface of G4 formed by Pu24, as these compounds are

relatively strong G4 binders and are present in the *in situ* click reaction mixture in excess. Thus, the pyridostatin alkynes and azides block other possible G4 interaction sites for the complementary fragments (non-pyridostatin alkynes or azides). The same conception also applies to the arising pyridostatin derived click products, whose presence may lead to depletion of all G4 present, therefore stopping the co-catalysis. Interestingly, if the *in situ* click reaction results in a pyridostatin click product that is a weaker binder, it may leave the G4 sooner, thus facilitating G4 co-catalysis, and we observe increased click product formation. Conversely, pyridostatin click products that behave as stronger binders exhibit lower formation yields. This represents major limitation of the *in situ* click approach for our study regarding pyridostatin chemistry. For illustration, the click products **96-101**, which exhibited either full restoration or mild increase of FRET activity compared to the original pyridostatin molecule **4** and its derivative **114**, did not show increase of the A/B or A/C ratio during *in situ* click experiments. On the other hand, pyridostatin click products **110** and **113** that originated from the 4-(piperazin-1-yl)aniline-derived alkyne **29a** exhibited moderately increased A/B and A/C ratios within the respective set of homologous compounds. Based on all these findings, we can conclude that the *in situ* click approach is not entirely favourable for KTGS employing already strong ligands as pyridostatin derivatives that utilize most of the binding capacity of the target molecule (Pu24). For that reason, it was assumed by the team that it was not necessary to perform control *in situ* click experiments with G4 non-forming ds26 oligonucleotide.

Similarly as for the FRET results at 2 μ M (Table 9.8 and 9.9), there was no overall trend regarding influence of the alkynyl or the azidoalkyl linker length on *in situ* formation of the pyridostatin derived click products. This was most probably due to the low co-catalytic activity of Pu24 on ligand formation for the respective fragment combinations discussed above. The only exception was represented by already discussed click products **110** and **113**, both originating from the 4-(piperazin-1-yl)aniline-derived alkyne **29a** with three methylene units in the alkynyl linker, which exhibited moderately increased A/B and A/C ratios within the respective set of homologous compounds.

To sum up, KTGS regarding CuAAC of pyridostatin-alkynes and pyridostatin-azides with selected cationic azides **7-9**, **30**, homologous azides **77-78**, and alkynes **27a**, **28a**, **29a** containing the 4-(piperazin-1-yl)aniline moiety did not demonstrate any significant co-catalytic effect of the Pu24 oligonucleotide on click product formation. We assume that the *in situ* click approach was not suitable for pyridostatin chemistry since pyridostatin derivatives are generally strong G4 binders and prevent co-catalysis by stacking on the G4 surface. Moreover, stacking of the emerging click products on the Pu24-formed G4 most probably led to their depletion from the reaction mixture for the LC-MS analysis. For all these reasons, the control *in situ* click experiments with G4 non-forming ds26 oligonucleotide were not performed.

10 Conclusions

1. The design of the 4-(4-methylpiperazin-1-yl)aniline-based fragment library started with evaluation of various compound **2** derivatives for their *c-MYC* G4 binding ability. Both the FRET and PEA results proved necessity of the unsubstituted NH₂ group at the position 1 of the aromatic ring for interaction of the ligand with *c-MYC*. Moreover, significantly increased stabilization potential was revealed for compound **15** as a direct bis-amino derivative of **2** that possessed an additional NH₂ group in position 2 of the aromatic ring. Therefore, two series of 4-(4-methylpiperazin-1-yl)aniline-derived compounds were synthesized: a mono-amino series directly inspired by the compound **2**, and a bis-amino series that contained an additional amino moiety in position 2 of the aromatic ring. Both of these series were modified for click chemistry by *N*⁴-alkynylation on the piperazine moiety and subjected to CuAAC with various cationic azides. In both the FRET and PEA biology assays, all *o*-aminoaniline compounds of the bis-amino series and especially the respective *o*-aminoaniline click products **55** and **56** exhibited increased *c-MYC* binding ability when compared to the original molecule **2**. On the other hand, the activity of the mono-amino compounds was either negligible or only partially restored. According to the chemical-shift perturbation ¹H NMR, *o*-aminoaniline derivatives of the bis-amino series were generally classified as strong *c-MYC* binders, while most compounds of the mono-amino series were classified as non-binders. All these SAR results further confirmed necessity of the additional amino moiety in position 2 of the aromatic ring for increased *c-MYC* binding and displayed the *o*-aminoaniline derivatives of the bis-amino series as substantially stronger *c-MYC* ligands than their mono-amino analogues. Furthermore, KTGS regarding the mono-amino click products revealed substantially increased *in situ* formation for compounds that originated from alkyne with the maximal length of the alkynyl linker (three methylene units). This finding suggests that increased flexibility of the click product molecule is necessary for its effective interaction with the *c-MYC* target.

2. Construction of the pyridostatin based fragment library started with synthesis of homologous sets of pyridostatin-alkynes and pyridostatin-azides, all of which contained a -NMe₂ moiety in the quinolone side chains as a modification. In the final synthetic step concerning the carboxyl-amine coupling, PyOxim was used as a coupling reagent with the aid of Oxyma as an accelerating agent and DIPEA as a base. This reaction was new regarding synthesis of pyridostatins and led to substantial yield increase when compared to the procedure described in the literature. All these pyridostatin derivatives were subjected to CuAAC with selected cationic azides and homologous azides and alkynes containing the 4-(4-methylpiperazin-1-yl)aniline moiety.

According to the FRET melting assay, both the pyridostatin-alkynes and pyridostatin-azides, as well as most of the respective click products, showed only partially restored activity compared to pyridostatin and its analogue containing -NMe₂ functionalities. No synergy between the pyridostatin and the 4-(4-methylpiperazin-1-yl)aniline moiety in *c-MYC* G4 binding was found as these two moieties most probably bind to the G4 surface in the same way. Only click products originated from pyridostatin-alkynes and cationic azides bearing primary amino moiety bound to an aliphatic chain exhibited fully restored or slightly increased FRET activity compared to standard pyridostatin molecules. Therefore, it is concluded that the presence of the primary amino group on a flexible linker as a part of the pyridine *O*-substituent is an important feature for interaction of pyridostatins with the *c-MYC* G4. Nevertheless, the overall FRET activity of pyridostatin-alkynes, pyridostatin-azides, and the respective click products towards *c-MYC* was generally lower than the FRET activity towards the telomeric H-Telo concerning similar pyridostatin derivatives prepared by the Balasubramanian research group [39]. Moreover, the FRET stabilization potential of the basic pyridostatin molecule (**4**) towards the *c-MYC* G4 (ΔT_m of 11.9 ± 0.7 °C at 2 μ M) was considerably lower than towards H-Telo (ΔT_m of 35.4 ± 1.2 °C at 1 μ M), reported by Di Antonio *et al* [39]. Based on all these results, we can conclude that pyridostatin, as well as its derivatives, exhibit higher binding affinity towards H-Telo than towards *c-MYC*, although they are not entirely selective. As no remarkable FRET activity increase at 2 μ M compared to the parent pyridostatin compound was observed for the pyridostatin derived fragment library, it was decided not to perform FRET at lower concentrations, the PEA biochemical assay, or the chemical-shift perturbation ¹H NMR. Finally, KTGS employing CuAAC did not demonstrate any significant co-catalytic effect of the oligonucleotide Pu24 on pyridostatin click product formation. All pyridostatin derivatives, being strong G4 binders, most probably stacked to the G4 surface and thus slowed or prevented co-catalysis. For that reason, control *in situ* click experiments with the G4 non-forming ds26 oligonucleotide were not performed.

11 Experimental part

11.1 General information

All chemicals were obtained from the following commercial sources: Sigma-Aldrich (Merck) Generi Biotech, PENTA, abcr GmbH, VWR international, ACT Chemical, and TCI (Tokyo Chemical Industry). The structure of oligonucleotides used for determination of *c-MYC* binding and co-catalysis and the respective commercial sources are as follows:

- The Pu24 oligonucleotide used for *in situ* click experiments, STD ^1H NMR and chemical-shift perturbation ^1H NMR was obtained in lyophilized form from Generi Biotech.
Pu24: 5'-TGA GGG TGG GGA GGG TGG GGA AGG-3'
- The ds26 oligonucleotide used for control *in situ* click measurements was obtained in lyophilized form from Generi Biotech.
ds26: 5'-TAT AGC TAT ATT TTT TTA TAG CTA TA -3'
- The template strand containing the Pu27 oligonucleotide sequence and the reverse primer used for PEA were obtained from Generi Biotech.
Template strand Pu27: 5'-CTGCGATCCGTCAGTCCAACATGCTATACT-
-GGGGAGGGTGGGGAGGGTGGGGAAGG-
TTAGCGGCACGCAATTGCTAGCGTGAGTCG-3'
Reverse primer: 5'-CGACTCACGCTAGCAATTGCGTG-3'
- The dual-labelled oligonucleotide Fc-myc25T used for the FRET metling assay was obtained from Sigma-Aldrich.
Fc-myc25T: 5'-(6-FAM)-TGGGGAGGGTGGGGAGGGTGGGGAA-(6-TAMRA)-3'
- The dual-labelled oligonucleotide FdsT used for the control FRET metling assay was obtained from Sigma-Aldrich.
FdsT: 5'-(6-FAM)-TATAGCTATATTTTTTATAGCTATA-(6-TAMRA)-3'

The column chromatography was performed on silica gel (Sigma S-0507, 40-63 μm). Thin-layer chromatography (TLC) analysis was performed on silica-gel plates with a fluorescent indicator (Silica gel 60 F₂₅₄-coated aluminum sheets, Merck, cat. no. 5554) and the spots were visualized with UV lamp ENF-240C/FE (Spectrolin) at 254/365 nm. Sonication was performed with SONOREX Ultrasonic baths sonicator (BANDELIN). Centrifugation was carried out with a laboratory centrifuge LMC-3000 (BioSan). Synthesis employing MW irradiation was facilitated by the Microwave Synthesizer Discover (CEM Corporation).

The ^1H and ^{13}C NMR spectra were recorded on the Bruker Avance 400 instrument (^1H at 400 MHz and ^{13}C at 100.6 MHz) and on the Bruker Avance 500 instrument (^1H at 500 MHz and ^{13}C at 125.7 MHz), in CDCl_3 (referenced to TMS as an internal standard), in $\text{DMSO}-d_6$ (referenced to the solvent signal $\delta(^1\text{H}) = 2.50$ ppm and $\delta(^{13}\text{C}) = 39.70$ ppm, respectively), or in $\text{MeOD}-d_4$ (referenced to the solvent signal $\delta(^1\text{H}) = 3.31$ ppm and $\delta(^{13}\text{C}) = 49.00$ ppm, respectively). The complete assignment of signals was based on H,H-COSY, H,C-HSQC, and H,C-HMBC correlation experiments. Chemical shifts are given in δ -scale as parts per million (ppm); coupling constants (J) are given in Hz. Furthermore, the chemical-shift perturbation ^1H NMR experiments were performed by Dr. Lukáš Vrzal from the IOCB research group of Dr. Pavlína Maloy Řezáčová; the NMR spectra were recorded on the Bruker Avance IIITM HD 600 MHz instrument equipped with a Cryoprobe and a sample changer.

The mass spectra were obtained at the Mass Spectrometry Facility operated by IOCB. For compound characterization, ESI mass spectra were recorded on a Q-ToF micro (Waters) and the HR MS were taken on a LTQ Orbitrap XL (Thermo Fisher Scientific) spectrometer. Additionally, LC-MS analysis of *in situ* click experiment samples was performed by Dr. Martin Hubálek using Luna Omega Polar C18 column 150x1.0 mm (Phenomenex), Mass Spectrometer (Synapt G2, Waters) coupled to a LC system (I-class, Waters), and Quantlynx application of MassLynx 4.1 (Waters).

The IR spectra were measured on the Nicolett 6700 FT-IR spectrometer (Thermo Scientific) either in KBr pellets, in CCl_4 or in CHCl_3 solution. The melting points were determined by Kofler melting point apparatus with microscope KBT300 (SANTIAGO) and are uncorrected. Elemental analysis was performed on a PE 2400 Series II CHNS/O Analyzer (Perkin Elmer, USA).

For vortexing of *in situ* click mixtures in Eppendorf tubes, Vortex-Genie 2 (Scientific Industries, Inc.) was used. Their shaking at constant temperature was performed using Thermo Shaker TS-100C (BIOSAN).

Both the FRET melting assay and PEA were performed by Dr. Jaroslav Kozák from the research group of Dr. Lenka Maletínská.

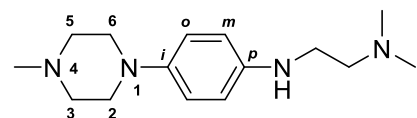
11.2 Synthetic part – reaction procedures and spectroscopic data

11.2.1 Synthesis of 4-(4-methylpiperazin-1-yl)aniline derivatives alkylated on the aniline amino group

The general procedure for synthesis of alkylation products of 4-(4-methylpiperazin-1-yl)aniline **25**; procedure according to ref. [135]

4-(4-Methylpiperazin-1-yl)aniline (**2**, 100 mg, 0.52 mmol) and alkylating agent (0.52 mmol) were dissolved in DMF (1.0 mL) and TEA (0.182 μ L, 1.31 mmol) was added. The reaction mixture was irradiated by MW for 30 min at 150 W at 150°C. To quench the reaction, water (0.1 mL) was added. The solvent was removed *in vacuo*, the residue was codistilled with toluene/methanol three times, then purified on a preparative TLC plate in methanol/ethyl acetate (2/1).

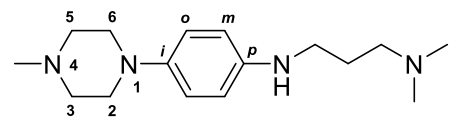
*N*¹,*N*¹-Dimethyl-*N*²-(4-(4-methylpiperazin-1-yl)phenyl)ethane-1,2-diamine (**25**).



Alkylating agent: 2-chloro-*N,N*-dimethylethylamine hydrochloride (75 mg, 0.52 mmol). Brown amorphous solid (63 mg, yield 46 %).

¹H NMR (400 MHz, MeOD-*d*₄): δ 6.89 (m, 2H, H-*o*-Ph), 6.65 (m, 2H, H-*m*-Ph), 3.19 (t, 2H, $J_{\text{vic}} = 6.8$ Hz, HNCH₂CH₂NMe₂), 3.05 (t, 4H, $J_{\text{vic}} = 5.0$ Hz, H-2,6), 2.62 (t, 4H, $J_{\text{vic}} = 4.9$ Hz, H-3,5), 2.56 (t, 2H, $J_{\text{vic}} = 6.7$ Hz, HNCH₂CH₂NMe₂), 2.34 (s, 3H, NCH₃), 2.30 (s, 6H, N(CH₃)₂); NH signal not detected. ¹³C NMR (100.6 MHz, MeOD-*d*₄): δ 144.89 (C-*i*-Ph), 144.47 (C-*p*-Ph), 120.24 (CH-*o*-Ph), 115.36 (CH-*m*-Ph), 59.33 (HNCH₂CH₂NMe₂), 56.08 (CH₂-3,5), 52.10 (CH₂-2,6), 46.07 (NCH₃), 45.59 (N(CH₃)₂), 43.23 (HNCH₂CH₂NMe₂). HRMS (ESI⁺) calcd. for C₁₅H₂₇N₄⁺ (M+H⁺): 263.22302, found: 263.22307. *R*_f (3/2 DCM/MeOH) = 0.26.

*N*¹,*N*¹-Dimethyl-*N*³-(4-(4-methylpiperazin-1-yl)phenyl)propane-1,3-diamine (**26**).



Alkylating agent: 3-chloro-*N,N*-dimethylpropylamine hydrochloride (83 mg, 0.52 mmol). Brown amorphous solid (75 mg, yield 51 %).

¹H NMR (400 MHz, MeOD-*d*₄): δ 6.88 (m, 2H, H-*o*-Ph), 6.65 (m, 2H, H-*m*-Ph), 3.09 (t, 2H, $J_{\text{vic}} = 7.1$ Hz, HNCH₂CH₂CH₂NMe₂), 3.04 (t, 4H, $J_{\text{vic}} = 4.7$ Hz, H-2,6), 2.62 (t, 4H, $J_{\text{vic}} = 4.9$ Hz, H-3,5), 2.47 (t, 2H, $J_{\text{vic}} = 7.7$ Hz, HNCH₂CH₂CH₂NMe₂), 2.34 (s, 3H, NCH₃), 2.28 (s, 6H, N(CH₃)₂), 1.79 (p, 2H, $J_{\text{vic}} = 7.4$ Hz, HNCH₂CH₂CH₂NMe₂); NH signal not detected. ¹³C NMR (100.6 MHz, MeOD-*d*₄): δ 144.82 (C-*i*-Ph), 144.49 (C-*p*-Ph), 120.21 (CH-*o*-Ph), 115.51 (CH-*m*-Ph), 58.50 (HNCH₂CH₂CH₂NMe₂), 56.08 (CH₂-3,5), 52.11 (CH₂-2,6), 46.06 (NCH₃), 45.36 (N(CH₃)₂), 43.12 (HNCH₂CH₂CH₂NMe₂), 30.78 (HNCH₂CH₂CH₂NMe₂). HRMS (ESI⁺) calcd. for C₁₆H₂₉N₄⁺ (M+H⁺): 277.23867, found: 277.23885. *R*_f (3/2 DCM/MeOH) = 0.26.

11.2.2 Synthesis of the mono-amino series

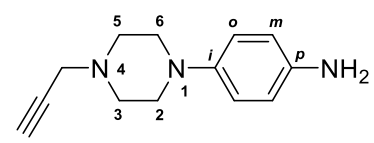
The general procedure for synthesis of alkylation products of 4-(piperazin-1-yl)aniline **27a**, **28a**, and **29a**; procedure according to ref. [136]

4-(Piperazin-1-yl)aniline (**13**, 2.500 g, 14.1 mmol) and K₂CO₃ (19.493 g, 141.0 mmol) were dissolved in DMF (120 mL) under argon atmosphere and stirred for 15 min at r.t. Afterwards, alkynyl halide (14.1 mmol) was added and the reaction mixture was stirred under argon at r.t. for 48 h. The salt was filtered off over Celite and the solvent was removed *in vacuo*. A silica gel column chromatography in (0-5% methanol/dichloromethane) afforded a monoalkylated main product (**27a**, **28a**, and **29a**) and a dialkylated side product (**27b**, **28b**, and **29b**).

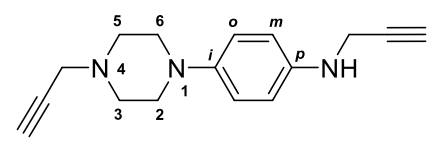
Synthesis of alkylation products **27a** and **27b**

Reactants: 4-(piperazin-1-yl)aniline (**13**, 2.500 g, 14.1 mmol), 3-bromoprop-1-yne (80% solution in toluene, 1.57 mL, 14.1 mmol), and K₂CO₃ (19.493 g, 141.0 mmol).

4-(4-(prop-2-yn-1-yl)piperazin-1-yl)aniline (**27a**) [137].

 Yellow crystalline solid (1.849 g, yield 61 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 6.68 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.54 (bs, 2H, NH₂), 3.29 (d, 2H, ⁴*J* = 2.5 Hz, NCH₂CCH), 3.16 (t, 1H, ⁴*J* = 2.4 Hz, NCH₂CCH), 2.91 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.56 (t, 4H, *J*_{vic} = 4.9 Hz, H-3,5). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 142.32 (C-*i*-Ph), 142.13 (C-*p*-Ph), 117.97 (CH-*o*-Ph), 114.69 (CH-*m*-Ph), 79.35 (NCH₂CCH), 75.67 (NCH₂CCH), 51.37 (CH₂-3,5), 50.23 (CH₂-2,6), 46.02 (NCH₂CCH). FTIR (CHCl₃, cm⁻¹) ν_{max}: 3440, 3366, 3307, 3103, 2940, 2890, 2824, 2125, 1624, 1612, 1584, 1515, 1455, 1383, 1271, 1138, 1054, 1006, 922, 826, 635. HRMS (EI⁺) calcd. for C₁₃H₁₇N₃⁺ (M⁺): 215.1422, found: 215.1426. R_f (0.25% TEA in 1/1 EtOAc/MeOH) = 0.53.

N-(prop-2-yn-1-yl)-4-(4-(prop-2-yn-1-yl)piperazin-1-yl)aniline (**27b**).

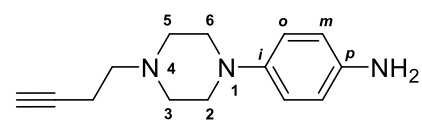
 White crystalline solid (106 mg, yield 3 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 6.77 (m, 2H, H-*o*-Ph), 6.57 (m, 2H, H-*m*-Ph), 5.46 (t, 1H, *J*_{vic} = 6.4 Hz, NH), 3.78 (dd, 2H, *J*_{vic} = 6.4 Hz, ⁴*J* = 2.4 Hz, NHCH₂CCH), 3.30 (d, 2H, ⁴*J* = 2.5 Hz, NCH₂CCH), 3.16 (t, 1H, ⁴*J* = 2.4 Hz, NCH₂CCH), 3.00 (t, 1H, ⁴*J* = 2.4 Hz, NHCH₂CCH), 2.95 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.57 (t, 4H, *J*_{vic} = 5.0 Hz, H-3,5). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 143.01 (C-*i*-Ph), 141.62 (C-*p*-Ph), 117.70 (CH-*o*-Ph), 113.71 (CH-*m*-Ph), 82.73 (NHCH₂CCH), 79.33 (NCH₂CCH), 75.70 (NCH₂CCH), 72.75 (NHCH₂CCH), 51.33 (CH₂-3,5), 49.98 (CH₂-2,6), 46.02 (NCH₂CCH), 32.75 (NHCH₂CCH). FTIR (CHCl₃, cm⁻¹) ν_{max}: 3419, 3307, 3105,

2941, 2825, 2117, 1619, 1584, 1516, 1455, 1411, 1382, 1318, 1275, 1229, 1138, 1082, 1006, 958, 822, 636, 496, 413. **HRMS** (EI⁺) calcd. for C₁₆H₁₉N₃⁺ (M⁺): 253.1579, found: 253.1577. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.67.

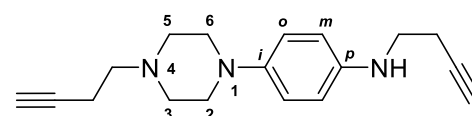
Synthesis of alkylation products 28a and 28b

Reactants: 4-(piperazin-1-yl)aniline (**13**, 2.500 g, 14.1 mmol), 4-bromobut-1-yne (1.32 mL, 14.1 mmol), and K₂CO₃ (19.493 g, 141.0 mmol).

4-(4-(But-3-yn-1-yl)piperazin-1-yl)aniline (28a).

 Yellow crystalline solid (1.450 g, yield 45 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 6.67 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.54 (bs, 2H, NH₂), 2.88 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.78 (t, 1H, ⁴*J* = 2.6 Hz, NCH₂CH₂CCH), 2.42-2.56 (m, 6H, H-3,5, NCH₂CH₂CCH), 2.34 (td, 2H, *J*_{vic} = 6.8 Hz, ⁴*J* = 2.7 Hz, NCH₂CH₂CCH). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 142.38 (C-*i*-Ph), 142.09 (C-*p*-Ph), 117.87 (CH-*o*-Ph), 114.70 (CH-*m*-Ph), 83.22 (NCH₂CH₂CCH), 71.73 (NCH₂CH₂CCH), 56.51 (NCH₂CH₂CCH), 52.59 (CH₂-3,5), 50.29 (CH₂-2,6), 16.19 (NCH₂CH₂CCH). **FTIR** (CHCl₃, cm⁻¹) *v*_{max}: 3441, 3367, 3308, 3103, 2951, 2886, 2825, 2119, 1623, 1611, 1583, 1515, 1458, 1262, 1139, 1006, 926, 826, 642. **HRMS** (EI⁺) calcd. for C₁₄H₁₉N₃⁺ (M⁺): 229.1579, found: 229.1577. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.57.

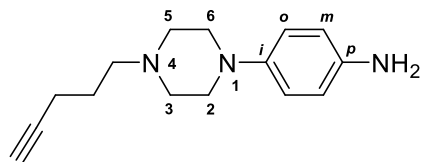
N-(But-3-yn-1-yl)-4-(4-(but-3-yn-1-yl)piperazin-1-yl)aniline (28b).

 Yellow crystalline solid (109 mg, yield 3 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 6.74 (m, 2H, H-*o*-Ph), 6.50 (m, 2H, H-*m*-Ph), 5.15 (t, 1H, *J*_{vic} = 6.3 Hz, NH), 3.12 (q, 2H, *J*_{vic} = 6.9 Hz, NHCH₂CH₂CCH), 2.90 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.83 (t, 1H, ⁴*J* = 2.7 Hz, NHCH₂CH₂CCH), 2.79 (t, 1H, ⁴*J* = 2.6 Hz, NCH₂CH₂CCH), 2.45-2.56 (m, 6H, H-3,5, NCH₂CH₂CCH), 2.30-2.39 (m, 4H, NCH₂CH₂CCH, NHCH₂CH₂CCH). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 142.59 (C-*i*-Ph), 142.10 (C-*p*-Ph), 117.91 (CH-*o*-Ph), 113.00 (CH-*m*-Ph), 83.20 (NCH₂CH₂CCH), 82.81 (NHCH₂CH₂CCH), 72.06 (NHCH₂CH₂CCH), 71.70 (NCH₂CH₂CCH), 56.49 (NCH₂CH₂CCH), 52.54 (CH₂-3,5), 50.15 (CH₂-2,6), 42.70 (NHCH₂CH₂CCH), 18.53 (NHCH₂CH₂CCH), 16.18 (NCH₂CH₂CCH). **FTIR** (CHCl₃, cm⁻¹) *v*_{max}: 3436, 3395, 3308, 3056, 3033, 2927, 2826, 2779, 2119, 1618, 1516, 1478, 1466, 1457, 1451, 1435, 1376, 1358, 1174, 1137, 1010, 1004, 925, 822, 642, 540. **HRMS** (EI⁺) calcd. for C₁₈H₂₃N₃⁺ (M⁺): 281.1892, found: 281.1895. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.70.

Synthesis of alkylation products 29a and 29b

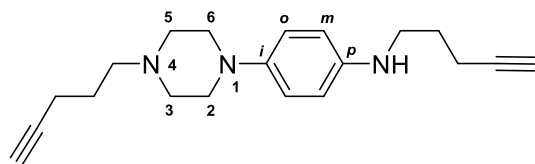
Reactants: 4-(piperazin-1-yl)aniline (**13**, 2.500 g, 14.1 mmol), 5-iodopent-1-yne (1.71 mL, 14.1 mmol), and K₂CO₃ (19.493 g, 141.0 mmol).

4-(4-(Pent-4-yn-1-yl)piperazin-1-yl)aniline (**29a**).



Yellow crystalline solid (1.706 g, yield 50 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 6.67 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.53 (bs, 2H, NH₂), 2.89 (t, 4H, *J*_{vic} = 4.9 Hz, H-2,6), 2.75 (t, 1H, ⁴*J* = 2.7 Hz, NCH₂CH₂CH₂CCH), 2.46 (t, 4H, *J*_{vic} = 5.0 Hz, H-3,5), 2.36 (t, 2H, *J*_{vic} = 7.2 Hz, NCH₂CH₂CH₂CCH), 2.18 (td, 2H, *J*_{vic} = 7.1 Hz, ⁴*J* = 2.7 Hz, NCH₂CH₂CH₂CCH), 1.62 (p, 2H, *J*_{vic} = 7.1 Hz, NCH₂CH₂CH₂CCH). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 142.43 (C-*i*-Ph), 142.05 (C-*p*-Ph), 117.82 (CH-*o*-Ph), 114.72 (CH-*m*-Ph), 84.42 (NCH₂CH₂CH₂CCH), 71.21 (NCH₂CH₂CH₂CCH), 56.61 (NCH₂CH₂CH₂CCH), 53.04 (CH₂-3,5), 50.36 (CH₂-2,6), 25.40 (NCH₂CH₂CH₂CCH), 15.66 (NCH₂CH₂CH₂CCH). FTIR (CHCl₃, cm⁻¹) ν_{max}: 3441, 3366, 3308, 3103, 2952, 2886, 2823, 1623, 1611, 1583, 1515, 1457, 1269, 1138, 1007, 923, 826, 640. HRMS (EI⁺) calcd. for C₁₅H₂₁N₃⁺ (M⁺): 243.1735, found: 243.1738. R_f (0.25% TEA in 1/1 EtOAc/MeOH) = 0.62.

N-(Pent-4-yn-1-yl)-4-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (**29b**).

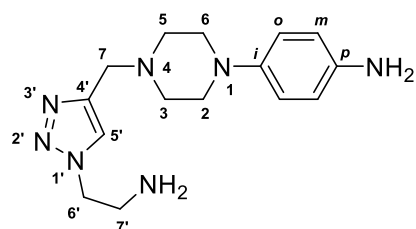


Yellow crystalline solid (747 mg, yield 17 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 6.73 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 5.10 (t, 1H, *J*_{vic} = 6.3 Hz, NH), 3.01 (q, 2H, *J*_{vic} = 6.4 Hz, NHCH₂CH₂CH₂CCH), 2.90 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.78 (t, 1H, ⁴*J* = 2.6 Hz, NHCH₂CH₂CH₂CCH), 2.75 (t, 1H, ⁴*J* = 2.7 Hz, NCH₂CH₂CH₂CCH), 2.46 (t, 4H, *J*_{vic} = 5.0 Hz, H-3,5), 2.37 (t, 2H, *J*_{vic} = 7.2 Hz, NCH₂CH₂CH₂CCH), 2.25 (td, 2H, *J*_{vic} = 7.1 Hz, ⁴*J* = 2.7 Hz, NHCH₂CH₂CH₂CCH), 2.19 (td, 2H, *J*_{vic} = 7.1 Hz, ⁴*J* = 2.7 Hz, NCH₂CH₂CH₂CCH), 1.69 (p, 2H, *J*_{vic} = 7.0 Hz, NHCH₂CH₂CH₂CCH), 1.61 (p, 2H, *J*_{vic} = 7.2 Hz, NCH₂CH₂CH₂CCH). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 142.87 (C-*p*-Ph), 142.33 (C-*i*-Ph), 117.89 (CH-*o*-Ph), 112.79 (CH-*m*-Ph), 84.40 (NCH₂CH₂CH₂CCH), 84.32 (NHCH₂CH₂CH₂CCH), 71.29 (NHCH₂CH₂CH₂CCH), 71.20 (NCH₂CH₂CH₂CCH), 56.61 (NCH₂CH₂CH₂CCH), 53.02 (CH₂-3,5), 50.31 (CH₂-2,6), 42.37 (NHCH₂CH₂CH₂CCH), 27.78 (NHCH₂CH₂CH₂CCH), 25.40 (NCH₂CH₂CH₂CCH), 15.66 (NCH₂CH₂CH₂CCH), 15.52 (NHCH₂CH₂CH₂CCH). FTIR (CHCl₃, cm⁻¹) ν_{max}: 3446, 3412, 3308, 3103, 2950, 2823, 2118, 1618, 1582, 1516, 1456, 1411, 1377, 1321, 1271, 1231, 1138, 1097, 1005, 951, 639, 495, 415. HRMS (EI⁺) calcd. for C₂₀H₂₇N₃⁺ (M⁺): 309.2205, found: 309.2207. R_f (0.25% TEA in 1/1 EtOAc/MeOH) = 0.74.

The general procedure for synthesis of click reaction products 31-42; procedure according to ref. [39]

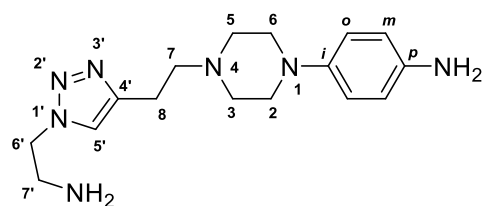
Alkyne (**27a**, **28a**, and **29a**, 0.230 mmol) was dissolved in *tert*-butanol/H₂O (1:2, 1.5 mL) under argon atmosphere, CuSO₄ (100 mM solution, 0.230 mL) and Na-ascorbate (100 mM solution, 0.690 mL) were added and the mixture was allowed to stir at r.t. for 15 min. Afterwards, azide (0.230 mmol) was added and the reaction mixture was kept stirring at r.t. for 24 h under argon. The solvent was removed under reduced pressure and the residue was put on a silica gel column in methanol (0.4 M NH₃ solution, 5-20 %)/ethyl acetate, which afforded the desired click product **31-42**.

4-(4-((1-(2-Aminoethyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)aniline (31**).**



Reactants: 4-(4-(prop-2-yn-1-yl)piperazin-1-yl)aniline (**27a**, 50.0 mg, 0.230 mmol), 2-azidoethan-1-aminium chloride (**7**, 28.3 mg, 0.230 mmol), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow amorphous solid (34.5 mg, yield 50 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.99 (s, 1H, H-5'), 6.66 (m, 2H, H-*o*-Ph), 6.47 (m, 2H, H-*m*-Ph), 4.31 (t, 2H, *J*(6',7') = 6.3 Hz, H-6'), 3.58 (s, 2H, H-7), 2.98 (t, 2H, *J*(7',6') = 6.3 Hz, H-7'), 2.88 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.47-2.55 (m, 4H, H-3,5, signal overlapped by DMSO peak); NH₂ signals not detected. ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 143.11 (C-4'), 142.39 (C-*i*-Ph), 142.07 (C-*p*-Ph), 123.98 (CH-5'), 117.86 (CH-*o*-Ph), 114.71 (CH-*m*-Ph), 52.65 (CH₂-7), 52.55 (CH₂-3,5), 52.15 (CH₂-6'), 50.28 (CH₂-2,6), 41.62 (CH₂-7'). FTIR (KBr, cm⁻¹) ν_{max}: 3414, 3347, 3133, 2752, 2691, 1630, 1581, 1514, 1453, 1375, 1327, 1292, 1273, 1224, 1168, 1029, 706. HRMS (ESI⁺) calcd. for C₁₅H₂₃N₇Na⁺ (M+Na⁺): 324.19071, found: 324.19052; calcd. for C₁₅H₂₄N₇⁺ (M+H⁺): 302.20877, found: 302.20859. R_f (0.25% TEA in 1/1 EtOAc/MeOH) = 0.15.

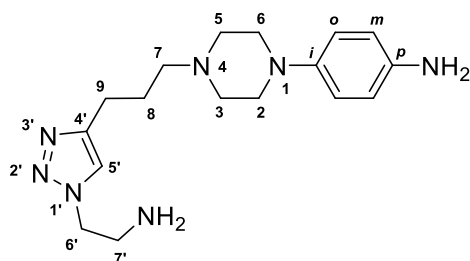
4-(4-(2-(1-(2-Aminoethyl)-1*H*-1,2,3-triazol-4-yl)ethyl)piperazin-1-yl)aniline (32**).**



Reactants: 4-(4-(but-3-yn-1-yl)piperazin-1-yl)aniline (**28a**, 52.8 mg, 0.230 mmol), 2-azidoethan-1-aminium chloride (**7**, 28.3 mg, 0.230 mmol), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow crystalline solid (40.6 mg, yield 56 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.89 (s, 1H, H-5'), 6.68 (m, 2H, H-*o*-Ph), 6.49 (m, 2H, H-*m*-Ph), 4.33 (t, 2H, *J*(6',7') = 6.3 Hz, H-6'), 3.01 (t, 2H, *J*(7',6') = 6.3 Hz, H-7'), 2.91 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.80 (t, 2H, *J*(8,7) = 7.7 Hz, H-8), 2.59 (t, 2H, *J*(7,8) = 7.8 Hz, H-7), 2.55 (t, 4H, *J*_{vic} = 4.9 Hz, H-3,5); NH₂ signals not detected. ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 144.98 (C-4'), 142.44 (C-*i*-Ph), 142.06 (C-*p*-Ph), 122.51 (CH-5'),

117.84 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.49 (CH₂-7), 52.84 (CH₂-3,5), 51.22 (CH₂-6'), 50.33 (CH₂-2,6), 41.18 (CH₂-7'), 23.04 (CH₂-8). **FTIR** (KBr, cm⁻¹) ν_{max} : 3410, 3383, 2816, 2749, 1631, 1612, 1577, 1515, 1457, 1449, 1384, 1230, 1171, 1135, 954, 942, 889, 826, 649. **HRMS** (ESI⁺) calcd. for C₁₆H₂₆N₇⁺ (M+H⁺): 316.22442, found: 316.22425. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.15.

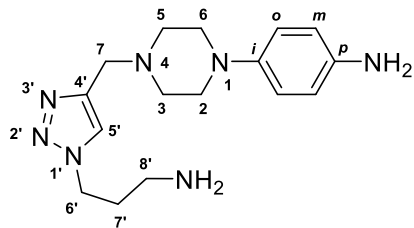
4-(4-(3-(1-(2-Aminoethyl)-1*H*-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)aniline (33).



Reactants: 4-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (**29a**, 55.8 mg, 0.230 mmol), 2-azidoethan-1-aminium chloride (**7**, 28.3 mg, 0.230 mmol), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow amorphous solid (49.9 mg, yield 66 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.94 (s, 1H, H-5'), 6.68 (m, 2H, H-*o*-Ph), 6.49 (m,

2H, H-*m*-Ph), 4.53 (t, 2H, $J(6',7') = 6.2$ Hz, H-6'), 3.25 (t, 2H, $J(7',6') = 6.2$ Hz, H-7'), 2.92 (t, 4H, $J_{\text{vic}} = 5.0$ Hz, H-2,6), 2.66 (t, 2H, $J(9,8) = 7.5$ Hz, H-9), 2.49-2.57 (m, 4H, H-3,5), 2.41 (t, 2H, $J(7,8) = 7.3$ Hz, H-7), 1.79 (p, 2H, $J(8,7) = J(8,9) = 7.4$ Hz, H-8); NH₂ signals not detected. **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 146.80 (C-4'), 142.34 (C-*i*-Ph), 142.10 (C-*p*-Ph), 122.47 (CH-5'), 117.84 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.01 (CH₂-7), 52.90 (CH₂-3,5), 50.22 (CH₂-2,6), 47.67 (CH₂-6'), 39.07 (CH₂-7'), 25.97 (CH₂-8), 22.88 (CH₂-9). **FTIR** (KBr, cm⁻¹) ν_{max} : 3530, 3433, 3254, 3136, 3004, 2815, 1631, 1611, 1577, 1515, 1454, 1445, 1385, 1306, 1282, 1229, 1176, 1137, 1006, 954, 891, 823, 782, 688, 649. **HRMS** (ESI⁺) calcd. for C₁₇H₂₈N₇⁺ (M+H⁺): 330.24007, found: 330.24020. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.15.

4-(4-((1-(3-Aminopropyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)aniline (34).

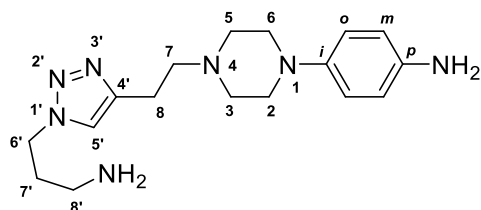


Reactants: 4-(4-(prop-2-yn-1-yl)piperazin-1-yl)aniline (**27a**, 50.0 mg, 0.230 mmol), 3-azidopropan-1-amine (**8**, 0.023 mL, 0.230 mmol), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Brown amorphous solid (30.6 mg, yield 42 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 8.01 (s, 1H, H-5'),

6.66 (m, 2H, H-*o*-Ph), 6.47 (m, 2H, H-*m*-Ph), 4.41 (t, 2H, $J(6',7') = 6.8$ Hz, H-6'), 3.58 (s, 2H, H-7), 2.88 (t, 4H, $J_{\text{vic}} = 5.0$ Hz, H-2,6), 2.61 (t, 2H, $J(8',7') = 7.0$ Hz, H-8'), 2.43-2.53 (m, 4H, H-3,5, signal overlapped by DMSO peak), 1.96 (p, 2H, $J(7',6') = J(7',8') = 6.9$ Hz, H-7'); NH₂ signals not detected. **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 143.27 (C-4'), 142.38 (C-*i*-Ph), 142.08 (C-*p*-Ph), 123.76 (CH-5'), 117.86 (CH-*o*-Ph), 114.71 (CH-*m*-Ph), 52.61 (CH₂-7), 52.53 (CH₂-3,5), 50.28 (CH₂-2,6), 46.83 (CH₂-6'), 37.54 (CH₂-8'), 31.31 (CH₂-7'). **FTIR** (KBr, cm⁻¹) ν_{max} : 3422, 3225, 3131, 3032, 2924, 2816, 2682, 2598,

1630, 1580, 1514, 1453, 1377, 1327, 1273, 1224, 1168, 1029, 706. **HRMS** (ESI+) calcd. for $C_{16}H_{26}N_7^+$ ($M+H^+$): 316.22442, found: 316.22414. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.16.

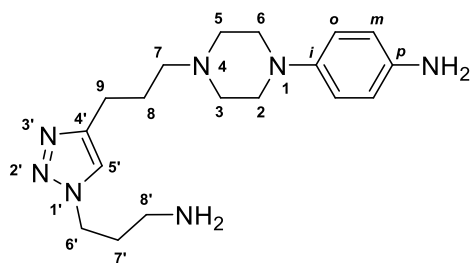
4-(4-(2-(1-(3-Aminopropyl)-1H-1,2,3-triazol-4-yl)ethyl)piperazin-1-yl)aniline (35).



