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Příprava inhibitorů chřipkové neuraminidasy a polymerasy

Preparation of Influenza Neuraminidase and Polymerase Inhibitors

Doctoral thesis

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Prohlášení:

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V Praze, 2.12. 2020

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List of abbreviations

2NA	2-Naphthylalanine
2-Pic	2-Picoline (2-methylpyridine)
A	Alanine
AA	Amino acid
Ac	Acetyl
Acm	*S*-Acetamidomethyl
Aib	2-Aminoisobutyric acid
APT	Attached proton test
Boc	*tert*-Butyloxycarbonyl
C	Cysteine
CI	Chemical ionization
COSY	Correlation spectroscopy
CPP	Cell penetrating peptides
CuAAC	Copper catalyzed alkyne-azide cycloaddition
D	Aspartic acid
DANA	2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid
DCE	1,2-Dichloroethane
DCM	Dichloromethane
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DIAD	Diisopropyl azodicarboxylate
DIANA	DNA-linked inhibitor antibody assay
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-Dimethyl formamide
DNA	Deoxyribonucleic acid
DPPA	Diphenylphosphoryl azide
EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
F	Phenylalanine
Fmoc	Fluorenylmethyloxycarbonyl
GSH	Glutathione
GST	Glutathione S-transferase
HA	Hemaglutinin
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HBTU	2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEK	Human Embryonic Kidney
HMBC	Heteronuclear Multiple Bond Correlation
HRMS	High-resolution mass spectrometry
HSQC	Heteronuclear single quantum coherence
I	Isoleucine

K	Lysine
L	Leucine
Leu	Leucine
Lys	Lysine
M	Methionine
MDCK	Madin-Darby Canine Kidney
MUNANA	2'-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid
N	Asparagine
NA	Neuraminidase
NBS	*N*-Bromosuccinimide
NMM	*N*-Methylmorpholine
NMP	*N*-Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance
P	Proline
PA	Polymerase acid
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PBD	Protein Data Bank
PCR	Polymerase chain reaction
Phe	Phenylalanine
PPI	Protein-protein interaction
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
Q	Glutamine
R	Arginine
S	Serine
SAR	Structure activity relationship
SPPS	Solid phase peptide synthesis
T	Threonine
TAT	HIV-1 trans-activator of transcription protein
TBTA	Tris(benzyltriazolylmethyl)amine
TBTU	2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
TEA	Triethylamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Trt	Trityl
Ts	Tosyl
V	Valine
Y	Tyrosine
Z	Caboxybenzyl group

Abstract

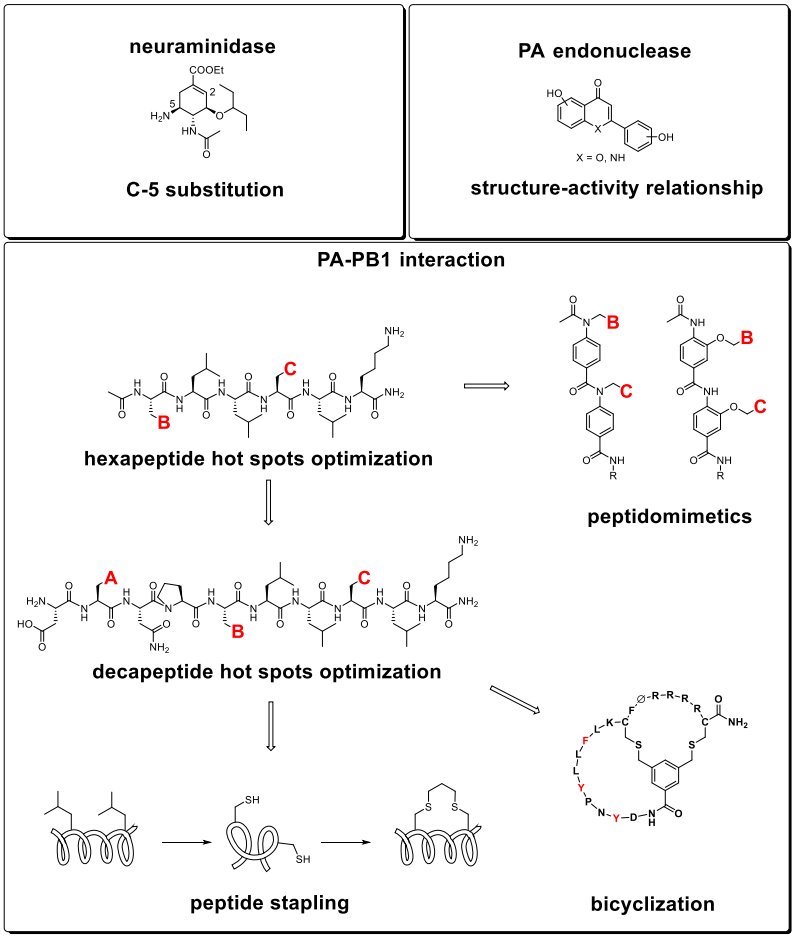
Influenza is an infectious disease caused by the influenza virus. This virus causes a severe viral infection that spreads easily from person to person in yearly pandemics. Vaccination is the most effective way to prevent the infection, however, due to the high rate in mutations of the virus, the vaccine needs to be often reformulated. Another option to combat influenza is based on administration of antiviral drugs. Clinical studies of isolated influenza strains („avian flu“ H5N1, 2004; „swine flu“ H1N1, 2009) revealed resistance towards known influenza neuraminidase inhibitors (zanamivir, oseltamivir). The resistance is caused by structural changes close to the enzymatic site. This calls for the development of new neuraminidase inhibitors as well for development of inhibitors targeting different influenza enzymes.