Reactants: 4-(4-(but-3-yn-1-yl)piperazin-1-yl)aniline (**28a**, 52.8 mg, 0.230 mmol), 3-azidopropan-1-amine (**8**, 0.023 mL, 0.230 mmol), $CuSO_4$ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow crystalline solid (39.4 mg, yield 52 %). **¹H NMR** (400 MHz, $DMSO-d_6$):

δ 7.87 (s, 1H, H-5'), 6.68 (m, 2H, H-*o*-Ph), 6.49 (m, 2H, H-*m*-Ph), 4.36 (t, 2H, $J(6',7') = 7.0$ Hz, H-6'), 2.91 (t, 4H, $J_{vic} = 4.9$ Hz, H-2,6), 2.79 (t, 2H, $J(8,7) = 7.7$ Hz, H-8), 2.58 (t, 2H, $J(7,8) = 7.9$ Hz, H-7), 2.54 (t, 4H, $J = 5.0$ Hz, H-3,5), 2.47-2.53 (m, 2H, H-8', signal overlapped by DMSO peak), 1.85 (p, 2H, $J(7',6') = J(7',8') = 6.9$ Hz, H-7'); NH_2 signals not detected. **¹³C NMR** (100.6 MHz, $DMSO-d_6$): δ 145.03 (C-4'), 142.44 (C-*i*-Ph), 142.04 (C-*p*-Ph), 122.15 (CH-5'), 117.83 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.52 (CH₂-7), 52.83 (CH₂-3,5), 50.34 (CH₂-2,6), 46.91 (CH₂-6'), 38.07 (CH₂-8'), 32.86 (CH₂-7'), 23.05 (CH₂-8). **FTIR** (KBr, cm^{-1}) ν_{max} : 3357, 3288, 3202, 3116, 2946, 2879, 2813, 1581, 1554, 1516, 1457, 1265, 1176, 1137, 1004, 924. **HRMS** (ESI+) calcd. for $C_{17}H_{27}N_7Na^+$ ($M+Na^+$): 352.22202, found: 352.22205; calcd. for $C_{17}H_{28}N_7^+$ ($M+H^+$): 330.24007, found: 330.24018. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.15.

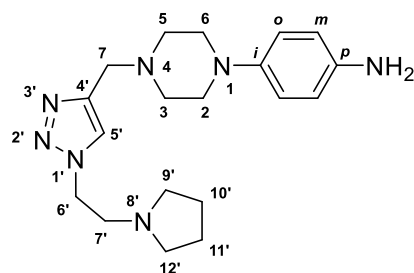
4-(4-(3-(1-(3-Aminopropyl)-1H-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)aniline (36).



Reactants: 4-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (**29a**, 55.8 mg, 0.230 mmol), 3-azidopropan-1-amine (**8**, 0.023 mL, 0.230 mmol), $CuSO_4$ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow amorphous solid (39.3 mg, yield 50 %). **¹H NMR** (400 MHz, $DMSO-d_6$): δ 7.85 (s, 1H, H-5'), 6.67 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.35 (t, 2H, $J(6',7') = 7.0$ Hz, H-6'), 2.89 (t, 4H, $J_{vic} = 5.0$ Hz, H-2,6), 2.62 (t, 2H, $J(9,8) = 7.6$ Hz, H-9), 2.49-2.53 (m, 2H, H-8', signal overlapped by DMSO peak), 2.47 (t, 4H, $J_{vic} = 4.7$ Hz, H-3,5), 2.33 (t, 2H, $J(7,8) = 7.2$ Hz, H-7), 1.88 (p, 2H, $J(7',6') = J(7',8') = 6.9$ Hz, H-7'), 1.76 (p, 2H, $J(8,7) = J(8,9) = 7.5$ Hz, H-8); NH_2 signals not detected. **¹³C NMR** (100.6 MHz, $DMSO-d_6$): δ 146.61 (C-4'), 142.47 (C-*i*-Ph), 142.03 (C-*p*-Ph), 121.78 (CH-5'), 117.81 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.20 (CH₂-7), 53.03 (CH₂-3,5), 50.38 (CH₂-2,6), 46.94 (CH₂-6'), 38.28 (CH₂-8'), 33.27 (CH₂-7'), 26.26 (CH₂-8), 22.94 (CH₂-9). **FTIR** (KBr, cm^{-1}) ν_{max} : 3334, 3221, 3132, 2941, 2876, 2815, 1610, 1581, 1552, 1515, 1455, 1272, 1137, 1006,

923. **HRMS** (ESI⁺) calcd. for C₁₈H₃₀N₇⁺ (M+H⁺): 344.25572, found: 344.25578. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.13.

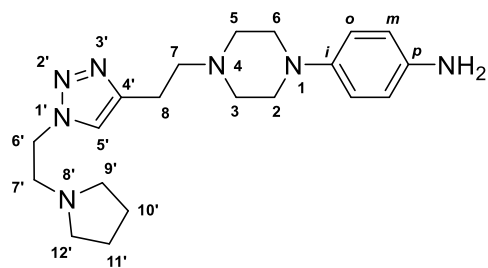
4-(4-((1-(2-(Pyrrolidin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)aniline (37).



Reactants: 4-(4-(prop-2-yn-1-yl)piperazin-1-yl)aniline (**27a**, 50.0 mg, 0.230 mmol), 1-(2-azidoethyl)pyrrolidine (**9**, 0.033 mL, 0.230 mmol, 1.0 eq.), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow amorphous solid (49.6 mg, yield 60 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.99 (s, 1H, H-5'), 6.66 (m, 2H, H-*o*-Ph), 6.47 (m, 2H, H-*m*-Ph), 4.43 (t, 2H,

J(6',7') = 6.5 Hz, H-6'), 3.58 (s, 2H, H-7), 2.88 (t, 4H, *J*_{vic} = 4.8 Hz, H-2,6), 2.85 (t, 2H, *J*(7',6') = 6.5 Hz, H-7'), 2.48-2.54 (m, 4H, H-3,5, signal overlapped by DMSO peak), 2.45 (m, 4H, H-9',12'), 1.64 (m, 4H, H-10',11'); NH₂ signal not detected. **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 143.09 (C-4'), 142.38 (C-*i*-Ph), 142.08 (C-*p*-Ph), 123.95 (CH-5'), 117.86 (CH-*o*-Ph), 114.70 (CH-*m*-Ph), 55.02 (CH₂-7'), 53.35 (CH₂-9',12'), 52.62 (CH₂-7), 52.54 (CH₂-3,5), 50.27 (CH₂-2,6), 48.51 (CH₂-6'), 23.12 (CH₂-10',11'). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3415, 3340, 3134, 3032, 2812, 1630, 1580, 1514, 1453, 1375, 1328, 1274, 1225, 1149, 1027, 707. **HRMS** (ESI⁺) calcd. for C₁₉H₂₉N₇Na⁺ (M+Na⁺): 378.23767, found: 378.23756; calcd. for C₁₉H₃₀N₇⁺ (M+H⁺): 356.25572, found: 356.25565. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.15.

4-(4-(2-(1-(2-(Pyrrolidin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)ethyl)piperazin-1-yl)aniline (38).

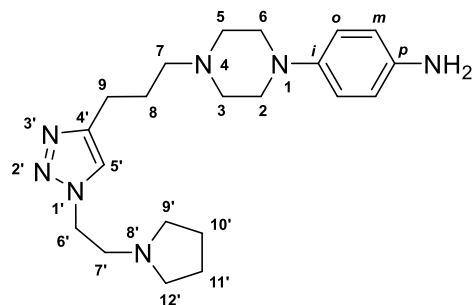


Reactants: 4-(4-(but-3-yn-1-yl)piperazin-1-yl)aniline (**28a**, 52.8 mg, 0.230 mmol), 1-(2-azidoethyl)pyrrolidine (**9**, 0.033 mL, 0.230 mmol), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow crystalline solid (45.4 mg, yield 53 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.88 (s, 1H, H-5'), 6.68 (m, 2H, H-*o*-Ph), 6.48

(m, 2H, H-*m*-Ph), 4.40 (t, 2H, *J*(6',7') = 6.4 Hz, H-6'), 2.90 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.82 (t, 2H, *J*(7',6') = 6.4 Hz, H-7'), 2.80 (t, 2H, *J*(8,7) = 7.7 Hz, H-8), 2.58 (t, 2H, *J*(7,8) = 7.7 Hz, H-7), 2.54 (t, 4H, *J*_{vic} = 4.8 Hz, H-3,5), 2.45 (m, 4H, H-9',12'), 1.65 (m, 4H, H-10',11'); NH₂ signal not detected. **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 144.90 (C-4'), 142.44 (C-*i*-Ph), 142.05 (C-*p*-Ph), 122.41 (CH-5'), 117.81 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.42 (CH₂-7), 55.09 (CH₂-7'), 53.36 (CH₂-9',12'), 52.82 (CH₂-3,5), 50.34 (CH₂-2,6), 48.43 (CH₂-6'), 23.12 (CH₂-10',11'), 23.03 (CH₂-8). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3428, 3281, 2756, 2303, 1658, 1631, 1611, 1574, 1516, 1467, 1447, 1384, 1352, 1279, 1229, 1171, 1121, 1017, 954, 891,

649, 572. **HRMS** (ESI+) calcd. for $C_{20}H_{32}N_7^+$ ($M+H^+$): 370.27137, found: 370.27141. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.16.

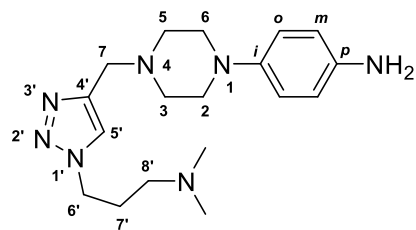
4-(4-(3-(1-(2-(Pyrrolidin-1-yl)ethyl)-1H-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)aniline (39).



Reactants: 4-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (**29a**, 55.8 mg, 0.230 mmol), 1-(2-azidoethyl)pyrrolidine (**9**, 0.033 mL, 0.230 mmol), $CuSO_4$ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Brown amorphous solid (58.5 mg, yield 67 %). **¹H NMR** (400 MHz, $DMSO-d_6$): δ 7.85 (s, 1H, H-5'), 6.67 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.39 (t, 2H, $J(6',7') = 6.5$ Hz, H-6'), 2.89 (t, 4H, $J_{vic} = 5.0$

Hz, H-2,6), 2.83 (t, 2H, $J(7',6') = 6.5$ Hz, H-7'), 2.63 (t, 2H, $J(9,8) = 7.5$ Hz, H-9), 2.40-2.50 (m, 8H, H-3,5, H-9',12'), 2.33 (t, 2H, $J(7,8) = 7.3$ Hz, H-7), 1.76 (p, 2H, $J(8,7) = J(8,9) = 7.5$ Hz, H-8), 1.65 (m, 4H, H-10',11'); NH_2 signal not detected. **¹³C NMR** (100.6 MHz, $DMSO-d_6$): δ 146.44 (C-4'), 142.46 (C-*i*-Ph), 142.06 (C-*p*-Ph), 122.05 (CH-5'), 117.82 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.15 (CH₂-7), 55.12 (CH₂-7'), 53.39 (CH₂-9',12'), 53.04 (CH₂-3,5), 50.39 (CH₂-2,6), 48.45 (CH₂-6'), 26.22 (CH₂-8), 23.14 (CH₂-10',11'), 22.92 (CH₂-9). **FTIR** (KBr, cm^{-1}) ν_{max} : 3499, 3431, 3228, 3134, 3067, 3001, 2811, 2782, 1631, 1611, 1578, 1515, 1456, 1446, 1384, 1272, 1228, 1008, 956, 823, 774, 649. **HRMS** (ESI+) calcd. for $C_{21}H_{33}N_7Na^+$ ($M+Na^+$): 406.26897, found: 406.26865; calcd. for $C_{21}H_{34}N_7^+$ ($M+H^+$): 384.28702, found: 384.28666. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.16.

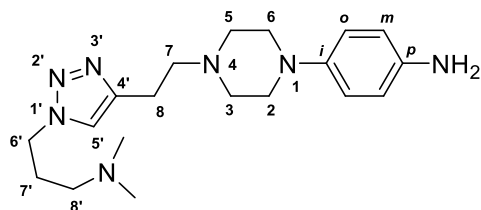
4-(4-((1-(3-(Dimethylamino)propyl)-1H-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)aniline (40).



Reactants: 4-(4-(prop-2-yn-1-yl)piperazin-1-yl)aniline (**27a**, 50.0 mg, 0.230 mmol), 3-azido-*N,N*-dimethylpropan-1-amine (**30**, 0.030 mL, 0.230 mmol), $CuSO_4$ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Brown amorphous solid (39.8 mg, yield 50 %). **¹H NMR** (400 MHz, $DMSO-d_6$): δ 7.99 (s, 1H, H-5'), 6.66 (m, 2H, H-*o*-Ph), 6.47 (m, 2H, H-*m*-Ph), 4.34 (t, 2H, $J(6',7') = 7.1$ Hz, H-6'), 3.58 (s, 2H, H-7), 2.88 (t, 4H, $J_{vic} = 4.9$ Hz, H-2,6), 2.48-2.53 (m, 4H, H-3,5, signal overlapped by DMSO peak), 2.16 (t, 2H, $J(8',7') = 6.9$ Hz, H-8'), 2.11 (s, 6H, CH₃), 1.93 (p, 2H, $J(7',6') = J(7',8') = 7.0$ Hz, H-7'); NH_2 signal not detected. **¹³C NMR** (100.6 MHz, $DMSO-d_6$): δ 143.21 (C-4'), 142.39 (C-*i*-Ph), 142.09 (C-*p*-Ph), 123.74 (CH-5'), 117.87 (CH-*o*-Ph), 114.71 (CH-*m*-Ph), 55.69 (CH₂-8'), 52.62 (CH₂-7), 52.52 (CH₂-3,5), 50.28 (CH₂-2,6), 47.41 (CH₂-6'), 45.06 (CH₃), 27.73 (CH₂-7'). **FTIR** (KBr, cm^{-1}) ν_{max} : 3424, 3325, 3134, 3033, 2777, 1629, 1578, 1515, 1454, 1377, 1326, 1271, 1225, 1143, 1029, 922, 825, 704, 541.

HRMS (ESI⁺) calcd. for C₁₈H₂₉N₇Na⁺ (M+Na⁺): 366.23767, found: 366.23755; calcd. for C₁₈H₃₀N₇⁺ (M+H⁺): 344.25572, found: 344.25562. **R_f** (1/1 EtOAc/MeOH) = 0.09.

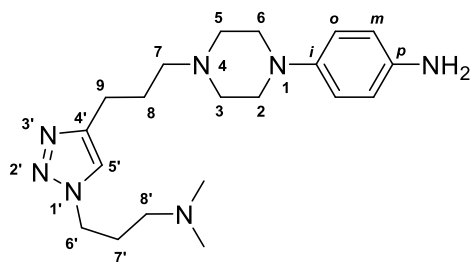
4-(4-(2-(1-(3-(Dimethylamino)propyl)-1*H*-1,2,3-triazol-4-yl)ethyl)piperazin-1-yl)aniline (41).



Reactants: 4-(4-(but-3-yn-1-yl)piperazin-1-yl)aniline (**28a**, 52.8 mg, 0.230 mmol), 3-azido-*N,N*-dimethylpropan-1-amine (**30**, 0.030 mL, 0.230 mmol), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Brown amorphous solid (37.5 mg, yield 46 %). **¹H NMR** (400 MHz,

DMSO-*d*₆): δ 7.86 (s, 1H, H-5'), 6.67 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.31 (t, 2H, *J*(6',7') = 7.1 Hz, H-6'), 2.90 (t, 4H, *J*_{vic} = 4.9 Hz, H-2,6), 2.80 (t, 2H, *J*(8,7) = 7.6 Hz, H-8), 2.58 (t, 2H, *J*(7,8) = 7.7 Hz, H-7), 2.54 (t, 4H, *J*_{vic} = 4.9 Hz, H-3,5), 2.15 (t, 2H, *J*(8',7') = 6.9 Hz, H-8'), 2.11 (s, 6H, CH₃), 1.90 (p, 2H, *J*(7',6') = *J*(7',8') = 7.2 Hz, H-7'); NH₂ signal not detected. **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 145.03 (C-4'), 142.44 (C-*i*-Ph), 142.05 (C-*p*-Ph), 122.20 (CH-5'), 117.83 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.48 (CH₂-7), 55.66 (CH₂-8'), 52.83 (CH₂-3,5), 50.34 (CH₂-2,6), 47.29 (CH₂-6'), 45.08 (CH₃), 27.81 (CH₂-7'), 23.04 (CH₂-8). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3414, 3298, 3115, 2765, 1631, 1578, 1518, 1458, 1382, 1331, 1271, 1233, 1151, 1030, 706, 530. **HRMS** (ESI⁺) calcd. for C₁₉H₃₁N₇Na⁺ (M+Na⁺): 380.25332, found: 380.25311; calcd. for C₁₉H₃₂N₇⁺ (M+H⁺): 358.27137, found: 358.27130. **R_f** (1/1 EtOAc/MeOH) = 0.09.

4-(4-(3-(1-(3-(Dimethylamino)propyl)-1*H*-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)aniline (42).

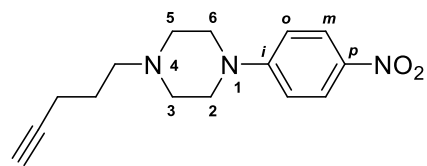


Reactants: 4-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (**29a**, 55.8 mg, 0.230 mmol), 3-azido-*N,N*-dimethylpropan-1-amine (**30**, 0.030 mL, 0.230 mmol), CuSO₄ (100 mM solution, 0.230 mL), and Na-ascorbate (100 mM solution, 0.690 mL). Yellow amorphous solid (72.8 mg, yield 85 %). **¹H NMR** (400 MHz,

DMSO-*d*₆): δ 7.84 (s, 1H, H-5'), 6.67 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.30 (t, 2H, *J*(6',7') = 7.1 Hz, H-6'), 2.89 (t, 4H, *J*_{vic} = 4.9 Hz, H-2,6), 2.63 (t, 2H, *J*(9,8) = 7.5 Hz, H-9), 2.46 (t, 4H, *J*_{vic} = 4.8 Hz, H-3,5), 2.33 (t, 2H, *J*(7,8) = 7.3 Hz, H-7), 2.15 (t, 2H, *J*(8',7') = 6.9 Hz, H-8'), 2.11 (s, 6H, CH₃), 1.91 (p, 2H, *J*(7',6') = *J*(7',8') = 7.0 Hz, H-7'), 1.77 (p, 2H, *J*(8,7) = *J*(8,9) = 7.4 Hz, H-8); NH₂ signal not detected. **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 146.60 (C-4'), 142.46 (C-*i*-Ph), 142.03 (C-*p*-Ph), 121.81 (CH-5'), 117.80 (CH-*o*-Ph), 114.73 (CH-*m*-Ph), 57.19 (CH₂-7), 55.70 (CH₂-8'), 53.03 (CH₂-3,5), 50.37 (CH₂-2,6), 47.29 (CH₂-6'), 45.09 (CH₃), 27.81 (CH₂-7'), 26.25 (CH₂-8), 22.94 (CH₂-9). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3419, 3348, 3220, 3132, 3032, 2769, 1628, 1578, 1515, 1457, 1377,

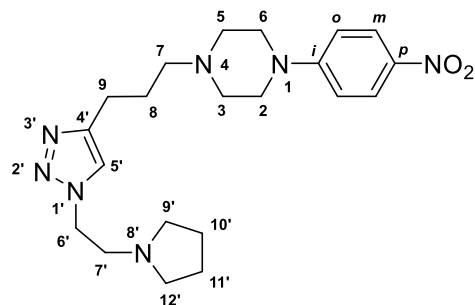
1331, 1268, 1228, 1151, 1025, 706, 538. **HRMS** (ESI+) calcd. for $C_{20}H_{34}N_7^+$ ($M+H^+$): 372.28702, found: 372.28671. **R_f** (1/1 EtOAc/MeOH) = 0.11.

1-(4-Nitrophenyl)-4-(pent-4-yn-1-yl)piperazine (44); procedure according to ref. [136].



The reaction conditions and the procedure is the same as for synthesis of compounds **27a**, **28a**, and **29a**. Reactants: 1-(4-nitrophenyl)piperazine (**43**, 300 mg, 1.45 mmol), 5-iodopent-1-yne (0.176 mL, 1.45 mmol), and K_2CO_3 (2.004 g, 14.5 mmol), dissolved in DMF (15 mL); reaction time 6 h. Orange crystalline solid (249 mg, yield 63 %). **¹H NMR** (400 MHz, $DMSO-d_6$): δ 8.04 (m, 2H, H-*m*-Ph), 7.02 (m, 2H, H-*o*-Ph), 3.44 (t, 4H, $J_{vic} = 5.2$ Hz, H-2,6), 2.76 (t, 1H, $^4J = 2.7$ Hz, $NCH_2CH_2CH_2CCH$), 2.47 (t, 4H, $J_{vic} = 5.2$ Hz, H-3,5), 2.38 (t, 2H, $J_{vic} = 7.2$ Hz, $NCH_2CH_2CH_2CCH$), 2.20 (td, 2H, $J_{vic} = 7.1$ Hz, $^4J = 2.7$ Hz, $NCH_2CH_2CH_2CCH$), 1.63 (p, 2H, $J_{vic} = 7.1$ Hz, $NCH_2CH_2CH_2CCH$). **¹³C NMR** (100.6 MHz, $DMSO-d_6$): δ 154.72 (C-*i*-Ph), 136.81 (C-*p*-Ph), 125.68 (CH-*m*-Ph), 112.55 (CH-*o*-Ph), 84.33 ($NCH_2CH_2CH_2CCH$), 71.24 ($NCH_2CH_2CH_2CCH$), 56.38 ($NCH_2CH_2CH_2CCH$), 52.29 (CH_2 -3,5), 46.30 (CH_2 -2,6), 25.28 ($NCH_2CH_2CH_2CCH$), 15.62 ($NCH_2CH_2CH_2CCH$). **FTIR** ($CHCl_3$, cm^{-1}) ν_{max} : 3308, 3094, 2823, 2788, 2117, 1599, 1506, 1452, 1383, 1331, 1246, 1140, 1117, 1006, 831, 693, 640, 538. **HRMS** (ESI+) calcd. for $C_{15}H_{20}O_2N_3^+$ ($M+H^+$): 274.15500, found: 274.15496. **R_f** (EtOAc) = 0.60.

1-(4-Nitrophenyl)-4-(3-(1-(2-(pyrrolidin-1-yl)ethyl)-1H-1,2,3-triazol-4-yl)propyl)piperazine (45); procedure according to ref. [39].



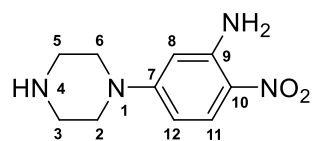
1-(4-Nitrophenyl)-4-(pent-4-yn-1-yl)piperazine (**44**, 50 mg, 0.183 mmol) was dissolved in $DMSO/H_2O$ (3:1, 2.4 mL) under argon atmosphere, $CuSO_4$ (9.1 mg, 0.036 mmol), and Na-ascorbate (14.5 mg, 0.073 mmol) were added and the mixture was allowed to stir at r.t. for 15 min. Afterwards, 1-(2-azidoethyl)pyrrolidine (**9**, 0.026 mL, 0.183 mmol) was added and the reaction mixture was stirred at r.t. for 24 h under argon.

The solvent was removed *in vacuo* and the residue was purified on silica gel column (0-10% MeOH/EtOAc). Orange crystalline solid (48 mg, yield 64 %). **¹H NMR** (400 MHz, $DMSO-d_6$): δ 8.05 (m, 2H, H-*m*-Ph), 7.85 (s, 1H, H-5'), 7.02 (m, 2H, H-*o*-Ph), 4.40 (t, 2H, $J(6',7') = 6.4$ Hz, H-6'), 3.44 (t, 4H, $J_{vic} = 5.1$ Hz, H-2,6), 2.83 (t, 2H, $J(7',6') = 6.4$ Hz, H-7'), 2.64 (t, 2H, $J(9,8) = 7.5$ Hz, H-9), 2.43-2.48 (m, 8H, H-3,5, H-9',12'), 2.35 (t, 2H, $J(7,8) = 7.3$ Hz, H-7), 1.78 (p, 2H, $J(8,7) = J(8,9) = 7.5$ Hz, H-8), 1.65 (m, 4H, H-10',11'). **¹³C NMR** (100.6 MHz, $DMSO-d_6$): δ 154.73 (C-*i*-Ph), 146.36 (C-4'), 136.79 (C-

p-Ph), 125.68 (CH-*m*-Ph), 122.04 (CH-5'), 112.55 (CH-*o*-Ph), 56.92 (CH₂-7), 55.09 (CH₂-7'), 53.36 (CH₂-9',12'), 52.28 (CH₂-3,5), 48.45 (CH₂-6'), 46.32 (CH₂-2,6), 26.08 (CH₂-8), 23.12 (CH₂-10',11'), 22.87 (CH₂-9). **FTIR** (CHCl₃, cm⁻¹) $\nu_{\max}$: 3137, 3075, 2950, 2928, 2812, 2785, 1598, 1506, 1494, 1326, 1295, 1245, 1117, 1100, 1001, 956, 853, 832, 755, 694, 663, 630, 539, 501. **HRMS** (ESI+) calcd. for C₂₁H₃₂O₂N₇⁺ (M+H⁺): 414.26120, found: 414.26132. **R_f** (EtOAc) = 0.24.

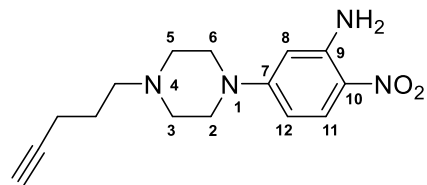
11.2.3 Synthesis of the bis-amino series

2-Nitro-5-(piperazin-1-yl)aniline (47) [139].



5-Chloro-2-nitroaniline (**46**, 1.400 g, 8.11 mmol) was dissolved in dry DMF (80 mL) under argon atmosphere and K₂CO₃ (2.960 g, 21.4 mmol) and piperazine (**1**, 1.844 g, 21.4 mmol) were added. The reaction was heated at 120°C for 24 h. DMF was removed under reduced pressure and the solid was extracted into ethyl acetate using water/ethyl acetate system. The organic layer was dried over MgSO₄, evaporated and centrifuged with diethylether. The solvent was decanted and the solid was dried on a vacuum pump. Orange crystalline solid (1.182 g, yield 66 %). **Mp** = 175-176 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.79 (d, 1H, J (11,12) = 9.7 Hz, H-11), 7.25 (s, 2H, NH₂), 6.36 (dd, 1H, J (12,11) = 9.8 Hz, J (12,8) = 2.7 Hz, H-12), 6.18 (d, 1H, J (8,12) = 2.7 Hz, H-8), 3.23 (t, 4H, J_{vic} = 5.1 Hz, H-2,6), 2.77 (t, 4H, J_{vic} = 5.1 Hz, H-3,5); NH signal not detected. **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 155.33 (C-7), 148.36 (C-9), 127.13 (CH-11), 122.75 (C-10), 105.33 (CH-12), 97.11 (CH-8), 47.45 (CH₂-2,6), 45.30 (CH₂-3,5). **FTIR** (CHCl₃, cm⁻¹) $\nu_{\max}$: 3513, 3387, 1618, 1587, 1569, 1440, 1379, 1354, 1321, 1254, 1157, 1023, 1010, 972, 687, 567. **HRMS** (ESI+) calcd. for C₁₀H₁₄O₂N₄Na⁺ (M+Na⁺): 245.10090, found: 245.10085; calcd. for C₁₀H₁₅O₂N₄⁺ (M+H⁺): 223.11895, found: 223.11890. **R_f** (MeOH) = 0.12.

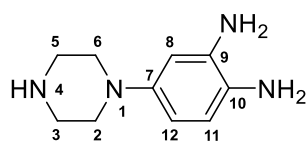
2-Nitro-5-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (52); procedure according to ref. [136].



2-Nitro-5-(piperazin-1-yl)aniline (**47**, 400 mg, 1.80 mmol) and K₂CO₃ (323 mg, 2.34 mmol) were resuspended in anhydrous CH₃CN (12 mL). 5-iodopent-1-yne (0.273 mL, 2.25 mmol) was added and the reaction mixture was stirred and heated to 60°C for 24 h. After cooling to r.t., the solvent was removed *in vacuo*, the residue was dissolved in dichloromethane and washed with brine solution in water. The organic layer was collected, dried over MgSO₄ and the solvent was removed *in vacuo*. The residue was dried on a vacuum pump without further purification. Yellow crystalline solid (504 mg, yield 97 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.80 (d, 1H, J (11,12) = 9.8 Hz, H-11), 7.26 (s, 2H, NH₂), 6.38 (dd, 1H, J (12,11) = 9.8 Hz, J (12,8) = 2.7 Hz, H-12), 6.21 (d, 1H, J (8,12) = 2.7 Hz, H-8), 3.30 (t, 4H, J_{vic} = 5.2 Hz, H-2,6), 2.76 (t, 1H, ^4J = 2.6 Hz,

NCH₂CH₂CH₂CCH), 2.45 (t, 4H, $J_{\text{vic}} = 5.1$ Hz, H-3,5), 2.37 (t, 2H, $J_{\text{vic}} = 7.2$ Hz, NCH₂CH₂CH₂CCH), 2.20 (td, 2H, $J_{\text{vic}} = 7.1$ Hz, $^4J = 2.7$ Hz, NCH₂CH₂CH₂CCH), 1.63 (p, 2H, $J_{\text{vic}} = 7.1$ Hz, NCH₂CH₂CH₂CCH). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 154.95 (C-7), 148.31 (C-9), 127.14 (CH-11), 122.88 (C-10), 105.36 (CH-12), 97.40 (CH-8), 84.36 (NCH₂CH₂CH₂CCH), 71.28 (NCH₂CH₂CH₂CCH), 56.43 (NCH₂CH₂CH₂CCH), 52.33 (CH₂-3,5), 46.23 (CH₂-2,6), 25.30 (NCH₂CH₂CH₂CCH), 15.64 (NCH₂CH₂CH₂CCH). FTIR (CHCl₃, cm⁻¹) ν_{max} : 3513, 3387, 3308, 1618, 1565, 1453, 1379, 1322, 1287, 1274, 1251, 1142, 1019, 1007, 974, 819, 638. HRMS (ESI+) calcd. for C₁₅H₂₀O₂N₄Na⁺ (M+Na⁺): 311.14785, found: 311.14789; calcd. for C₁₅H₂₁O₂N₄⁺ (M+H⁺): 289.16590, found: 289.16594. **R_f** (EtOAc) = 0.52.

4-(Piperazin-1-yl)benzene-1,2-diamine (**48**) [139].

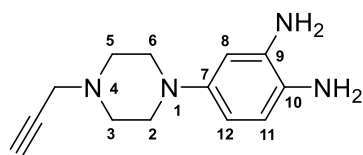


2-Nitro-5-(piperazin-1-yl)aniline (**47**, 1.520 g, 6.84 mmol) was resuspended in anhydrous ethanol (60 mL) under argon atmosphere. Pd/C (10 %, 152 mg, 0.143 mmol of Pd) was added and the reaction mixture was put under hydrogen atmosphere (1 atm) and stirred at r.t. for 24 h. The mixture was filtered over Celite, the solvent was removed *in vacuo* and the residue was dried on a vacuum pump. Brown crystalline solid (1.279 g, yield 98 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 6.39 (d, 1H, $J(11,12) = 8.3$ Hz, H-11), 6.20 (d, 1H, $J(8,12) = 2.6$ Hz, H-8), 6.00 (dd, 1H, $J(12,11) = 8.3$ Hz, $J(12,8) = 2.7$ Hz, H-12), 4.33 (s, 2H, NH₂), 3.84-4.05 (bs, 1H, NH), 2.77 (s, 8H, H-2,6, H-3,5); NH₂ signal not detected. ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 144.80 (C-7), 135.85 (C-9), 128.24 (C-10), 115.41 (CH-12), 105.80 (CH-12), 104.48 (CH-8), 51.64 (CH₂-2,6), 45.93 (CH₂-3,5). FTIR (CHCl₃, cm⁻¹) ν_{max} : 3444, 3423, 1625, 1582, 1517, 1441, 1376, 1322, 1272, 1252, 1117, 1029, 693, 570. HRMS (ESI+) calcd. for C₁₀H₁₇N₄⁺ (M+H⁺): 193.14477, found: 193.14480. **R_f** (2/1 EtOAc/MeOH) = 0.09.

The general procedure for synthesis of alkylation products of 4-(piperazin-1-yl)aniline **49-51**; procedure according to ref. [136]

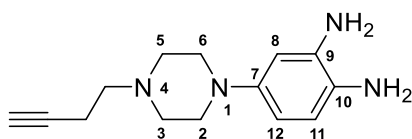
4-(Piperazin-1-yl)benzene-1,2-diamine (**48**, 50 mg, 0.26 mmol) and K₂CO₃ (54 mg, 0.39 mmol) were dissolved in DMF (5 mL) under argon atmosphere and allowed to stir at r.t. for 10 minutes. Afterwards, alkynyl halide (0.26 mmol) was added and the reaction mixture was stirred under argon at r.t. for 6 h. The solvent was removed *in vacuo* and the residue was put on a silica gel column in methanol (0-0.5 %)/TEA (0.25 %)/ethyl acetate, which afforded the desired product **49-51**.

4-(4-(Prop-2-yn-1-yl)piperazin-1-yl)benzene-1,2-diamine (49).



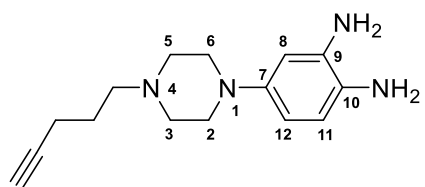
Alkylating agent: 3-bromoprop-1-yne (80% solution in toluene, 0.031 mL, 0.26 mmol). Beige amorphous solid (38.4 mg, yield 64 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 6.40 (d, 1H, *J*(11,12) = 8.3 Hz, H-11), 6.21 (d, 1H, *J*(8,12) = 2.6 Hz, H-8), 6.02 (dd, 1H, *J*(12,11) = 8.3 Hz, *J*(12,8) = 2.7 Hz, H-12), 4.07-4.83 (vbs, 4H, 2xNH₂), 3.29 (d, 2H, ⁴*J* = 2.5 Hz, NCH₂CCH), 3.17 (t, 1H, ⁴*J* = 2.4 Hz, NCH₂CCH), 2.89 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.55 (t, 4H, *J*_{vic} = 4.9 Hz, H-3,5). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 143.91 (C-7), 135.89 (C-9), 128.29 (C-10), 115.40 (CH-11), 105.84 (CH-12), 104.46 (CH-8), 79.38 (NCH₂CCH), 75.71 (NCH₂CCH), 51.43 (CH₂-3,5), 50.10 (CH₂-2,6), 46.04 (NCH₂CCH). **HRMS** (ESI⁺) calcd. for C₁₃H₁₉N₄⁺ (M+H⁺): 231.16042, found: 231.16045. **R_f** (0.25% TEA in 2/1 EtOAc/MeOH) = 0.43.

4-(4-(But-3-yn-1-yl)piperazin-1-yl)benzene-1,2-diamine (50).



Alkylating agent: 4-bromobut-1-yne (0.025 mL, 0.26 mmol). Beige amorphous solid (21.9 mg, yield 35 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 6.41 (d, 1H, *J*(11,12) = 8.4 Hz, H-11), 6.21 (d, 1H, *J*(8,12) = 2.6 Hz, H-8), 6.02 (dd, 1H, *J*(12,11) = 8.3 Hz, *J*(12,8) = 2.7 Hz, H-12), 4.25-4.85 (vbs, 4H, 2xNH₂), 2.87 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.80 (t, 1H, ⁴*J* = 2.6 Hz, NCH₂CH₂CCH), 2.47-2.54 (m, 6H, H-3,5, NCH₂CH₂CCH), 2.35 (td, 2H, *J*_{vic} = 7.4 Hz, ⁴*J* = 2.6 Hz, NCH₂CH₂CCH). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 144.02 (C-7), 136.01 (C-9), 127.88 (C-10), 115.59 (CH-11), 105.72 (CH-12), 104.37 (CH-8), 83.07 (NCH₂CH₂CCH), 71.83 (NCH₂CH₂CCH), 56.39 (NCH₂CH₂CCH), 52.55 (CH₂-3,5), 50.00 (CH₂-2,6), 16.07 (NCH₂CH₂CCH). **HRMS** (ESI⁺) calcd. for C₁₄H₂₁N₄⁺ (M+H⁺): 245.17607, found: 245.17606. **R_f** (0.25% TEA in 2/1 EtOAc/MeOH) = 0.40.

4-(4-(Pent-4-yn-1-yl)piperazin-1-yl)benzene-1,2-diamine (51).



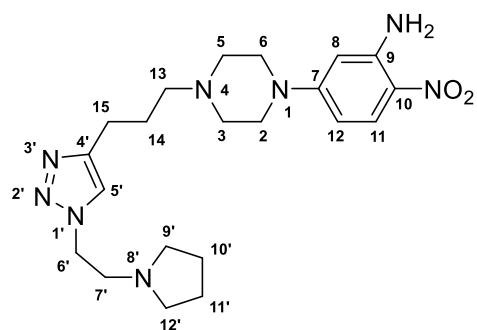
Alkylating agent: 5-iodopent-1-yne (0.032 mL, 0.26 mmol). Beige amorphous solid (19.7 mg, yield 29 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 6.39 (d, 1H, *J*(11,12) = 8.3 Hz, H-11), 6.21 (d, 1H, *J*(8,12) = 2.6 Hz, H-8), 6.01 (dd, 1H, *J*(12,11) = 8.3 Hz, *J*(12,8) = 2.6 Hz, H-12), 4.06-4.62 (vbs, 4H, 2xNH₂), 2.86 (t, 4H, *J*_{vic} = 5.0 Hz, H-2,6), 2.75 (t, 1H, ⁴*J* = 2.7 Hz, NCH₂CH₂CH₂CCH), 2.45 (t, 4H, *J*_{vic} = 5.0 Hz, H-3,5), 2.36 (t, 2H, *J*_{vic} = 7.2 Hz, NCH₂CH₂CH₂CCH), 2.18 (td, 2H, *J*_{vic} = 7.2 Hz, ⁴*J* = 2.7 Hz, NCH₂CH₂CH₂CCH), 1.61 (p, 2H, *J*_{vic} = 7.1 Hz, NCH₂CH₂CH₂CCH). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 143.98 (C-7), 135.87 (C-9), 128.29 (C-10), 115.38 (CH-11), 105.64 (CH-12), 104.37 (CH-8), 84.41 (NCH₂CH₂CH₂CCH), 71.18

(NCH₂CH₂CH₂CCH), 56.63 (NCH₂CH₂CH₂CCH), 53.07 (CH₂-3,5), 50.21 (CH₂-2,6), 25.40 (NCH₂CH₂CH₂CCH), 15.66 (NCH₂CH₂CH₂CCH). **HRMS** (ESI+) calcd. for C₁₅H₂₃N₄⁺ (M+H⁺): 259.19172, found: 259.19173. **R_f** (0.25% TEA in 2/1 EtOAc/MeOH) = 0.36.

The general procedure for synthesis of click reaction products 53-54; procedure according to ref. [39]

The reaction conditions and the procedure is the same as for click reaction products **31-42**, using compound **52** as an alkyne; solvent: 2/1 H₂O/*tert*-butanol (3.0 mL).

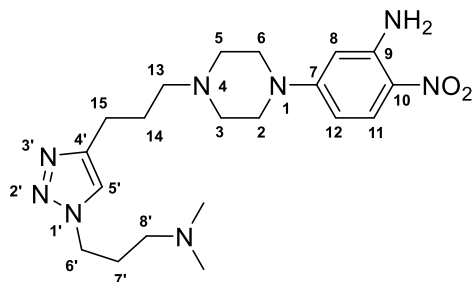
2-Nitro-5-(4-(3-(1-(2-(pyrrolidin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)aniline (53).



Reactants: 2-nitro-5-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (**52**, 100 mg, 0.347 mmol), 1-(2-azidoethyl)pyrrolidine (**9**, 0.049 mL, 0.347 mmol), CuSO₄ (100 mM solution, 0.350 mL), and Na-ascorbate (100 mM solution, 1.050 mL). Yellow amorphous solid (123 mg, yield 82 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.85 (s, 1H, H-5'), 7.80 (d, 1H, *J*(11,12) = 9.8 Hz, H-11), 7.26 (s, 2H, NH₂), 6.38 (dd, 1H, *J*(12,11) = 9.8 Hz,

J(12,8) = 2.7 Hz, H-12), 6.21 (d, 1H, *J*(8,12) = 2.7 Hz, H-8), 4.39 (t, 2H, *J*(6',7') = 6.5 Hz, H-6'), 3.31 (t, 4H, *J*_{vic} = 5.1 Hz, H-2,6), 2.83 (t, 2H, *J*(7',6') = 6.5 Hz, H-7'), 2.63 (t, 2H, *J*(15,14) = 7.5 Hz, H-15), 2.41-2.48 (m, 8H, H-3,5, H-9',12'), 2.34 (t, 2H, *J*(13,14) = 7.3 Hz, H-13), 1.77 (p, 2H, *J*(14,13) = *J*(14,15) = 7.5 Hz, H-14), 1.65 (m, 4H, H-10',11'). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 154.95 (C-7), 148.31 (C-9), 146.36 (C-4'), 127.13 (CH-11), 122.89 (C-10), 122.04 (CH-5'), 105.34 (CH-12), 97.40 (CH-8), 56.96 (CH₂-13), 55.10 (CH₂-7'), 53.37 (CH₂-9',12'), 52.31 (CH₂-3,5), 48.45 (CH₂-6'), 46.25 (CH₂-2,6), 26.08 (CH₂-14), 23.12 (CH₂-10',11'), 22.88 (CH₂-15). **FTIR** (KBr, cm⁻¹) ν_{max}: 3447, 3372, 3142, 2810, 1619, 1505, 1477, 1454, 1383, 1323, 1271, 1138, 1115, 1005, 972, 815, 754, 663, 540. **HRMS** (ESI+) calcd. for C₂₁H₃₂O₂N₈Na⁺ (M+Na⁺): 451.25404, found: 451.25375; calcd. for C₂₁H₃₃O₂N₈⁺ (M+H⁺): 429.27210, found: 429.27170. **R_f** (1/1 EtOAc/MeOH) = 0.17.

5-(4-(3-(1-(3-(Dimethylamino)propyl)-1H-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)-2-nitroaniline (54).



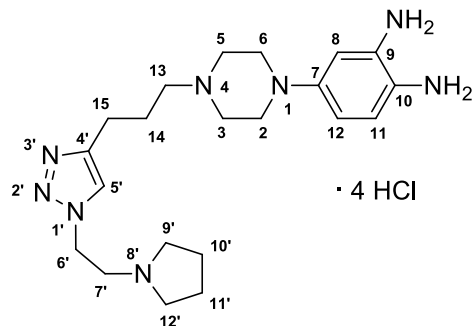
Reactants: 2-nitro-5-(4-(pent-4-yn-1-yl)piperazin-1-yl)aniline (**52**, 100 mg, 0.347 mmol), 3-azido-*N,N*-dimethylpropan-1-amine (**30**, 0.045 mL, 0.347 mmol), CuSO₄ (100 mM solution, 0.350 mL), and Na-ascorbate (100 mM solution, 1.050 mL). Yellow amorphous solid (120.1 mg, yield 83 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.85 (s, 1H, H-5'), 7.80 (d, 1H,

J(11,12) = 9.8 Hz, H-11), 7.25 (s, 2H, NH₂), 6.39 (dd, 1H, *J*(12,11) = 9.8 Hz, *J*(12,8) = 2.7 Hz, H-12), 6.21 (d, 1H, *J*(8,12) = 2.6 Hz, H-8), 4.30 (t, 2H, *J*(6',7') = 7.1 Hz, H-6'), 3.31 (t, 4H, *J*_{vic} = 5.2 Hz, H-2,6), 2.63 (t, 2H, *J*(15,14) = 7.5 Hz, H-15), 2.45 (t, 4H, *J*_{vic} = 5.1 Hz, H-3,5), 2.34 (t, 2H, *J*(13,14) = 7.3 Hz, H-13), 2.16 (t, 2H, *J*(8',7') = 6.9 Hz, H-8'), 2.11 (s, 6H, CH₃), 1.91 (p, 2H, *J*(7',6') = *J*(7',8') = 7.0 Hz, H-7'), 1.77 (p, 2H, *J*(14,13) = *J*(14,15) = 7.5 Hz, H-14). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 154.96 (C-7), 148.30 (C-9), 146.54 (C-4'), 127.13 (CH-11), 122.89 (C-10), 121.83 (CH-5'), 105.34 (CH-12), 97.40 (CH-8), 57.00 (CH₂-13), 55.69 (CH₂-8'), 52.31 (CH₂-3,5), 47.29 (CH₂-6'), 46.25 (CH₂-2,6), 45.07 (CH₃), 27.79 (CH₂-7'), 26.12 (CH₂-14), 22.90 (CH₂-15). **FTIR** (KBr, cm⁻¹) ν_{max}: 3454, 3351, 3138, 2768, 1619, 1567, 1440, 1382, 1323, 1262, 1164, 1117, 1097, 1004, 972, 827, 754, 664. **HRMS** (ESI+) calcd. for C₂₀H₃₂O₂N₈Na⁺ (M+Na⁺): 439.25404, found: 439.25383; calcd. for C₂₀H₃₃O₂N₈⁺ (M+H⁺): 417.27210, found: 417.27202. **R_f** (1/1 EtOAc/MeOH) = 0.18.

The general procedure for synthesis of diamino click products 55-56

The compound **53-54** (0.100 mmol) and Pd/C (10 %, 52.7 mg, 0.050 mmol of Pd) was resuspended in ethanol (absolute, 3.0 mL) under argon. The atmosphere was changed to H₂ (1 atm) and the reaction mixture was hydrogenated at r.t. for 2.5 h. Afterwards, HCl/ethanol (1.25 M, 1.5 mL, 1.88 mmol) was added. Pd/C was filtered off over Celite and the solvent was removed *in vacuo*. The residue was dissolved in methanol and washed with diethylether by centrifugation, the solvent was decanted and the procedure was repeated 3x. After that, the residue was resuspended in diethylether, centrifugated, the solvent was decanted and the residue was dried on a vacuum pump to afford the product **55-56**.

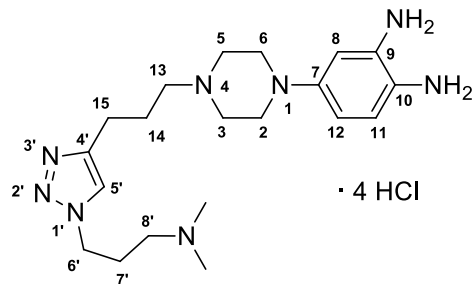
4-(4-(3-(1-(2-(Pyrrolidin-1-yl)ethyl)-1H-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)benzene-1,2-diamine tetrahydrochloride (55).



Reactants: 2-nitro-5-(4-(3-(1-(2-(pyrrolidin-1-yl)ethyl)-1H-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)aniline (**53**, 42.8 mg, 0.100 mmol) and Pd/C (52.7 mg). Purple crystalline solid (45.6 mg, yield 84 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 8.10 (s, 1H, H-5'), 7.10 (d, 1H, *J*(11,12) = 8.7 Hz, H-11), 6.51 (d, 1H, *J*(8,12) = 2.6 Hz, H-8), 6.40 (dd, 1H, *J*(12,11) = 8.6 Hz, *J*(12,8) = 2.6 Hz, H-12), 4.81 (t, 2H, *J*(6',7') = 6.3, H-6'), 3.69 (m, 2H,

H-7') 3.51-3.68 (m, H-2a, H-3a, H-5a, H-6a), 3.42-3.52 (m, 2H, H-9'a, H-12'a) 3.04-3.26 (m, 6H, H-2b, H-3b, H-5b, H-6b, H-13), 2.91-3.01 (m, 2H, H-9'b, H-12'b), 2.72 (t, 2H, *J*(15,14) = 7.1 Hz, H-15), 2.05-2.18 (m, 2H, H-14), 1.91-2.03, 1.78-1.90 (2x m, 2x 2H, H-10', H-11'); NH₂ signals not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 149.53 (C-7), 145.60 (C-4'), 124.39 (CH-11), 123.04 (CH-5'), 117.57 (C-10), 106.56 (CH-12), 104.53 (CH-8), 54.72 (CH₂-13), 53.07 (CH₂-9',12'), 52.46 (CH₂-7'), 50.37 (CH₂-3,5), 45.34 (CH₂-6'), 45.20 (CH₂-2,6), 22.82 (CH₂-14), 22.64 (CH₂-10',11'), 22.21 (CH₂-15); C-9 signal not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3415, 3217, 3136, 3030, 2479, 2687, 1825, 1621, 1618, 1516, 1444, 1379, 1267, 1239, 1169, 1118, 1030, 707, 568. HRMS (ESI+) calcd. for C₂₁H₃₅N₈⁺ (M+H⁺): 399.29792, found: 399.29766. Anal calcd. for C₂₁H₃₈Cl₄N₈ (%): C 46.33; H 7.04; Cl 26.05; N 20.58. Found: C 46.08; H 6.99; Cl 26.43; N 20.46. R_f (0.5% TEA in 1/1 EtOAc/MeOH) = 0.50.

4-(4-(3-(1-(3-(Dimethylamino)propyl)-1H-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)benzene-1,2-diamine tetrahydrochloride (56).



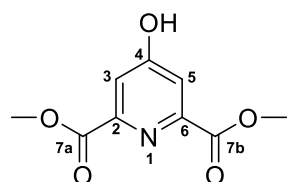
Reactants: 5-(4-(3-(1-(3-(dimethylamino)propyl)-1H-1,2,3-triazol-4-yl)propyl)piperazin-1-yl)-2-nitroaniline (**54**, 41.7 mg, 0.100 mmol) and Pd/C (52.7 mg). Purple crystalline solid (17.8 mg, yield 34 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 8.03 (s, 1H, H-5'), 7.07 (d, 1H, *J*(11,12) = 8.6 Hz, H-11), 6.48 (d, 1H, *J*(8,12) = 2.6 Hz, H-8), 6.37 (dd, 1H, *J*(12,11) = 8.6 Hz, *J*(12,8)

= 2.6 Hz, H-12), 4.61-5.49 (vbs, 8H, 2x NH₃⁺, 2x NH⁺), 4.44 (t, 2H, *J*(6',7') = 6.8 Hz, H-6'), 3.65, 3.54 (2x d, 2x 2H, *J*_{gem} = 12.0 Hz, H-2a, H-3a, H-5a, H-6a), 3.07-3.21 (m, 6H, H-2b, H-3b, H-5b, H-6b, H-13), 2.99-3.07 (m, 2H, H-8'), 2.68-2.75 (m, 8H, 2x CH₃, H-15), 2.25 (p, 2H, *J*(7',6') = *J*(7',8') = 7.3 Hz, H-7'), 2.11 (p, 2H, *J*(14,13) = *J*(14,15) = 7.8 Hz, H-14). ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 149.59 (C-7), 145.44 (C-4'), 140.83 (C-9), 124.35 (CH-11), 122.33 (CH-5'), 112.03 (C-10), 106.48 (CH-12), 104.39 (CH-8), 54.80 (CH₂-13), 53.69 (CH₂-8'), 50.42 (CH₂-3,5), 46.56 (CH₂-6'), 45.36 (CH₂-2,6), 41.94 (2x

CH₃), 24.62 (CH₂-7'), 22.89 (CH₂-14), 22.24 (CH₂-15). **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3501, 3429, 3134, 2732, 2473-2689, 1625, 1445, 1378, 1262, 1225, 1169, 1116, 1029, 712, 572. **HRMS** (ESI+) calcd. for C₂₀H₃₅N₈⁺ (M+H⁺): 387.29792, found: 387.29753. **Anal** calcd. for C₂₀H₃₈Cl₄N₈ (%): C 45.12; H 7.19; Cl 26.64; N 21.05. Found: C 44.88; H 7.06; Cl 26.97; N 20.91. **R_f** (0.25% TEA in 1/1 EtOAc/MeOH) = 0.32.

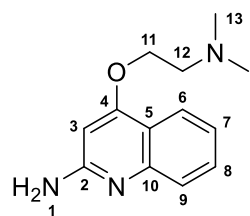
11.2.4 Synthesis of pyridostatin derivatives

Dimethyl 4-hydroxypyridine-2,6-dicarboxylate (**64**) [129, 130]; procedure according to ref. [141].



Chelidamic acid monohydrate (**63**, 5.0 g, 27.3 mmol) was resuspended in methanol (absolute, 70 mL) and H₂SO₄ (96 %, 1.0 mL, 18.0 mmol), was added as a catalyst. The reaction mixture was stirred and heated at 70 °C overnight. After cooling to r.t., saturated aqueous solution of Na₂CO₃ was added to adjust pH to 7 upon stirring. Then, HCl (35 %) was added dropwise to adjust pH to 1. The reaction was extracted between H₂O and CH₂Cl₂, the organic layer was washed with H₂O (1x) and dried over MgSO₄. The salts were filtered off and the solvent was removed *in vacuo*. White crystalline solid (3.887 g, yield 67 %). **Mp** = 142-144 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 11.58 (s, 1H, OH), 7.58 (s, 2H, H-3,5), 3.88 (s, 6H, CH₃). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 165.85 (C-4), 164.76 (C-7a,7b), 149.28 (C-2,6), 115.21 (CH-3,5), 52.56 (CH₃). **HRMS** (ESI+) calcd. for C₉H₉O₅NNa⁺ (M+Na⁺): 234.03729, found: 234.03701; calcd. for C₉H₁₀O₅N⁺ (M+H⁺): 212.05535, found: 212.05518. **R_f** (EtOAc) = 0.30. The spectra are in accordance with ref. [129].

4-(2-(Dimethylamino)ethoxy)quinolin-2-amine (**75**) [130].



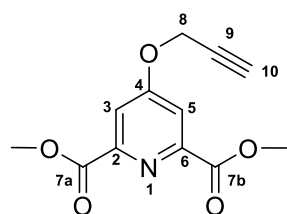
2-Aminoquinolin-4-ol (**76**, 3.70 g, 23.1 mmol), Ph₃P (12.11 g, 46.2 mmol), and 2-(dimethylamino)ethan-1-ol (3.50 mL, 34.7 mmol) were dissolved in dry THF (400 mL) under argon atmosphere and cooled to 0 °C. DIAD (4.55 mL, 23.1 mmol) was added and the solution was stirred at 0 °C for 30 min. Afterwards, the reaction mixture was removed from the cooling bath and left stirring at r.t. for 3 days under argon atmosphere. The solvent was removed *in vacuo* and the residue was purified by CombiFlash on silica gel column in ethyl acetate (100 %) to remove Ph₃P and Ph₃PO and then in EtOAc/MeOH/Et₃N (17/2/1). White crystalline solid (3.0 g, yield 56 %). **Mp** = 136-137 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.81 (dd, 1H, $J(6,7)$ = 8.1 Hz, $J(6,8)$ = 1.2 Hz, H-6), 7.44 (ddd, 1H, $J(8,9)$ = 8.3 Hz, $J(8,7)$ = 6.7 Hz, $J(8,6)$ = 1.2 Hz, H-8), 7.38 (dd, 1H, $J(9,8)$ = 8.3 Hz, $J(9,7)$ = 1.4 Hz, H-9), 7.10 (ddd, 1H, $J(7,6)$ = 8.1 Hz, $J(7,8)$ = 6.7 Hz, $J(7,9)$ = 1.4 Hz, H-7), 6.27 (bs, 2H, NH₂), 6.20 (s, 1H, H-3), 4.17 (t, 2H, $J(11,12)$ = 5.6 Hz, H-11), 2.77 (t, 2H, $J(12,11)$ = 5.6 Hz, H-12), 2.27 (s, 6H, H-13). **¹³C NMR** (100.6 MHz, DMSO-*d*₆):

δ 160.79 (C-4), 159.48 (C-2), 148.88 (C-10), 129.34 (CH-8), 124.91 (CH-9), 121.22 (CH-7), 120.34 (CH-6), 116.77 (C-5), 90.61 (CH-3), 66.19 (CH₂-11), 57.25 (CH₂-12), 45.59 (CH₃-13). **HRMS** (ESI⁺) calcd. for C₁₃H₁₈ON₃⁺ (M+H⁺): 232.14444, found: 232.14476. **R_f** (17/2/1 EtOAc/MeOH/TEA) = 0.90. The spectra are in accordance with ref. [130].

The general procedure for *O*-alkylation of 4-hydroxypyridine-2,6-dicarboxylate (65-69); procedure according to ref. [39, 130]

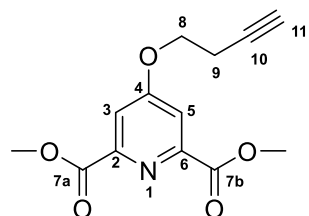
Ph₃P (995 mg, 3.79 mmol) was dissolved in dry THF (10 ml) under argon atmosphere and cooled to -8 °C. DIAD (0.72 mL, 3.55 mmol) was added dropwise to form a yellow suspension and the reaction mixture was stirred at -8 °C for 30 min. Afterwards, the alkylating agent (alkynyl alcohol or azidoalcohol, 3.56 mmol) was resuspended in dry THF (10 mL), added to the suspension and stirred at -8 °C for 5 min. Finally, **64** (0.500 g, 2.37 mmol) was added to the reaction mixture as a solution in dry THF (20 mL) at -8 °C, stirred for 15 min and then removed from the cooling bath and left stirring at r.t. overnight under argon atmosphere. The solvent was removed *in vacuo* and the residue was purified by CombiFlash on silica gel column in ethyl acetate (0-60 %)/hexane (45 min in total, 0-5 min 100% hexane and 0% EtOAc, 5-45 min EtOAc 0-60 %).

Dimethyl 4-(prop-2-yn-1-yloxy)pyridine-2,6-dicarboxylate (65) [39].



Alkylating agent: prop-2-yn-1-ol (205 μ l). White crystalline solid (566 mg, yield 96 %). **Mp** = 152.5-153 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.82 (s, 2H, H-3,5), 5.12 (d, 2H, *J*(8,10) = 2.4 Hz, H-8), 3.91 (s, 6H, CH₃), 3.75 (t, 1H, *J*(10,8) = 2.4 Hz, H-10). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 165.13 (C-4), 164.48 (C-7a,7b), 149.35 (C-2,6), 114.52 (CH-3,5), 79.88 (CH-10), 77.62 (C-9), 56.51 (CH₂-8), 52.76 (CH₃). **HRMS** (ESI⁺) calcd. for C₁₂H₁₁O₅NNa⁺ (M+Na⁺): 272.05294, found: 272.05302; calcd. for C₁₂H₁₂O₅N⁺ (M+H⁺): 250.07100, found: 250.07107. **R_f** (EtOAc) = 0.71. The spectra are in accordance with ref. [39].

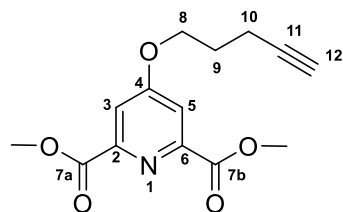
Dimethyl 4-(but-3-yn-1-yloxy)pyridine-2,6-dicarboxylate (66).



Alkylating agent: but-3-yn-1-ol (270 μ l). White crystalline solid (530 mg, yield 85 %). **Mp** = 100-101 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 7.74 (s, 2H, H-3,5), 4.33 (t, 2H, *J*(8,9) = 6.3 Hz, H-8), 3.91 (s, 6H, CH₃), 2.92 (t, 1H, *J*(11,9) = 2.6 Hz, H-11), 2.70 (td, 2H, *J*(9,8) = 6.3 Hz, *J*(9,11) = 2.6 Hz, H-9). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 166.01 (C-4), 164.53 (C-7a,7b), 149.41 (C-2,6), 114.14 (CH-3,5), 80.79 (C-10), 72.75 (CH₂-11), 66.81 (CH₂-8), 52.70 (CH₃), 18.53 (CH₂-9).

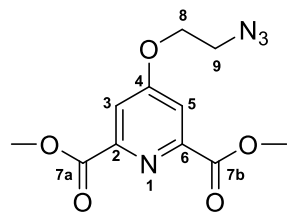
FTIR (KBr, cm^{-1}) ν_{max} : 3261, 2156, 1717, 1599, 1568, 1442, 1378, 1261, 1154, 1111, 997, 981, 785, 696. **HRMS** (ESI+) calcd. for $\text{C}_{13}\text{H}_{13}\text{O}_5\text{NNa}^+$ ($\text{M}+\text{Na}^+$): 286.06859, found: 286.06866; calcd. for $\text{C}_{13}\text{H}_{14}\text{O}_5\text{N}^+$ ($\text{M}+\text{H}^+$): 264.08665, found: 264.08674. R_f (EtOAc) = 0.74.

Dimethyl 4-(pent-4-yn-1-yloxy)pyridine-2,6-dicarboxylate (67).



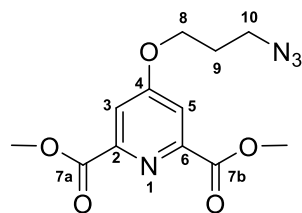
Alkylating agent: pent-4-yn-1-ol (330 μl). White crystalline solid (589 mg, yield 90 %). **mp** = 102-104 $^{\circ}\text{C}$. **^1H NMR** (400 MHz, $\text{DMSO}-d_6$): δ 7.74 (s, 2H, H-3,5), 4.28 (t, 2H, $J(8,9) = 6.2$ Hz, H-8), 3.90 (s, 6H, CH_3), 2.83 (t, 1H, $J(12,10) = 2.7$ Hz, H-12), 2.35 (td, 2H, $J(10,9) = 7.1$ Hz, $J(10,12) = 2.7$ Hz, H-10), 1.94 (p, 2H, $J(9,8) = J(9,10) = 6.5$ Hz, H-9). **^{13}C NMR** (100.6 MHz, $\text{DMSO}-d_6$): δ 166.28 (C-4), 164.57 (C-7a,7b), 149.39 (C-2,6), 114.08 (CH-3,5), 83.37 (C-11), 71.80 (CH-12), 67.32 (CH_2 -8), 52.68 (CH_3), 27.21 (CH_2 -9), 14.32 (CH_2 -10). **FTIR** (KBr, cm^{-1}) ν_{max} : 3270, 2120, 1726, 1718, 1601, 1565, 1442, 1269, 1259, 1045. **HRMS** (ESI+) calcd. for $\text{C}_{14}\text{H}_{15}\text{O}_5\text{NNa}^+$ ($\text{M}+\text{Na}^+$): 300.08424, found: 300.08452; calcd. for $\text{C}_{14}\text{H}_{16}\text{O}_5\text{N}^+$ ($\text{M}+\text{H}^+$): 278.10230, found: 278.10251. R_f (EtOAc) = 0.76.

Dimethyl 4-(2-azidoethoxy)pyridine-2,6-dicarboxylate (68).



Alkylating agent: 2-azidoethan-1-ol (270 μl). White crystalline solid (475 mg, yield 71 %). **mp** = 87-88 $^{\circ}\text{C}$. **^1H NMR** (400 MHz, $\text{DMSO}-d_6$): δ 7.76 (s, 2H, H-3,5), 4.45 (t, 2H, $J(8,9) = 4.8$ Hz, H-8), 3.91 (s, 6H, CH_3), 3.73 (t, 2H, $J(9,8) = 4.8$ Hz, H-9). **^{13}C NMR** (100.6 MHz, $\text{DMSO}-d_6$): δ 165.89 (C-4), 164.51 (C-7a,7b), 149.46 (C-2,6), 114.14 (CH-3,5), 67.94 (CH_2 -8), 52.72 (CH_3), 49.21 (CH_2 -9). **FTIR** (CHCl_3 , cm^{-1}) ν_{max} : 3017, 2956, 2915, 2849, 2107, 1739, 1729, 1593, 1567, 1466, 1449, 1440, 1305, 1259, 1156, 1108, 1053, 543. **HRMS** (ESI+) calcd. for $\text{C}_{11}\text{H}_{12}\text{O}_5\text{N}_4\text{Na}^+$ ($\text{M}+\text{Na}^+$): 303.06999, found: 303.06976. R_f (EtOAc) = 0.64.

Dimethyl 4-(3-azidopropoxy)pyridine-2,6-dicarboxylate (69).



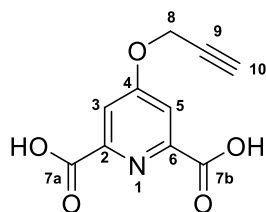
Alkylating agent: 3-azidopropan-1-ol (330 μl). Colorless amorphous solid (470 mg, yield 68 %). **^1H NMR** (400 MHz, $\text{DMSO}-d_6$): δ 7.74 (s, 2H, H-3,5), 4.29 (t, 2H, $J(8,9) = 6.1$ Hz, H-8), 3.90 (s, 6H, CH_3), 3.53 (t, 2H, $J(10,9) = 6.7$ Hz, H-10), 2.02 (p, 2H, $J(9,8) = J(9,10) = 6.4$ Hz, H-9). **^{13}C NMR** (100.6 MHz, $\text{DMSO}-d_6$): δ 166.21 (C-4), 164.57 (C-7a,7b), 149.38 (C-2,6), 114.13 (CH-3,5), 66.06 (CH_2 -8), 52.69 (CH_3), 47.45 (CH_2 -10), 27.67 (CH_2 -9). **FTIR** (CHCl_3 , cm^{-1}) ν_{max} : 3094, 2954, 2847, 2100, 1747, 1725, 1596, 1568, 1450, 1440, 1359, 1303, 1254, 1156, 1107, 1044, 880, 588,

559. **HRMS** (ESI+) calcd. for $C_{12}H_{14}O_5N_4Na^+$ ($M+Na^+$): 317.08564, found: 317.08537; calcd. for $C_{12}H_{15}O_5N_4^+$ ($M+H^+$): 295.10370, found: 295.10348. **R_f** (EtOAc) = 0.70.

The general procedure for ester hydrolysis of *O*-alkylated 4-hydroxypyridine-2,6-dicarboxylate (70-74); procedure according to ref. [39]

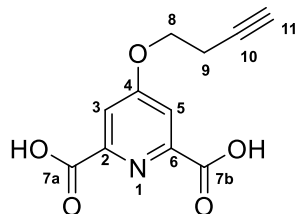
The compound **65-69** (1.50 mmol) was dissolved in ethanol (absolute, 120 mL), solid KOH (3.36 g, 60.0 mmol) was added and the reaction mixture was stirred and heated to 60 °C for 1.5 h. The solvent was removed *in vacuo*, the residue was diluted with H₂O (50 mL) and HCl (35 %) was added dropwise to adjust pH to 1. Water was extracted by ethyl acetate (3x 70 mL), the organic phase was collected and dried with Na₂SO₄. The salts were filtered off and the solvent was removed *in vacuo*.

4-(Prop-2-yn-1-yloxy)pyridine-2,6-dicarboxylic acid (70) [39].



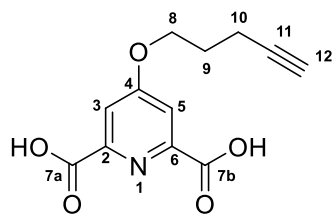
Reactants: **65** (373 mg, 1.50 mmol) and KOH (3.36 g, 60.0 mmol). White crystalline solid (317 mg, yield 96 %). **Mp** = 105-106 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 13.27 (vbs, 2H, OH), 7.79 (s, 2H, H-3,5), 5.10 (d, 2H, *J*(8,10) = 2.3 Hz, H-8), 3.74 (t, 1H, *J* = 2.3 Hz, H-10). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 165.36 (C-4), 165.22 (C-7a,7b), 149.82 (C-2,6), 113.98 (CH-3,5), 79.81 (CH-10), 77.71 (C-9), 56.44 (CH₂-8). **HRMS** (ESI+) calcd. for $C_{10}H_7O_5NNa^+$ ($M+Na^+$): 244.02164, found: 244.02150; calcd. for $C_{10}H_8O_5N^+$ ($M+H^+$): 222.03970, found: 222.03958. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.31. The spectra are in accordance with ref. [39].

4-(But-3-yn-1-yloxy)pyridine-2,6-dicarboxylic acid (71).



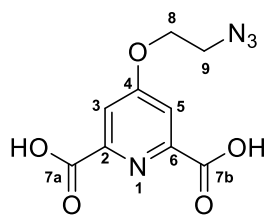
Reactants: **66** (395 mg, 1.50 mmol) and KOH (3.36 g, 60.0 mmol). White crystalline solid (296 mg, yield 84 %). **Mp** = 159-161 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 13.18 (vbs, 2H, OH), 7.71 (s, 2H, H-3,5), 4.32 (t, 2H, *J*(8,9) = 6.3 Hz, H-8), 2.91 (t, 1H, *J*(11,9) = 2.6 Hz, H-11), 2.70 (td, 2H, *J*(9,8) = 6.3 Hz, *J*(9,11) = 2.6 Hz, H-9). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 166.27 (C-4), 165.25 (C-7a,7b), 149.82 (C-2,6), 113.57 (CH-3,5), 80.83 (C-10), 72.75 (CH-11), 66.76 (CH₂-8), 18.56 (CH₂-9). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3490, 3288, 3236, 2585, 2135, 1751, 1732, 1724, 1606, 1563, 1446, 1419, 1282, 1037. **HRMS** (ESI+) calcd. for $C_{11}H_9O_5NNa^+$ ($M+Na^+$): 258.03729, found: 258.03735; calcd. for $C_{11}H_{10}O_5N^+$ ($M+H^+$): 236.05535, found: 236.05553. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.32.

4-(Pent-4-yn-1-yloxy)pyridine-2,6-dicarboxylic acid (72).



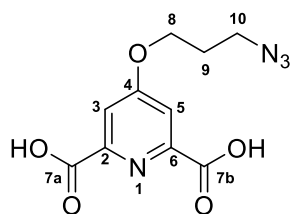
Reactants: **67** (416 mg, 1.50 mmol) and KOH (3.36 g, 60.0 mmol). White crystalline solid (328 mg, yield 88 %). **Mp** = 160-161 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 13.17 (vbs, 2H, OH), 7.71 (s, 2H, H-3,5), 4.28 (t, 2H, *J*(8,9) = 6.0 Hz, H-8), 2.83 (t, 1H, *J*(12,10) = 2.7 Hz, H-12), 2.35 (td, 2H, *J*(10,9) = 7.1 Hz, *J*(10,12) = 2.6 Hz, H-10), 1.94 (p, 2H, *J*(9,8) = *J*(9,10) = 6.6 Hz, H-9). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 166.54 (C-4), 165.26 (C-7a,7b), 149.75 (C-2,6), 113.51 (CH-3,5), 83.40 (C-11), 71.79 (CH-12), 67.27 (CH₂-8), 27.24 (CH₂-9), 14.35 (CH₂-10). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3276, 3216, 2589, 2115, 1747, 1733, 1728, 1603, 1565, 1445, 1418, 1276, 1047. **HRMS** (ESI⁺) calcd. for C₁₂H₁₁O₅NNa⁺ (M+Na⁺): 272.05294, found: 272.05310; calcd. for C₁₂H₁₂O₅N⁺ (M+H⁺): 250.07100, found: 250.07117. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.35.

4-(2-Azidoethoxy)pyridine-2,6-dicarboxylic acid (73).



Reactants: **68** (420 mg, 1.50 mmol) and KOH (3.36 g, 60.0 mmol). White crystalline solid (358 mg, yield 95 %). **Mp** = 78-79 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 13.15 (vbs, 2H, OH), 7.73 (s, 2H, H-3,5), 4.44 (t, 2H, *J*(8,9) = 4.6 Hz, H-8), 3.72 (t, 2H, *J*(9,8) = 4.6 Hz, H-9). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 166.14 (C-4), 165.24 (C-7a,7b), 149.90 (C-2,6), 113.59 (CH-3,5), 67.87 (CH₂-8), 49.22 (CH₂-9). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3257, 3094, 2927, 2857, 2580, 2107, 1733, 1672, 1602, 1575, 1507, 1455, 1439, 1223, 1062, 999, 934, 695, 680. **HRMS** (ESI⁺) calcd. for C₉H₈O₅N₄Na⁺ (M+Na⁺): 275.03869, found: 275.03846. **R_f** (EtOAc) = 0.12.

4-(3-Azidopropoxy)pyridine-2,6-dicarboxylic acid (74).

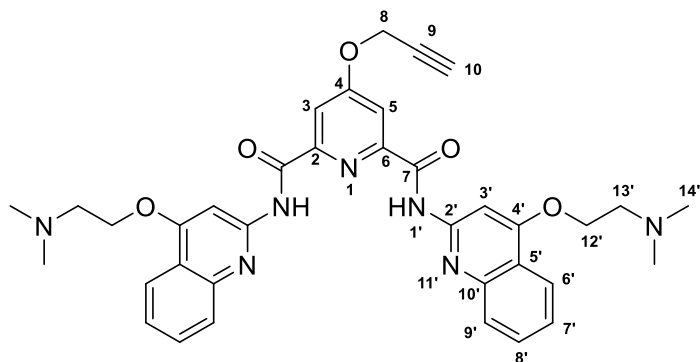


Reactants: **69** (441 mg, 1.50 mmol) and KOH (3.36 g, 60.0 mmol). White crystalline solid (316 mg, yield 79 %). **Mp** = 69-70 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 13.19 (vbs, 2H, OH), 7.72 (s, 2H, H-3,5), 4.29 (t, 2H, *J*(8,9) = 6.0 Hz, H-8), 3.53 (t, 2H, *J*(10,9) = 6.7 Hz, H-10), 2.02 (p, 2H, *J*(9,8) = *J*(9,10) = 6.3 Hz, H-9). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 166.47 (C-4), 165.27 (C-7a,7b), 149.76 (C-2,6), 113.58 (CH-3,5), 66.00 (CH₂-8), 47.49 (CH₂-10), 27.70 (CH₂-9). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3423, 3280, 3100, 2929, 2857, 2573, 2101, 1751, 1727, 1627, 1603, 1566, 1510, 1458, 1447, 1378, 1306, 1280, 1201, 1040, 988, 887, 779, 688, 557. **HRMS** (ESI⁺) calcd. for C₁₀H₁₀O₅N₄Na⁺ (M+Na⁺): 289.05434, found: 289.05445. **R_f** (EtOAc) = 0.14.

The general procedure for amide bond formation (58-62); procedure according to ref. [142, 143]

The compound **70-74** (1.00 mmol), **75** (0.497 g, 2.15 mmol), PyOxim (1.134 g, 2.15 mmol), and ethyl-(2-cyanoacrylate) (0.306 g, 2.15 mmol) were dissolved in dry DMF (15 mL) under argon atmosphere and DIPEA (870 μ l, 5.00 mmol) was added dropwise. The reaction mixture was stirred at r.t. overnight under argon. Part of the reaction mixture (0.5 mL) was transferred to a glass bottle (8 mL of total volume), which was then filled with diethylether. The resulting suspension was sonicated and centrifuged for 3 min at 3000 revolutions per minute (RPM). The solvent was decanted, the solid was resuspended in another 0.5 mL of the reaction mixture and the procedure was repeated until the total volume of the original reaction mixture was depleted. Afterwards, the solid was resuspended in methanol (1 mL), the bottle was filled with diethylether and the resulting suspension was sonicated and centrifuged for 3 min at 3000 RPM. The procedure was repeated five times. Finally, the solid was resuspended in diethylether, sonicated and centrifuged for 3 min at 3000 RPM. The solvent was decanted and the solid was dried on a vacuum pump.

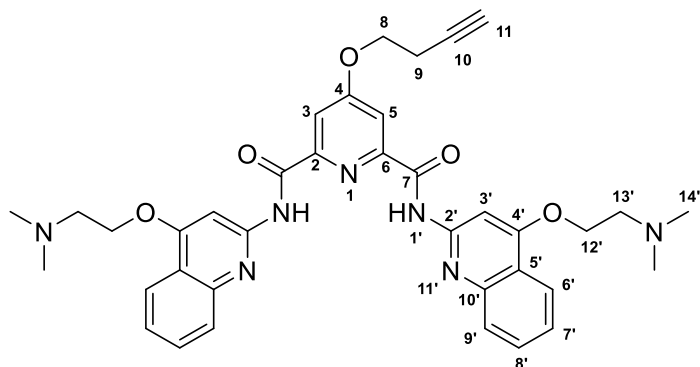
***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-(prop-2-yn-1-yloxy)pyridine-2,6-dicarboxamide (58).**



Reactants: **70** (0.221 g, 1.00 mmol), **75** (0.497 g, 2.15 mmol), PyOxim (1.134 g, 2.15 mmol), ethyl-(2-cyanoacrylate) (0.306 g, 2.15 mmol), and DIPEA (870 μ l, 5.00 mmol). Beige crystalline solid (0.344 g, yield 53 %). **Mp** = 203-205 °C. **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.12 (s, 2H, NH), 8.29 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5

Hz, H-6'), 8.18 (s, 2H, H-3'), 8.01 (s, 2H, H-3,5), 7.96 (d, 2H, *J*(9',8') = 8.4 Hz, H-9'), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.1 Hz, H-7'), 5.20 (d, 2H, *J*(8,10) = 2.3 Hz, H-8), 4.61 – 4.66 (m, 4H, H-12'), 3.81 (t, 1H, *J*(10,8) = 2.3 Hz, H-10), 3.53 – 3.60 (m, 4H, H-13'), 2.85 (s, 12H, H-14'). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 166.19 (C-4), 163.37 (C-7), 161.75 (C-4'), 152.55 (C-2'), 151.26 (C-2,6), 147.22 (C-10'), 130.97 (CH-8'), 127.04 (CH-9'), 124.89 (CH-7'), 122.43 (CH-6'), 119.22 (C-5'), 112.73 (CH-3,5), 95.39 (CH-3'), 80.20 (CH-10), 77.93 (C-9), 63.96 (CH₂-12'), 56.72 (CH₂-8), 55.97 (CH₂-13'), 43.71 (CH₃-14'). **FTIR** (KBr, cm⁻¹) ν_{max} : 3433, 3370, 3291, 3092, 2126, 1694, 1596, 1582, 1526, 1504, 1455, 1415, 1193, 1041, 1026, 999, 739, 683. **HRMS** (ESI+) calcd. for C₃₆H₃₈O₅N₇⁺ (M+H⁺): 648.29289, found: 648.29334. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.78.

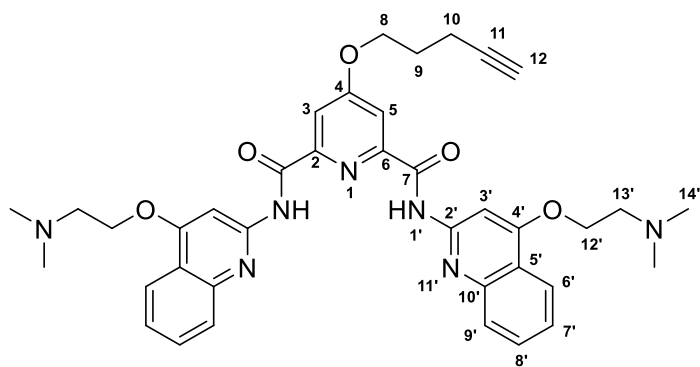
4-(But-3-yn-1-yloxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (59).



Reactants: **71** (0.235 g, 1.00 mmol), **75** (0.497 g, 2.15 mmol), PyOxim (1.134 g, 2.15 mmol), ethyl-(2-cyanoacrylate) (0.306 g, 2.15 mmol), and DIPEA (870 μ l, 5.00 mmol). Beige crystalline solid (0.311 g, yield 47 %). **Mp** = 195-197 °C. **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.08 (s, 2H, NH), 8.24 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5

Hz, H-6'), 8.15 (s, 2H, H-3'), 7.95 (ddd, 2H, *J*(9',8') = 8.5 Hz, *J*(9',7') = 1.2 Hz, *J*(9',6') = 0.6 Hz, H-9'), 7.92 (s, 2H, H-3,5), 7.79 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.55 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.2 Hz, H-7'), 4.55 – 4.59 (m, 4H, H-12'), 4.41 (t, 2H, *J*(8,9) = 6.4 Hz, H-8), 3.37 – 3.47 (bm, 4H, H-13'), 2.98 (t, 1H, *J*(11,9) = 2.7 Hz, H-11), 2.77 (td, 2H, *J*(9,8) = 6.4 Hz, *J*(9,11) = 2.7 Hz, H-9), 2.71 (s, 12H, H-14'). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.06 (C-4), 163.41 (C-7), 161.87 (C-4'), 152.57 (C-2'), 151.30 (C-2,6), 147.22 (C-10'), 130.91 (CH-8'), 127.05 (CH-9'), 124.87 (CH-7'), 122.30 (CH-6'), 119.26 (C-5'), 112.27 (CH-3,5), 95.33 (CH-3'), 81.05 (C-10), 73.08 (CH-11), 67.02 (CH₂-8), 64.72 (CH₂-12'), 56.32 (CH₂-13'), 44.23 (CH₃-14'), 18.77 (CH₂-9). **FTIR** (KBr, cm⁻¹) ν_{max} : 3431, 3293, 1694, 1596, 1581, 1526, 1503, 1454, 1417, 1192, 1044, 1025, 999, 739, 683. **HRMS** (ESI+) calcd. for C₃₇H₃₉O₅N₇Na⁺ (M+Na⁺): 684.29049, found: 684.29093; calcd. for C₃₇H₄₀O₅N₇⁺ (M+H⁺): 662.30854, found: 662.30900. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.80.

***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-(pent-4-yn-1-yloxy)pyridine-2,6-dicarboxamide (60).**

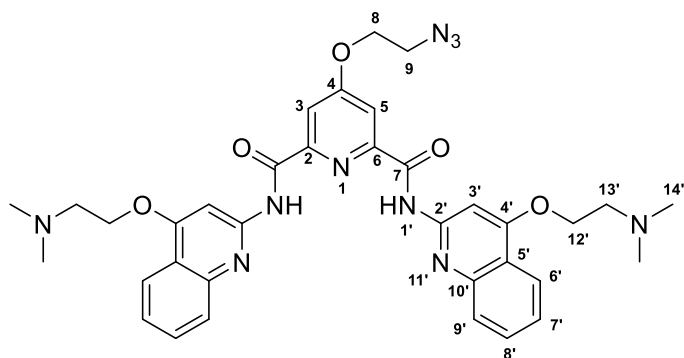


Reactants: **72** (0.249 g, 1.00 mmol), **75** (0.497 g, 2.15 mmol), PyOxim (1.134 g, 2.15 mmol), ethyl-(2-cyanoacrylate) (0.306 g, 2.15 mmol), and DIPEA (870 μ l, 5.00 mmol). Beige crystalline solid (0.344 g, yield 51 %). **Mp** = 186-188 °C. **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.32 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5

Hz, H-6'), 8.15 (s, 2H, H-3'), 7.97 (d, 2H, *J*(9',8') = 8.4 Hz, H-9'), 7.93 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.57 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9

Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.64 – 4.69 (m, 4H, H-12'), 4.38 (t, 2H, $J(8,9) = 6.2$ Hz, H-8), 3.67 – 3.72 (bm, 4H, H-13'), 2.94 (s, 12H, H-14'), 2.89 (t, 1H, $J(12,10) = 2.7$ Hz, H-12), 2.42 (td, 2H, $J(10,9) = 7.1$ Hz, $J(10,12) = 2.7$ Hz, H-10), 2.02 (tt, 2H, $J(9,10) = 7.1$ Hz, $J(9,8) = 6.2$ Hz, H-9). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.35 (C-4), 163.48 (C-7), 161.63 (C-4'), 152.54 (C-2'), 151.27 (C-2,6), 147.21 (C-10'), 130.99 (CH-8'), 127.02 (CH-9'), 124.88 (CH-7'), 122.51 (CH-6'), 119.18 (C-5'), 112.23 (CH-3,5), 95.44 (CH-3'), 83.59 (C-11), 72.11 (CH-12), 67.57 (CH₂-8), 63.40 (CH₂-12'), 55.71 (CH₂-13'), 43.33 (CH₃-14'), 27.40 (CH₂-9), 14.61 (CH₂-10). **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3297, 1694, 1596, 1581, 1524, 1503, 1454, 1416, 1194, 1042, 1027, 998, 739, 682. **HRMS** (ESI⁺) calcd. for C₃₈H₄₂O₅N₇⁺ (M+H⁺): 676.32419, found: 676.32463. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.83.

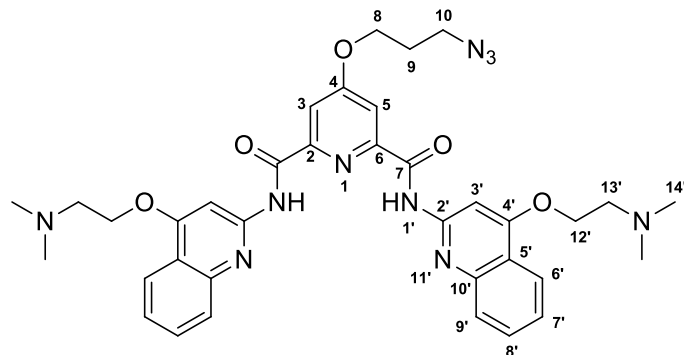
4-(2-Azidoethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (61).