This Thesis is focused on design and synthesis of new inhibitors of influenza neuraminidase and RNA-dependent RNA polymerase, namely PA subunit and the assembly of PA-PB1 heterodimer enzymes (Scheme 1).

Influenza neuraminidase inhibitors were prepared by C-5 derivatization of oseltamivir followed by subsequent extension of its structure with binders of 150-cavity. Binding potencies of new oseltamivir derivatives against two influenza strains were determined.

The next part contributed to the elucidation of the anti-influenza effect of quercetin and related compounds as RNA-dependent RNA polymerase inhibitors. The mechanism of action of flavonoids as PA endonuclease inhibitors was discussed. This work features the extensive structure-activity relationship study accomplished on flavonoids as PA endonuclease inhibitors. Submicromolar inhibitors of PA endonuclease were successfully prepared and the Thesis covers numerous common synthetic approaches toward flavonoids.

The last part of the Thesis deals with preparation of PA-PB1 protein-protein interaction inhibitors. The crystal structure of PA C-terminal domain with a truncated decapeptide sequence derived from PB1 N-terminal subunit was used to design peptidomimetics based on the oligobenzamide scaffold. Phenylalanine and two tyrosine residues were identified to be crucial for the nanomolar activity of the PB1-derived decapeptide. In the subsequent work, the original hot spots were optimized using solid-phase peptide synthesis of hexapeptide inhibitors.



Scheme 1. Outline of three different topics of this thesis and the different strategies used.

The aim of this optimization was to increase the solubility of compounds while maintaining or even improving the inhibitory activities. The knowledge gathered was then applied in the design of new decapeptide inhibitors. Another crystal structure of optimized decapeptide with PA C-terminal domain was solved. Gained structural information was used to design stapled decapeptides with the intention to increase binding efficiency. Since the peptide inhibitors are inefficient in entering the cells, the peptide inhibitors were equipped with cell-penetrating sequence. These combined peptides were than cyclized around a central fragment to increase the proteolytic stability and cell-penetrating ability. Upon entering the infected cell, these bicyclic peptides should become linear and inhibit formation of PA-PB1 heterodimer.

Keywords: *neuraminidase, polymerase, enzyme, inhibition, synthesis, design*

Abstrakt

Chřipka je infekční onemocnění způsobené virem chřipky. Tento vir způsobuje závažnou virovou infekci, která se snadno přenáší z osoby na osobu během sezonních pandemií. Nejefektivnější způsob jak zabránit infekci, je očkování, nicméně kvůli vysoké mutační schopnosti viru se musí každý rok vakcína přeformulovat. Další způsob, jak bojovat proti chřipce, je podávání antivirotik. Klinické studie izolovaných kmenů ptačí chřipky (H5N1, 2004) a prasečí chřipky (H1N1, 2009) ukázaly na zvyšující se rezistenci vůči známým neuraminidasovým inhibitorům (oseltamivir, zanamivir). Tato resistence je zapříčiněna strukturními změnami v blízkosti katalytického místa enzymu. Výše zmíněné skutečnosti jsou motivací pro vývoj nových neuraminidasových inhibitorů a také pro vývoj inhibitorů, které cílí na jiné chřipkové enzymy.

Tato práce se zabývá návrhem a přípravou nových inhibitorů chřipkové neuraminidasy a RNA-dependentní RNA polymerasy (konkrétně na PA podjednotku a skladbu PA-PB1 heterodimeru, Schéma 1).