Reactants: **73** (0.252 g, 1.00 mmol), **75** (0.497 g, 2.15 mmol), PyOxim (1.134 g, 2.15 mmol), ethyl-(2-cyanoacrylate) (0.306 g, 2.15 mmol), and DIPEA (870 μ l, 5.00 mmol). Beige crystalline solid (0.351 g, yield 52 %). **Mp** = 179-180 °C. **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.12 (s, 2H, NH), 8.30 (dd, 2H, $J(6',7') = 8.4$ Hz, $J(6',8') = 1.5$ Hz,

H-6'), 8.17 (s, 2H, H-3'), 7.96 (ddd, 2H, $J(9',8') = 8.6$ Hz, $J(9',7') = 1.2$ Hz, $J(9',6') = 0.6$ Hz, H-9'), 7.95 (s, 2H, H-3,5), 7.81 (ddd, 2H, $J(8',9') = 8.6$ Hz, $J(8',7') = 6.9$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.57 (ddd, 2H, $J(7',6') = 8.4$ Hz, $J(7',8') = 6.9$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.62 – 4.67 (m, 4H, H-12'), 4.52 – 4.55 (m, 2H, H-8), 3.79 – 3.82 (m, 2H, H-9), 3.60 – 3.66 (m, 4H, H-13'), 2.89 (s, 12H, H-14'). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 166.97 (C-4), 163.40 (C-7), 161.69 (C-4'), 152.53 (C-2'), 151.34 (C-2,6), 147.21 (C-10'), 130.99 (CH-8'), 127.03 (CH-9'), 124.89 (CH-7'), 122.46 (CH-6'), 119.20 (C-5'), 112.31 (CH-3,5), 95.42 (CH-3'), 68.16 (CH₂-8), 63.67 (CH₂-12'), 55.87 (CH₂-13'), 49.45 (CH₂-9), 43.55 (CH₃-14'). **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3359, 3092, 2943, 2824, 2770, 2111, 1695, 1596, 1580, 1529, 1504, 1469, 1453, 1423, 1384, 1360, 1192, 1161, 1052, 1011, 999, 881, 683, 559. **HRMS** (ESI⁺) calcd. for C₃₅H₃₉O₅N₁₀⁺ (M+H⁺): 679.30994, found: 679.30977. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.75.

4-(3-Azidopropoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (62).



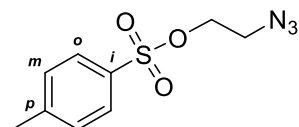
Reactants: **74** (0.266 g, 1.00 mmol), **75** (0.497 g, 2.15 mmol), PyOxim (1.134 g, 2.15 mmol), ethyl-(2-cyanoacrylate) (0.306 g, 2.15 mmol), and DIPEA (870 μ l, 5.00 mmol). Beige crystalline solid (0.491 g, yield 71 %). **Mp** = 182-183 °C. **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.09 (s, 2H, NH), 8.26 (dd, 2H, *J*(6',7') = 8.4 Hz, H-6'), 8.15 (s, 2H,

H-3'), 7.96 (d, 2H, *J*(9',8') = 8.6 Hz, H-9'), 7.93 (s, 2H, H-3,5), 7.80 (ddd, 2H, *J*(8',9') = 8.6 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.55 (ddd, 2H, *J*(7',6') = 8.4 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.2 Hz, H-7'), 4.57 – 4.62 (m, 4H, H-12'), 4.39 (t, 2H, *J*(8,9) = 6.1 Hz, H-8), 3.59 (t, 2H, *J*(10,9) = 6.6 Hz, H-10), 3.45 – 3.54 (bm, 4H, H-13'), 2.79 (s, 12H, H-14'), 2.09 (tt, 2H, *J*(9,10) = 6.6 Hz, *J*(9,8) = 6.1 Hz, H-9). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.26 (C-4), 163.46 (C-7), 161.77 (C-4'), 152.55 (C-2'), 151.26 (C-2,6), 147.21 (C-10'), 130.94 (CH-8'), 127.04 (CH-9'), 124.87 (CH-7'), 122.37 (CH-6'), 119.23 (C-5'), 112.28 (CH-3,5), 95.38 (CH-3'), 66.33 (CH₂-8), 64.19 (CH₂-12'), 56.08 (CH₂-13'), 47.77 (CH₂-10), 43.88 (CH₃-14'), 27.93 (CH₂-9). **FTIR** (KBr, cm⁻¹) ν_{max} : 3433, 3282, 2769, 2732, 2117, 2101, 1687, 1594, 1567, 1526, 1502, 1453, 1379, 1353, 1305, 1187, 1160, 1018, 998, 559. **HRMS** (ESI⁺) calcd. for C₃₆H₄₁O₅N₁₀⁺ (M+H⁺): 693.32559, found: 693.32562. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.73.

The general procedure for synthesis of azide synthons **81-82**

Ditosylate **79-80** (10.0 mmol) and NaN₃ (0.650 g, 10.0 mmol) were dissolved in CH₃CN (200 mL) and the reaction mixture was kept stirring at reflux (85°C) for 48 h. Salts (TsONa) were filtered off and the solvent was removed *in vacuo*. The residue was put on a silica gel column in EtOAc (5-10 %)/hexane, which afforded the desired azide (**81-82**).

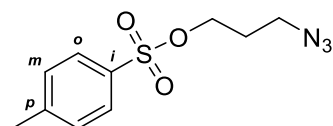
2-Azidoethyl-4-methylbenzenesulfonate (81) [144].



Reactants: ethylene bis(*p*-toluenesulfonate) (**79**, 3.700 g, 10.0 mmol) and NaN₃ (0.650 g, 10.0 mmol). Colourless liquid (0.772 g, yield 32 %). **¹H NMR** (400 MHz, CDCl₃): δ 7.82 (m, 2H, H-*o*-Ph), 7.37 (m, 2H, H-*m*-Ph), 4.16 (t, 2H, *J*_{vic} = 5.2 Hz, OCH₂CH₂N₃), 3.49 (t, 2H, *J*_{vic} = 5.2 Hz, OCH₂CH₂N₃), 2.46 (s, 3H, CH₃). **¹³C NMR** (100.6 MHz, CDCl₃): δ 145.38 (C-*i*-Ph), 132.76 (C-*p*-Ph), 130.14 (CH-*m*-Ph), 128.12 (CH-*o*-Ph), 68.15 (OCH₂CH₂N₃), 49.73 (OCH₂CH₂N₃), 21.82 (CH₃). **FTIR** (CCl₄, cm⁻¹) ν_{max} : 3068, 3035, 2954, 2926,

2863, 2141, 2111, 2090, 2066, 1599, 1496, 1457, 1440, 1400, 1383, 1376, 1349, 1307, 1299, 1293, 1269, 1211, 1190, 1179, 1119, 1099, 1025, 1021, 998, 917, 844, 705, 664, 635, 578, 554. **HRMS** (ESI+) calcd. for $C_9H_{11}O_3N_3NaS^+$ ($M+Na^+$): 264.04133, found: 264.04128. R_f (7/3 hexane/EtOAc) = 0.58.

3-Azidopropyl-4-methylbenzenesulfonate (**82**) [145].

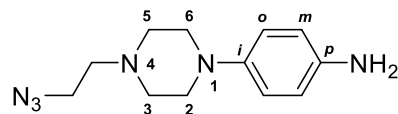


Reactants: 1,3-propanediol bis(*p*-toluenesulfonate) (**80**, 3.850 g, 10.0 mmol) and NaN_3 (0.650 g, 10.0 mmol). Colourless liquid (1.224 g, yield 48 %). **^1H NMR** (400 MHz, $CDCl_3$): δ 7.80 (m, 2H, H-*o*-Ph), 7.36 (m, 2H, H-*m*-Ph), 4.11 (t, 2H, J_{vic} = 6.0 Hz, $OCH_2CH_2CH_2N_3$), 3.38 (t, 2H, J_{vic} = 6.0 Hz, $OCH_2CH_2CH_2N_3$), 2.46 (s, 3H, CH_3), 1.90 (p, 2H, J_{vic} = 6.0 Hz, $OCH_2CH_2CH_2N_3$). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 145.18 (C-*i*-Ph), 132.94 (C-*p*-Ph), 130.09 (CH-*m*-Ph), 128.07 (CH-*o*-Ph), 67.12 ($OCH_2CH_2CH_2N_3$), 47.44 ($OCH_2CH_2CH_2N_3$), 28.62 ($OCH_2CH_2CH_2N_3$), 21.81 (CH_3). **FTIR** (CCl_4 , cm^{-1}) ν_{max} : 3068, 2165, 2100, 1599, 1496, 1399, 1386, 1372, 1307, 1190, 1179, 1099, 831, 664. **HRMS** (APCI+) calcd. for $C_{10}H_{14}O_3N_3S^+$ ($M+H^+$): 256.07504, found: 256.07510. R_f (1/1 hexane/EtOAc) = 0.73.

The general procedure for synthesis of azides containing of 4-(piperazin-1-yl)aniline moiety **77-78**; procedure according to ref. [136]

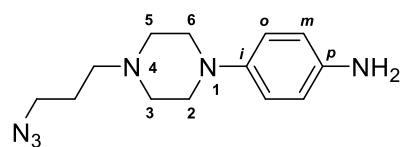
4-(Piperazin-1-yl)aniline (**13**, 0.532 g, 3.00 mmol) and K_2CO_3 (4.146 g, 30.0 mmol) were dissolved in DMF (50 mL) under argon atmosphere and allowed to stir for 10 min at r.t. Afterwards, azide (**81-82**, 3.00 mmol) was added and the reaction was stirred at r.t. for 24 h under argon. K_2CO_3 was filtered off over Celite and the solvent was removed *in vacuo*. The residue was put on a silica gel column in ethyl acetate (20-50 %)/hexane, which afforded the desired compound (**77-78**).

4-(4-(2-Azidoethyl)piperazin-1-yl)aniline (**77**).



Reactants: 4-(piperazin-1-yl)aniline (**13**, 0.532 g, 3.00 mmol), K_2CO_3 (4.146 g, 30.0 mmol), and 2-azidoethyl-4-methylbenzenesulfonate (**81**, 0.724 g, 3.00 mmol). Brown amorphous solid (0.214 g, yield 29 %). **^1H NMR** (400 MHz, $DMSO-d_6$): δ 6.68 (m, 2H, H-*o*-Ph), 6.48 (m, 2H, H-*m*-Ph), 4.56 (vbs, 2H, NH_2), 3.37 (t, 2H, J_{vic} = 5.9 Hz, $NCH_2CH_2N_3$), 2.90 (t, 4H, J_{vic} = 4.9 Hz, H-2,6), 2.53-2.60 (m, 6H, H-3,5, $NCH_2CH_2N_3$). **^{13}C NMR** (100.6 MHz, $DMSO-d_6$): δ 142.67 (C-*i*-Ph), 142.20 (C-*p*-Ph), 118.11 (CH-*o*-Ph), 115.08 (CH-*m*-Ph), 56.88 ($NCH_2CH_2N_3$), 52.93 (CH_2 -3,5), 50.55 (CH_2 -2,6), 47.33 ($NCH_2CH_2N_3$). **FTIR** (CCl_4 , cm^{-1}) ν_{max} : 3430, 3340, 3276, 2928, 2820, 2111, 1652, 1626, 1577, 1515, 1438, 1380, 1280, 1188, 1143, 1128, 824, 707, 538. **HRMS** (ESI+) calcd. for $C_{12}H_{19}N_6^+$ ($M+H^+$): 247.16657, found: 247.16655. R_f (EtOAc) = 0.31.

27a, 28a, and 29a 4-(4-(3-Azidopropyl)piperazin-1-yl)aniline (78).

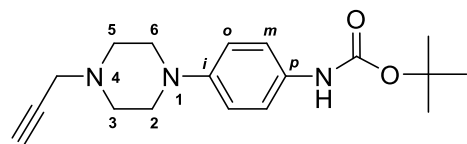


Reactants: 4-(piperazin-1-yl)aniline (**13**, 0.532 g, 3.00 mmol), K_2CO_3 (4.146 g, 30.0 mmol), and 3-azidopropyl-4-methylbenzenesulfonate (**82**, 0.766 g, 3.00 mmol). Brown amorphous solid (0.172 g, yield 22 %). ^1H NMR (400 MHz, $CDCl_3$): δ 6.81 (m, 2H, H-*o*-Ph), 6.65 (m, 2H, H-*m*-Ph), 3.37 (t, 2H, $J_{vic} = 6.8$ Hz, $NCH_2CH_2CH_2N_3$), 3.06 (t, 4H, $J_{vic} = 5.0$ Hz, H-2,6), 2.61 (t, 4H, $J_{vic} = 4.9$ Hz, H-3,5), 2.49 (t, 2H, $J_{vic} = 7.2$ Hz, $NCH_2CH_2CH_2N_3$), 1.82 (p, 2H, $J_{vic} = 7.0$ Hz, $NCH_2CH_2CH_2N_3$); NH_2 signal not detected. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 144.60 (C-*i*-Ph), 140.29 (C-*p*-Ph), 118.74 (CH-*o*-Ph), 116.34 (CH-*m*-Ph), 55.43 ($NCH_2CH_2CH_2N_3$), 53.47 (CH_2 -3,5), 50.97 (CH_2 -2,6), 49.72 ($NCH_2CH_2CH_2N_3$), 26.35 ($NCH_2CH_2CH_2N_3$). FTIR (CCl_4 , cm^{-1}) ν_{max} : 3460, 3382, 3065, 3036, 3007, 2948, 2880, 2818, 2778, 2097, 1623, 1610, 1584, 1515, 1456, 1449, 1377, 1270, 1171, 1112, 1084, 1056, 1017, 823, 640, 556, 529. HRMS (ESI+) calcd. for $C_{13}H_{21}N_6^+$ ($M+H^+$): 261.18222, found: 261.18227. R_f (EtOAc) = 0.33.

The general procedure for Boc-protection of BSF7-derived alkynes and azides (89-93); procedure according to ref. [146]

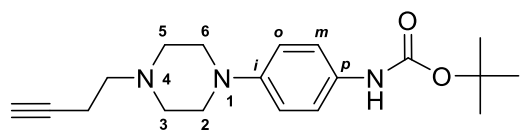
The compound **27a**, **28a**, **29a**, **77**, **78** (1.00 mmol) and di-*t*-butyl dicarbonate (0.327 g, 1.50 mmol) were dissolved in ethanol (abs., 25 mL) and TEA (0.84 mL, 6.00 mmol) was added dropwise. The reaction mixture was stirred under argon at r.t. overnight. The solvent was removed *in vacuo* and the residue was extracted between ethyl acetate and water. The organic phase was dried over $MgSO_4$ and the solvent was removed *in vacuo*.

***t*-Butyl (4-(4-(prop-2-yn-1-yl)piperazin-1-yl)phenyl)carbamate (89).**



Reactants: **27a** (0.215 g, 1.00 mmol), di-*t*-butyl dicarbonate (0.327 g, 1.50 mmol), and TEA (0.84 mL, 6.00 mmol). Yellowish crystalline solid (0.290 g, yield 92 %). $M_p = 165$ -166 $^{\circ}C$. ^1H NMR (400 MHz, $DMSO-d_6$): δ 9.01 (s, 1H, NH), 7.28 (m, 2H, H-*m*-Ph), 6.83 (m, 2H, H-*o*-Ph), 3.31 (d, 2H, $^4J = 2.3$ Hz, NCH_2CCH), 3.16 (t, 1H, $^4J = 2.4$ Hz, NCH_2CCH), 3.05 (t, 4H, $J_{vic} = 4.9$ Hz, H-2,6), 2.57 (t, 4H, $J_{vic} = 4.9$ Hz, H-3,5), 1.45 (s, 9H, 3x CH_3). ^{13}C NMR (100.6 MHz, $DMSO-d_6$): δ 152.89 (C=O), 146.39 (C-*i*-Ph), 131.61 (C-*p*-Ph), 119.26 (CH-*m*-Ph), 116.03 (CH-*o*-Ph), 79.24 (NCH_2CCH), 78.48 (C-*t*-Bu), 75.75 (NCH_2CCH), 51.12 (CH_2 -3,5), 48.84 (CH_2 -2,6), 46.00 (NCH_2CCH), 28.17 (3x CH_3). FTIR ($CHCl_3$, cm^{-1}) ν_{max} : 3363, 3308, 2985, 2974, 2930, 2881, 2825, 2755, 2119, 1697, 1617, 1589, 1524, 1513, 1418, 1315, 1232, 1168, 1138, 1097, 1007, 815, 649. HRMS (ESI+) calcd. for $C_{18}H_{25}O_2N_3Na^+$ ($M+Na^+$): 338.18390, found: 338.18398; calcd. for $C_{18}H_{26}O_2N_3^+$ ($M+H^+$): 316.20195, found: 316.20208. R_f (EtOAc) = 0.70.

***t*-Butyl (4-(4-(but-3-yn-1-yl)piperazin-1-yl)phenyl)carbamate (90).**

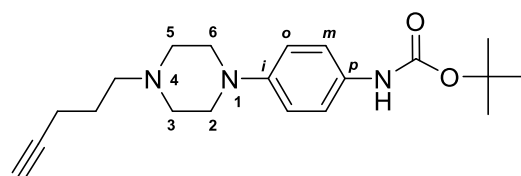


Reactants: **28a** (0.229 g, 1.00 mmol), di-*t*-butyl dicarbonate (0.327 g, 1.50 mmol), and TEA (0.84 mL, 6.00 mmol).

White crystalline solid (0.296 g, yield 90 %). **Mp** = 151-

152 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 9.01 (s, 1H, NH), 7.27 (m, 2H, H-*m*-Ph), 6.82 (m, 2H, H-*o*-Ph), 3.01 (t, 4H, *J*_{vic} = 4.9 Hz, H-2,6), 2.78 (t, 1H, ⁴*J* = 2.6 Hz, NCH₂CH₂CCH), 2.53 (t, 4H, *J*_{vic} = 4.9 Hz, H-3,5), 2.48-2.52 (m, 2H, NCH₂CH₂CCH, signal overlapped by DMSO peak), 2.35 (td, 2H, *J*_{vic} = 7.3 Hz, ⁴*J* = 2.5 Hz, NCH₂CH₂CCH), 1.45 (s, 9H, 3x CH₃). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 152.90 (C=O), 146.45 (C-*i*-Ph), 131.56 (C-*p*-Ph), 119.29 (CH-*m*-Ph), 115.92 (CH-*o*-Ph), 83.18 (NCH₂CH₂CCH), 78.48 (C-*t*-Bu), 71.72 (NCH₂CH₂CCH), 56.44 (NCH₂CH₂CCH), 52.34 (CH₂-3,5), 48.89 (CH₂-2,6), 28.18 (3x CH₃), 16.18 (NCH₂CH₂CCH). **FTIR** (CHCl₃, cm⁻¹) *v*_{max}: 3367, 3301, 2985, 2949, 2823, 2878, 2743, 2117, 1691, 1615, 1589, 1524, 1509, 1416, 1368, 1320, 1312, 1237, 1160, 1136, 1105, 1007, 821, 722, 642, 622. **HRMS** (ESI+) calcd. for C₁₉H₂₇O₂N₃Na⁺ (M+Na⁺): 352.19955, found: 352.19929; calcd. for C₁₉H₂₈O₂N₃⁺ (M+H⁺): 330.21760, found: 330.21738. **R_f** (EtOAc) = 0.72.

***t*-Butyl (4-(4-(pent-4-yn-1-yl)piperazin-1-yl)phenyl)carbamate (91).**

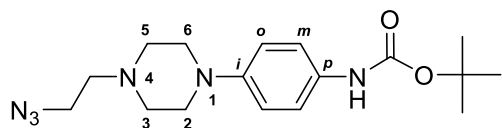


Reactants: **29a** (0.243 g, 1.00 mmol), di-*t*-butyl dicarbonate (0.327 g, 1.50 mmol), and TEA (0.84 mL, 6.00 mmol). Yellowish crystalline solid (0.288 g, yield 84 %).

Mp = 144-145 °C. **¹H NMR** (400 MHz, DMSO-*d*₆): δ 9.01

(s, 1H, NH), 7.27 (m, 2H, H-*m*-Ph), 6.82 (m, 2H, H-*o*-Ph), 3.02 (t, 4H, *J*_{vic} = 4.9 Hz, H-2,6), 2.75 (t, 1H, ⁴*J* = 2.7 Hz, NCH₂CH₂CH₂CCH), 2.48 (m, 4H, H-3,5, signal overlapped by DMSO peak), 2.37 (t, 2H, *J*_{vic} = 7.2 Hz, NCH₂CH₂CH₂CCH), 2.19 (td, 2H, *J*_{vic} = 7.1 Hz, ⁴*J* = 2.6 Hz, NCH₂CH₂CH₂CCH), 1.62 (p, 2H, *J*_{vic} = 7.2 Hz, NCH₂CH₂CH₂CCH), 1.45 (s, 9H, 3x CH₃). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 152.89 (C=O), 146.49 (C-*i*-Ph), 131.51 (C-*p*-Ph), 119.29 (CH-*m*-Ph), 115.87 (CH-*o*-Ph), 84.38 (NCH₂CH₂CH₂CCH), 78.47 (C-*t*-Bu), 71.19 (NCH₂CH₂CH₂CCH), 56.57 (NCH₂CH₂CH₂CCH), 52.78 (CH₂-3,5), 48.96 (CH₂-2,6), 28.17 (3x CH₃), 25.37 (NCH₂CH₂CH₂CCH), 15.64 (NCH₂CH₂CH₂CCH). **FTIR** (CCl₄, cm⁻¹) *v*_{max}: 3364, 3277, 2981, 2954, 2879, 2819, 2746, 2115, 1698, 1615, 1589, 1523, 1415, 1390, 1379, 1365, 1316, 1233, 1162, 1138, 1108, 1011, 821. **HRMS** (ESI+) calcd. for C₂₀H₂₉O₂N₃Na⁺ (M+Na⁺): 366.21520, found: 366.21543; calcd. for C₂₀H₃₀O₂N₃⁺ (M+H⁺): 344.23325, found: 344.23354. **R_f** (EtOAc) = 0.67.

***t*-Butyl (4-(4-(2-azidoethyl)piperazin-1-yl)phenyl)carbamate (92).**

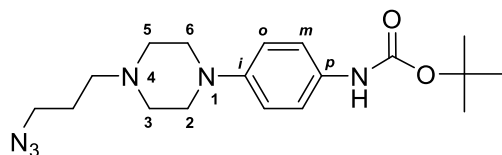


Reactants: **77** (0.246 g, 1.00 mmol), di-*t*-butyl dicarbonate (0.327 g, 1.50 mmol), and TEA (0.84 mL, 6.00 mmol).

Brown amorphous solid (0.263 g, yield 76 %). **¹H NMR**

(400 MHz, DMSO-*d*₆): δ 9.01 (s, 1H, NH), 7.28 (m, 2H, H-*m*-Ph), 6.84 (m, 2H, H-*o*-Ph), 3.38 (t, 2H, *J*_{vic} = 5.8 Hz, NCH₂CH₂N₃), 3.03 (t, 4H, *J*_{vic} = 4.9 Hz, H-2,6), 2.55-2.61 (m, 6H, H-3,5, NCH₂CH₂N₃), 1.45 (s, 9H, 3x CH₃). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 152.89 (C=O), 146.43 (C-*i*-Ph), 131.59 (C-*p*-Ph), 119.28 (CH-*m*-Ph), 115.94 (CH-*o*-Ph), 78.47 (C-*t*-Bu), 56.69 (NCH₂CH₂N₃), 52.52 (CH₂-3,5), 48.97 (CH₂-2,6), 47.10 (NCH₂CH₂N₃), 28.17 (3x CH₃). **FTIR** (CHCl₃, cm⁻¹) ν_{max}: 3361, 2975, 2954, 2930, 2871, 2823, 2750, 2101, 1720, 1689, 1615, 1589, 1524, 1509, 1415, 1391, 1370, 1366, 1319, 1314, 1237, 1161, 1139, 1010, 821, 721, 639, 627. **HRMS** (ESI+) calcd. for C₁₇H₂₆O₂N₆Na⁺ (M+Na⁺): 369.20095, found: 369.20072; calcd. for C₁₇H₂₇O₂N₆⁺ (M+H⁺): 347.21900, found: 347.21889. **R_f** (EtOAc) = 0.79.

***t*-Butyl (4-(4-(3-azidopropyl)piperazin-1-yl)phenyl)carbamate (93).**



Reactants: **78** (0.260 g, 1.00 mmol), di-*t*-butyl dicarbonate (0.327 g, 1.50 mmol), and TEA (0.84 mL, 6.00 mmol).

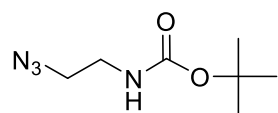
Beige amorphous solid (0.210 g, yield 58 %). **¹H NMR** (400 MHz, DMSO-*d*₆): δ 9.01 (s, 1H, NH), 7.27 (m, 2H, H-*m*-Ph),

6.83 (m, 2H, H-*o*-Ph), 3.37 (t, 2H, *J*_{vic} = 6.7 Hz, NCH₂CH₂CH₂N₃), 3.02 (t, 4H, *J*_{vic} = 4.9 Hz, H-2,6), 2.46-2.51 (m, 4H, H-3,5, signal overlapped by DMSO peak), 2.38 (t, 2H, *J*_{vic} = 7.0 Hz, NCH₂CH₂CH₂N₃), 1.72 (p, 2H, *J*_{vic} = 6.8 Hz, NCH₂CH₂CH₂N₃), 1.45 (s, 9H, 3x CH₃). **¹³C NMR** (100.6 MHz, DMSO-*d*₆): δ 152.90 (C=O), 146.47 (C-*i*-Ph), 131.53 (C-*p*-Ph), 119.29 (CH-*m*-Ph), 115.88 (CH-*o*-Ph), 78.47 (C-*t*-Bu), 54.64 (NCH₂CH₂CH₂N₃), 52.69 (CH₂-3,5), 48.95 (CH₂-2,6), 48.89 (NCH₂CH₂CH₂N₃), 28.18 (3x CH₃), 25.63 (NCH₂CH₂CH₂N₃). **FTIR** (CHCl₃, cm⁻¹) ν_{max}: 3359, 2983, 2949, 2876, 2822, 2750, 2100, 1693, 1616, 1590, 1525, 1510, 1415, 1390, 1367, 1320, 1313, 1235, 1164, 1130, 1009, 821, 721, 641. **HRMS** (ESI+) calcd. for C₁₈H₂₈O₂N₆Na⁺ (M+Na⁺): 383.21660, found: 383.21634; calcd. for C₁₈H₂₉O₂N₆⁺ (M+H⁺): 361.23465, found: 361.23441. **R_f** (EtOAc) = 0.69.

The general procedure for Boc-protection of aliphatic azidoamines (94-95)

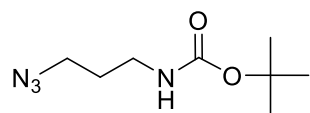
Azidoamine **7-8** (1.00 mmol) and di-*t*-butyl dicarbonate (0.240 g, 1.10 mmol) were dissolved in THF (10 mL) and stirred for 30 min at r.t. under argon atmosphere. Afterwards, TEA (0.84 mL, 6.00 mmol) was added dropwise and the reaction mixture was stirred under argon at r.t. overnight. The solvent was removed *in vacuo* and the residue was dried without further purification on a vacuum pump.

t-Butyl (2-azidoethyl)carbamate (**94**) [148].



Reactants: 2-azidoethan-1-amine (**7**, 0.086 g, 1.00 mmol), di-*t*-butyl dicarbonate (0.240 g, 1.10 mmol), and TEA (0.84 mL, 6.00 mmol). Colourless oil (0.163 g, yield 88 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.03 (t, 1H, *J*_{vic} = 5.1 Hz, NH), 3.29 (t, 2H, *J*_{vic} = 5.8 Hz, NHCH₂CH₂N₃), 3.10 (t, 2H, *J*_{vic} = 5.3 Hz, NHCH₂CH₂N₃), 1.38 (s, 9H, 3x CH₃). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 155.58 (C=O), 77.86 (C-*t*-Bu), 50.02 (NHCH₂CH₂N₃), 39.59 (NHCH₂CH₂N₃), 28.19 (3x CH₃). *R*_f (EtOAc) = 0.21. The spectra are in accordance with ref. [148].

t-Butyl (3-azidopropyl)carbamate (**95**) [149].

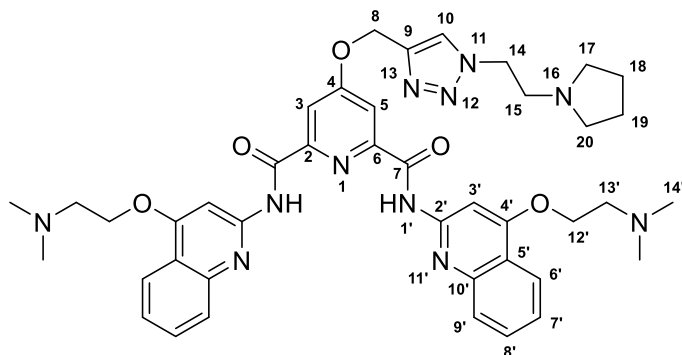


Reactants: 3-azidopropan-1-amine (**8**, 0.100 g, 1.00 mmol), di-*t*-butyl dicarbonate (0.240 g, 1.10 mmol), and TEA (0.84 mL, 6.00 mmol). Colourless oil (0.188 g, yield 94 %). ¹H NMR (400 MHz, DMSO-*d*₆): δ 6.86 (t, 1H, *J*_{vic} = 5.0 Hz, NH), 3.33 (t, 2H, *J*_{vic} = 6.8 Hz, NHCH₂CH₂CH₂N₃), 2.97 (q, 2H, *J*_{vic} = 5.9 Hz, NHCH₂CH₂CH₂N₃), 1.62 (p, 2H, *J*_{vic} = 6.8 Hz, NHCH₂CH₂CH₂N₃), 1.37 (s, 9H, 3x CH₃). ¹³C NMR (100.6 MHz, DMSO-*d*₆): δ 155.69 (C=O, determined by HMBC), 77.54 (C-*t*-Bu), 48.35 (NHCH₂CH₂CH₂N₃), 37.19 (NHCH₂CH₂CH₂N₃), 28.71 (NHCH₂CH₂CH₂N₃), 28.22 (3x CH₃). *R*_f (EtOAc) = 0.26. The spectra are in accordance with ref. [149].

The general procedure for synthesis of pyridostatin click products with tertiary azidoamines (83-88); procedure according to ref. [39]

The compound **58-60** (0.06 mmol) was dissolved in *tert*-butanol/H₂O (1:2, 1.5 mL) under argon atmosphere, CuSO₄ (100 mM solution, 0.120 mL) and Na-ascorbate (100 mM solution, 0.360 mL) were added and the mixture was allowed to stir at r.t. for 15 min. Afterwards, azide (0.12 mmol) was added and the reaction mixture was kept stirring at r.t. for 24 h under argon. The solvent was removed under reduced pressure and the residue was purified by a combi flash chromatograph on a reverse-phase column in methanol (0.4 M NH₃ solution, 5-20 %)/EtOAc, which afforded the desired click product **83-88**.

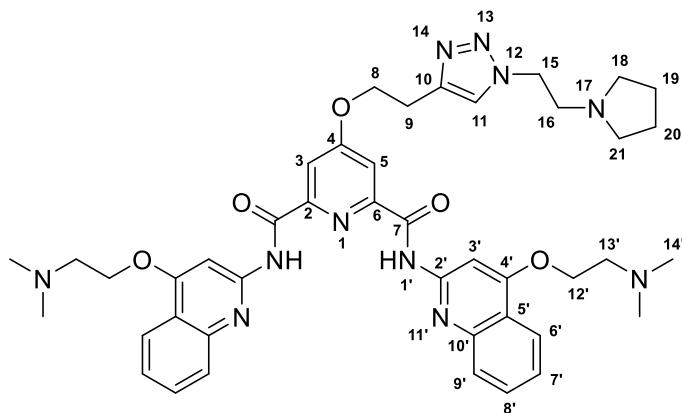
***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-((1-(2-(pyrrolidin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)pyridine-2,6-dicarboxamide (83).**



Reactants: **58** (0.039 g, 0.06 mmol), 1-(2-azidoethyl)pyrrolidine (**9**, 0.017 mL, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Brown amorphous solid (0.039 g, yield 82 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.43 (s, 1H, H-10), 8.35 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5

Hz, H-6'), 8.18 (s, 2H, H-3'), 8.07 (s, 2H, H-3,5), 7.97 (d, 2H, *J*(9',8') = 8.3 Hz, H-9'), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.55 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.3 Hz, H-7'), 5.56 (s, 2H, H-8), 4.84 (t, 2H, *J*(14,15) = 6.3 Hz, H-14), 4.69 (m, 4H, H-12'), 3.72-3.82 (m, 6H, H-13', H-15), 3.55 (m, 2H, H-17a, H-20a), 3.04 (m, 2H, H-17b, H-20b), 2.99 (s, 12H, H-14'), 2.00 (m, 2H, H-18a, H-19a), 1.86 (m, 2H, H-18b, H-19b). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 166.75 (C-4), 163.22 (C-7), 161.64 (C-4'), 152.26 (C-2'), 151.05 (C-2,6), 146.85 (C-10'), 141.83 (C-9), 130.84 (CH-8'), 126.67 (CH-9'), 125.85 (CH-10), 124.68 (CH-7'), 122.41 (CH-6'), 118.98 (C-5'), 112.47 (CH-3,5), 95.18 (CH-3'), 62.97 (CH₂-12'), 61.91 (CH₂-8), 55.24 (CH₂-13'), 53.59 (CH₂-17,20), 52.70 (CH₂-15), 45.64 (CH₂-14), 42.79 (CH₃-14'), 22.53 (CH₂-18,19). **FTIR** (KBr, cm⁻¹) *v*_{max}: 3251, 3135, 2925, 2855, 1681, 1605, 1581, 1562, 1528, 1452, 1360, 1203, 1046, 998, 882, 721. **HRMS** (ESI+) calcd. for C₄₂H₅₀O₅N₁₁⁺ (M+H⁺): 788.39909, found: 788.39924. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.55.

***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-(2-(1-(2-(pyrrolidin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)ethoxy)pyridine-2,6-dicarboxamide (84).**

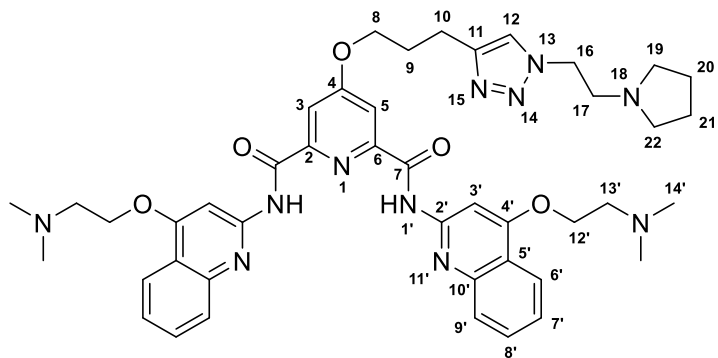


Reactants: **59** (0.039 g, 0.06 mmol), 1-(2-azidoethyl)pyrrolidine (**9**, 0.017 mL, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Brown amorphous solid (0.019 g, yield 39 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.13-8.18 (m, 3H, H-3', H-11), 7.96 (dd, 2H,

J(9',8') = 8.4 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.94 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') =

6.8 Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.56 (ddd, 2H, $J(7',6') = 8.4$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.77 (t, 2H, $J(15,16) = 6.2$ Hz, H-15), 4.68 (m, 4H, H-12'), 4.60 (t, 2H, $J(8,9) = 6.3$ Hz, H-8), 3.70-3.80 (m, 6H, H-13', H-16), 3.54 (m, 2H, H-18a, H-21a), 3.25 (t, 2H, $J(9,8) = 6.1$ Hz, H-9), 3.04 (m, 2H, H-18b, H-21b), 2.99 (s, 12H, H-14'), 2.00 (m, 2H, H-19a, H-20a), 1.85 (m, 2H, H-19b, H-20b). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.05 (C-4), 163.27 (C-7), 161.45 (C-4'), 152.27 (C-2'), 151.08 (C-2,6), 146.96 (C-10'), 143.48 (C-10), 130.83 (CH-8'), 126.76 (CH-9'), 124.70 (CH-7'), 123.70 (CH-11), 122.40 (CH-6'), 118.98 (C-5'), 112.11 (CH-3,5), 95.23 (CH-3'), 67.62 (CH₂-8), 62.93 (CH₂-12'), 55.29 (CH₂-13'), 53.63 (CH₂-18,21), 52.83 (CH₂-16), 45.47 (CH₂-15), 42.85 (CH₃-14'), 25.07 (CH₂-9), 22.52 (CH₂-19,20). **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3264, 3135, 2926, 2855, 1690, 1682, 1596, 1580, 1563, 1530, 1453, 1358, 1203, 1048, 999, 882, 721. **HRMS** (ESI+) calcd. for C₄₃H₅₂O₅N₁₁⁺ (M+H⁺): 802.41474, found: 802.41406. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.54.

***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-(3-(1-(2-(pyrrolidin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)propoxy)pyridine-2,6-dicarboxamide (85).**

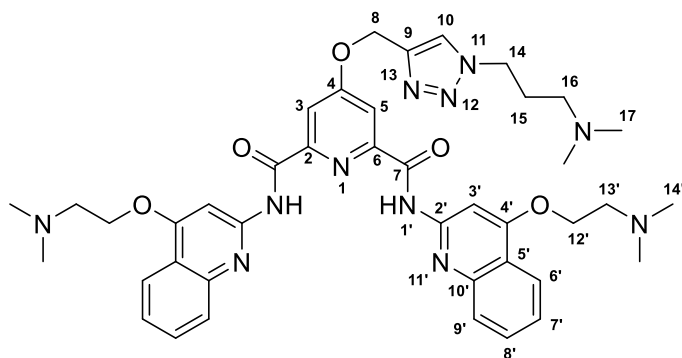


Reactants: **60** (0.039 g, 0.06 mmol), 1-(2-azidoethyl)pyrrolidine (**9**, 0.017 mL, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Brown amorphous solid (0.018 g, yield 37 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.35 (dd, 2H, $J(6',7') = 8.3$ Hz, $J(6',8') = 1.5$ Hz, H-

6'), 8.16 (s, 2H, H-3'), 8.04 (s, 1H, H-12), 7.96 (dd, 2H, $J(9',8') = 8.4$ Hz, $J(9',7') = 1.2$ Hz, H-9'), 7.93 (s, 2H, H-3,5), 7.81 (ddd, 2H, $J(8',9') = 8.4$ Hz, $J(8',7') = 6.9$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.55 (ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.9$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.75 (t, 2H, $J(16,17) = 6.3$ Hz, H-16), 4.68 (m, 4H, H-12'), 4.39 (t, 2H, $J(8,9) = 6.9$ Hz, H-8), 3.69-6.80 (m, 6H, H-13', H-17), 3.53 (m, 2H, H-19a, H-22a), 3.04 (m, 2H, H-19b, H-22b), 2.99 (s, 12H, H-14'), 2.88 (t, 2H, $J(10,9) = 7.4$ Hz, H-10), 2.18 (p, 2H, $J(9,8) = J(9,10) = 7.2$ Hz, H-9), 2.00 (m, 2H, H-20a, H-21a), 1.85 (m, 2H, H-20b, H-21b). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.25 (C-4), 163.30 (C-7), 161.45 (C-4'), 152.28 (C-2'), 151.06 (C-2,6), 146.97 (C-10'), 146.35 (C-11), 130.82 (CH-8'), 126.77 (CH-9'), 124.69 (CH-7'), 122.86 (CH-12), 122.39 (CH-6'), 118.98 (C-5'), 112.07 (CH-3,5), 95.24 (CH-3'), 68.05 (CH₂-8), 62.92 (CH₂-12'), 55.29 (CH₂-13'), 53.62 (CH₂-19,22), 52.83 (CH₂-17), 45.39 (CH₂-16), 42.85 (CH₃-14'), 28.01 (CH₂-9), 22.52 (CH₂-20,21), 21.41 (CH₂-10). **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3342, 3135, 2929, 1697, 1983, 1596, 1581, 1560, 1525, 1505,

1453, 1357, 1206, 1050, 998, 881, 722. **HRMS** (ESI⁺) calcd. for C₄₄H₅₄O₅N₁₁⁺ (M+H⁺): 816.43039, found: 816.42987. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.54.

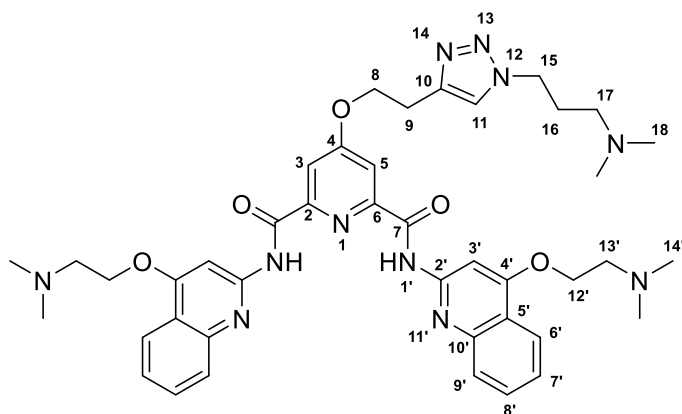
***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-((1-(3-(dimethylamino)propyl)-1*H*-1,2,3-triazol-4-yl)methoxy)pyridine-2,6-dicarboxamide (86).**



Reactants: **58** (0.039 g, 0.06 mmol), 3-azido-*N,N*-dimethylpropan-1-amine (**30**, 0.016 mL, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Brown amorphous solid (0.028 g, yield 60 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.32-8.40 (m, 3H, H-6', H-10), 8.18 (s, 2H, H-3'), 8.07 (s, 2H,

H-3,5), 7.97 (d, 2H, *J*(9',8') = 8.3 Hz, H-9'), 7.81 (m, 2H, H-8'), 7.55 (m, 2H, H-7'), 5.54 (s, 2H, H-8), 4.69 (m, 4H, H-12'), 4.50 (m, 2H, H-14), 3.77 (m, 4H, H-13'), 3.11 (m, 2H, H-16), 2.99 (s, 12H, H-14'), 2.79 (s, 6H, H-17), 2.25 (m, 2H, H-15). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 166.79 (C-4), 163.24 (C-7), 161.48 (C-4'), 152.30 (C-2'), 151.08 (C-2,6), 146.95 (C-10'), 141.58 (C-9), 130.83 (CH-8'), 126.75 (CH-9'), 125.31 (CH-10), 124.68 (CH-7'), 122.41 (CH-6'), 118.99 (C-5'), 112.47 (CH-3,5), 95.20 (CH-3'), 62.95 (CH₂-12'), 62.00 (CH₂-8), 55.28 (CH₂-13'), 54.00 (CH₂-16), 46.76 (CH₂-14), 42.83 (CH₃-14'), 42.25 (CH₃-17), 24.70 (CH₂-15). **FTIR** (KBr, cm⁻¹) ν_{max}: 3390, 3135, 2930, 2896, 1685, 1596, 1582, 1522, 1505, 1466, 1453, 1360, 1203, 1049, 1002, 881, 721. **HRMS** (ESI⁺) calcd. for C₄₁H₅₀O₅N₁₁⁺ (M+H⁺): 776.39909, found: 776.39894. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.23.