Inhibitory chřipkové neuraminidasy byly připraveny pomocí modifikace oseltamiviru v pozici C-5. Oselatamivir byl následně modifikován skupinami, které byly navržené tak, aby vytvářely interakce v kavitě-150. Byly změřeny aktivity nových derivátů oseltamiviru proti dvěma kmenům chřipky.

Další část disertační práce přispěla k objasnění mechanismu účinku kvercetinu (a strukturně podobných látek) jako inhibitoru RNA-dependentní RNA polymerasy. Je zde diskutován mechanismus účinku flavonoidů jako inhibitorů PA podjednotky. Tato disertační práce představila rozsáhlé studium vztahu mezi strukturou a biologickou aktivitou flavonoidů jako inhibitorů PA podjednotky chřipkové polymerasy. Byly připraveny inhibitory chřipkové PA endonukleasy se submikromolární aktivitou a tato práce obsahuje několik syntetických přístupů pro přípravu flavonoidů.

Poslední část disertační práce je zaměřena na přípravu inhibitorů PA-PB1 protein-proteinové interakce. Na základě krystalové struktury PB1 odvozeného dekapeptidu s PA C-terminální podjednotkou byla navržena a připravena peptidomimetika vycházející z oligobenzamidového strukturního motivu. Fenylalanin a dva tyrosiny byly identifikovány jako nezbytné pro aktivitu PB1 odvozeného dekapeptidového inhibitoru PA-PB1 interakce.

Následně byly pomocí peptidové syntézy na pevné fázi optimalizovány hexapeptidové inhibitory tak, aby při zachování či zlepšení inhibičního účinku byla zlepšena jejich rozpustnost. Data získaná při optimalizaci hexapeptidů byla použita pro optimalizaci dekapeptidů.

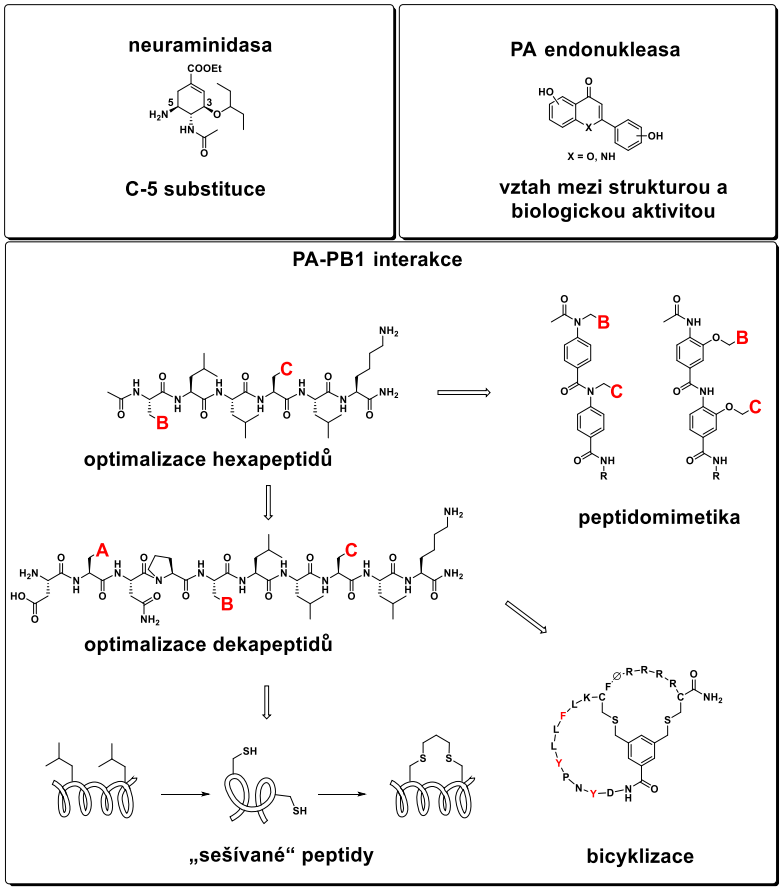


Schéma 1. Vyobrazení tří témat této disertační práce včetně použitých strategií.