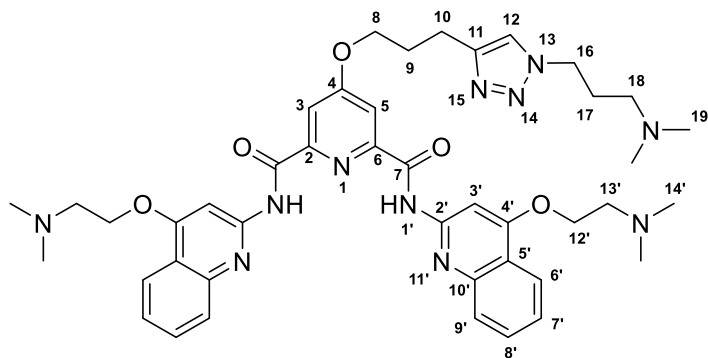
***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-(2-(1-(3-(dimethylamino)propyl)-1*H*-1,2,3-triazol-4-yl)ethoxy)pyridine-2,6-dicarboxamide (87).**



Reactants: **59** (0.039 g, 0.06 mmol), 3-azido-*N,N*-dimethylpropan-1-amine (**30**, 0.016 mL, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Brown amorphous solid (0.016 g, yield 33 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.15

(s, 2H, H-3'), 8.09 (s, 1H, H-11), 7.96 (dd, 2H, $J(9',8') = 8.4$ Hz, $J(9',7') = 1.2$ Hz, H-9'), 7.94 (s, 2H, H-3,5), 7.81 (ddd, 2H, $J(8',9') = 8.3$ Hz, $J(8',7') = 6.8$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.56 (ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.68 (m, 4H, H-12'), 4.59 (t, 2H, $J(8,9) = 6.2$ Hz, H-8), 4.44 (t, 2H, $J(15,16) = 6.9$ Hz, H-15), 3.76 (m, 4H, H-13'), 3.23 (t, 2H, $J(9,8) = 6.3$ Hz, H-9), 3.05-3.11 (m, 2H, H-17), 2.99 (s, 12H, H-14'), 2.79 (s, 6H, H-18), 2.22 (p, 2H, $J(16,15) = J(16,17) = 7.3$ Hz, H-16). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.05 (C-4), 163.27 (C-7), 161.43 (C-4'), 152.28 (C-2'), 151.07 (C-2,6), 146.98 (C-10'), 143.13 (C-10), 130.82 (CH-8'), 126.77 (CH-9'), 124.69 (CH-7'), 123.10 (CH-11), 122.38 (CH-6'), 118.98 (C-5'), 112.12 (CH-3,5), 95.21 (CH-3'), 67.65 (CH₂-8), 62.92 (CH₂-12'), 55.29 (CH₂-13'), 54.06 (CH₂-17), 46.52 (CH₂-15), 42.85 (CH₃-14'), 42.25 (CH₃-18), 25.08 (CH₂-9), 24.74 (CH₂-16). **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3358, 3135, 2929, 2896, 1686, 1595, 1522, 1505, 1467, 1453, 1357, 1204, 1050, 997, 881, 722. **HRMS** (ESI+) calcd. for C₄₂H₅₂O₅N₁₁⁺ (M+H⁺): 790.41474, found: 790.41466. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.27.

***N*²,*N*⁶-Bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)-4-(3-(1-(3-(dimethylamino)propyl)-1*H*-1,2,3-triazol-4-yl)propoxy)pyridine-2,6-dicarboxamide (88).**



Reactants: **60** (0.039 g, 0.06 mmol), 3-azido-*N,N*-dimethylpropan-1-amine (**30**, 0.016 mL, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Brown amorphous solid (0.030 g, yield 63 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.35 (dd, 2H, $J(6',7') = 8.3$ Hz,

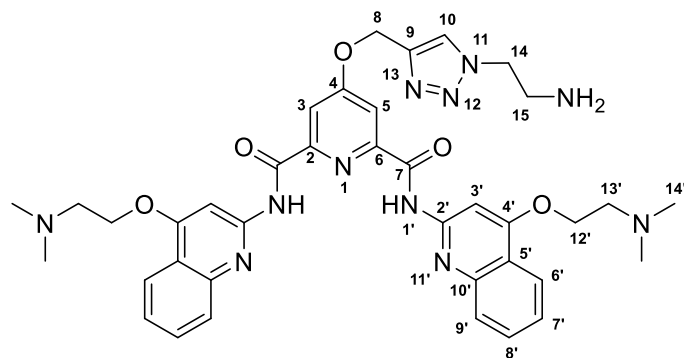
$J(6',8') = 1.5$ Hz, H-6'), 8.16 (s, 2H, H-3'), 7.99 (s, 1H, H-12), 7.96 (d, 2H, $J(9',8') = 8.4$ Hz, H-9'), 7.93 (s, 2H, H-3,5), 7.81 (ddd, 2H, $J(8',9') = 8.3$ Hz, $J(8',7') = 6.9$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.55 (ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.68 (m, 4H, H-12'), 4.36-4.44 (m, 4H, H-16, H-8), 3.76 (m, 4H, H-13'), 3.04-3.10 (m, 2H, H-18), 2.99 (s, 12H, H-14'), 2.86 (t, 2H, $J(10,9) = 7.5$ Hz, H-10), 2.79 (s, 6H, H-19), 2.13-2.25 (m, 4H, H-17, H-9). **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.26 (C-4), 163.30 (C-7), 161.44 (C-4'), 152.30 (C-2'), 151.06 (C-2,6), 146.98 (C-10'), 146.44 (C-11), 130.82 (CH-8'), 126.77 (CH-9'), 124.68 (CH-7'), 122.39 (CH-6'), 122.26 (CH-12), 118.99 (C-5'), 112.07 (CH-3,5), 95.24 (CH-3'), 68.10 (CH₂-8), 62.93 (CH₂-12'), 55.29 (CH₂-13'), 54.05 (CH₂-18), 46.46 (CH₂-16), 42.85 (CH₃-14'), 42.25 (CH₃-19), 28.09 (CH₂-9), 24.72 (CH₂-17), 21.45 (CH₂-10). **FTIR** (KBr, cm⁻¹) $\nu_{\max}$: 3138, 2925, 2854, 1687, 1596, 1524, 1505, 1470, 1453, 1358, 1203, 1041, 998, 879, 720. **HRMS**

(ESI+) calcd. for $C_{43}H_{54}O_5N_{11}^+$ ($M+H^+$): 804.43039, found: 804.43051. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.27.

The general procedure for synthesis of pyridostatin click products with Boc-protected azidoamines (procedure according to ref. [39]), followed by Boc-deprotection

The procedure for click reaction of an alkyne (**58-60**, **89-91**) with the respective azide (**92-95**, **61-62**) was the same as in the case of compounds **83-88**. After drying on a vacuum pump, the click product was dissolved in dichloromethane (1.0 mL), TFA (100 μ L) was added dropwise and the reaction mixture was stirred at r.t. for 1 h. The reaction mixture was transferred to a vial (2.5 mL volume) and the solvent with TFA was removed by N_2 flow. The residue was put under high vacuum overnight. The next day, the residue was dissolved in methanol (50 μ L) and the bottle was filled with diethylether. The mixture was sonicated and then centrifuged for 3 min at 3000 RPM. The liquid phase was decanted and the procedure was repeated 3x. The residue was dried on high vacuum overnight to yield the desired click products **96-101**, **102-107**, and **108-113**.

4-((1-(2-Aminoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (96**).**

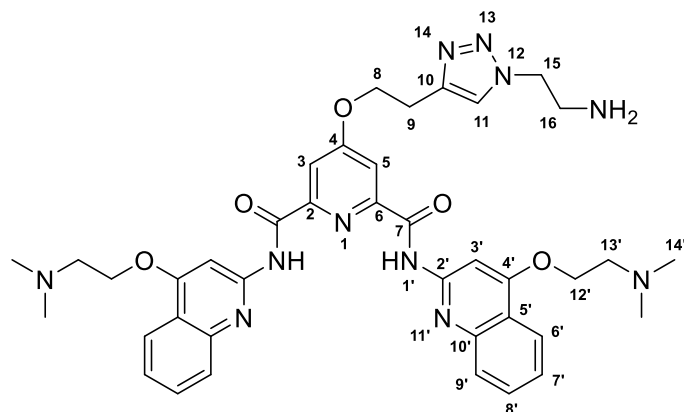


Reactants: **58** (0.039 g, 0.06 mmol), **94** (22 μ L, 0.12 mmol), $CuSO_4$ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.024 g, yield 55 %). **¹H NMR** (500 MHz, $DMSO-d_6$): δ 12.10 (s, 2H, NH), 8.40 (s, 1H, H-10), 8.35 (dd, 2H, $J(6',7') = 8.3$ Hz, $J(6',8') = 1.5$ Hz, H-6'), 8.18 (s, 2H, H-3'), 8.07 (s,

2H, H-3,5), 7.97 (dd, 2H, $J(9',8') = 8.4$ Hz, $J(9',7') = 1.2$ Hz, H-9'), 7.81 (ddd, 2H, $J(8',9') = 8.4$ Hz, $J(8',7') = 6.9$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.56 (ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.9$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 5.55 (s, 2H, H-8), 4.64-4.71 (m, 6H, H-12', H-14), 3.76 (m, 4H, H-13'), 3.37-3.43 (m, 2H, H-15, signal overlapped by H_2O peak), 2.98 (s, 12H, H-14'); NH_2 signal not detected. **¹³C NMR** (125.7 MHz, $DMSO-d_6$): δ 166.80 (C-4), 163.26 (C-7), 161.46 (C-4'), 152.32 (C-2'), 151.10 (C-2,6), 147.03 (C-10'), 141.67 (C-9), 130.84 (CH-8'), 126.81 (CH-9'), 125.86 (CH-10), 124.70 (CH-7'), 122.40 (CH-6'), 119.00 (C-5'), 112.45 (CH-3,5), 95.20 (CH-3'), 62.95 (CH_2 -12'), 61.92 (CH_2 -8), 55.32 (CH_2 -13'), 47.10 (CH_2 -14), 42.88 (CH_3 -14'), 38.47 (CH_2 -15). **FTIR** (KBr, cm^{-1}) ν_{max} : 1682, 1595, 1535, 1505, 1470, 1454,

1417, 1390, 1359, 1330, 1203, 1185, 1130, 1038, 1021, 1000, 884, 834, 721, 683. **HRMS** (ESI+) calcd. for $C_{38}H_{44}O_5N_{11}^+$ ($M+H^+$): 734.35214, found: 734.35226. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.09.

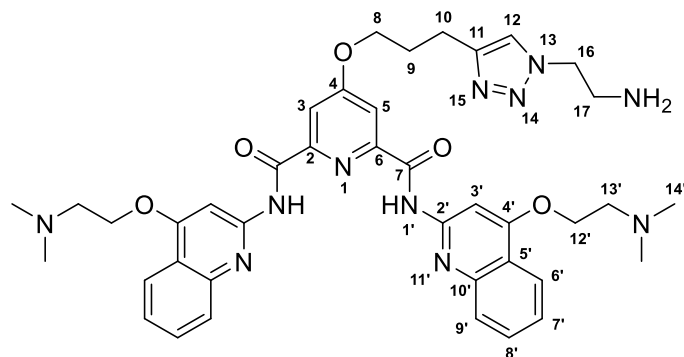
4-(2-(1-(2-Aminoethyl)-1*H*-1,2,3-triazol-4-yl)ethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (97).



Reactants: **59** (0.039 g, 0.06 mmol), **94** (22 μ L, 0.12 mmol), $CuSO_4$ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.022 g, yield 48 %). **¹H NMR** (500 MHz, $DMSO-d_6$): δ 12.08 (s, 2H, NH), 8.34 (dd, 2H, $J(6',7') = 8.3$ Hz, $J(6',8') = 1.5$ Hz, H-6'), 8.15 (s, 2H, H-3'), 8.12 (s, 1H, H-11), 7.96 (dd, 2H, $J(9',8') = 8.4$ Hz, $J(9',7') = 1.2$ Hz,

H-9'), 7.94 (s, 2H, H-3,5), 7.81 (ddd, 2H, $J(8',9') = 8.4$ Hz, $J(8',7') = 6.8$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.55 (ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.67 (m, 4H, H-12'), 4.56-4.64 (m, 4H, H-15, H-8), 3.76 (m, 4H, H-13'), 3.32-3.40 (m, 2H, H-16, signal overlapped by H_2O peak), 3.24 (t, 2H, $J(9,8) = 6.4$ Hz, H-9), 2.98 (s, 12H, H-14'); NH_2 signal not detected. **¹³C NMR** (125.7 MHz, $DMSO-d_6$): δ 167.12 (C-4), 163.32 (C-7), 161.49 (C-4'), 152.32 (C-2'), 151.11 (C-2,6), 147.06 (C-10'), 143.39 (C-10), 130.90 (CH-8'), 126.86 (CH-9'), 124.78 (CH-7'), 123.66 (CH-11), 122.43 (CH-6'), 119.04 (C-5'), 112.17 (CH-3,5), 95.27 (CH-3'), 67.69 (CH_2 -8), 62.99 (CH_2 -12'), 55.39 (CH_2 -13'), 46.90 (CH_2 -15), 42.95 (CH_3 -14'), 38.59 (CH_2 -16), 25.14 (CH_2 -9). **FTIR** (KBr, cm^{-1}) ν_{max} : 1687, 1596, 1531, 1505, 1471, 1454, 1418, 1399, 1354, 1328, 1203, 1181, 1130, 1048, 1026, 999, 883, 721, 684. **HRMS** (ESI+) calcd. for $C_{39}H_{46}O_5N_{11}^+$ ($M+H^+$): 748.36779, found: 748.36787. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.11.

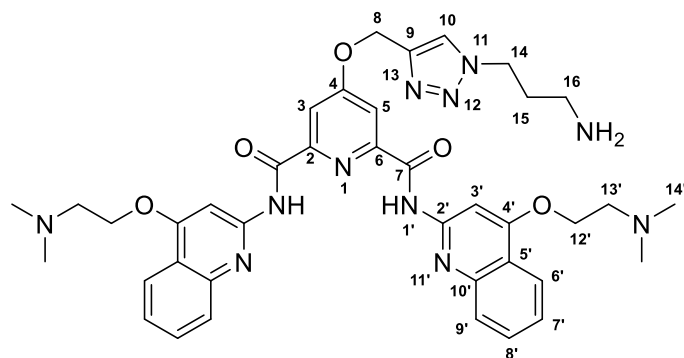
4-(3-(1-(2-Aminoethyl)-1*H*-1,2,3-triazol-4-yl)propoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (98).



Reactants: **60** (0.039 g, 0.06 mmol), **94** (22 μ L, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.033 g, yield 71 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.08 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.15 (s, 2H, H-3'), 8.01 (s, 1H, H-12), 7.96

(dd, 2H, *J*(9',8') = 8.3 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.93 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.8 Hz, *J*(7',9') = 1.2 Hz, H-7'), 4.67 (m, 4H, H-12'), 4.59 (t, 2H, *J*(16,17) = 6.1 Hz, H-16), 4.39 (t, 2H, *J*(8,9) = 6.8 Hz, H-8), 3.75 (m, 4H, H-13'), 3.30-3.38 (m, 2H, H-17, signal overlapped by H₂O peak), 2.98 (s, 12H, H-14'), 2.87 (t, 2H, *J*(10,9) = 7.5 Hz, H-10), 2.18 (p, 2H, *J*(9,8) = *J*(9,10) = 7.2 Hz, H-9); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 167.33 (C-4), 163.35 (C-7), 161.49 (C-4'), 152.34 (C-2'), 151.10 (C-2,6), 147.06 (C-10'), 146.49 (C-11), 130.90 (CH-8'), 126.87 (CH-9'), 124.78 (CH-7'), 122.82 (CH-12), 122.44 (CH-6'), 119.04 (C-5'), 112.14 (CH-3,5), 95.30 (CH-3'), 68.12 (CH₂-8), 63.00 (CH₂-12'), 55.40 (CH₂-13'), 46.84 (CH₂-16), 42.96 (CH₃-14'), 38.59 (CH₂-17), 28.08 (CH₂-9), 21.46 (CH₂-10). FTIR (KBr, cm⁻¹) ν_{max} : 1682, 1596, 1532, 1504, 1470, 1454, 1417, 1391, 1359, 1328, 1203, 1181, 1044, 1000, 883, 833, 721, 684, 625. HRMS (ESI⁺) calcd. for C₄₀H₄₈O₅N₁₁⁺ (M+H⁺): 762.38344, found: 762.38369. *R*_f (9/9/2 EtOAc/MeOH/TEA) = 0.10.

4-((1-(3-Aminopropyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (99).

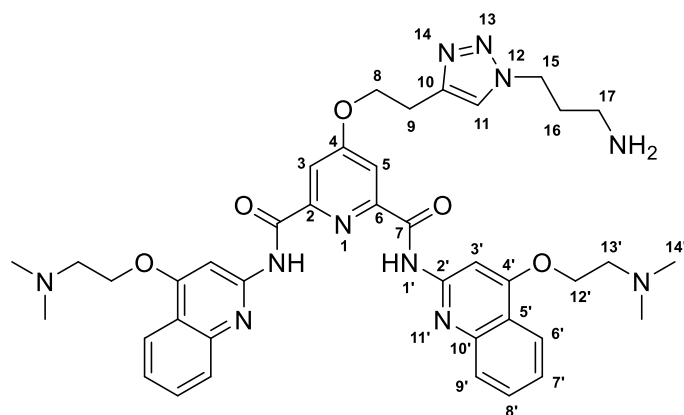


Reactants: **58** (0.039 g, 0.06 mmol), **95** (24 μ L, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.029 g, yield 65 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.32-8.37 (m, 3H, H-10, H-6'), 8.18 (s, 2H, H-3'), 8.07 (s, 2H, H-3,5), 7.96 (dd, 2H, *J*(9',8') = 8.3 Hz,

J(9',7') = 1.2 Hz, H-9'), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56

(ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 5.53 (s, 2H, H-8), 4.69 (m, 4H, H-12'), 4.51 (t, 2H, $J(14,15) = 7.0$ Hz, H-14), 3.76 (m, 4H, H-13'), 2.98 (s, 12H, H-14'), 2.84 (m, 2H, H-16), 2.14 (p, $J(15,14) = J(15,16) = 7.2$ Hz, H-15); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 166.80 (C-4), 163.25 (C-7), 161.44 (C-4'), 152.31 (C-2'), 151.09 (C-2,6), 147.02 (C-10'), 141.56 (C-9), 130.83 (CH-8'), 126.80 (CH-9'), 125.32 (CH-10), 124.68 (CH-7'), 122.39 (CH-6'), 118.99 (C-5'), 112.43 (CH-3,5), 95.21 (CH-3'), 62.94 (CH₂-12'), 61.98 (CH₂-8), 55.32 (CH₂-13'), 46.71 (CH₂-14), 42.88 (CH₃-14'), 36.37 (CH₂-16), 27.83 (CH₂-15). **FTIR** (KBr, cm⁻¹) ν_{max} : 3431, 1682, 1595, 1532, 1505, 1469, 1454, 1416, 1390, 1359, 1203, 1130, 1000, 883, 834, 721, 683, 642. **HRMS** (ESI+) calcd. for C₃₉H₄₆O₅N₁₁⁺ (M+H⁺): 748.36779, found: 748.36815. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.06.

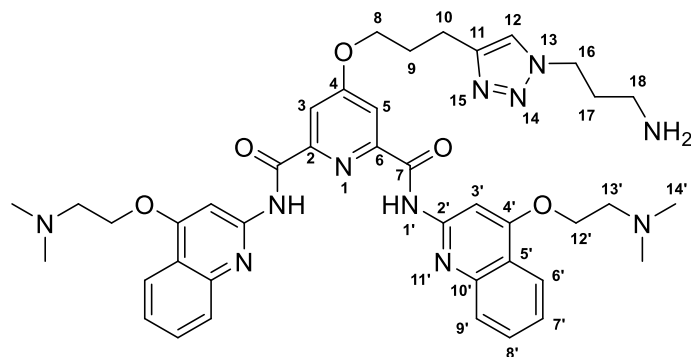
4-(2-(1-(3-Aminopropyl)-1*H*-1,2,3-triazol-4-yl)ethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (100).



Reactants: **59** (0.039 g, 0.06 mmol), **95** (24 μL, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.029 g, yield 64 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.09 (s, 2H, NH), 8.34 (dd, 2H, $J(6',7') = 8.4$ Hz, $J(6',8') = 1.5$ Hz, H-6'), 8.16 (s, 2H, H-3'), 8.07 (s, 1H, H-11), 7.96 (dd, 2H, $J(9',8') = 8.4$ Hz, $J(9',7') = 1.2$ Hz,

H-9'), 7.94 (s, 2H, H-3,5), 7.81 (ddd, 2H, $J(8',9') = 8.4$ Hz, $J(8',7') = 6.8$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.56 (ddd, 2H, $J(7',6') = 8.4$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 4.68 (m, 4H, H-12'), 4.59 (t, 2H, $J(8,9) = 6.5$ Hz, H-8), 4.46 (t, 2H, $J(15,16) = 7.0$ Hz, H-15), 3.76 (m, 4H, H-13'), 3.23 (t, 2H, $J(9,8) = 6.5$ Hz, H-9), 2.98 (s, 12H, H-14'), 2.83 (m, 2H, H-17), 2.11 (p, $J(16,15) = J(16,17) = 7.3$ Hz, H-16); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 167.06 (C-4), 163.28 (C-7), 161.43 (C-4'), 152.30 (C-2'), 151.08 (C-2,6), 147.02 (C-10'), 143.40 (C-10), 130.82 (CH-8'), 126.81 (CH-9'), 124.68 (CH-7'), 123.58 (CH-11, signal determined by HSQC), 122.39 (CH-6'), 118.99 (C-5'), 112.12 (CH-3,5), 95.23 (CH-3'), 67.68 (CH₂-8), 62.96 (CH₂-12'), 55.33 (CH₂-13'), 46.48 (CH₂-15), 42.89 (CH₃-14'), 36.40 (CH₂-17), 27.88 (CH₂-16), 25.08 (CH₂-9). **FTIR** (KBr, cm⁻¹) ν_{max} : 3430, 1686, 1596, 1534, 1505, 1473, 1454, 1417, 1392, 1352, 1203, 1130, 1044, 998, 882, 833, 721, 683. **HRMS** (ESI+) calcd. for C₄₀H₄₈O₅N₁₁⁺ (M+H⁺): 762.38344, found: 762.38331. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.06.

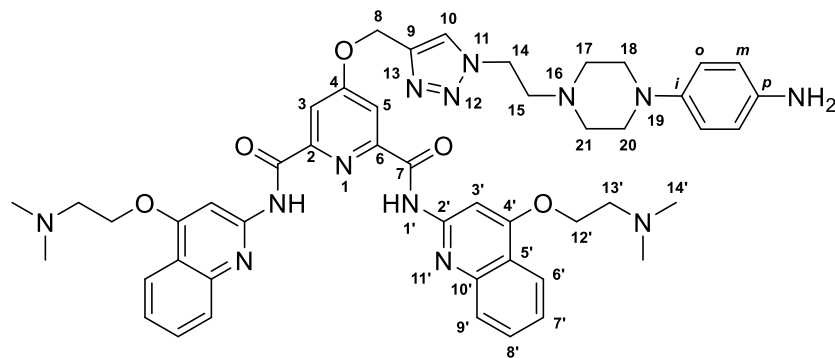
4-(3-(1-(3-Aminopropyl)-1*H*-1,2,3-triazol-4-yl)propoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (101).



Reactants: **60** (0.039 g, 0.06 mmol), **95** (24 μ L, 0.12 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.033 g, yield 70 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.16 (s, 2H, H-3'), 7.94-7.99 (m, 3H, H-12,

H-9'), 7.93 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.8 Hz, *J*(7',9') = 1.2 Hz, H-7'), 4.68 (m, 4H, H-12'), 4.43 (t, 2H, *J*(16,15) = 6.8 Hz, H-16), 4.39 (t, 2H, *J*(8,9) = 6.5 Hz, H-8), 3.76 (m, 4H, H-13'), 2.99 (s, 12H, H-14'), 2.77-2.90 (m, 4H, H-10, H-18), 2.17 (p, 2H, *J*(9,8) = *J*(9,10) = 6.6 Hz, H-9), 2.10 (p, 2H, *J*(17,16) = *J*(17,18) = 7.1 Hz, H-17); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 167.26 (C-4), 163.31 (C-7), 161.43 (C-4'), 152.31 (C-2'), 151.07 (C-2,6), 147.02 (C-10'), 146.46 (C-11), 130.82 (CH-8'), 126.81 (CH-9'), 124.70 (CH-7'), 122.38 (CH-6'), 122.34 (CH-12), 119.00 (C-5'), 112.08 (CH-3,5), 95.26 (CH-3'), 68.12 (CH₂-8), 62.97 (CH₂-12'), 55.32 (CH₂-13'), 46.42 (CH₂-16), 42.90 (CH₃-14'), 36.40 (CH₂-18), 28.13 (CH₂-9), 27.85 (CH₂-17), 21.45 (CH₂-10). FTIR (KBr, cm⁻¹) ν_{max} : 3433, 1683, 1596, 1527, 1504, 1471, 1454, 1416, 1359, 1203, 1130, 1041, 998, 879, 834, 721, 684. HRMS (ESI⁺) calcd. for C₄₁H₅₀O₅N₁₁⁺ (M+H⁺): 776.39909, found: 776.39895. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.06.

4-((1-(2-(4-(4-Aminophenyl)piperazin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (102).

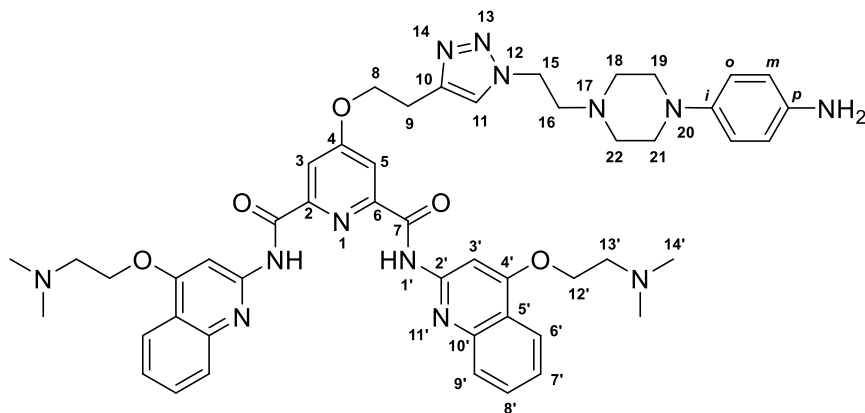


Reactants: **58** (0.039 g, 0.06 mmol), **92** (0.031 g, 0.09 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.019 g, yield 36 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH),

8.39 (s, 1H, H-10), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.18 (s, 2H, H-3'), 8.07 (s, 2H, H-3,5), 7.96 (d, 2H, *J*(9',8') = 8.3 Hz, H-9'), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') =

1.5 Hz, H-8'), 7.56 (ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.3$ Hz, H-7'), 6.96 (m, 4H, H-*o*-Ph, H-*m*-Ph), 5.54 (s, 2H, H-8), 4.71 (m, 2H, H-14), 4.68 (m, 4H, H-12'), 3.76 (m, 4H, H-13'), 3.35-3.46 (m, 8H, H-17,18,20,21), 3.20 (m, 2H, H-15), 2.98 (s, 12H, H-14'); NH₂ signal not detected. **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 166.80 (C-4), 163.26 (C-7), 161.45 (C-4'), 158.11 (C-*i*-Ph), 157.87 (C-*p*-Ph), 152.33 (C-2'), 151.08 (C-2,6), 147.02 (C-10'), 141.49 (C-9), 130.85 (CH-8'), 126.81 (CH-9'), 125.70 (CH-10), 124.70 (CH-7'), 122.41 (CH-6'), 119.00 (C-5'), 116.88 (CH-*o*-Ph, CH-*m*-Ph), 112.47 (CH-3,5), 95.21 (CH-3'), 62.94 (CH₂-12'), 62.01 (CH₂-8), 55.33 (CH₂-13'), 52.62 (CH₂-15), 45.48 (CH₂-14), 42.88 (CH₃-14'); CH₂-17,21 and CH₂-18,20 signals not detected. **FTIR** (KBr, cm⁻¹) ν_{max}: 3430, 3150, 2829, 1686, 1595, 1570, 1525, 1505, 1465, 1454, 1415, 1359, 1323, 1202, 1129, 999, 833, 721, 683, 624. **HRMS** (ESI⁺) calcd. for C₄₈H₅₆O₅N₁₃⁺ (M+H⁺): 894.45219, found: 894.45160. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.20.

4-(2-(1-(2-(4-(4-Aminophenyl)piperazin-1-yl)ethyl)-1*H*-1,2,3-triazol-4-yl)ethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (103).

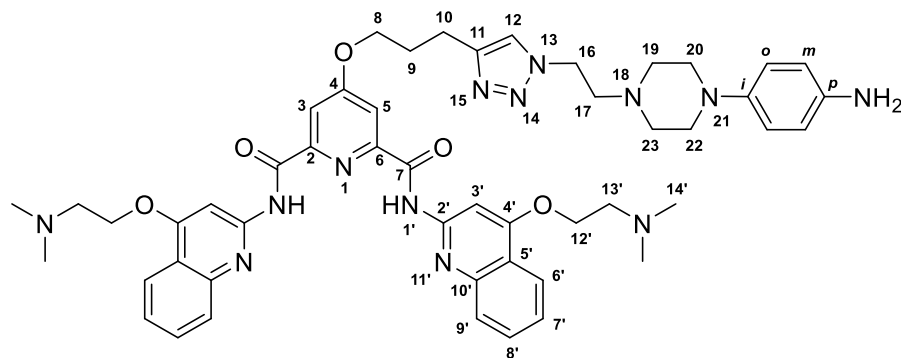


Reactants: **59** (0.039 g, 0.06 mmol), **92** (0.031 g, 0.09 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.018 g, yield 33 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.34

(dd, 2H, $J(6',7') = 8.3$ Hz, $J(6',8') = 1.5$ Hz, H-6'), 8.16 (s, 2H, H-3'), 8.12 (s, 1H, H-11), 7.96 (dd, 2H, $J(9',8') = 8.3$ Hz, $J(9',7') = 1.2$ Hz, H-9'), 7.94 (s, 2H, H-3,5), 7.81 (ddd, 2H, $J(8',9') = 8.3$ Hz, $J(8',7') = 6.8$ Hz, $J(8',6') = 1.5$ Hz, H-8'), 7.56 (ddd, 2H, $J(7',6') = 8.3$ Hz, $J(7',8') = 6.8$ Hz, $J(7',9') = 1.2$ Hz, H-7'), 6.93 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.67 (m, 4H, H-12'), 4.60 (t, 2H, $J(8,9) = 6.3$ Hz, H-8), 3.76 (m, 4H, H-13'), 3.38-3.52 (m, 8H, H-18,19,21,22), 3.14-3.30 (m, 6H, H-9, H-15, H-16), 2.99 (s, 12H, H-14'); NH₂ signal not detected. **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.07 (C-4), 163.29 (C-7), 161.43 (C-4'), 158.01 (C-*i*-Ph), 157.77 (C-*p*-Ph), 152.31 (C-2'), 151.08 (C-2,6), 147.02 (C-10'), 143.03 (C-10), 130.85 (CH-8'), 126.82 (CH-9'), 124.71 (CH-7'), 123.51 (CH-11), 122.40 (CH-6'), 118.99 (C-5'), 117.03 (CH-*o*-Ph, CH-*m*-Ph), 112.13 (CH-3,5), 95.24 (CH-3'), 67.69 (CH₂-8), 62.93 (CH₂-12'), 55.34 (CH₂-13'), 52.74 (CH₂-16), 45.73 (CH₂-15), 42.90 (CH₃-14'), 25.11 (CH₂-9); CH₂-18,22 and CH₂-19,21 signals not detected. **FTIR** (KBr, cm⁻¹) ν_{max}: 3355, 3133, 2837, 1682, 1596, 1570, 1530, 1505, 1470, 1453, 1418,

1329, 1203, 1130, 1050, 999, 836, 722, 684, 625. **HRMS** (ESI⁺) calcd. for C₄₉H₅₈O₅N₁₃⁺ (M+H⁺): 908.46784, found: 908.46745. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.22.

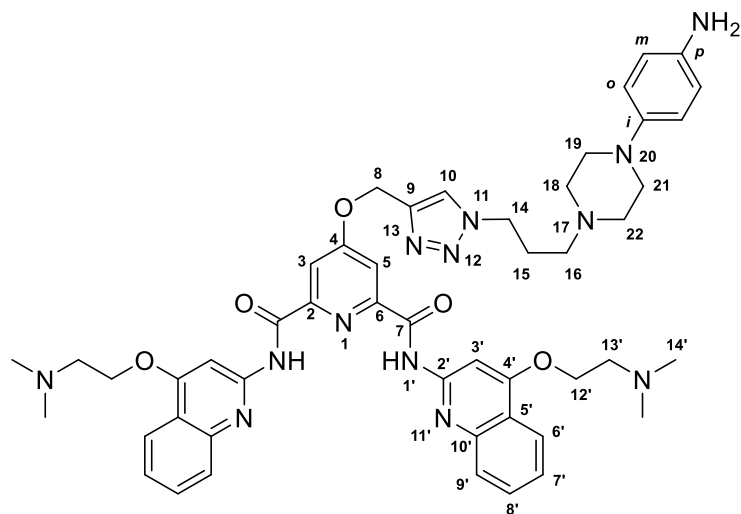
4-(3-(1-(2-(4-(4-Aminophenyl)piperazin-1-yl)ethyl)-1H-1,2,3-triazol-4-yl)propoxy)-N²,N⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (104).



Reactants: **60** (0.039 g, 0.06 mmol), **92** (0.031 g, 0.09 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.016 g, yield 29 %). **¹H NMR**

(500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.16 (s, 2H, H-3'), 8.01 (s, 1H, H-12), 7.96 (d, 2H, *J*(9',8') = 8.3 Hz, H-9'), 7.92 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.8 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.92 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.61-4.72 (m, 6H, H-12', H-16), 4.38 (t, 2H, *J*(8,9) = 6.5 Hz, H-8), 3.76 (m, 4H, H-13'), 3.39-3.53 (m, 8H, H-19,20,22,23), 3.18 (m, 2H, H-17), 2.98 (s, 12H, H-14'), 2.86 (t, 2H, *J*(10,9) = 7.5 Hz, H-10), 2.17 (p, 2H, *J*(9,8) = *J*(9,10) = 6.9 Hz, H-9); NH₂ signal not detected. **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.27 (C-4), 163.32 (C-7), 161.43 (C-4'), 158.01 (C-*i*-Ph), 157.75 (C-*p*-Ph), 152.33 (C-2'), 151.07 (C-2,6), 147.02 (C-10'), 130.85 (CH-8'), 126.83 (CH-9'), 124.71 (CH-7'), 122.40 (CH-6'), 118.99 (C-5'), 117.42 (CH-*o*-Ph, CH-*m*-Ph), 112.07 (CH-3,5), 95.26 (CH-3'), 68.11 (CH₂-8), 62.94 (CH₂-12'), 55.35 (CH₂-13'), 45.75 (CH₂-16), 42.91 (CH₃-14'), 28.12 (CH₂-9), 21.47 (CH₂-10); C-11, CH-12, CH₂-17, CH₂-19,23, and CH₂-20,22 signals not detected. **FTIR** (KBr, cm⁻¹) ν_{max}: 3428, 3129, 2830, 1687, 1595, 1581, 1530, 1504, 1470, 1454, 1415, 1357, 1329, 1201, 1128, 1041, 998, 832, 721, 683, 624. **HRMS** (ESI⁺) calcd. for C₅₀H₆₀O₅N₁₃⁺ (M+H⁺): 922.48349, found: 922.48291. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.22.

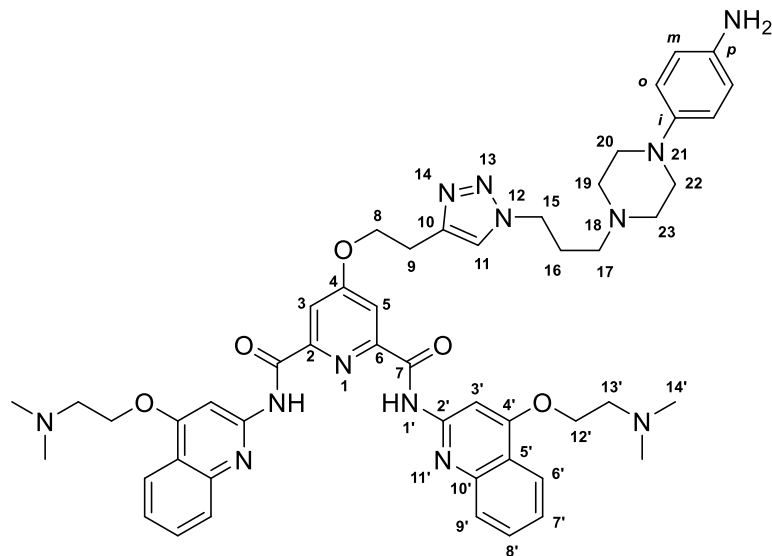
4-((1-(3-(4-(4-Aminophenyl)piperazin-1-yl)propyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (105).



Reactants: **58** (0.039 g, 0.06 mmol), **93** (0.033 g, 0.09 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.025 g, yield 45 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.39 (s, 1H, H-10), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.18 (s, 2H, H-3'), 8.07 (s, 2H, H-3,5), 7.96 (d, 2H, *J*(9',8') = 8.3 Hz, H-9'), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz,

J(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.8 Hz, *J*(7',9') = 1.3 Hz, H-7'), 6.92 (m, 4H, H-*o*-Ph, H-*m*-Ph), 5.54 (s, 2H, H-8), 4.68 (m, 4H, H-12'), 4.52 (t, 2H, *J*(14,15) = 6.8 Hz, H-14), 3.76 (m, 4H, H-13'), 3.36-3.48 (m, 8H, H-18,19,21,22), 3.17 (m, 2H, H-16), 2.98 (s, 12H, H-14'), 2.30 (p, 2H, *J*(15,14) = *J*(15,16) = 7.3 Hz, H-15); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 166.80 (C-4), 163.27 (C-7), 161.46 (C-4'), 158.14 (C-*i*-Ph), 157.89 (C-*p*-Ph), 152.33 (C-2'), 151.09 (C-2,6), 147.02 (C-10'), 141.58 (C-9), 130.86 (CH-8'), 126.82 (CH-9'), 125.35 (CH-10), 124.72 (CH-7'), 122.42 (CH-6'), 119.00 (C-5'), 117.40 (CH-*o*-Ph, CH-*m*-Ph), 112.47 (CH-3,5), 95.21 (CH-3'), 62.94 (CH₂-12'), 62.03 (CH₂-8), 55.34 (CH₂-13'), 52.99 (CH₂-16), 46.95 (CH₂-14), 42.89 (CH₃-14'), 24.48 (CH₂-15); CH₂-18,22 and CH₂-19,21 signals not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3350, 3307, 3130, 2834, 1684, 1595, 1525, 1506, 1470, 1454, 1415, 1359, 1331, 1202, 1128, 992, 884, 833, 721, 683. HRMS (ESI⁺) calcd. for C₄₉H₅₈O₅N₁₃⁺ (M+H⁺): 908.46784, found: 908.46773. R_f (9/9/2 EtOAc/MeOH/TEA) = 0.20.

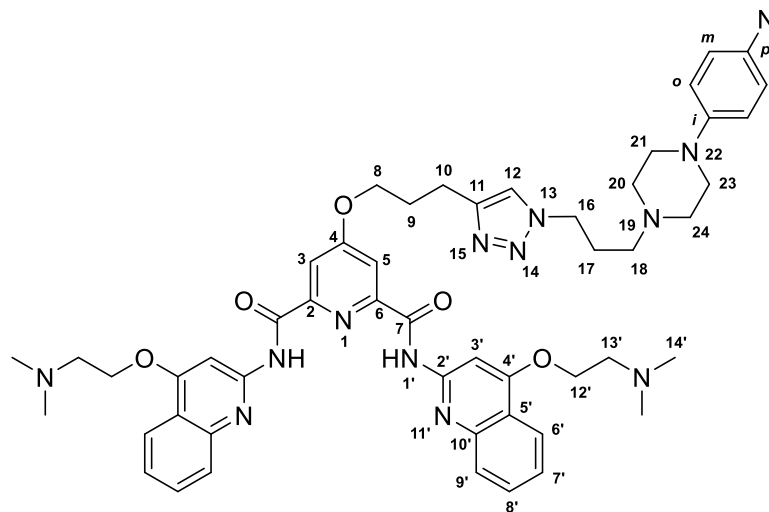
4-(2-(1-(3-(4-(4-Aminophenyl)piperazin-1-yl)propyl)-1*H*-1,2,3-triazol-4-yl)ethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (106).



Reactants: **59** (0.039 g, 0.06 mmol), **93** (0.033 g, 0.09 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.022 g, yield 39 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.15 (s, 2H, H-3'), 8.10 (s, 1H, H-11), 7.93-7.98 (m, 4H, H-9', H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz,

H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.8 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.92 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.67 (m, 4H, H-12'), 4.59 (t, 2H, *J*(8,9) = 6.2 Hz, H-8), 4.47 (t, 2H, *J*(15,16) = 6.8 Hz, H-15), 3.75 (m, 4H, H-13'), 3.37-3.52 (m, 8H, H-19,20,22,23), 3.23 (t, 2H, *J*(9,8) = 6.2 Hz, H-9), 3.15 (t, 2H, *J*(17,16) = 7.6 Hz, H-17), 2.98 (s, 12H, H-14'), 2.27 (p, 2H, *J*(16,15) = *J*(16,17) = 7.3 Hz, H-16); NH₂ signal not detected. **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 167.08 (C-4), 163.30 (C-7), 161.44 (C-4'), 157.96 (C-*i*-Ph), 157.74 (C-*p*-Ph), 152.31 (C-2'), 151.09 (C-2,6), 147.02 (C-10'), 143.00 (C-10, signal determined by HMBC), 130.86 (CH-8'), 126.82 (CH-9'), 124.72 (CH-7'), 123.21 (CH-11), 122.41 (CH-6'), 119.00 (C-5'), 117.33 (CH-*o*-Ph, CH-*m*-Ph), 112.13 (CH-3,5), 95.23 (CH-3'), 67.68 (CH₂-8), 62.92 (CH₂-12'), 55.33 (CH₂-13'), 53.03 (CH₂-17), 46.71 (CH₂-15), 42.89 (CH₃-14'), 25.12 (CH₂-9), 24.45 (CH₂-16); CH₂-19,23 and CH₂-20,22 signals not detected. **FTIR** (KBr, cm⁻¹) ν_{max}: 3364, 3132, 1684, 1596, 1525, 1506, 1470, 1454, 1417, 1357, 1329, 1202, 1129, 1050, 999, 882, 834, 721, 684. **HRMS** (ESI+) calcd. for C₅₀H₆₀O₅N₁₃⁺ (M+H⁺): 922.48349, found: 922.48287. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.20.