Byla vyřešena nová krystalová struktura optimalizovaného dekapeptidu s PA C-terminální podjednotkou. Nové strukturní informace byly použity pro návrh a přípravu tzv. „sešívaných“ peptidů za účelem zlepšení vazebné interakce. Peptidové inhibitory postrádají schopnost efektivního průchodu buněčnou membránou. Za tímto účelem byly peptidové inhibitory vybaveny peptidovou sekvencí, která umožňuje průchod buněčnou membránou. Tyto kombinované peptidy byly cyklizovány kolem centrálního fragmentu za účelem průchodu buněčnou membránou a zvýšení metabolické stability. Po průchodu peptidů do nakažené buňky by se tyto bicyklické peptidy měly stát lineárními a následně zablokovat vznik PA-PB1 heterodimeru.

Klíčová slova: *neuraminidasa, polymerasa, enzym, inhibice, syntéza, design*

1. Introduction

1.1. Influenza virus

Influenza is acute respiratory disease caused by Influenza virus in birds and mammals. The disease is characterized by sudden fever, cough, nasal congestion and fatigue. Approximately 33 % of infected people are asymptotical.[1] Influenza spreads easily from person to person in seasonal pandemics. These seasonal pandemics are every year responsible for three to five million cases of severe acute viral infection and 290 000 to 650 000 deaths worldwide.[2] Since the 1500s, the influenza virus was responsible for 22 pandemics. During the last century, several influenza pandemics occurred and each was caused by a new different strain of influenza virus. The most severe pandemics were Spanish flu (1918-20, strain H1N1) killing from 20 to 50 million humans worldwide, the Asian flu (1957, strain H2N2) with 1-2 million causalities and Hong-Kong flu (1968, H3N2) with 700 000 causalities. High-risk groups are children under 5 years old, elderly people, pregnant woman, and people whose immunity was compromised. Influenza viruses damage and infect the mucous membrane of the upper respiratory tract and as a result, a second bacterial infection may occur in lower respiratory tract. Death of patient is caused by secondary bacterial pneumonia infection.

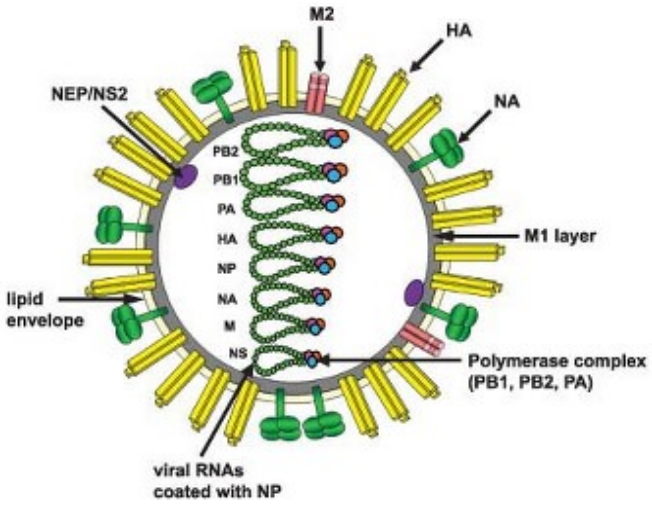


Figure 1. Schematic representation of influenza virus particle.[3]

The influenza virus is RNA virus of the *Orthomyxoviridae* family which is characterized by negative-sense, single-stranded, and segmented RNA genome. Influenza viruses make up four out of seven genera of the *Orthomyxoviridae* family. The four influenza viruses A, B, C and D are classified according to nucleoprotein and antigenic determinants on its Matrix

protein. All A- and B-type influenza viruses possess eight RNA segments. For influenza C and D viruses the major glycoprotein, HEF (hemagglutinin-esterase fusion) combines the function of neuraminidase and hemagglutinin and thus influenza C viruses possess one less RNA segment. Each segmented viral-RNA encode protein(s) that are essential for viral replication and each genomic RNA segment exists in the form of a ribonucleoprotein (RNP) complex in association with nucleoproteins and an RNA-dependent RNA polymerase (Figure 1). Very recent publication suggest that even though influenza viruses C and D possess seven-segmented RNA genome its RNPs are arranged in the 1+7 pattern regardless of the number of RNA segments in their genome.[4]

Influenza A viruses have a lipid membrane derived from the host cell and this envelope contains the hemagglutinin, the neuraminidase, and the M2 ion channel protein (Figure 1).[3] Beneath the envelope there is the matrix protein (M1) and the core of the viral particle harbors the ribonucleoprotein complex (RNP) consisting of the nucleoprotein (NP), viral RNA segments and the RNA-dependent RNA polymerase complex.[5] The viral particle also contains nonstructural protein 1 (NS1) and nuclear export protein (NEP).[6]

Influenza A viruses are further subdivided based on antigenic properties of its surface glycoprotein enzymes neuraminidase and hemagglutinin. There are now eighteen HAs[7] and eleven NAs identified so far. Influenza A viruses are known to infect mammals and birds. Influenza B viruses are subdivided into two lineages: the B/Victoria/2/87- and B/Yamagata/16/88.[8] Influenza B viruses are known to infect only seals and human.[9]

Both influenza viruses A and B encode two surface glycoproteins HAs and NAs. These glycoproteins are major antigenic determinants of influenza viruses and are under immunological pressure, leading to antigenic variants that are positively selected to avoid immune detection.[10] A major change in antigenicity happens during antigenic shift, which is caused by viral genome reassortment, or the transfer of genomic segments between different viral strains in co-infected cells within an organism.[3] Another antigenic variation is called antigenic drift and is a result of accumulation of point mutation in HA and NA genes. These two antigenic variations can explain the proclivity of influenza A viruses to mutate and also the ability to cause pandemics.[11]

Influenza A and B viruses are responsible for seasonal outbreaks and influenza C is less common but is still a significant cause of upper respiratory tract disease in children under 6

years old.[12] Influenza A viruses attracts the most attention because they are responsible for the most of the pandemic outbreaks.

1.2. Influenza viral life cycle

This Thesis focuses on the preparation of Influenza virus inhibitors. Therefore, it is necessary to have the knowledge of the virus life cycle that is briefly described below.

The first step of Influenza A virus life cycle is binding to the host cells. The viral hemagglutinin (HA) binds to sialic acids on host cell glycoproteins or glycolipids. The HA of human influenza A viruses preferentially binds to sialic acid linked to galactose by α(2,6) glycosidic bond (Figure 2).[13] This linkage is predominant on human epithelial cells in the upper respiratory track. In contrast, HA of the avian influenza preferentially bind to sialic acid, which is bound by α(2,3) glycosidic bond and it is found in the epithelial cells of the duck intestines where avian influenza replicates. Differences in hemagglutinin specificity are determinant of influenza A virus host range.[14]

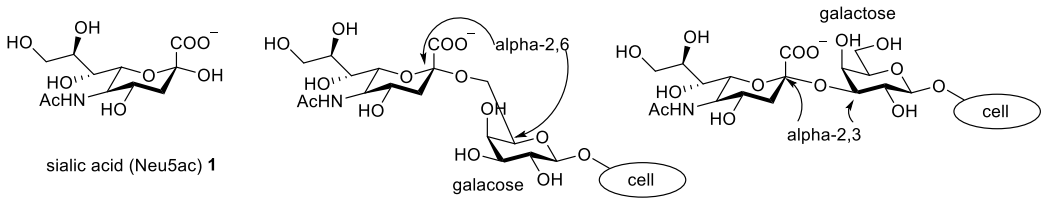


Figure 2. Example of α-2,6 and α-2,3 glycosidic bonds of sialic acid and galactose.

Following receptor binding, influenza A viruses enter cells through receptor-mediated endocytosis. At the low pH of the late endosome, HA undergoes an irreversible conformational shift resulting in the fusion of the viral and endosomal membranes (Figure 3). The acidic environment of the endosome also opens up the M2 ion channels. Opening the M2 ion channels acidifies the viral core. This acidic environment in the virion releases the vRNP from M1.[15] vRNP is now free to enter the host cells cytoplasm and is subsequently imported to the nucleus. This transport is mediated by the cellular nuclear import factors. Viral RNA replication is catalyzed by viral polymerase and starts with the synthesis of a copy of the vRNA, called complementary RNA (cRNA).