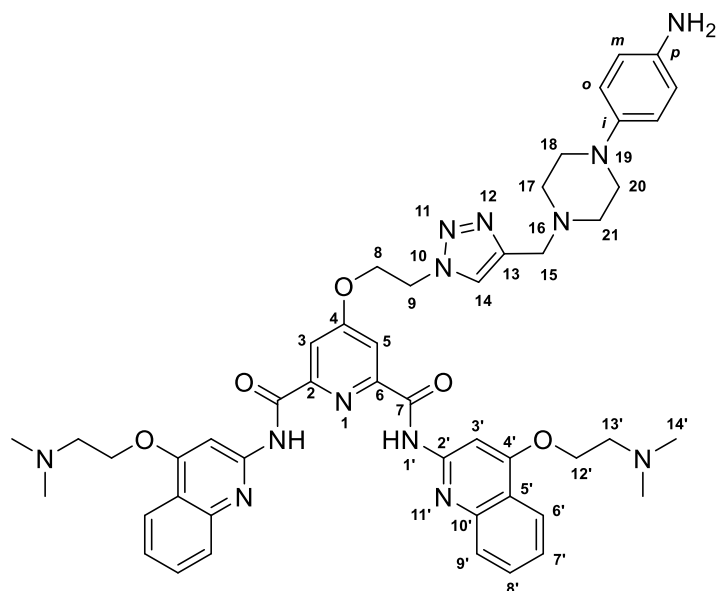
4-(3-(1-(3-(4-(4-Aminophenyl)piperazin-1-yl)propyl)-1*H*-1,2,3-triazol-4-yl)propoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (107).



Reactants: **60** (0.039 g, 0.06 mmol), **93** (0.033 g, 0.09 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.024 g, yield 42 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.16 (s, 2H, H-3'), 8.00 (s, 1H, H-12), 7.96 (d, 2H, *J*(9',8')

= 8.3 Hz, H-9'), 7.93 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.8 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.89 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.68 (m, 4H, H-12'), 4.44 (t, 2H, *J*(16,17) = 6.0 Hz, H-16), 4.39 (t, 2H, *J*(8,9) = 6.6 Hz, H-8), 3.76 (m, 4H, H-13'), 3.38-3.52 (m, 8H, H-20,21,23,24), 3.13 (m, 2H, H-18), 2.98 (s, 12H, H-14'), 2.86 (t, 2H, *J*(10,9) = 7.4 Hz, H-10), 2.26 (m, 2H, H-17), 2.18 (p, 2H, *J*(9,8) = *J*(9,10) = 7.0 Hz, H-9); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 167.28 (C-4), 163.32 (C-7), 161.44 (C-4'), 158.10 (C-*i*-Ph), 157.85 (C-*p*-Ph), 152.32 (C-2'), 151.07 (C-2,6), 147.03 (C-10'), 130.85 (CH-8'), 126.82 (CH-9'), 124.71 (CH-7'), 123.30 (CH-12), 122.41 (CH-6'), 119.00 (C-5'), 117.48 (CH-*o*-Ph, CH-*m*-Ph), 112.08 (CH-3,5), 95.25 (CH-3'), 68.13 (CH₂-8), 62.94 (CH₂-12'), 55.33 (CH₂-13'), 53.03 (CH₂-18), 46.65 (CH₂-16), 42.89 (CH₃-14'), 28.13 (CH₂-9), 24.51 (CH₂-17), 21.49 (CH₂-10); C-11, CH₂-20,24, and CH₂-21,23 signals not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3430, 3130, 2833, 1687, 1596, 1525, 1506, 1470, 1454, 1416, 1359, 1328, 1203, 1128, 1042, 998, 880, 832, 721, 684, 623. HRMS (ESI⁺) calcd. for C₅₁H₆₂O₅N₁₃⁺ (M+H⁺): 936.49914, found: 936.49841. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.18.

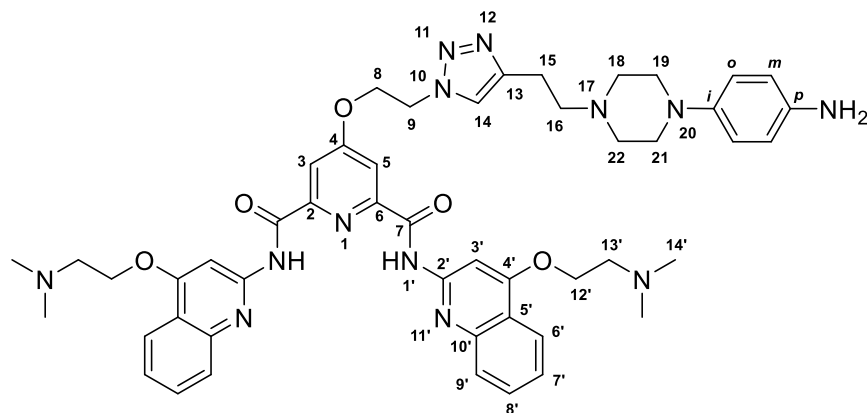
4-(2-(4-((4-(4-Aminophenyl)piperazin-1-yl)methyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (108).



Reactants: **89** (0.038 g, 0.12 mmol), **61** (0.041 g, 0.06 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.026 g, yield 48 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.42 (s, 1H, H-14), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.14 (s, 2H, H-3'), 7.96 (dd, 2H, *J*(9',8') = 8.3 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.92 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-

8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.89 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.96 (m, 2H, H-9), 4.81 (m, 2H, H-8), 4.66 (m, 4H, H-12'), 3.75 (m, 4H, H-13'), 3.37-3.47 (m, 8H, H-17,18,20,21), 3.24 (m, 2H, H-15), 2.98 (s, 12H, H-14'); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 166.62 (C-4), 163.19 (C-7), 161.45 (C-4'), 158.13 (C-*i*-Ph), 157.88 (C-*p*-Ph), 152.27 (C-2'), 151.15 (C-2,6), 147.01 (C-10'), 145.56 (C-13), 130.87 (CH-8'), 126.82 (CH-9'), 126.62 (CH-14), 124.74 (CH-7'), 122.42 (CH-6'), 119.00 (C-5'), 117.41 (CH-*o*-Ph, CH-*m*-Ph), 112.19 (CH-3,5), 95.22 (CH-3'), 67.12 (CH₂-8), 62.92 (CH₂-12'), 55.32 (CH₂-13'), 50.76 (CH₂-15), 48.88 (CH₂-9), 42.88 (CH₃-14'); CH₂-17,21 and CH₂-18,20 signals not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3416, 3356, 3100, 2833, 1688, 1595, 1580, 1530, 1506, 1460, 1416, 1328, 1202, 1127, 1048, 999, 886, 832, 721, 683, 625. HRMS (ESI⁺) calcd. for C₄₈H₅₆O₅N₁₃⁺ (M+H⁺): 894.45219, found: 894.45099. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.20.

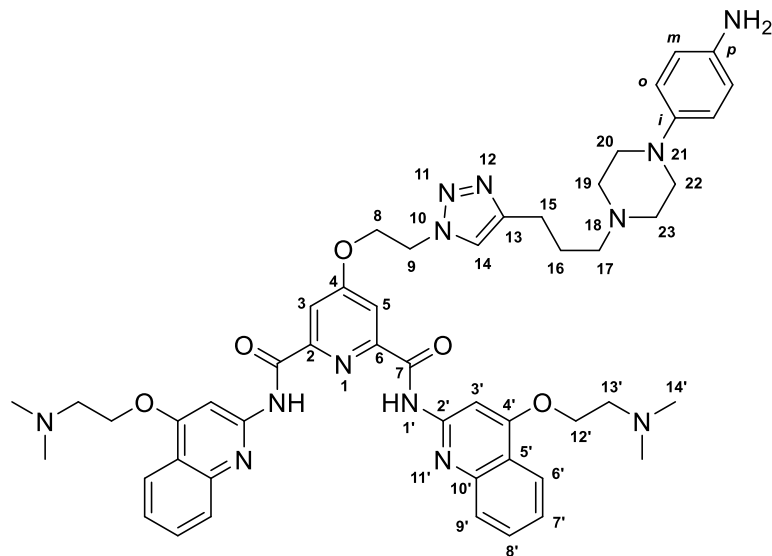
4-(2-(4-(2-(4-(4-Aminophenyl)piperazin-1-yl)ethyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (109).



Reactants: **90** (0.040 g, 0.12 mmol), **61** (0.041 g, 0.06 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.029 g, yield 53 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.34

(dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.18 (s, 1H, H-14), 8.14 (s, 2H, H-3'), 7.96 (dd, 2H, *J*(9',8') = 8.3 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.92 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.92 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.89 (m, 2H, H-9), 4.78 (m, 2H, H-8), 4.67 (m, 4H, H-12'), 3.76 (m, 4H, H-13'), 3.39-3.51 (m, 10H, H-16, H-18,19,21,22), 3.13 (t, 2H, *J*(15,16) = 7.7 Hz, H-15), 2.98 (s, 12H, H-14'); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 166.63 (C-4), 163.20 (C-7), 161.45 (C-4'), 158.14 (C-*i*-Ph), 157.87 (C-*p*-Ph), 152.28 (C-2'), 151.13 (C-2,6), 147.02 (C-10'), 143.69 (C-13), 130.86 (CH-8'), 126.82 (CH-14), 124.73 (CH-7'), 123.47 (CH-14), 122.41 (CH-6'), 119.00 (C-5'), 117.47 (CH-*m*-Ph), 117.41 (CH-*o*-Ph), 112.19 (CH-3,5), 95.24 (CH-3'), 67.20 (CH₂-8), 62.93 (CH₂-12'), 55.31 (CH₂-13'), 54.54 (CH₂-16), 48.67 (CH₂-9), 42.88 (CH₃-14'), 20.23 (CH₂-15); CH₂-18,22 and CH₂-19,21 signals not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3354, 3100, 2832, 1685, 1595, 1585, 1523, 1506, 1465, 1453, 1418, 1360, 1327, 1202, 1130, 1048, 1027, 999, 832, 721, 684, 625. HRMS (ESI⁺) calcd. for C₄₉H₅₈O₅N₁₃⁺ (M+H⁺): 908.46784, found: 908.46790. *R*_f (9/9/2 EtOAc/MeOH/TEA) = 0.15.

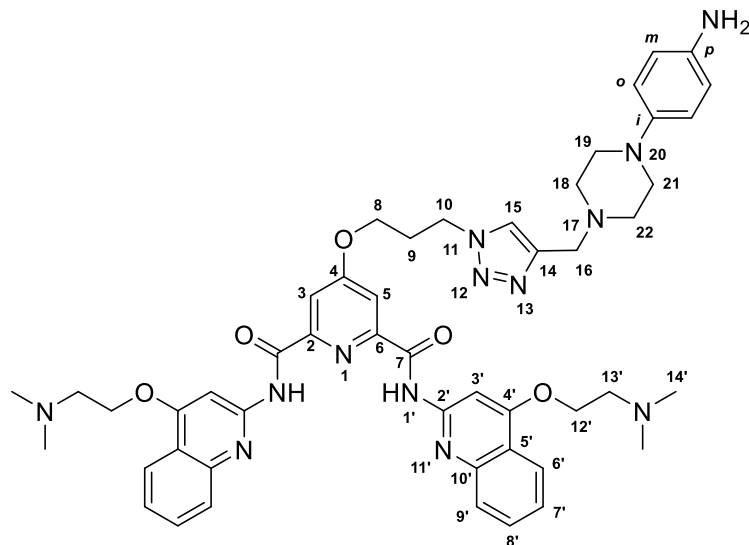
4-(2-(4-(3-(4-(4-Aminophenyl)piperazin-1-yl)propyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (110).



Reactants: **91** (0.041 g, 0.12 mmol), **61** (0.041 g, 0.06 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.024 g, yield 43 %). **¹H NMR** (500 MHz, DMSO-*d*₆): δ 12.10 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.14 (s, 2H, H-3'), 8.09 (s, 1H, H-14), 7.96 (dd, 2H, *J*(9',8') = 8.3 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.92 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9')

= 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.89 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.86 (t, 2H, *J*(9,8) = 5.8 Hz, H-9), 4.77 (t, 2H, *J*(8,9) = 5.9 Hz, H-8), 4.67 (m, 4H, H-12'), 3.75 (m, 4H, H-13'), 3.37-3.50 (m, 8H, H-19,20,22,23), 3.20 (t, 2H, *J*(17,16) = 7.9 Hz, H-17), 2.98 (s, 12H, H-14'), 2.72 (t, 2H, *J*(15,16) = 7.3 Hz, H-15), 2.04 (p, 2H, *J*(16,15) = *J*(16,17) = 7.5 Hz, H-16); NH₂ signal not detected. **¹³C NMR** (125.7 MHz, DMSO-*d*₆): δ 166.66 (C-4), 163.20 (C-7), 161.45 (C-4'), 158.17 (C-*i*-Ph), 157.91 (C-*p*-Ph), 152.28 (C-2'), 151.12 (C-2,6), 147.01 (C-10'), 130.86 (CH-8'), 126.81 (CH-14), 124.73 (CH-7'), 122.41 (CH-6'), 119.00 (C-5'), 117.47 (CH-*m*-Ph), 117.21 (CH-*o*-Ph), 112.18 (CH-3,5), 95.22 (CH-3'), 67.24 (CH₂-8), 62.92 (CH₂-12'), 55.30 (CH₂-13'), 55.14 (CH₂-17), 48.57 (CH₂-9), 42.87 (CH₃-14'), 23.23 (CH₂-16), 22.17 (CH₂-15); CH₂-19,23 and CH₂-20,22 signals not detected. **FTIR** (KBr, cm⁻¹) *v*_{max}: 3427, 3362, 3140, 2890, 2838, 1688, 1595, 1582, 1525, 1505, 1470, 1454, 1415, 1359, 1327, 1201, 1128, 1048, 1027, 998, 831, 721, 683, 625. **HRMS** (ESI⁺) calcd. for C₅₀H₆₀O₅N₁₃⁺ (M+H⁺): 922.48349, found: 922.48226. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.16.

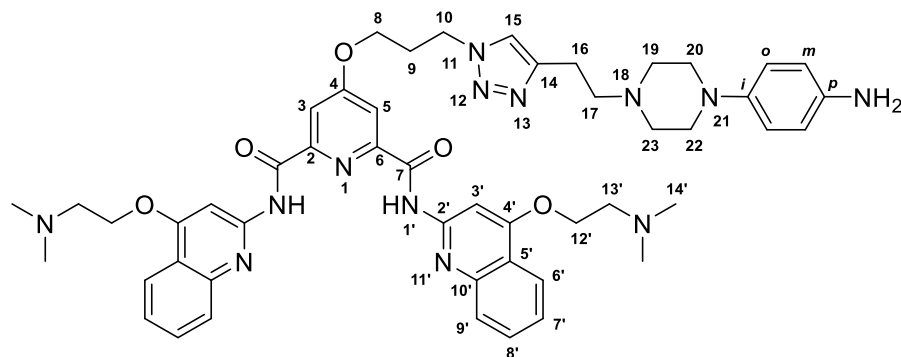
4-(3-(4-((4-(4-Aminophenyl)piperazin-1-yl)methyl)-1*H*-1,2,3-triazol-1-yl)propoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (111).



Reactants: **89** (0.038 g, 0.12 mmol), **62** (0.042 g, 0.06 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.037 g, yield 67 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.32-8.37 (m, 3H, H-15, H-6'), 8.15 (s, 2H, H-3'), 7.96 (dd, 2H, *J*(9',8') = 8.3 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.92 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9

Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.90 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.64-4.70 (m, 6H, H-12', H-10), 4.37 (t, 2H, *J*(8,9) = 5.8 Hz, H-8), 3.75 (m, 4H, H-13'), 3.38-3.49 (m, 8H, H-18,19,21,22), 3.24 (s, 2H, H-16), 2.98 (s, 12H, H-14'), 2.43 (p, 2H, *J*(9,8) = *J*(9,10) = 6.6 Hz, H-9); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 167.05 (C-4), 163.29 (C-7), 161.43 (C-4'), 158.00 (C-*i*-Ph), 157.74 (C-*p*-Ph), 152.29 (C-2'), 151.08 (C-2,6), 147.02 (C-10'), 145.21 (C-14), 130.86 (CH-8'), 126.82 (CH-9'), 126.47 (CH-15), 124.73 (CH-7'), 122.40 (CH-6'), 118.99 (C-5'), 117.40 (CH-*o*-Ph, CH-*m*-Ph), 112.09 (CH-3,5), 95.24 (CH-3'), 65.92 (CH₂-8), 62.90 (CH₂-12'), 55.35 (CH₂-13'), 50.73 (CH₂-16), 46.65 (CH₂-10), 42.91 (CH₃-14'), 29.07 (CH₂-9); CH₂-18,22 and CH₂-19,21 signals not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3355, 3100, 2833, 1685, 1595, 1580, 1530, 1505, 1470, 1454, 1416, 1358, 1328, 1201, 1126, 1045, 1025, 999, 833, 721, 684, 625. HRMS (ESI+) calcd. for C₄₉H₅₈O₅N₁₃⁺ (M+H⁺): 908.46784, found: 908.46681. **R_f** (9/9/2 EtOAc/MeOH/TEA) = 0.19.

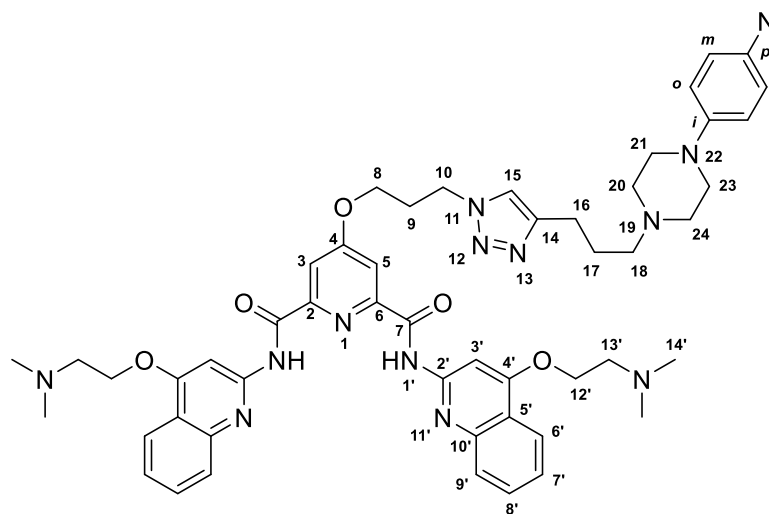
4-(3-(4-(2-(4-(4-Aminophenyl)piperazin-1-yl)ethyl)-1*H*-1,2,3-triazol-1-yl)propoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (112).



Reactants: **90** (0.040 g, 0.12 mmol), **62** (0.042 g, 0.06 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.034 g, yield 62 %). ¹H NMR

(500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.34 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.15 (s, 2H, H-3'), 8.10 (s, 1H, H-15), 7.96 (dd, 2H, *J*(9',8') = 8.3 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.90 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.4 Hz, *J*(8',7') = 6.9 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.9 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.89 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.67 (m, 4H, H-12'), 4.60 (t, 2H, *J*(10,9) = 6.9 Hz, H-10), 4.35 (t, 2H, *J*(8,9) = 6.1 Hz, H-8), 3.75 (m, 4H, H-13'), 3.37-3.52 (m, 10H, H-17, H-19,20,22,23), 3.12 (t, 2H, *J*(16,17) = 7.8 Hz, H-16), 2.98 (s, 12H, H-14'), 2.39 (p, 2H, *J*(9,8) = *J*(9,10) = 6.5 Hz, H-9); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 167.04 (C-4), 163.29 (C-7), 161.44 (C-4'), 157.99 (C-*i*-Ph), 157.70 (C-*p*-Ph), 152.30 (C-2'), 151.06 (C-2,6), 147.02 (C-10'), 142.31 (C-14), 130.86 (CH-8'), 126.82 (CH-14), 124.73 (CH-7'), 123.01 (CH-15), 122.41 (CH-6'), 119.00 (C-5'), 117.72 (CH-*o*-Ph, CH-*m*-Ph), 112.08 (CH-3,5), 95.25 (CH-3'), 65.95 (CH₂-8), 62.92 (CH₂-12'), 55.34 (CH₂-13'), 54.63 (CH₂-17), 46.43 (CH₂-10), 42.90 (CH₃-14'), 29.11 (CH₂-9), 20.31 (CH₂-16); CH₂-19,23 and CH₂-20,22 signals not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3413, 3140, 2974, 2928, 2896, 1684, 1596, 1581, 1524, 1505, 1470, 1453, 1358, 1328, 1204, 1130, 1049, 998, 881, 835, 722, 683. HRMS (ESI+) calcd. for C₅₀H₆₀O₅N₁₃⁺ (M+H⁺): 922.48349, found: 922.48276. R_f (9/9/2 EtOAc/MeOH/TEA) = 0.17.

4-(3-(4-(3-(4-(4-Aminophenyl)piperazin-1-yl)propyl)-1*H*-1,2,3-triazol-1-yl)propoxy)-*N*²,*N*⁶-bis(4-(2-(dimethylamino)ethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide (113).



Reactants: **91** (0.041 g, 0.12 mmol), **62** (0.042 g, 0.06 mmol), CuSO₄ (100 mM solution, 0.120 mL), and Na-ascorbate (100 mM solution, 0.360 mL). Beige crystalline solid (0.028 g, yield 49 %). ¹H NMR (500 MHz, DMSO-*d*₆): δ 12.11 (s, 2H, NH), 8.35 (dd, 2H, *J*(6',7') = 8.3 Hz, *J*(6',8') = 1.5 Hz, H-6'), 8.15 (s, 2H, H-3'), 8.02 (s, 1H, H-15), 7.96 (dd, 2H, *J*(9',8')

= 8.3 Hz, *J*(9',7') = 1.2 Hz, H-9'), 7.91 (s, 2H, H-3,5), 7.81 (ddd, 2H, *J*(8',9') = 8.3 Hz, *J*(8',7') = 6.8 Hz, *J*(8',6') = 1.5 Hz, H-8'), 7.56 (ddd, 2H, *J*(7',6') = 8.3 Hz, *J*(7',8') = 6.8 Hz, *J*(7',9') = 1.2 Hz, H-7'), 6.90 (m, 4H, H-*o*-Ph, H-*m*-Ph), 4.68 (m, 4H, H-12'), 4.58 (t, 2H, *J*(10,9) = 6.9 Hz, H-10), 4.34 (t, 2H, *J*(8,9) = 6.1 Hz, H-8), 3.76 (m, 4H, H-13'), 3.37-3.47 (m, 8H, H-20,21,23,24), 3.20 (t, 2H, *J*(18,17) = 7.8 Hz, H-18), 2.98 (s, 12H, H-14'), 2.72 (t, 2H, *J*(16,17) = 7.3 Hz, H-16), 2.22 (p, 2H, *J*(9,8) = *J*(9,10) = 6.4 Hz, H-9), 2.04 (p, 2H, *J*(17,16) = *J*(17,18) = 7.6 Hz, H-17); NH₂ signal not detected. ¹³C NMR (125.7 MHz, DMSO-*d*₆): δ 167.52 (C-4), 163.75 (C-7), 161.90 (C-4'), 158.65 (C-*i*-Ph), 158.41 (C-*p*-Ph), 152.77 (C-2'), 151.53 (C-2,6), 147.49 (C-10'), 145.90 (C-14), 131.31 (CH-8'), 127.28 (CH-9'), 125.18 (CH-7'), 122.88 (CH-6'), 122.83 (CH-15), 119.46 (C-5'), 117.90 (CH-*o*-Ph, CH-*m*-Ph), 112.55 (CH-3,5), 95.72 (CH-3'), 66.43 (CH₂-8), 63.39 (CH₂-12'), 55.77 (CH₂-13'), 55.57 (CH₂-18), 46.75 (CH₂-10), 43.33 (CH₃-14'), 29.59 (CH₂-9), 23.70 (CH₂-17), 22.67 (CH₂-16); CH₂-20,24 and CH₂-21,23 signals not detected. FTIR (KBr, cm⁻¹) ν_{max}: 3434, 3140, 2891, 2836, 1685, 1595, 1582, 1525, 1505, 1470, 1454, 1418, 1358, 1328, 1202, 1127, 1044, 1026, 998, 881, 833, 721, 683, 621. HRMS (ESI⁺) calcd. for C₅₁H₆₂O₅N₁₃⁺ (M+H⁺): 936.49914, found: 936.49777. R_f (9/9/2 EtOAc/MeOH/TEA) = 0.18.

11.3 Biochemical evaluation

11.3.1 Control FRET experiments for the bis-amino series

The control FRET experiments were performed with the G4 non-forming FdsT oligonucleotide template at 25 μ M and 50 μ M concentration for all compounds of the bis-amino series. The data regarding the control measurements, which are displayed in Chart 9.3 (Results and discussion, Chapter 9.1.2.2.2), are summarized in Table 11.1.

Compound	ΔT_m (°C) FRET for FdsT (control)	
	25 μ M	50 μ M
2	-0.1 $\pm$ 0.2	-0.1 $\pm$ 0.2
14	-0.1 $\pm$ 0.0	-0.1 $\pm$ 0.1
15	0.4 $\pm$ 0.3	0.8 $\pm$ 0.1
57	-0.1 $\pm$ 0.1	0.0 $\pm$ 0.2
48	0.8 $\pm$ 0.0	3.2 $\pm$ 0.4
47	-0.1 $\pm$ 0.0	-0.1 $\pm$ 0.1
49	0.0 $\pm$ 0.0	-0.1 $\pm$ 0.2
50	0.0 $\pm$ 0.0	0.0 $\pm$ 0.1
51	0.5 $\pm$ 0.1	1.5 $\pm$ 0.4
52	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1
55	0.3 $\pm$ 0.1	1.1 $\pm$ 0.1
53	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1
56	0.1 $\pm$ 0.1	1.0 $\pm$ 0.1
54	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1

*Table 11.1: Activity of the bis-amino series in control FRET experiments with FdsT at 25 μ M and 50 μ M, compared to reference molecules **2** and **14** of the mono-amino series.*

11.3.2 *In situ* click experiments

11.3.2.1 The mono-amino click products: Experiment X-Z – methodology and results

The 1mM solution of each oligonucleotide (Pu24 or ds26) was prepared by dissolving commercially lyophilized sample. The Pu24 lyophilizate (6722.0 µg, 873 nmol) was dissolved in 66 µL of Tris-HCl buffer (200 mM, pH 7.4), 44 µL of KCl (2 M), and 763 µL of water to obtain a 1mM solution of Pu24. The ds26 oligonucleotide (6974.6 µg, 878.5 nmol) was dissolved in 66.4 µL of Tris-HCl buffer (200 mM, pH 7.4), 44.3 µL of KCl (2 M) and 767.8 µL of water. The mixture was centrifuged, heated at 95 °C for 10 min, and then shaken at 300 RPM overnight upon slow cooling to r.t. For each alkyne or azide, a stock solution in DMSO/H₂O (1:4) was prepared so that concentration of the respective alkyne was 100 mM and concentration of the respective azide was 150 mM. To avoid any discrepancy caused by pipetting, a solution mixture containing three alkynes **27a**, **28a**, and **29a** (20 mM each alkyne, 60 mM in total) and two azides (either **7** and **30**, or **8** and **9**, or **9** and **30** ; 30 mM each azide, 60 mM in total) was prepared by mixing an equal volume (100 µl) of each stock solution.

For each combination (Experiment X-Z) of alkynes and azides, three simultaneous *in situ* click sub-experiments were performed: with oligonucleotide (A), without oligonucleotide (B), and a control sub-experiment (C). Apart from that, each sub-experiment A, B, and C was done in three parallel performances. The reaction mixture for every individual *in situ* click sub-experiment contained three alkynes (1.0 mM each), two azides (1.5 mM each), Tris-HCl buffer (15 mM, pH 7.4), KCl (100 mM), CuSO₄ (1.5 mM), sodium ascorbate (3.0 mM), and either oligonucleotide (0.1 mM in Tris-HCl/KCl buffer) (sub-experiments A and C) or water (sub-experiment B). Regarding the control sub-experiment (C), oligonucleotide was added to the reaction mixture only 1 minute before quenching the reaction. The exact mixture composition of each sub-experiment A, B, and C is specified in the following section (Table 11.2-11.4).

The reaction mixture was shaken at 300 RPM and at 22 °C for 2 hours. Afterwards, TFA (40% in H₂O) was added to quench the reaction by DNA denaturation. The composition analysis of each reaction mixture by LC-MS was performed by Dr. Martin Hubálek, who also provided the raw data. For each click product, a ratio of the integral intensities measured by LC-MS was calculated between the sub-experiments A and B (the A/B ratio) and between the sub-experiments A and C (the A/C ratio). The average integral intensities, standard deviation (STDEV), and the A/B and A/C ratios for Pu24 co-catalysis are summarized in Tables 11.5-11.7 and for ds26 co-catalysis (control experiments) in Tables 11.8-11.10. Charts 11.1-11.3 display the integral intensity of A, B, and C sub-experiments for Pu24 co-catalysis and, analogously, Charts 11.4-11.6 display the respective integral intensity for ds26 co-catalysis (control experiments). Finally, Table 11.11 outlines the A/B and A/C formation ratios for the control *in situ* click experiments with ds26.

General procedure for *in situ* click experiment co-catalyzed by an oligonucleotide (sub-experiment A)

In a 1.5 mL Eppendorf tube, 15 μ L of Tris-HCl buffer (100 mM, pH 7.4), 20 μ L of KCl solution (500 mM), 10 μ L of oligonucleotide solution (1 mM), 25 μ L of azide/alkyne solution mixture, 15 μ L of CuSO₄ (10 mM), and at the end, 15 μ L of Na-ascorbate (20 mM) was added to obtain a reaction mixture of total volume of 100 μ L. Composition of the resulting reaction mixture is summarized in Table 11.2. The solution was vortexed and then shaken at 300 RPM at 22.0 °C for 2 h. Afterwards, 20 μ L of TFA (40% aq. solution) was added to quench the reaction, the mixture was vortexed and then analyzed by LC-MS (17 min).

Component	c _{init} [mM]	V [μ L]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	15
KCl	500	20	10000	100
Oligo.	1	10	10	0.1
Alkyne 27a	20	5	100	1
Alkyne 28a	20	5	100	1
Alkyne 29a	20	5	100	1
Azide I	30	5	150	1.5
Azide II	30	5	150	1.5
CuSO ₄	10	15	150	1.5
NaAsc	20	15	300	3
Total volume:		100		

Table 11.2: Composition of the reaction mixture in sub-experiment A – *in situ* click experiment co-catalyzed by an oligonucleotide. Azides I-II: 7 and 30 (X), or 8 and 9 (Y), or 9 and 30 (Z).

General procedure for *in situ* click experiment without any oligonucleotide (sub-experiment B)

In a 1.5 mL Eppendorf tube, 15 μ L of Tris-HCl buffer (100 mM, pH 7.4), 20 μ L of KCl solution (500 mM), 10 μ L of water, 25 μ L of azide/alkyne solution mixture, 15 μ L of CuSO₄ (10 mM), and at the end, 15 μ L of Na-ascorbate (20 mM) was added to obtain a reaction mixture of total volume of 100 μ L. Composition of the resulting reaction mixture is summarized in Table 11.3. The solution was vortexed and then shaken at 300 RPM at 22.0 °C for 2 h. Afterwards, 20 μ L of TFA (40% aq. solution) was added to quench the reaction, the mixture was vortexed and then analyzed by LC-MS (17 min).

Component	c _{init} [mM]	V [μL]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	15
KCl	500	20	10000	100
H ₂ O	-	10	-	-
Alkyne 27a	20	5	100	1
Alkyne 28a	20	5	100	1
Alkyne 29a	20	5	100	1
Azide I	30	5	150	1.5
Azide II	30	5	150	1.5
CuSO ₄	10	15	150	1.5
NaAsc	20	15	300	3
Total volume:		100		

Table 11.3: Composition of the reaction mixture in sub-experiment B – *in situ* click experiment without oligonucleotide. Azides I-II: 7 and 30 (X), or 8 and 9 (Y), or 9 and 30 (Z).

General procedure for a control *in situ* click experiment (sub-experiment C)

In a 1.5 mL Eppendorf tube, 15 μL of Tris-HCl buffer (100 mM, pH 7.4), 20 μL of KCl solution (500 mM), 25 μL of azide/alkyne solution mixture, 15 μL of CuSO₄ (10 mM), and at the end, 15 μL of Na-ascorbate (20 mM) was added to obtain a reaction mixture of total volume of 90 μL. The solution was vortexed and then shaken at 300 RPM at 22.0 °C for 1 h 59 min. Then, 10 μL of oligonucleotide solution (1 mM) was added to obtain a reaction mixture of total volume of 100 μL, the resulting mixture was vortexed and incubated for 1 min. Composition of the resulting reaction mixture is summarized in Table 11.4. Afterwards, 20 μL of TFA (40% aq. solution) was added to quench the reaction, the mixture was vortexed and then analyzed by LC-MS (17 min).

Component	c _{init} [mM]	V [μL]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	15
KCl	500	20	10000	100
Oligo.*	1	10	10	0.1
Alkyne 27a	20	5	100	1
Alkyne 28a	20	5	100	1
Alkyne 29a	20	5	100	1
Azide I	30	5	150	1.5
Azide II	30	5	150	1.5
CuSO ₄	10	15	150	1.5
NaAsc	20	15	300	3
Total volume:		100		

Table 11.4: Composition of the reaction mixture in sub-experiment C – a control *in situ* click experiment. Azides I-II: 7 and 30 (X), or 8 and 9 (Y), or 9 and 30 (Z). * This component was added only 1 min. before quenching the reaction.

Results: integral intensities of click products formed in experiments X-1 – Z-1

Table 11.5 - Experiment X-1								
Click product			A (with Pu24)		B (without Pu24)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
31	302.21	1	40318.6	1686.0	21916.1	6565.8	30435.3	3636.6
40	344.26	1	56159.6	2616.4	7878.1	2514.8	11881.1	2110.3
32	316.22	2	73953.9	2647.3	44604.6	6285.8	56649.2	3428.9
41	358.27	2	118116.1	5094.5	22740.8	3950.9	38352.7	2125.3
33	330.24	3	145081.6	6587.1	33371.0	7587.8	49272.9	6650.3
42	372.29	3	128290.0	4411.9	5657.3	2744.1	11873.2	1939.5

Table 11.5: Average integral intensity of **31-33** and **40-42** click products formed and the respective STDEV in three sub-experiments: with Pu24 (A), without Pu24 (B), and control experiment (C).

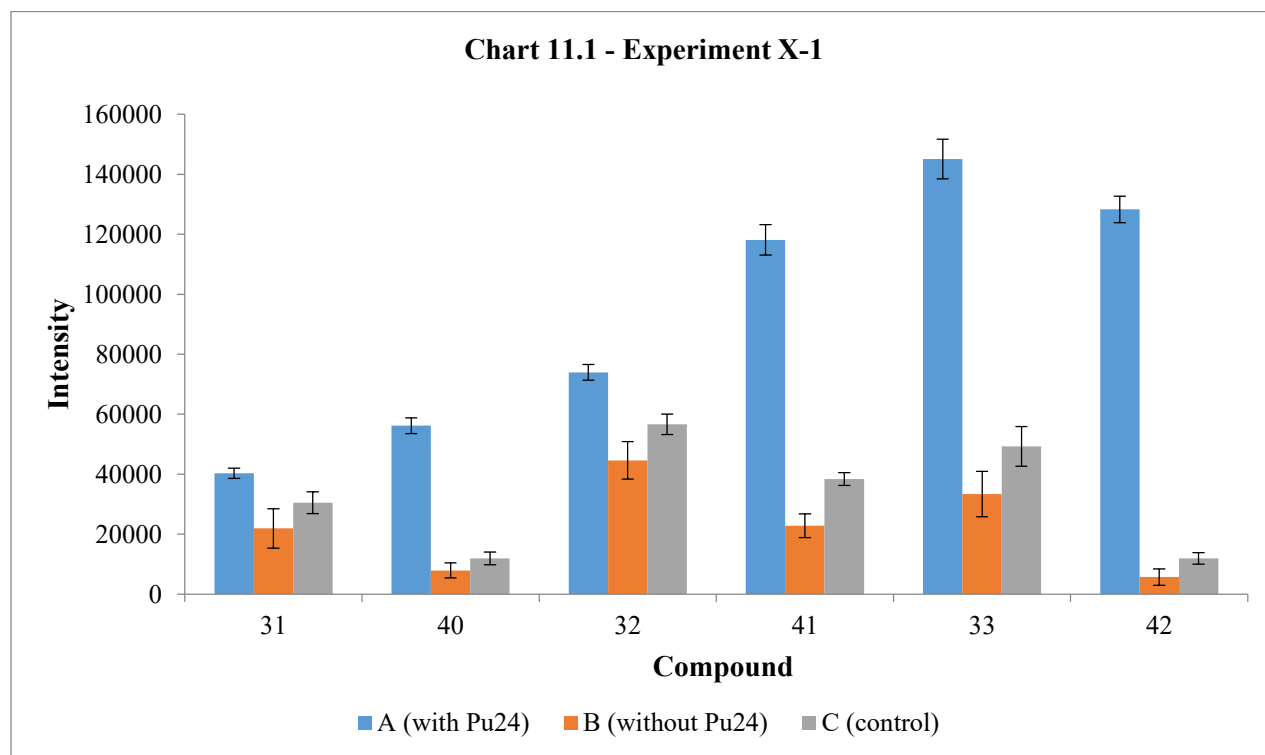


Chart 11.1: Comparison of average integral intensity of each click product in three sub-experiments: with Pu24 (A), without Pu24 (B), and control experiment (C).

Table 11.6 - Experiment Y-1								
Click product			A (with Pu24)		B (without Pu24)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
34	316.22	1	47337.5	2047.8	33690.0	8062.1	40520.9	1989.7
37	356.26	1	128563.7	6615.5	26719.3	5902.5	34238.2	2759.2
35	330.24	2	85272.3	5432.2	58936.9	10973.9	71327.4	6194.1
38	370.27	2	146986.3	1970.0	42946.7	4360.5	59812.7	2081.2
36	344.26	3	152927.7	4034.5	61240.3	14153.8	78797.7	1576.4
39	384.29	3	164211.7	4248.2	16792.1	5891.1	29761.6	1758.9

Table 11.6: Average integral intensity of **34-36** and **37-39** click products formed and the respective STDEV in three sub-experiments: with Pu24 (A), without Pu24 (B), and control experiment (C).

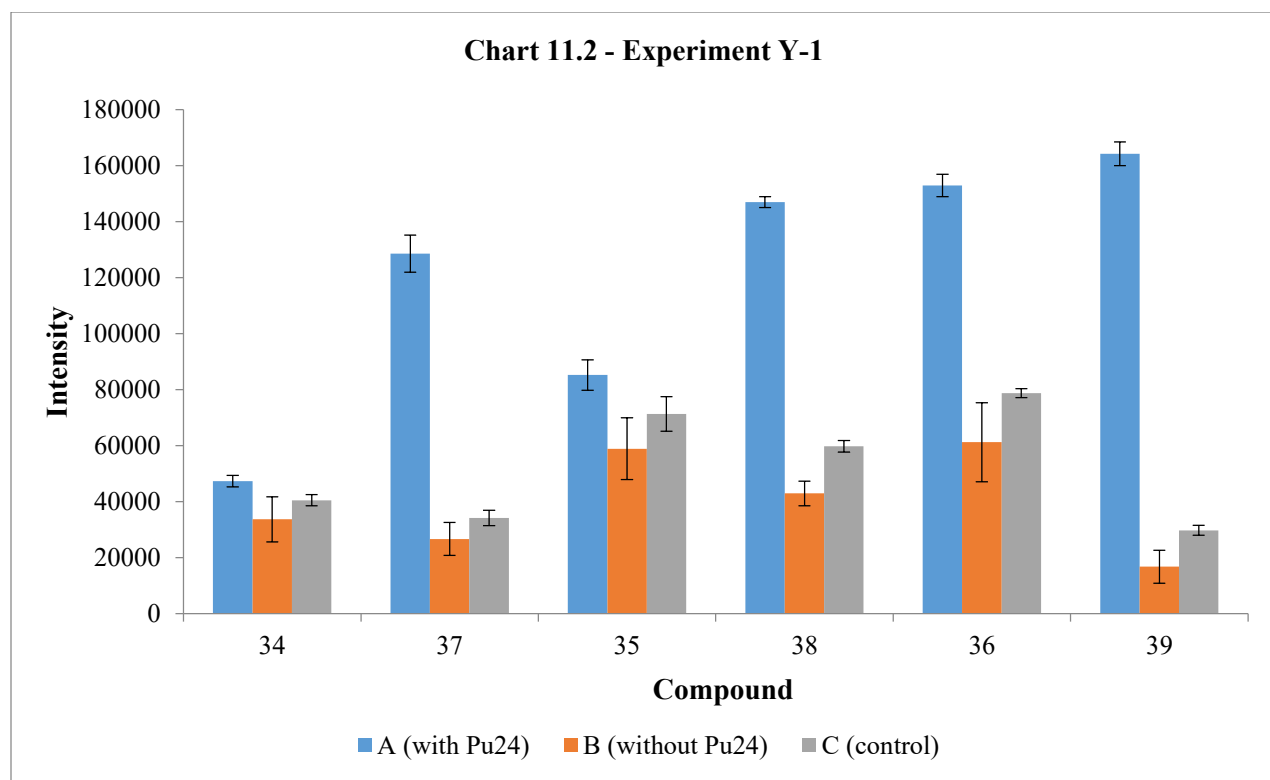


Chart 11.2: Comparison of average integral intensity of each click product in three sub-experiments: with Pu24 (A), without Pu24 (B), and control experiment (C).