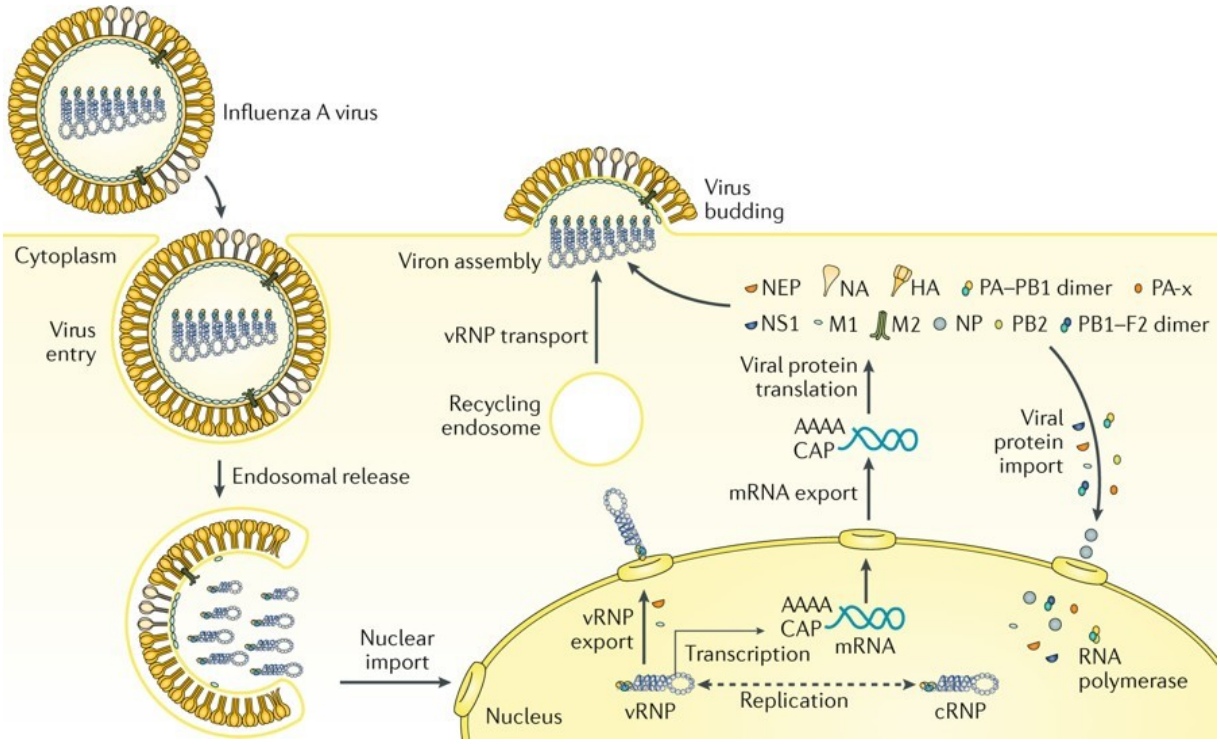


Figure 3. Schematic representation of influenza viral life cycle.[16]

The complementary RNA is then copied to produce viral RNA. Transcription of viral RNA is initiated by the binding of PB2 to the 5' end 7-methylguanosine (m7G) cap structure of the host mRNAs. The endonuclease activity of PA domain then 'snatches' the cap structure and the 10–13 nucleotides included with the cap serve as a primer for viral mRNA synthesis. The synthesis of viral mRNAs is carried out by the PB1 subunit with the polymerase activity. Influenza viral mRNAs are translated by the translation apparatus of the host cell. After their synthesis in the cytoplasm, the viral polymerase subunit (PA, PB1, PB2) proteins and NP are imported into the nucleus via their nuclear localization signals to catalyze the replication and transcription of vRNA. NEP, M1 and NS1 protein are also transported to nucleus to execute their roles in vRNP nuclear export. The vRNP is associated with NEP and M1 protein and are transported to the plasma membrane. Following their synthesis by the cellular translation apparatus, the HA, NA, and M2 proteins enter the endoplasmic reticulum where they are glycosylated (HA and NA) or palmitoylated (HA and M2). HA and NA associate with lipid rafts (membrane regions rich in sphingolipids and cholesterol) at the plasma membrane. These lipid rafts are the site of influenza virus budding. Enzymatic activity of the viral NA protein removes sialic acids from host cells and from glycoproteins on virions, allowing virus release and preventing virion aggregation.[17] Understanding the influenza viral life cycle is crucial for development of antivirals against influenza. In the time of writing the Thesis there were five

approved drugs for treatment of influenza: oseltamivir, peramivir, zanamivir, favipiravir and baloxavir. Amantadine and rimantadine (M2 ion channel inhibitors) are not recommended for use against circulating viruses.[18]

1.3. Neuraminidase

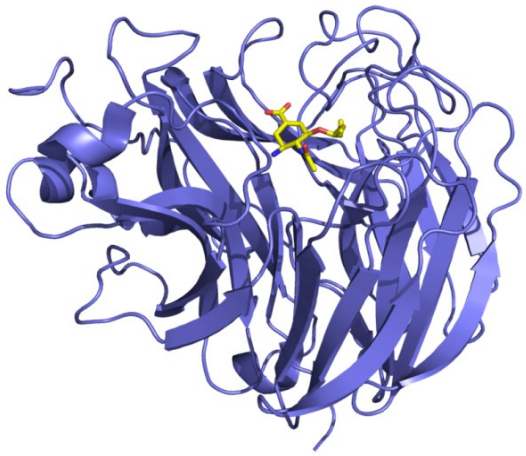


Figure 4. Single monomer of the N1 NA in complex with oseltamivir carboxylate.[19]

The neuraminidase is surface glycoprotein of influenza A and B viruses. Viruses C and D have hemagglutinin-esterase-fusion glycoprotein (HEF) that combines the functions of hemagglutinin and neuraminidase of influenza A and B.[20,21] Neuraminidase cleaves the glycosidic bond between sialic acid and the host glycoconjugate, facilitating viral shedding. Without this process, the viral particle would not be released from the plasma membrane and the infection would not spread. Moreover, NA cleaves the sialic acid from the viral particles as well to prevent recognition by the HA of the virus. Neuraminidases of the A viruses are further classified into two phylogenetically distinct groups (group 1: N1, N4, N5, N8 and group 2: N2, N3, N6, N7, N9). One of the differences between the two groups is the presence of the 150-cavity; this difference will be discussed in the following chapter. No subtypes were found for the NAs of B viruses.

Neuraminidase head is a homotetramer[22] and each monomer is composed of six topologically identical beta sheet arranged in a propeller formation (Figure 4).[19] For successful development of neuraminidase inhibitors it is crucial to know how the enzyme catalyzes the cleavage of the glycosidic bond.

In the first step the carboxylate group flips from the axial position into pseudoequatorial positions as a result of the charge-charge interactions with the side residues of arginines and steric constraint with residues such as Tyr406. In the second step proton donation occurs and results in a cleavage of a glycosidic bond and formation of the endocyclic sialosyl cation

transition-state intermediate **2**. This cationic intermediate is attacked by tyrosine and the protonation results in a second cationic transition state. After nucleophilic attack of water the α-anomer is released which than mutarotates to more thermodynamically stable β-anomer (Figure 5).[23,24] The knowledge of the transitional states was instrumental for the development of oseltamivir and zanamivir.

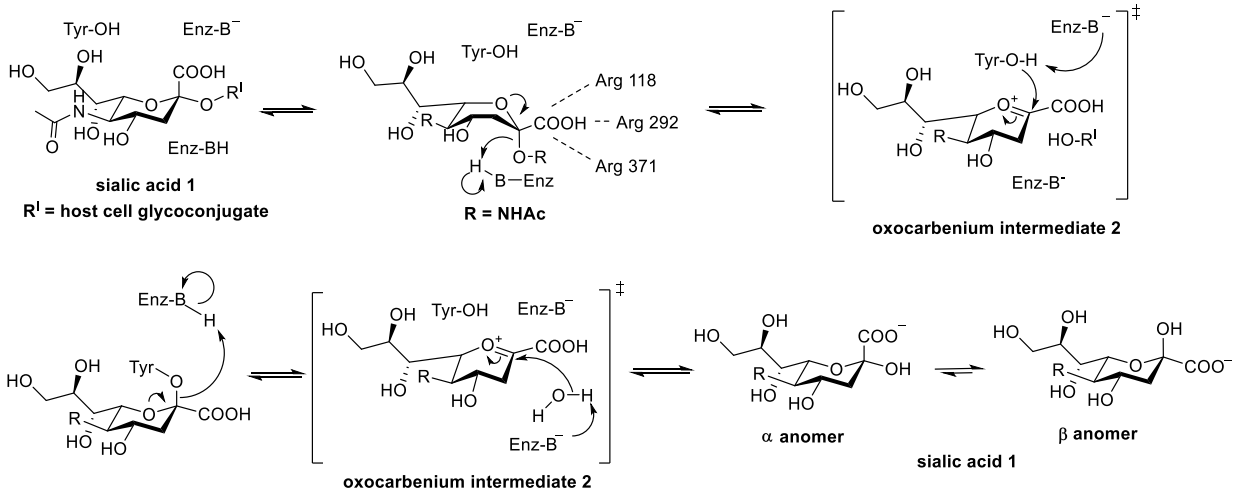


Figure 5. Proposed enzyme reaction mechanism of influenza A virus neuraminidase.[25]

1.4. Oseltamivir

Oseltamivir (sold under the name Tamiflu as a phosphonate salt) is an ethyl ester prodrug that is hydrolyzed by the hepatic esterases to produce the active free acid (Figure 6).