Table 11.7 - Experiment Z-1								
Click product			A (with Pu24)		B (without Pu24)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
37	356.26	1	82650.6	4985.0	15448.6	886.6	18579.7	1441.5
40	344.26	1	49443.7	1886.4	7454.7	1087.7	9751.6	874.3
38	370.27	2	132537.4	734.9	36019.9	1896.2	51028.8	1920.5
41	358.27	2	120268.9	2445.3	16353.9	985.9	27524.5	1972.4
39	384.29	3	136232.6	1417.0	8081.7	597.3	12994.1	627.6
42	372.29	3	90438.1	1568.5	1645.9	137.3	2118.8	114.9

Table 11.7: Average integral intensity of 37-39 and 40-42 click products formed and the respective STDEV in three sub-experiments: with Pu24 (A), without Pu24 (B), and control experiment (C).

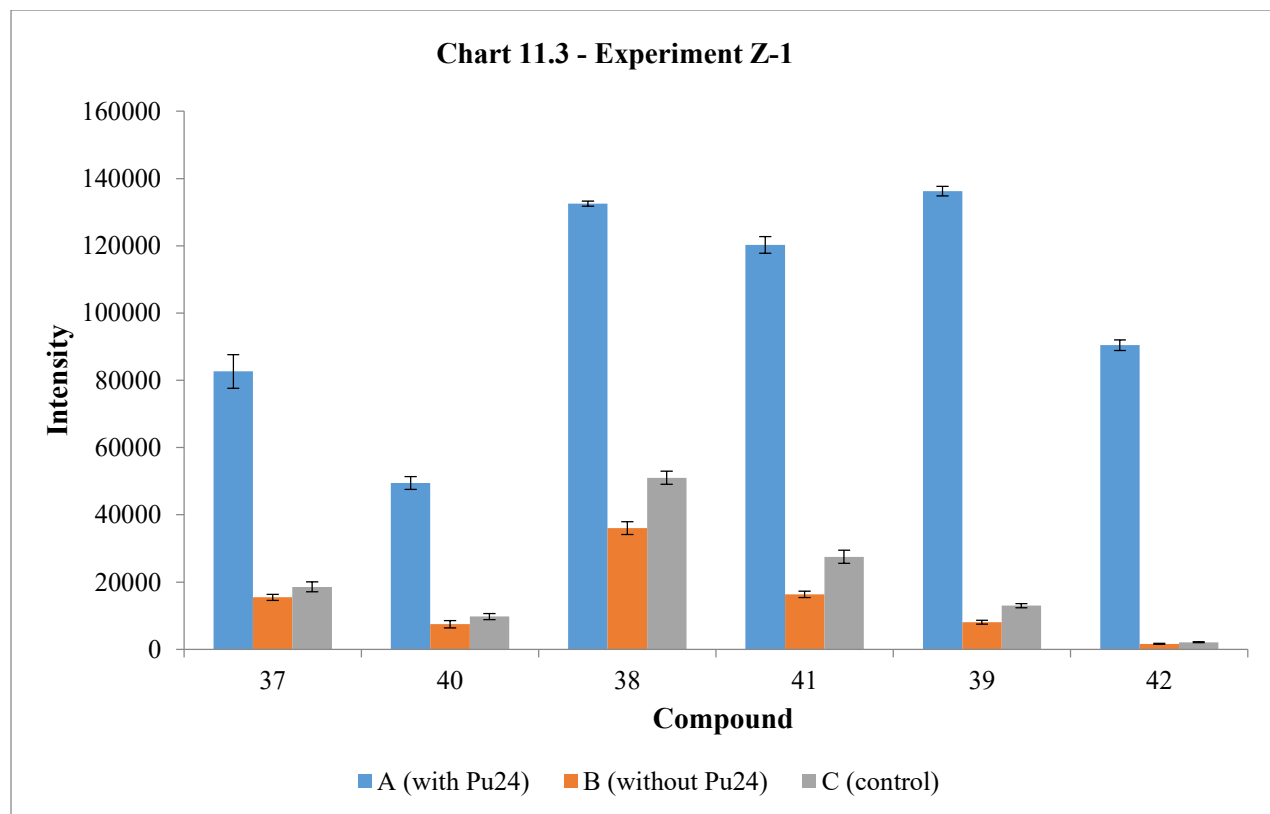


Chart 11.3: Comparison of average integral intensity of each click product in three sub-experiments: with Pu24 (A), without Pu24 (B), and control experiment (C).

Results: integral intensities of click products formed in experiments X-2 – Z-2

Table 11.8 - Experiment X-2								
Click product			A (with ds26)		B (without ds26)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
31	302.21	1	2258.1	383.7	5050.0	85.1	4341.7	156.9
40	344.26	1	1759.2	519.8	1672.8	408.7	2047.1	351.1
32	316.22	2	6894.1	719.1	11301.5	1042.2	11908.1	523.1
41	358.27	2	3254.1	275.4	3118.2	864.3	3323.9	294.4
33	330.24	3	9662.3	532.6	12093.9	764.6	12305.3	550.6
42	372.29	3	3960.9	587.3	3182.0	639.2	3584.4	339.2

Table 11.8: Average integral intensity of **31-33** and **40-42** click products formed and the respective STDEV in three sub-experiments: with oligonucleotide (A), without oligonucleotide (B), and control experiment (C).

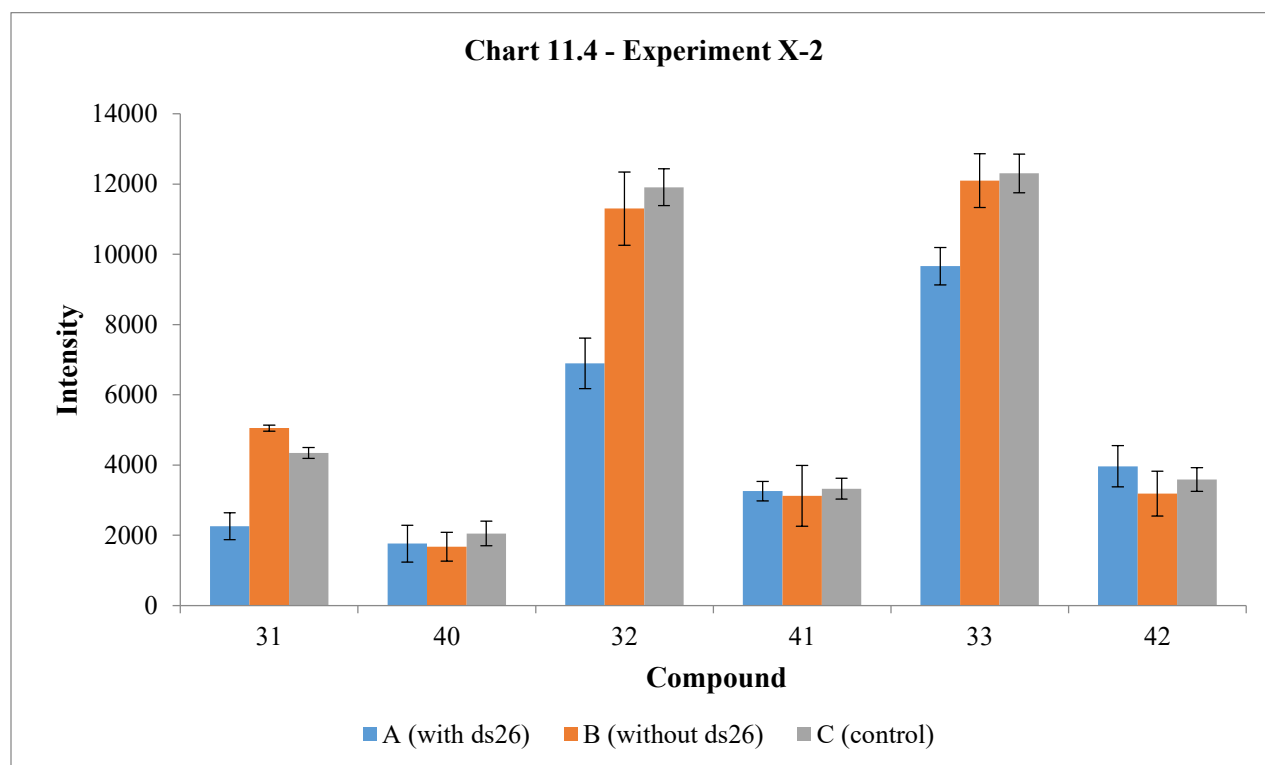


Chart 11.4: Comparison of average integral intensity of each click product in three sub-experiments: with ds26 (A), without ds26 (B), and control experiment (C).

Table 11.9 - Experiment Y-2								
Click product			A (with ds26)		B (without ds26)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
34	316.22	1	2340.4	152.7	4784.3	247.4	4292.9	450.5
37	356.26	1	3585.5	236.6	3556.9	123.7	3254.0	450.2
35	330.24	2	13799.2	904.9	12620.7	635.3	11581.9	1158.5
38	370.27	2	6084.3	480.0	5302.8	520.6	4836.2	334.4
36	344.26	3	5016.2	933.9	4927.6	312.6	3971.7	522.3
39	384.29	3	3186.0	227.9	3427.3	461.6	3002.4	768.7

Table 11.9: Average integral intensity of **34-36** and **37-39** click products formed and the respective STDEV in three sub-experiments: with ds26 (A), without ds26 (B), and control experiment (C).

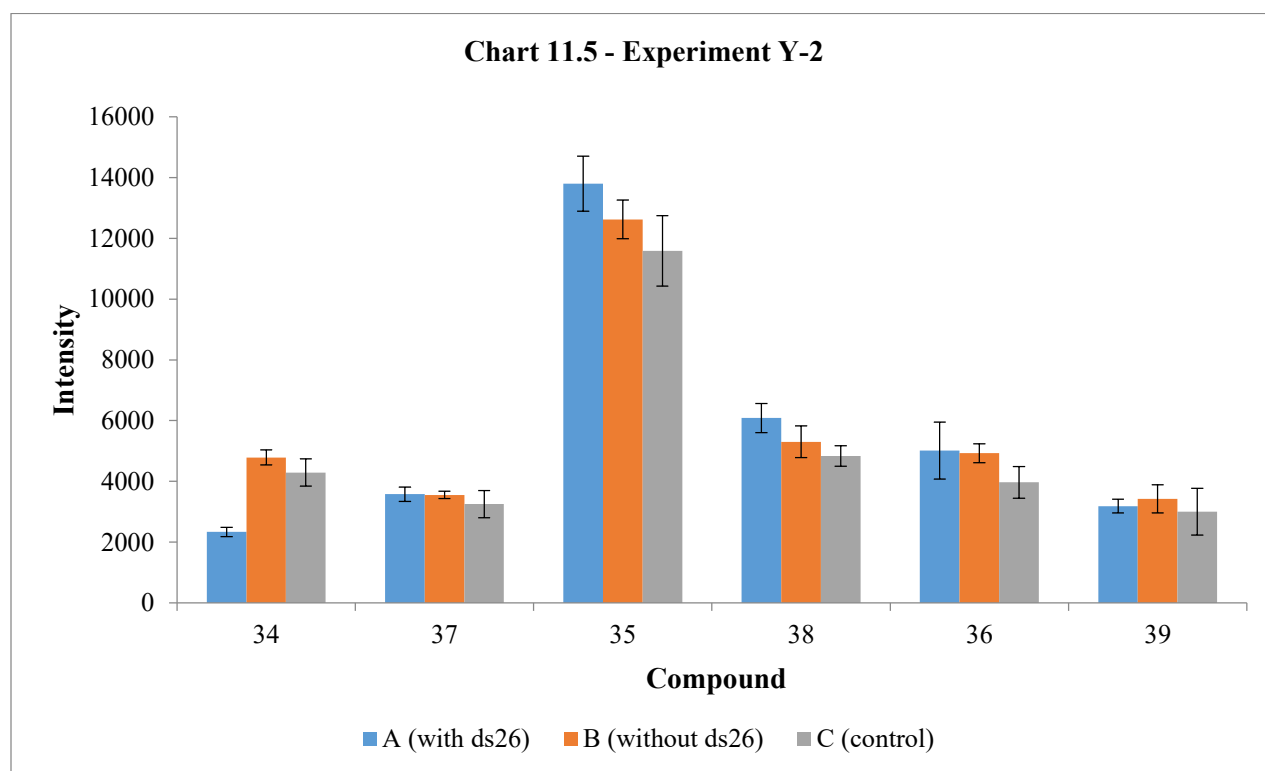


Chart 11.5: Comparison of average integral intensity of each click product in three sub-experiments: with ds26 (A), without ds26 (B), and control experiment (C).

Table 11.10 - Experiment Z-2								
Click product			A (with ds26)		B (without ds26)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
37	356.26	1	437.0	41.6	673.1	157.6	595.7	95.8
40	344.26	1	514.7	95.6	409.6	114.2	682.9	145.6
38	370.27	2	2328.7	131.5	2189.0	161.1	2084.0	59.1
41	358.27	2	910.3	156.4	862.2	144.4	1044.7	155.6
39	384.29	3	229.7	13.3	273.7	34.4	233.4	70.4
42	372.29	3	162.7	75.3	171.1	24.0	159.5	47.2

Table 11.10: Average integral intensity of 37-39 and 40-42 click products formed and the respective STDEV in three sub-experiments: with ds26 (A), without ds26 (B), and control experiment (C).

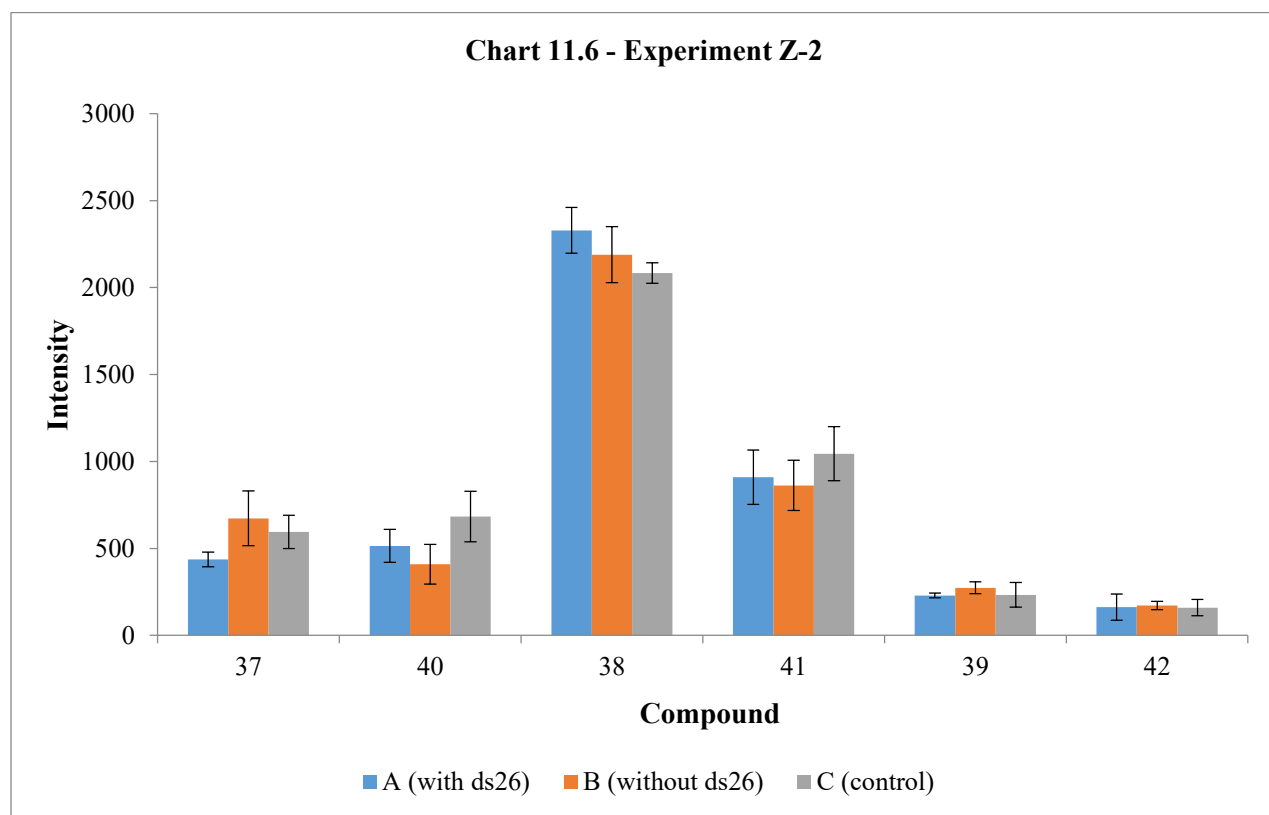


Chart 11.6: Comparison of average integral intensity of each click product in three sub-experiments: with ds26 (A), without ds26 (B), and control experiment (C).

Results: A/B and A/C ratios regarding click product formation in experiments X-2 – Z-2

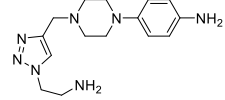
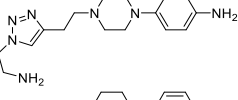
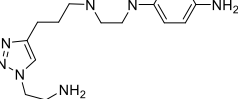
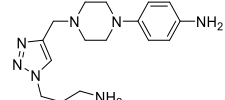
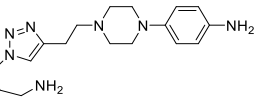
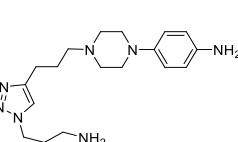
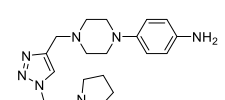
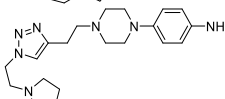
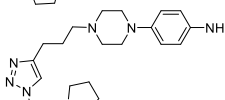
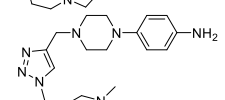
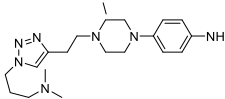
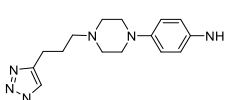
Compound	CH ₂ units	Experiment X-2		Experiment Y-2		Experiment Z-2	
		A/B	A/C	A/B	A/C	A/B	A/C
31 	1	0.5	0.5	-	-	-	-
32 	2	0.6	0.6	-	-	-	-
33 	3	0.8	0.8	-	-	-	-
34 	1	-	-	0.5	0.6	-	-
35 	2	-	-	1.1	1.2	-	-
36 	3	-	-	1.0	1.3	-	-
37 	1	-	-	1.0	1.1	0.7	0.7
38 	2	-	-	1.2	1.3	1.1	1.1
39 	3	-	-	0.9	1.1	0.8	1.0
40 	1	1.1	0.9	-	-	1.3	0.8
41 	2	1.0	1.0	-	-	1.1	0.9
42 	3	1.3	1.1	-	-	1.0	1.0

Table 11.11: Control in situ click experiments performed with ds26: the relative formation yields of compounds 31-42 given by reaction ratios. X-2, Y-2, and Z-2 are the combination reactions of three alkynes with the corresponding azides. A, B, and C are in situ click sub-experiments in the presence of ds26, without ds26, and a control reaction, respectively.

11.3.2.2 The pyridostatin click products: Experiment D-G – methodology and results

For the purpose of *in situ* click experiments with pyridostatin derived fragments, we used the 1mM solutions of the Pu24 oligonucleotide that was prepared for mono-amino click products (Chapter 11.3.2.1). For each alkyne or azide, a stock solution in DMSO/H₂O (1:4) was prepared so that concentration of the compound was 100 mM. To avoid any discrepancy caused by pipetting, for each experiment D-G, a solution mixture containing the fragments that should click together *in situ* was prepared. In the case of experiments D and E, equal volume of each 100mM stock solution was mixed so that the final concentration of each azide (**8**, **9**, and **78** in experiment D; **7**, **30**, and **77** in experiment E) and each pyridostatin-alkyne (**58-60**) in the solution mixture was 16.67 mM (50 mM in total for azides, 50 mM in total for pyridostatin-alkynes). In the case of experiments F and G, equal volume of each alkyne (**27a**, **28a**, **29a**) 100mM stock solution was mixed with three-times larger volume of the respective pyridostatin-azide (**61** in experiment F; **62** in experiment G) 100mM stock solution so that the final concentration in the solution mixture was 16.67 mM for each alkyne (50 mM in total for alkynes) and 50 mM for the pyridostatin-azide.

For each combination (Experiment D-G) of alkynes and azides, three simultaneous *in situ* click sub-experiments were performed: with Pu24 (A), without Pu24 (B), and a control sub-experiment (C). Apart from that, each sub-experiment A, B, and C was done in three parallel performances. The reaction mixture for every individual *in situ* click experiment contained the azide/alkyne solution mixture (D, E, F, or G), Tris-HCl buffer (15 mM, pH 7.4), KCl (100 mM), CuSO₄ (1.5 mM), sodium ascorbate (3.0 mM), and either Pu24 (0.1 mM in Tris-HCl/KCl buffer) (sub-experiments A and C) or water (sub-experiment B). Regarding the control sub-experiment (C), the Pu24 oligonucleotide was added to the reaction mixture only 1 minute before quenching the reaction. The exact mixture composition of each sub-experiment A, B, and C is specified in the following section (Table 11.12-11.17).

The reaction mixture was shaken at 300 RPM and at 22 °C for 2 hours. Afterwards, TFA (40% in H₂O) was added to quench the reaction by DNA denaturation. The composition analysis of each reaction mixture by LC-MS was performed by Dr. Martin Hubálek, who also provided the raw data. For each click product, a ratio of the integral intensities measured by LC-MS was calculated between the sub-experiments A and B (the A/B ratio) and between the sub-experiments A and C (the A/C ratio). The average integral intensities, STDEV, and the A/B and A/C ratios for Pu24 co-catalysis are summarized in Tables 11.18-11.21 and displayed in Charts 11.7-11.10.

General procedure for *in situ* click sub-experiment co-catalyzed by an oligonucleotide (sub-experiment A)

In a 1.5 mL Eppendorf tube, 15 μ L of Tris-HCl buffer (100 mM, pH 7.4), 20 μ L of KCl solution (500 mM), 10 μ L of the oligonucleotide Pu24 solution (1 mM), 30 μ L of azide/alkyne solution mixture, 15 μ L of CuSO₄ (10 mM), and at the end, 15 μ L of Na-ascorbate (20 mM) was added to obtain a reaction mixture of total volume of 105 μ L. Composition of the resulting reaction mixture for experiments D and E is summarized in Table 11.12, for experiments F and G in Table 11.13. The solution was vortexed and then shaken at 300 RPM at 22.0 °C for 2 h. Afterwards, 20 μ L of TFA (40% aq. solution) was added to quench the reaction, the mixture was vortexed and then analyzed by LC-MS (17 min).

Component	c _{init} [mM]	V [μ L]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	14.29
KCl	500	20	10000	95.24
Pu24	1	10	10	0.1
Alkyne 58	16.67	5	83.35	0.79
Alkyne 59	16.67	5	83.35	0.79
Alkyne 60	16.67	5	83.35	0.79
Azide I	16.67	5	83.35	0.79
Azide II	16.67	5	83.35	0.79
Azide III	16.67	5	83.35	0.79
CuSO ₄	10	15	150	1.43
NaAsc	20	15	300	2.86
Total volume:		105		

Table 11.12: Experiments D and E: composition of the reaction mixture in sub-experiment A – co-catalyzed by Pu24. Azides I-III: 8, 9, and 78 (D); or 7, 30, and 77 (E).

Component	c _{init} [mM]	V [μ L]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	14.29
KCl	500	20	10000	95.24
Pu24	1	10	10	0.1
Alkyne 27a	16.67	5	83.35	0.79
Alkyne 28a	16.67	5	83.35	0.79
Alkyne 29a	16.67	5	83.35	0.79
Azide	16.67	15	250	2.38
CuSO ₄	10	15	150	1.43
NaAsc	20	15	300	2.86
Total volume:		105		

Table 11.13: Experiments F and G: composition of the reaction mixture in sub-experiment A – co-catalyzed by Pu24. Azide: 61 (F), or 62 (G).

General procedure for *in situ* click sub-experiment without any oligonucleotide (sub-experiment B)

In a 1.5 mL Eppendorf tube, 15 μ L of Tris-HCl buffer (100 mM, pH 7.4), 20 μ L of KCl solution (500 mM), 10 μ L of water, 30 μ L of azide/alkyne solution mixture, 15 μ L of CuSO₄ (10 mM), and at the end, 15 μ L of Na-ascorbate (20 mM) was added to obtain a reaction mixture of total volume of 105 μ L. Composition of the resulting reaction mixture for experiments D and E is summarized in Table 11.14, for experiments F and G in Table 11.15. The solution was vortexed and then shaken at 300 RPM at 22.0 °C for 2 h. Afterwards, 20 μ L of TFA (40% aq. solution) was added to quench the reaction, the mixture was vortexed and then analyzed by LC-MS (17 min).

Component	c _{init} [mM]	V [μ L]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	14.29
KCl	500	20	10000	95.24
H ₂ O	-	10	-	-
Alkyne 58	16.67	5	83.35	0.79
Alkyne 59	16.67	5	83.35	0.79
Alkyne 60	16.67	5	83.35	0.79
Azide I	16.67	5	83.35	0.79
Azide II	16.67	5	83.35	0.79
Azide III	16.67	5	83.35	0.79
CuSO ₄	10	15	150	1.43
NaAsc	20	15	300	2.86
Total volume:		105		

Table 11.14: Experiments D and E: composition of the reaction mixture in sub-experiment B – in without Pu24. Azides I-III: 8, 9, and 78 (D); or 7, 30, and 77 (E).

Component	c _{init} [mM]	V [μ L]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	14.29
KCl	500	20	10000	95.24
H ₂ O	-	10	-	-
Alkyne 27a	16.67	5	83.35	0.79
Alkyne 28a	16.67	5	83.35	0.79
Alkyne 29a	16.67	5	83.35	0.79
Azide	16.67	15	250	2.38
CuSO ₄	10	15	150	1.43
NaAsc	20	15	300	2.86
Total volume:		105		

Table 11.15: Experiments F and G: composition of the reaction mixture in sub-experiment B – without Pu24. Azide: 61 (F), or 62 (G).

General procedure for a control *in situ* click sub-experiment (sub-experiment C)

In a 1.5 mL Eppendorf tube, 15 μ L of Tris-HCl buffer (100 mM, pH 7.4), 20 μ L of KCl solution (500 mM), 30 μ L of azide/alkyne solution mixture, 15 μ L of CuSO₄ (10 mM), and at the end, 15 μ L of Na-ascorbate (20 mM) was added to obtain a reaction mixture of total volume of 95 μ L. The solution was vortexed and then shaken at 300 RPM at 22.0 °C for 1 h 59 min. Then, 10 μ L of Pu24 solution (1 mM) was added to obtain a reaction mixture of total volume of 105 μ L, the resulting mixture was vortexed and incubated for 1 min. Composition of the resulting reaction mixture for experiments D and E is summarized in Table 11.16, for experiments F and G in Table 11.17. Afterwards, 20 μ L of TFA (40% aq. solution) was added to quench the reaction, the mixture was vortexed and then analyzed by LC-MS (17 min).

Component	c _{init} [mM]	V [μ L]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	14.29
KCl	500	20	10000	95.24
Pu24*	1	10	10	0.1
Alkyne 58	16.67	5	83.35	0.79
Alkyne 59	16.67	5	83.35	0.79
Alkyne 60	16.67	5	83.35	0.79
Azide I	16.67	5	83.35	0.79
Azide II	16.67	5	83.35	0.79
Azide III	16.67	5	83.35	0.79
CuSO ₄	10	15	150	1.43
NaAsc	20	15	300	2.86
Total volume:		105		

Table 11.16: Experiments D and E: composition of the reaction mixture in the control sub-experiment C. Azides I-III: **8**, **9**, and **78** (D); or **7**, **30**, and **77** (E). * Added only 1 min. before quenching the reaction.

Component	c _{init} [mM]	V [μ L]	n [nmol]	c _{fin} [mM]
Tris-HCl	100	15	1500	14.29
KCl	500	20	10000	95.24
Pu24*	1	10	10	0.1
Alkyne 27a	16.67	5	83.35	0.79
Alkyne 28a	16.67	5	83.35	0.79
Alkyne 29a	16.67	5	83.35	0.79
Azide	16.67	15	250	2.38
CuSO ₄	10	15	150	1.43
NaAsc	20	15	300	2.86
Total volume:		105		

Table 11.17: Experiments F and G: composition of the reaction mixture in the control sub-experiment C. * Added only 1 min. before quenching the reaction. Azide: **61** (F), or **62** (G).

Results: integral intensities of click products formed in experiments D-G

Table 11.18 - Experiment D									
Click product			A (with Pu24)		B (without Pu24)		C (control)		
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV	
83	788.40	1	58102.8	1863.9	48929.0	1764.0	48542.3	1491.5	
99	748.37	1	170347.8	19322.6	164527.6	5006.5	167106.6	7869.9	
105	908.47	1	81132.8	6049.1	98409.0	4880.2	93835.0	3320.6	
84	802.41	2	13118.2	944.7	14851.4	658.1	14858.3	1320.7	
100	762.38	2	29334.8	384.7	42580.8	533.7	42671.2	2999.8	
106	922.48	2	22776.8	968.2	32598.3	1303.7	32006.8	1861.1	
85	816.43	3	22839.0	1411.0	23136.2	511.9	23347.6	461.9	
101	776.40	3	56572.0	2376.7	68893.1	1380.2	70549.4	904.7	
107	936.50	3	55343.7	4410.6	61640.4	2811.7	63552.7	1429.5	

Table 11.18: Average integral intensity of **83-85**, **99-101**, and **105-107** click products formed and the respective STDEV in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

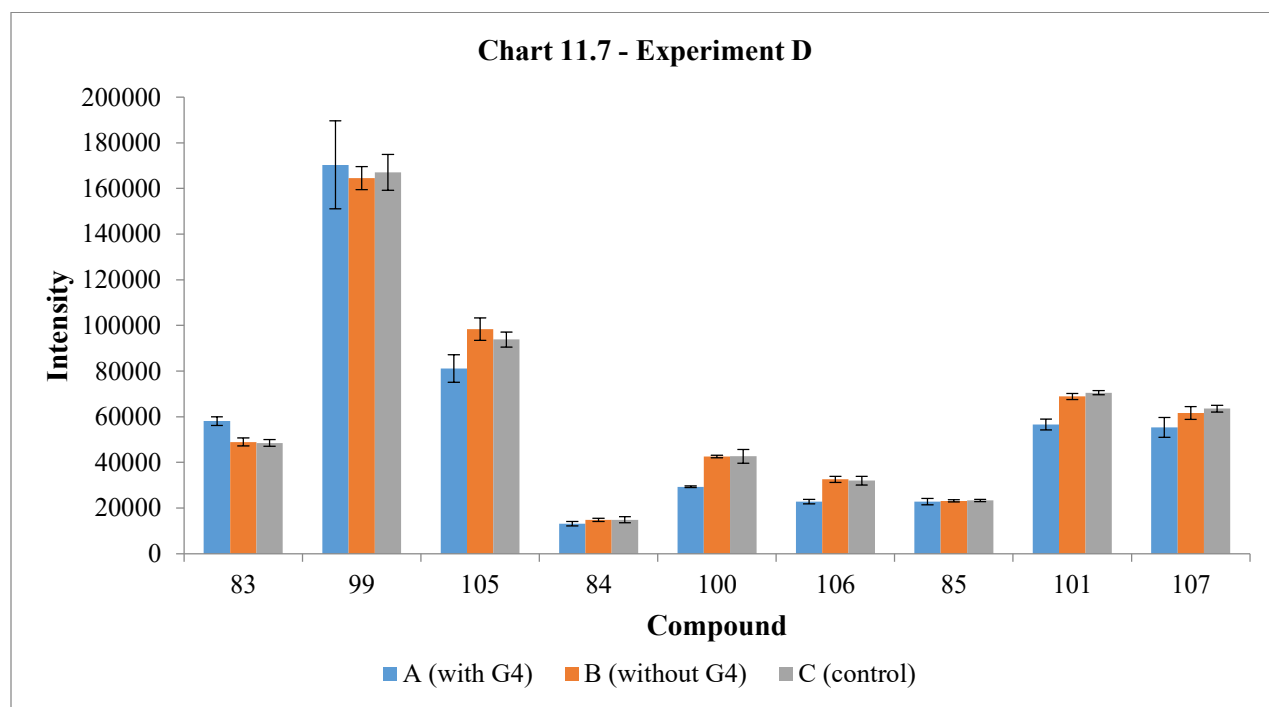


Chart 11.7: Comparison of average integral intensity of each click product in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

Table 11.19 - Experiment E								
Click product			A (with Pu24)		B (without Pu24)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
86	776.40	1	16466.7	383.0	14633.7	1391.1	16229.8	399.0
96	734.35	1	57967.3	1521.2	102184.0	9342.2	118494.6	5264.7
102	894.45	1	45844.5	240.0	80001.4	4937.4	71344.25	3421.8
87	790.41	2	3610.0	335.2	1865.5	120.2	3585.4	362.4
97	748.37	2	7253.3	371.4	9742.0	1104.9	15891.3	1019.3
103	908.47	2	8730.8	230.4	12637.1	880.8	14396.2	1315.0
88	804.43	3	10202.0	423.5	6801.2	248.0	8736.0	611.6
98	762.38	3	19930.0	534.8	22748.5	213.8	36910.3	3527.4
104	922.48	3	18192.8	378.8	29750.4	2724.4	30096.4	898.3

Table 11.19: Average integral intensity of **86-88**, **96-98**, and **102-104** click products formed and the respective STDEV in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

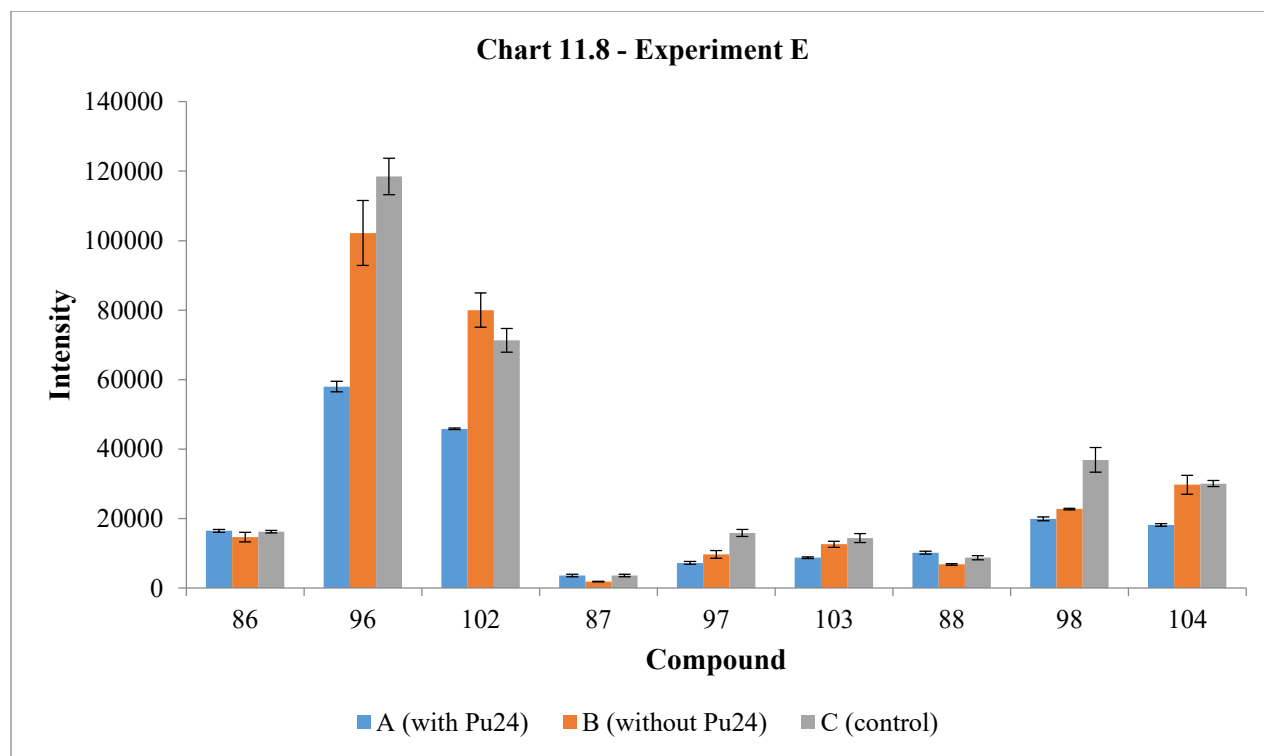


Chart 11.8: Comparison of average integral intensity of each click product in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

Table 11.20 - Experiment F								
Click product			A (with Pu24)		B (without Pu24)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
108	894.45	1	32478.9	3747.6	53396.5	785.8	44105.2	4520.8
109	908.47	2	53955.0	6431.9	67140.6	625.4	56824.2	5829.1
110	922.48	3	19295.6	570.4	13886.5	805.5	11411.5	55.3

Table 11.20: Average integral intensity of **108-110** click products formed and the respective STDEV in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

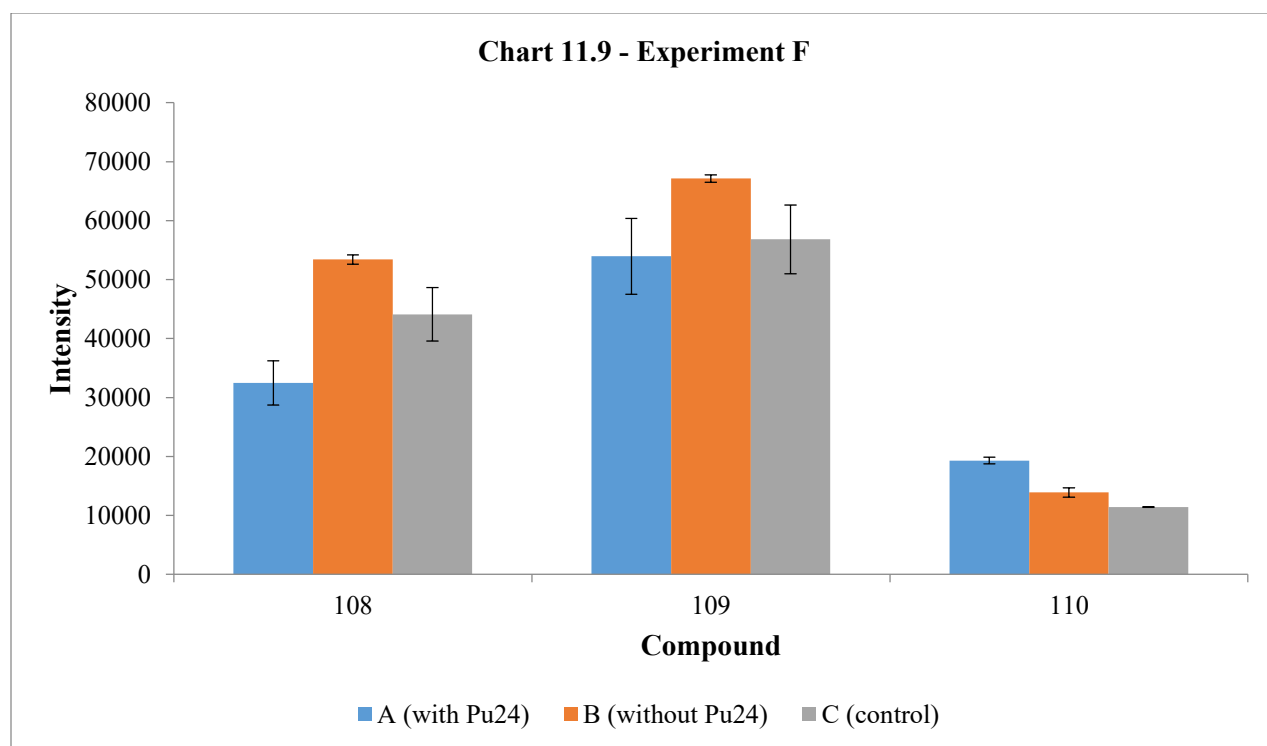


Chart 11.9: Comparison of average integral intensity of each click product in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

Table 11.21 – Experiment G								
Click product			A (with Pu24)		B (without Pu24)		C (control)	
Code	Mass (M+H ⁺)	CH ₂ units	Average	STDEV	Average	STDEV	Average	STDEV
111	908.47	1	30072.3	620.3	30276.3	480.8	28961.5	1764.7
112	922.48	2	72704.2	18969.1	84377.1	3206.0	74575.9	2228.0
113	936.50	3	18926.6	1843.1	13047.2	841.2	9855.5	548.1

Table 11.21: Average integral intensity of **111-113** click products formed and the respective STDEV in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

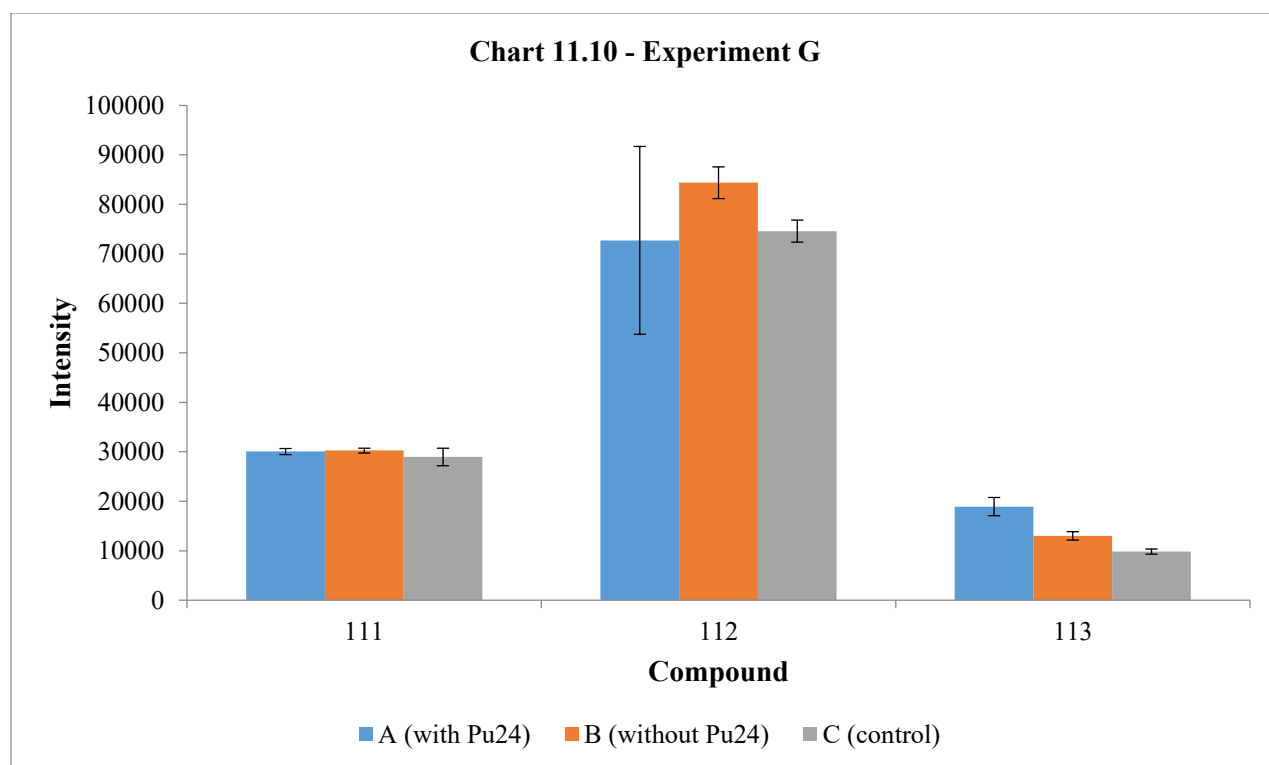


Chart 11.10: Comparison of average integral intensity of each click product in three sub-experiments: with Pu24 (A), without Pu24 (B), and control sub-experiment (C).

12 References

1. Csermely, P.; Korcsmáros, T.; Kiss, H. J. M.; London, G.; and Nussinov, R.: Structure and dynamics of molecular networks: A novel paradigm of drug discovery: A comprehensive review. *Pharmacol. Ther.* **2013**, *138*, 333-408.
2. Meghani, N. M.; Amin, H. H.; and Lee, B.-J.: Mechanistic applications of click chemistry for pharmaceutical drug discovery and drug delivery. *Drug Discov. Today* **2017**, *22*, 1604-1619.
3. Bosc, D.; Jakhlal, J.; Deprez, B.; and Deprez-Poulain, R.: Kinetic target-guided synthesis in drug discovery and chemical biology: a comprehensive facts and figures survey. *Future Med. Chem.* **2016**, *8*, 381-404.
4. Hu, X.; and Manetsch, R.: Kinetic target-guided synthesis. *Chem. Soc. Rev.* **2010**, *39*, 1316-1324.
5. Mayr, L. M.; and Fuerst, P.: The Future of High-Throughput Screening. *J. Biomol. Screen.* **2008**, *13*, 443-448.
6. Macarron, R.; Banks, M. N.; Bojanic, D.; Burns, D. J.; Cirovic, D. A.; Garyantes, T.; Green, D. V. S.; Hertzberg, R. P.; Janzen, W. P.; Paslay, J. W.; Schopfer, U.; and Sittampalam, G. S.: Impact of high-throughput screening in biomedical research. *Nat. Rev. Drug Discov.* **2011**, *10*, 188-195.
7. Rees, D. C.; Congreve, M.; Murray, C. W.; and Carr, R.: Fragment-based lead discovery. *Nat. Rev. Drug Discov.* **2004**, *3*, 660-672.
8. Price, A. J.; Howard, S.; and Cons, B. D.: Fragment-based drug discovery and its application to challenging drug targets. *Essays Biochem.* **2017**, *61*, 475-484.
9. Kirsch, P.; Hartman, A. M.; Hirsch, A. K. H.; and Empting, M.: Concepts and Core Principles of Fragment-Based Drug Design. *Molecules* **2019**, *24*, 4309.
10. Vrzal, L.: Fragment-based drug discovery. Dissertation, University of Chemistry and Technology, Prague, Czech Republic, Faculty of Food and Biochemical Technology, **2019**.
11. Chapman, P. B.; Hauschild, A.; Robert, C.; Haanen, J. B.; Ascierto, P.; Larkin, J.; Dummer, R.; Garbe, C.; Testori, A.; Maio, M.; Hogg, D.; and Lorigan, P.: Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation. *N. Engl. J. Med.* **2011**, *364*, 2507-2516.
12. Roberts, A. W.; Davids, M. S.; Pagel, J. M.; Kahl, B. S.; Puvvada, S. D.; Gerecitano, J. F.; Kipps, T. J.; Anderson, M. A.; Brown, J. R.; Gressick, L.; Wong, S.; and Dunbar, M.: Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *N. Engl. J. Med.* **2016**, *374*, 311-322.
13. Schulz, M. N.; and Hubbard, R. E.: Recent progress in fragment-based lead discovery. *Curr. Opin. Pharmacol.* **2009**, *9*, 615-621.

14. Erlanson, D. A.: Introduction to Fragment-Based Drug Discovery. *Top. Curr. Chem.* **2012**, 317, 1-32.
15. Jana, S.; Panda, D.; Saha, P.; Pantos, G. D.; and Dash, J.: Dynamic Generation of G-Quadruplex DNA Ligands by Target-Guided Combinatorial Chemistry on a Magnetic Nanoplatfrom. *J. Med. Chem.* **2019**, 62, 762-773.
16. Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radić, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; and Sharpless, K. B.: Click Chemistry In Situ: Acetylcholinesterase as a Reaction Vessel for the Selective Assembly of a Femtomolar Inhibitor from an Array of Building Blocks. *Angew. Chem. Int. Ed. Engl.* **2002**, 41, 1053-1057.
17. Whiting, M.; Muldoon, J.; Lin, Y.-C.; Silverman, S. M.; Lindstrom, W.; Olson, A. J.; Kolb, H. C.; Finn, M. G.; Sharpless, K. B.; Elder, J. H.; and Fokin, V. V.: Inhibitors of HIV-1 Protease by Using In Situ Click Chemistry. *Angew. Chem. Int. Ed. Engl.* **2006**, 45, 1435-1439.
18. Gelin, M.; Poncet-Montange, G.; Assairi, L.; Morellato, L.; Huteau, V.; Dugué, L.; Dussurget, O.; Pochet, S.; and Labesse, G.: Screening and In Situ Synthesis Using Crystals of a NAD Kinase Lead to a Potent Antistaphylococcal Compound. *Structure* **2012**, 20, 1107-1117.
19. Oueis, E.; Sabot, C.; and Renard, P.-Y.: New insights into the kinetic target-guided synthesis of protein ligands. *Chem. Commun.* **2015**, 51, 12158-12169.
20. Kolb, H. C.; Finn, M. G.; and Sharpless, K. B.: Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chem. Int. Ed. Engl.* **2001**, 40, 2004-2021.
21. Cesare Tron, G.; Pirali, T.; Billington, R. A.; Canonico, P. L.; Sorba, G.; and Genazzani, A. A.: Click Chemistry Reactions in Medicinal Chemistry: Applications of the 1,3-dipolar cycloaddition Between Azides and Alkynes. *Med. Res. Rev.* **2008**, 28, 278-308.
22. Boren, B. C.; Narayan, S.; Rasmussen, L. K.; Zhang, L.; Zhao, H.; Lin, Z.; Jia, G.; and Fokin, V. V.: Ruthenium-Catalyzed Azide-Alkyne Cycloaddition: Scope and Mechanism. *J. Am. Chem. Soc.* **2008**, 130, 8923-8930.
23. Michael, A.: Ueber die Einwirkung von Diazobenzolimid auf Acetylendicarbonsäuremethylester. *J. Prakt. Chem.* **1893**, 48, 94-95.
24. Huisgen, R.; Szeimies, G.; and Möbius, L.: 1,3-Dipolar cycloadditions. XXXII. Kinetics of the addition of organic azides to carbon-carbon multiple bonds. *Chem. Ber.* **1967**, 100, 2494-2507.
25. Hoyle, C. E.; and Bowman, C. N.: Thiol-Ene Click Chemistry. *Angew. Chem. Int. Ed.* **2010**, 49, 1540-1573.
26. Lowe, A. B.: Thiol-ene "click" reactions and recent applications in polymer and materials synthesis: a first update. *Polym. Chem.* **2014**, 5, 4820-4870.

27. Liu, H.; and Chung, H.: Visible-Light Induced Thiol-Ene Reaction on Natural Lignin. *ACS Sustainable Chem. Eng.* **2017**, *5*, 9160-9168.
28. Posner, T.: Beiträge zur Kenntniss der ungesättigten Verbindungen. II. Ueber die Addition von Mercaptanen an ungesättigte Kohlenwasserstoffe. *Ber. Dtsch. Chem. Ges.* **1905**, *38*, 646-657.
29. Oueis, E.; Nachon, F.; Sabot, C.; and Renard, P.-Y.: First enzymatic hydrolysis/thio-Michael addition cascade route to synthesis of AChE inhibitors. *Chem. Commun.* **2014**, *50*, 2043-2045.
30. Shangguan, N.; Katukojvala, S.; Greenberg, R.; and Williams, L. J.: The Reaction of Thio Acids with Azides: A New Mechanism and New Synthetic Applications. *J. Am. Chem. Soc.* **2003**, *125*, 7754-7755.
31. Kolakowski, R. V.; Shangguan, N.; Sauers, R. R.; and Williams, L. J.: Mechanism of Thio Acid/Azide Amidation. *J. Am. Chem. Soc.* **2006**, *128*, 5695-5702.
32. Namelikonda, N. K.; and Manetsch, R.: Sulfo-click reaction *via in situ* generated thioacids and its application in kinetic target-guided synthesis. *Chem. Commun.* **2012**, *48*, 1526-1528.
33. Rijkers, D. T. S.; Merks, R.; Yim, C.-B.; Brouwer, A. J.; and Liskamp, R. M. J.: 'Sulfo-click' for ligation as well as for site-specific conjugation with peptides, fluorophores, and metal chelators. *J. Pept. Sci.* **2010**, *16*, 1-5.
34. Carey, F. A.; and Sundberg, R. J.: Advanced Organic Chemistry, Part B: Reactions and Synthesis, Chapter 12: Oxidations, pp 1104. *Springer*, 5th Edition, USA **2007**. ISBN: 978-0-387-68354-6.
35. Maki, T.; Kawamura, A.; Kato, N.; and Ohkanda, J.: Chemical ligation of epoxide-containing fusococcins and peptide fragments guided by 14-3-3 protein. *Mol. Biosyst.* **2013**, *9*, 940-943.
36. Chase, J. F. A.; and Tubbs, P. K.: Conditions for the Self-Catalysed Inactivation of Carnitine Acetyltransferase. A Novel Form of Enzyme Inhibition. *Biochem. J.* **1969**, *111*, 225-235.
37. Nguyen, R.; and Huc, I.: Using an Enzyme's Active Site to Template Inhibitors. *Angew. Chem. Int. Ed. Engl.* **2001**, *40*, 1774-1776.
38. Mock, W. L.; Irra, T. A.; Wepsiec, J. P.; and Manimaran, T. L.: Cycloaddition induced by cucubituril. A case of Pauling principle catalysis. *J. Org. Chem.* **1983**, *48*, 3619-3620.
39. Di Antonio, M.; Biffi, G.; Mariani, A.; Raiber, E.-A.; Rodriguez, R.; and Balasubramanian, S.: Selective RNA Versus DNA G-Quadruplex Targeting by In Situ Click Chemistry. *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 11073-11078.
40. Bock, V. D.; Hiemstra, H.; and van Maarseveen, J. H.: Cu^I-Catalyzed Alkyne-Azide "Click" Cycloadditions from a Mechanistic and Synthetic Perspective. *Eur. J. Org. Chem.* **2006**, *2006*, 51-68.

41. Oueis, E.; Santoni, G.; Ronco, C.; Syzgantseva, O.; Tognetti, V.; Joubert, L.; Romieu, A.; Weik, M.; Jean, L.; Sabot, C.; Nachon, F.; and Renard, P.-Y.: Reaction site-driven regioselective synthesis of AChE inhibitors. *Org. Biomol. Chem.* **2014**, *12*, 156-161.
42. Manetsch, R.; Krasinski, A.; Radić, Z.; Raushel, J.; Taylor, P.; Sharpless, K. B.; and Kolb, H. C.: In Situ Click Chemistry: Enzyme Inhibitors Made to Their Own Specifications. *J. Am. Chem. Soc.* **2004**, *126*, 12809-12818.
43. Bourne, Y.; Kolb, H. C.; Radić, Z.; Sharpless, K. B.; Taylor, P.; and Marchot, P.: Freeze-frame inhibitor captures acetylcholinesterase in a unique conformation. *PNAS* **2004**, *101*, 1449-1454.
44. Krasinski, A.; Radić, Z.; Manetsch, R.; Raushel, J.; Taylor, P.; Sharpless, K. B.; and Kolb, H. C.: In Situ Selection of Lead Compounds by Click Chemistry: Target-Guided Optimization of Acetylcholinesterase Inhibitors. *J. Am. Chem. Soc.* **2005**, *127*, 6686-6692.
45. Mocharla, V. P.; Colasson, B.; Lee, L. V.; Röper, S.; Sharpless, K. B.; Wong, C.-H.; and Kolb, H. C.: In Situ Click Chemistry: Enzyme-Generated Inhibitors of Carbonic Anhydrase II. *Angew. Chem.* **2004**, *117*, 118-122.
46. Howell, S. J.; Spencer, N.; and Philp, D.: Recognition-mediated regiocontrol of a dipolar cycloaddition reaction. *Tetrahedron* **2001**, *57*, 4945-4954.
47. Hein, J. E.; and Fokin, V. V.: Copper-catalyzed azide-alkyne cycloaddition (CuAAC) and beyond: new reactivity of copper(I) acetylides. *Chem. Soc. Rev.* **2010**, *39*, 1302-1315.
48. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; and Sharpless, K. B.: A Stepwise Huisgen Cycloaddition process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 2596-2599.
49. Tornøe, C. W.; Christensen, C.; and Meldal, M.: Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. *J. Org. Chem.* **2002**, *67*, 3057-3064.
50. Worrell, B. T.; Malik, J. A.; and Fokin, V. V.: Direct Evidence of a Dinuclear Copper Intermediate in Cu(I)-Catalyzed Azide-Alkyne Cycloadditions. *Science* **2013**, *340*, 457-460.
51. Glassford, I.; Teijaro, C. N.; Daher, S. S.; Weil, A.; Small, M. C.; Redhu, S. K.; Colussi, D. J.; Jacobson, M. A.; Childers, W. E.; Buttar, B.; Nicholson, A. W.; MacKerell, A. D.; Cooperman, B. S.; and Andrade, R. B.: Ribosome-Templated Azide-Alkyne Cycloadditions: Synthesis of Potent Macrolide Antibiotics by In Situ Click Chemistry. *J. Am. Chem. Soc.* **2016**, *138*, 3136-3144.
52. El-Sagheer, A. H.; and Brown, T.: Click chemistry with DNA. *Chem. Soc. Rev.* **2010**, *39*, 1388-1405.

53. Meldal, M.: Polymer “Clicking” by CuAAC Reactions. *Macromol. Rapid Commun.* **2008**, *29*, 1016-1051.
54. Golas, P. L.; and Matyjaszewski, K.: Marrying click chemistry with polymerization: expanding the scope of polymeric materials. *Chem. Soc. Rev.* **2010**, *39*, 1338-1354.
55. Thirumurugan, P.; Matosiuk, D.; and Jozwiak, K.: Click Chemistry for Drug Development and Diverse Chemical–Biology Applications. *Chem. Rev.* **2013**, *113*, 4905-4979.
56. Kolb, H. C.; and Sharpless, K. B.: The growing impact of click chemistry on drug discovery. *Drug Discov. Today* **2003**, *8*, 1128-1137.
57. Saha, P.; Panda, D.; and Dash, J.: The application of click chemistry for targeting quadruplex nucleic acids. *Chem. Commun.* **2019**, *55*, 731-750.
58. Bochman, M. L.; Paeschke, K.; and Zakian, V. A.: DNA secondary structures: stability and function of G-quadruplex structures. *Nat. Rev. Genet.* **2012**, *13*, 770-780.
59. Sen, D.; and Gilbert, W.: Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. *Nature* **1988**, *334*, 364-366.
60. Biffi, G.; Tannahill, D.; McCafferty, J.; and Balasubramanian, S.: Quantitative Visualization of DNA G-quadruplex Structures in Human Cells. *Nat. Chem.* **2013**, *5*, 182-186.
61. Edwards, D. N.; Machwe, A.; Wang, Z.; and Orren, D. K.: Intramolecular Telomeric G-Quadruplexes Dramatically Inhibit DNA Synthesis by Replicative and Translesion Polymerases, Revealing their Potential to Lead to Genetic Change. *PLoS One* **2014**, *9*, e80664.
62. Cahoon, L. A.; and Seifert, H. S.: An Alternative DNA Structure is Necessary for Pilin Antigenic Variation in *Neisseria gonorrhoeae*. *Science* **2009**, *325*, 764-767.
63. Cheung, I.; Schertzer, M.; Rose, A.; and Lansdorp, P. M.: Disruption of *dog-1* in *Caenorhabditis elegans* triggers deletions upstream of guanine-rich DNA. *Nat. Genet.* **2002**, *31*, 405-409.
64. Rodriguez, R.; Miller, K. M.; Forment, J. V.; Bradshaw, C. R.; Nikan, M.; Britton, S.; Oelschlaegel, T.; Xhemalce, B.; Balasubramanian, S.; and Jackson, S. P.: Small-molecule-induced DNA damage identifies alternative DNA structures in human genes. *Nat. Chem. Biol.* **2012**, *8*, 301-310.
65. Siddiqui-Jain, A.; Grand, C. L.; Bearss, D. J.; and Hurley, L. H.: Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress *c-MYC* transcription. *PNAS* **2002**, *99*, 11593-11598.
66. Huppert, J. L.; and Balasubramanian, S.: G-quadruplexes in promoters throughout the human genome. *Nucleic Acids Res.* **2007**, *35*, 406-413.
67. Quin, Y.; and Hurley, L. H.: Structures, folding patterns, and functions of intramolecular DNA G-quadruplexes found in eukaryotic promoter regions. *Biochimie* **2008**, *90*, 1149-1171.

68. Balasubramanian, S.; and Neidle, S.: G-quadruplex nucleic acids as therapeutic targets. *Curr. Opin. Chem. Biol.* **2009**, *13*, 345-353.
69. Eddy, J.; and Maizels, N.: Conserved elements with potential to form polymorphic G-quadruplex structures in the first intron of human genes. *Nucleic Acid Res.* **2008**, *36*, 1321-1333.
70. Ma, D.-L.; Ma, V. P.-Y.; Leung, K.-H.; Zhong, H.-J.; He, H.-Z.; Chan, D. S.-H.; and Leung, C.-H.: Oncogene and Cancer – From Bench to Clinic, **2013**; *Open access peer-reviewed chapter: Structure-Based Approaches Targeting Oncogene Promoter G-Quadruplexes*, Chapter 1: Introduction. DOI: 10.5772/54809.
71. Monchaud, D.; and Tellaude-Fichou, M.-P.: A hitchhiker's guide to G-quadruplex ligands. *Org. Biomol. Chem.* **2008**, *6*, 627-636.
72. Su, Q.-B.; He, F.; Wang, X.-D.; Guan, S.; Xie, Z.-Y.; Wang, L.-Y.; Lu, Y.-J.; Gu, L.-Q.; Huang, Z.-S.; Chen, X.; Huang, M.; and Zhou, S.-F.: Biotransformation and pharmacokinetics of the novel anticancer drug, SYUIQ-5, in the rat. *Invest. New Drugs* **2008**, *26*, 119-137.
73. Diveshkumar, K. V.; Sakrikar, S.; Harikrishna, S.; Dhamodharan, V.; and Pradeepkumar, P. I.: Targeting promoter G-Quadruplex DNAs by Indenopyrimidine-Based Ligands. *ChemMedChem* **2014**, *9*, 2754-2765.
74. Jiang, Y.-L.; and Liu, Z.-P.: Metallo-Organic G-quadruplex Ligands in Anticancer Drug Design. *Mini Rev. Med. Chem.* **2010**, *10*, 726-736.
75. Campbell, N. H.; Patel, M.; Tofa, A. B.; Ghosh, R.; Parkinson, G. N.; and Neidle, S.: Selectivity in Ligand Recognition of G-Quadruplex Loops. *Biochemistry* **2009**, *48*, 1675-1680.
76. Campbell, N. H.; Parkinson, G. N.; Reszka, A. P.; and Neidle, S.: Structural Basis of DNA Quadruplex Recognition by an Acridine Drug. *J. Am. Chem. Soc.* **2008**, *130*, 6722-6724.
77. Phan, A. T.; Kuryavyi, V.; Gaw, H. Y.; and Patel, D. J.: Small-molecule interaction with a five-guanine-tract G-quadruplex structure from the human *MYC* promoter. *Nat. Chem. Biol.* **2005**, *1*, 167-173.
78. Marcu, K. B.; Bossone, S. A.; and Patel, A. J.: *myc* function and regulation. *Annu. Rev. Biochem.* **1992**, *61*, 809-860.
79. Dang, C. V.: c-MYC Target Genes Involved in Cell Growth, Apoptosis, and Metabolism. *Mol. Cell Biol.* **1999**, *19*, 1-11.
80. Pelengaris, S.; Rudolph, B.; and Littlewood, T.: Action of Myc *in vivo* – proliferation and apoptosis. *Curr. Opin. Genet. Dev.* **2000**, *10*, 100-105.
81. Jäättelä, M.: Multiple cell death pathways as regulators of tumor initiation and progression. *Oncogene* **2004**, *23*, 2746-2756.

82. Little, C. D.; Nau, M. M.; Carney, D. N.; Gazdar, A. F.; and Minna, J. D.: Amplification and expression of the *c-myc* oncogene in human lung cancer cell lines. *Nature* **1983**, *306*, 194-196.
83. Mariani-Costantini, R.; Escot, C.; Theillet, C.; Gentile, A.; Merlo, G.; Lidereau, R.; and Callahan, R.: *In Situ c-myc* Expression and Genomic Status of the *c-myc* Locus in Infiltrating Ductal Carcinomas of the Breast. *Cancer Res.* **1988**, *48*, 199-205.
84. Münzel, P.; Marx, D.; Köchel, H.; Schauer, A.; and Bock, K. W.: Genomic alterations of the *c-myc* protooncogene in relation to the overexpression of *c-erbB2* and Ki-67 in human breast and cervix carcinomas. *J. Cancer Res. Clin. Oncol.* **1991**, *117*, 603-607.
85. Augenlicht, L. H.; Wadler, S.; Corner, G.; Richards, C.; Ryan, L.; Multani, A. S.; Pathak, S.; Benson, A.; Haller, D.; and Heerdt, B. G.: Low-level *c-myc* amplification in human colonic carcinoma cell lines and tumors: a frequent, p53-independent mutation associated with improved outcome in a randomized multi-institutional trial. *Cancer Res.* **1997**, *57*, 1769-1775.
86. Yang, D.; and Hurley, L. H.: Structure of the Biologically Relevant G-Quadruplex in The c-MYC Promoter. *Nucleos. Nucleot. Nucl. Acids* **2006**, *25*, 951-968.
87. Seenisamy, J.; Rezler, E. M.; Powell, T. J.; Tye, D.; Gokhale, V.; Joshi, C. S.; Siddiqui-Jain, A.; and Hurley, L. H.: The Dynamic Character of the G-Quadruplex Element in the c-MYC Promoter and Modification by TMPyP4. *J. Am. Chem. Soc.* **2004**, *126*, 8702-8709.
88. Ambrus, A.; Chen, D.; Dai, J.; Jones, R. A.; and Yang, D.: Solution Structure of the Biologically Relevant G-Quadruplex Element in the Human c-MYC Promoter. Implications for G-Quadruplex Stabilization. *Biochemistry* **2005**, *44*, 2048-2058.
89. Phan, A. T.; Modi, Y. S.; and Patel, D. J.: Propeller-Type Parallel-Stranded G-Quadruplexes in the Human *c-myc* Promoter. *J. Am. Chem. Soc.* **2004**, *126*, 8710-8716.
90. Parkinson, G. N.; Lee, M. P. H.; and Neidle, S.: Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature* **2002**, *417*, 876-880.
91. Grand, C. L.; Han, H.; Muñoz, R. M.; Weitman, S.; Von Hoff, D. D.; Hurley, L. H.; and Bearss, D. J.: The Cationic Porphyrin TMPyP4 Down-Regulates c-MYC and Human Telomerase Reverse Transcriptase Expression and Inhibits Tumor Growth *in Vivo*. *Mol. Cancer Ther.* **2002**, *1*, 565-573.
92. Ou, T.-M.; Lu, Y.-J.; Zhang, C.; Huang, Z.-S.; Wang, X.-D.; Tan, J.-H.; Chen, Y.; Ma, D.-L.; Wong, K.-Y.; Tang, J. C.-O.; Chan, A. S.-C.; and Gu, L.-Q.: Stabilization of G-Quadruplex DNA and Down-Regulation of Oncogene *c-myc* by Quindoline Derivatives. *J. Med. Chem.* **2007**, *50*, 1465-1474.
93. Lu, Y.-J.; Ou, T.-M.; Tan, J.-H.; Hou, J.-Q.; Shao, W.-Y.; Peng, D.; Sun, N.; Wang, X.-D.; Wu, W.-B.; Bu, X.-Z.; Huang, Z.-S.; Ma, D.-L.; Wong, K.-Y.; and Gu, L. Q.: 5-*N*-Methylated

- Quindoline Derivatives as Telomeric G-Quadruplex Stabilizing Ligands: Effects of 5-*N* Positive Charge on Quadruplex Binding Affinity and Cell Proliferation. *J. Med. Chem.* **2008**, *51*, 6381-6392.
94. Ma, Y.; Ou, T.-M.; Hou, J.-Q.; Lu, Y.-J.; Tan, J.-H.; Gu, L.-Q.; and Huang, Z.-S.: 9-*N*-Substituted berberine derivatives: stabilization of G-quadruplex DNA and down-regulation of oncogene *c-myc*. *Bioorg. Med. Chem.* **2008**, *16*, 7582-7591.
 95. Bejugam, M.; Sewitz, S.; Shirude, P. S.; Rodriguez, R.; Shahid, R.; and Balasubramanian, S.: Trisubstituted Isoalloxazines as a New Class of G-Quadruplex Binding Ligands: Small Molecule Regulation of c-kit Oncogene Expression. *J. Am. Chem. Soc.* **2007**, *129*, 12926-12927.
 96. Wheelhouse, R. T.; Sun, D.; Han, H.; Han, F. X.; and Hurley, L. H.: Cationic Porphyrins as Telomerase Inhibitors: the Interaction of Tetra-(*N*-methyl-4-pyridyl)porphine with Quadruplex DNA. *J. Am. Chem. Soc.* **1998**, *120*, 3261-3262.
 97. Membrino, A.; Paramasivam, M.; Cogoi, S.; Alzeer, J.; Luedtke, N. W.; and Xodo, L. E.: Cellular uptake and binding of guanidine-modified phthalocyanines to *KRAS/HRAS* G-quadruplexes. *Chem. Commun.* **2010**, *46*, 625-627.
 98. Portugal, J.; and Waring, M. J.: Assignment of DNA binding sites for 4',6-diamidine-2-phenylindole and bisbenzimidazole (Hoechst 33258). A comparative footprinting study. *Biochim. Biophys. Acta, Gene Struct. Expression* **1988**, *949*, 158-168.
 99. Chen, B.-J.; Wu, Y.-L.; Tanaka, Y.; and Zhang, W.: Small Molecules Targeting c-Myc Oncogene: Promising Anti-Cancer Therapeutics. *Int. J. Biol. Sci.* **2014**, *10*, 1084-1096.
 100. Maiti, S.; Chaudhury, N. K.; and Chowdhury, S.: Hoechst 33258 binds to G-quadruplex in the promoter region of human *c-myc*. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 505-512.
 101. Dumat, B.; Bordeau, G.; Faurel-Paul, E.; Mahuteau-Betzer, F.; Saettel, N.; Bombled, M.; Metgé, G.; Charra, F.; Fiorini-Debuisschert, C.; and Teulade-Fichou, M.-P.: *N*-phenyl-carbazole-based two-photon fluorescent probes: Strong sequence dependence of the duplex vs quadruplex selectivity. *Biochimie* **2011**, *93*, 1209-1218.
 102. Rangan, A.; Fedoroff, O. Y.; and Hurley, L. H.: Induction of Duplex to G-quadruplex Transition in the *c-myc* Promoter Region by a Small Molecule. *J. Biol. Chem.* **2001**, *276*, 4640-4646.
 103. Mo, H.; Vita, M.; Crespin, M.; and Henriksson, M. A.: Myc Overexpression Enhances Apoptosis Induced by Small Molecules. *Cell Cycle* **2006**, *5*, 2191-2194.
 104. Duan, W.; Rangan, A.; Vankayalapati, H.; Kim, M.-Y.; Zeng, Q.; Sun, D.; Han, H.; Fedoroff, O. Y.; Nishioka, D.; Rha, S. Y.; Izbička, E.; Von Hoff, D. D.; and Hurley, L. H.: Design and Synthesis of Fluoroquinophenoxazines That Interact with Human Telomeric G-Quadruplexes and Their Biological Effects. *Mol. Cancer Ther.* **2001**, *1*, 103-120.

105. Asamitsu, S.; Obata, S.; Yu, Z.; Bando, T.; and Sugiyama, H.: Recent Progress of Targeted G-Quadruplex-Preferred Ligands Toward Cancer Therapy. *Molecules* **2019**, *24*, 429.
106. Drygin, D.; Siddiqui-Jain, A.; O'Brien, S.; Schwaebe, M.; Lin, A.; Bliesath, J.; Ho, C. B.; Proffitt, C.; Trent, K.; Whitten, J. P.; Lim, J. K. C.; Von Hoff, D.; Anderes, K.; and Rice, W. G.: Anticancer Activity of CX-3543: A Direct Inhibitor of rRNA Biogenesis. *Cancer Res.* **2009**, *69*, 7653-7661.
107. Morgan, R. K.; and Brooks, T. A.: Small-molecule Transcription Factor Inhibitors in Oncology. Chapter 7: Targeting Promoter G-Quadruplexes for Transcriptional Control. *The Royal Society of Chemistry*, 1st Edition, Cambridge, UK **2019**. ISBN: 978-1-78262-401-1.
108. Hurley, L. H.; and Guzman, M.: Combination cancer chemotherapy. Patent WO 2007/137000 A2, (29.11.2007).
109. Ma, Y.; Ou, T.-M.; Tan, J.-H.; Hou, J.-Q.; Huang, S.-L.; Gu, L.-Q.; and Huang, Z.-S.: Quinolino-benzo-[5,6]-dihydroisoquinolium compounds derived from berberine: A new class of highly selective ligands for G-quadruplex DNA in *c-myc* oncogene. *Eur. J. Med. Chem.* **2011**, *46*, 1906-1913.
110. Pagano, B.; Cosconati, S.; Gabelica, V.; Petraccone, L.; De Tito, S.; Marinelli, L.; La Pietra, V.; Di Leva, F. S.; Lauri, I.; Trotta, R.; Novellino, E.; Giancola, C.; and Randazzo, A.: State-of-the-Art Methodologies for the Discovery and Characterization of DNA G-Quadruplex Binders. *Curr. Pharm. Des.* **2012**, *18*, 1880-1899.
111. Jaumot, J.; and Gargallo, R.: Experimental Methods for Studying the Interactions between G-Quadruplex Structures and Ligands. *Curr. Pharm. Des.* **2012**, *18*, 1900-1916.
112. Sahoo, M.: Förster resonance energy transfer – A spectroscopic nanoruler: Principle and applications. *J. Photochem. Photobiol. C* **2011**, *12*, 20-30.
113. Huppert, J. L.: Four-stranded nucleic acids: structure, function and targeting of G-quadruplexes. *Chem. Soc. Rev.* **2008**, *37*, 1375-1384.
114. Murat, P.; Singh, Y.; and Defrancq, E.: Methods for investigating G-quadruplex DNA/ligand interactions. *Chem. Soc. Rev.* **2011**, *40*, 5293-5307.
115. Ueno, Y.; Jiao, G.-S.; and Burgess, K.: Preparation of 5- and 6-Carboxyfluorescein. *Synthesis* **2004**, *15*, 2591-2593.
116. Mudd, G.; Pérez Pi, I.; Fethers, N.; Dodd, P. G.; Barbeau, O. R.; and Auer, M.: A general synthetic route to isomerically pure functionalized rhodamine dyes. *Methods Appl. Fluoresc.* **2015**, *3*, 045002.

117. Hubálková, P.: Nové stabilizátory G-kvadruplexových struktur. Diploma thesis, University of Chemistry and Technology, Prague, Czech Republic, Faculty of Food and Biochemical Technology, **2014**, pp 16-17.
118. Renčiuk, D.; Zhou, J.; Beaurepaire, L.; Guédin, A.; Bourdoncle, A.; and Mergny, J.-L.: A FRET-based screening assay for nucleic acid ligands. *Methods* **2012**, *57*, 122-128.
119. Chien, A.; Edgar, D. B.; and Trela, J. M.: Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J. Bacteriol.* **1976**, *127*, 1550-1557.
120. Han, H.; Hurley, L. H.; and Salazar, M.: A DNA polymerase stop assay for G-quadruplex-interactive compounds. *Nucleic Acids Res.* **1999**, *27*, 537-542.
121. Guittat, L.; Lacroix, L.; Gomez, D.; Arimondo, P. B.; De Cian, A.; Pennarun, G.; Amrane, S.; Alberti, P.; Lemarteleur, T.; Aouali, N.; Morjani, H.; Trentesaux, C.; Saccà, B.; Mandine, E.; Boussin, F.; Mailliet, P.; Riou, J.-F.; and Mergny, J.-L.: Dynamical Genetics, Chapter 11: Quadruplex structures and quadruplex ligands, pp 199-236. *Research Signpost*, Kerala, India **2004**. ISBN: 81-7736-231-3.
122. Sun, D.; and Hurley, L. H.: Biochemical Techniques for the Characterization of G-Quadruplex Structures: EMSA, DMS Footprinting, and DNA Polymerase Stop Assay. *Methods Mol. Biol.* **2010**, *608*, 65-79.
123. Pellecchia, M.; Sem, D. S.; and Wüthrich, K.: NMR in drug discovery. *Nat. Rev. Drug Discov.* **2002**, *1*, 211-219.
124. Williamson, M. P.: Using chemical shift perturbation to characterise ligand binding. *Prog. Nucl. Mag. Res. Sp.* **2013**, *73*, 1-16.
125. Sidir, Y. G.; Sidir, I.; Berber, H.; Tasal, E.; and Ögretir, C.: Spectroscopic determination of Acid Dissociation Constants of Some Novel Drug Precursor 6-Acylbenzothiazolon Derivatives. *J. Chem. Eng. Data* **2010**, *55*, 4752-4756.
126. Ferraris, D.; Ko, Y.-S.; Pahutski, T.; Pargas Ficco, R.; Serdyuk, L.; Alemu, C.; Bradford, C.; Chiou, T.; Hoover, R.; Huang, S.; Lautar, S.; Liang, S.; Lin, Q.; Lu, M. X.-C.; Mooney, M.; Morgan, L.; Qian, Y.; Tran, S.; Williams, L. R.; Wu, Q. Y.; Zhang, J.; Zou, Y.; and Kalish, V.: Design and Synthesis of Poly ADP-ribose Polymerase-1Inhibitors. 2. Biological Evaluation of Aza-5[H]-phenanthridin-6-ones as Potent, Aqueous-Soluble Compounds for the Treatment of Ischemic Injuries. *J. Med. Chem.* **2003**, *46*, 3138-3151.
127. Sun, Q.-Z.; Xu, Y.; Liu, J.-J.; Zhang, C.-H.; Wang, Z.-R.; Zheng, R.-L.; Wang, W.-J.; Li, L.-L.; and Yang, S.-Y.: Structural modification of an EGFR inhibitor that showed weak off-target activity against RET leading to the discovery of a potent RET inhibitor. *Mol. Divers.* **2014**, *18*, 403-409.

128. Nasiri, H. R.; Bell, N. M.; McLuckie, K. I. E.; Husby, J.; Abell, C.; Neidle, S.; and Balasubramanian, S.: Targeting a c-MYC G-quadruplex DNA with a fragment library. *Chem. Commun.* **2014**, 50, 1704-1707.
129. Rodriguez, R.; Müller, S.; Yeoman, J. A.; Trentesaux, C.; Riou, J.-F.; and Balasubramanian, S.: A Novel Small Molecule That Alters Shelterin Integrity and Triggers a DNA-Damage Response at Telomeres. *J. Am. Chem. Soc.* **2008**, 130, 15758-15759.
130. Müller, S.; Sanders, D. A.; Di Antonio, M.; Matsis, S.; Riou, J.-F.; Rodriguez, R.; and Balasubramanian, S.: Pyridostatin analogues promote telomere dysfunction and long-term growth inhibition in human cancer cells. *Org. Biomol. Chem.* **2012**, 10, 6537-6546.
131. De Rache, A.; and Mergny, J.-L.: Assessment of selectivity of G-quadruplex ligands via an optimised FRET melting assay. *Biochimie* **2015**, 115, 194-202.
132. Feng, Y.; Yang, D.; Chen, H.; Cheng, W.; Wang, L.; Sun, H.; and Tang, Y.: Stabilization of G-quadruplex DNA and inhibition of Bcl-2 expression by a pyridostatin analog. *Bioorg. Med. Chem. Lett.* **2016**, 26, 1660-1663.
133. Mandal, S.; Kawamoto, Y.; Yue, Z.; Hashiya, K.; Cui, Y.; Bando, T.; Pandey, S.; Hoque, M. E.; Hossain, M. A.; Sugiyama, H.; and Mao, H.: Submolecular dissection reveals strong and specific binding of polyamide-pyridostatin conjugates to human telomere interface. *Nucleic Acids Res.* **2019**, 47, 3295-3305.
134. Ma, T.-Z.; Zhang, M.-J.; Liao, T.-C.; Li, J.-H.; Zou, M.; Wang, Z.-M.; and Zhou, C.-Q.: Dimers formed with the mixed-type G-quadruplex binder pyridostatin specifically recognize human telomere G-quadruplex dimers. *Org. Biomol. Chem.* **2020**, 18, 920-930.
135. Mahindroo, N.; Connelly, M. C.; Punchihewa, C.; Yang, L.; Yan, B.; and Fujii, N.: Amide Conjugates of Ketoprofen and Indole as Inhibitors of Gli1-Mediated Transcription in the Hedgehog Pathway. *Bioorg. Med. Chem.* **2010**, 18, 4801-4811.
136. Hryhorenko, E.; Sankaran, B.; Decory, T. R.; Tubbs, T.; Colt, L.; Remmerie, B. M.; Salter, R.; and Lin, R.: Antibodies to aripiprazole haptens and use thereof. Patent WO/2014/031635 (27.02.2014).
137. Risseuw, M. D. P.; De Clercq, D. J. H.; Lievens, S.; Hillaert, U.; Sinnaeve, D.; Van den Broeck, F.; Martins, J. C.; Tavernier, J.; and Van Calenbergh, S.: A “Clickable” MTX Reagent as a Practical Tool for Profiling Small-Molecule–Intracellular Target Interactions via MASPIT. *ChemMedChem* **2013**, 8, 521-526.
138. Anselmet, A.; Mayat, E.; Wietek, S.; Layer, P. G.; Payrastra, B.; and Massoulié, J.: Non-antisense cellular responses to oligonucleotides. *FEBS Lett.* **2002**, 510, 175-180.

139. De la Fuente, T.; Martín-Fontecha, M.; Sallander, J.; Benhamú, B.; Campillo, M.; Medina, R. A.; Pellissier, L. P.; Claeysen, S.; Dumuis, A.; Pardo, L.; and López-Rodríguez, M. L.: Benzimidazole Derivatives as New Serotonin 5-HT₆ Receptor Antagonists. Molecular Mechanisms of Receptor Inactivation. *J. Med. Chem.* **2010**, *53*, 1357-1369.
140. Coville, N. J.; and Neuse, E. W.: Oxidative cyclodehydrogenation of aromatic bis(o-aminoanils). *J. Org. Chem.* **1977**, *42*, 3485-3491.
141. Bourrie, B.; Casellas, P.; Ciapetti, P.; Derocq, J.-M.; Jegham, S.; Muneaux, Y.; and Wermuth, C.-G.: Pyridoindolone derivatives substituted in the 3-position by a phenyl, their preparation and their application in therapeutics. Patent US8012991 B2 (06.09.**2011**).
142. Subirós-Funosas, R.; El-Faham, A.; and Albericio, F.: PyOxP and PyOxB: the Oxyma-based novel family of phosphonium salts. *Org. Biomol. Chem.* **2010**, *8*, 3665-3673.
143. Fredriksen, K. A.; and Amedjkouh, M.: Investigation of Reactive Intermediates and Reaction Pathways in the Coupling Agent Mediated Phosphonamidation Reaction. *Eur. J. Org. Chem.* **2016**, *2016*, 474-482.
144. Macleod, F.; Lang, S.; and Murphy, J. A.: The 2-(2-Azidoethyl)cycloalkanone Strategy for Bridged Amides and Medium-Sized Cyclic Amine Derivatives in the Aubé-Schmidt Reaction. *Synlett* **2010**, *4*, 529-534.
145. Herrero, C.; Quaranta, A.; El Ghachtouli, S.; Vauzeilles, B.; Leibl, W.; and Aukauloo, A.: Carbon dioxide reduction via light activation of a ruthenium-Ni(cyclam) complex. *Phys. Chem. Chem. Phys.* **2014**, *16*, 12067-12072.
146. Daugan, A. C.-M.; and Kirilovsky, J. E.: Use of therapeutic benzamide derivatives. Patent WO/2001/097810 (27.12.**2001**).
147. Sinha, M. K.; Reany, O.; Yefet, M.; Botoshansky, M.; and Keinan, E.: Bistable Cucurbituril Rotaxanes Without Stoppers. *Chem. Eur. J.* **2012**, *18*, 5589-5605.
148. Fabre, B.; Pícha, J.; Vaněk, V.; Selicharová, I.; Chrudinová, M.; Collinsová, M.; Žáková, L.; Buděšínský, M.; and Jiráček, J.: Synthesis and Evaluation of a Library of Trifunctional Scaffold-Derived Compounds as Modulators of the Insulin Receptor. *ACS Comb. Sci.* **2016**, *18*, 710-722.
149. Ladd, E.; Sheikhi, A.; Li, N.; Van de Ven, T. G. M.; and Kakkar, A.: Design and Synthesis of Dendrimers with Facile Surface Group Functionalization, and an Evaluation of Their Bactericidal Efficacy. *Molecules* **2017**, *22*, 868.