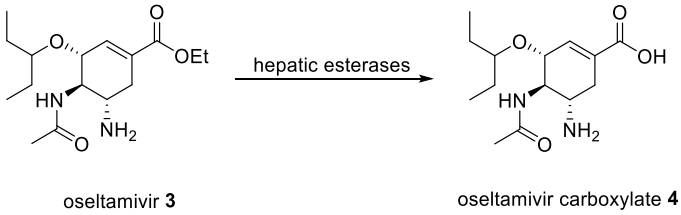


Figure 6. Hydrolysis of ethyl ester prodrug oseltamivir **3** to active oseltamivir carboxylate **4** by hepatic esterase.

This neuraminidase inhibitor was approved by the FDA and launched in 1999 by Gilead Sciences and F. Hoffman-La Roche. The most used NA inhibitor is oseltamivir and nowadays is still used for treatment and prophylaxis of influenza A and B. Oseltamivir was developed by intense optimization of 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid **5** (DANA) and zanamivir **6** structures with the goal to develop more lipophilic compound with enhanced oral

bioavailability. By replacing the glycerol moiety with pentyloxy moiety a change in the orientation of residues in the active site occurs. Gly276 is reoriented and binds with Arg224 and creates the hydrophobic pocket.[26] The double bond of oseltamivir mimics the oxocarbenium intermediate more closely than zanamivir (Figure 7).

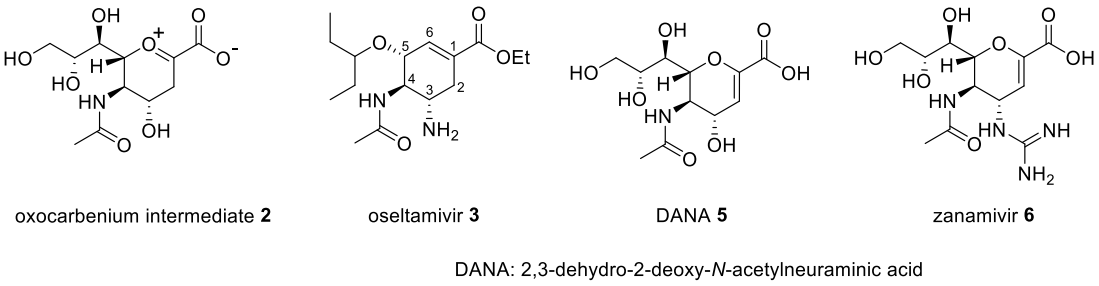


Figure 7. Structures of oxocarbenium ion intermediate **2**, oseltamivir **3**, DANA **5** and zanamivir **6** with schematic representation of the pockets in the neuraminidase active site.[27]

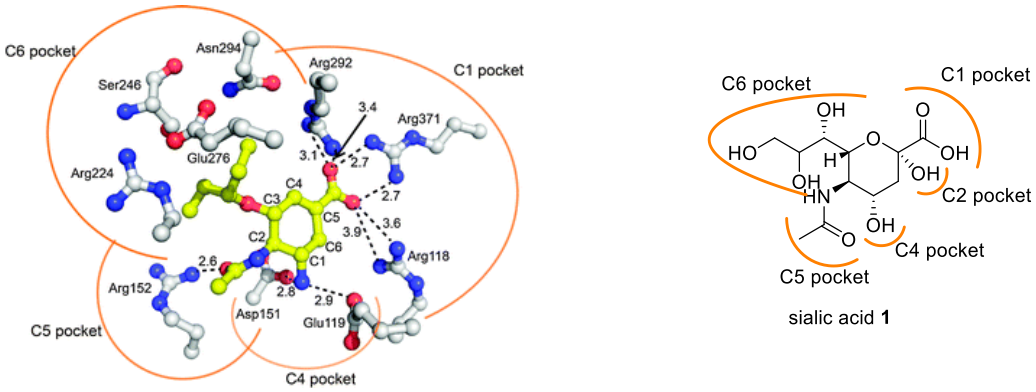


Figure 8. Left: Intermolecular interactions in the structure of 2009 neuraminidase H1N1 in complex with oseltamivir[27] and Right: Schematic representation of sialic acid in the same pocket (carbon: yellow for oseltamivir/white for NA; nitrogen blue; oxygen red).

Acid-base interaction binds the negatively charged carboxylate at C-1 of oseltamivir with three basic residues from C1 pocket of the NA (composed of amino acid residues Arg292, Arg71, Arg118). Positively charged amine at C-5 interacts with residues Glu119, Asp151 and Glu227 and the C-4 acetamide moiety binds Arg152 via hydrogen bond. The pentyloxy moiety is engaged in lipophilic interactions with residues Ile222, Arg224, Ala246 and Glu276 (Figure 8).

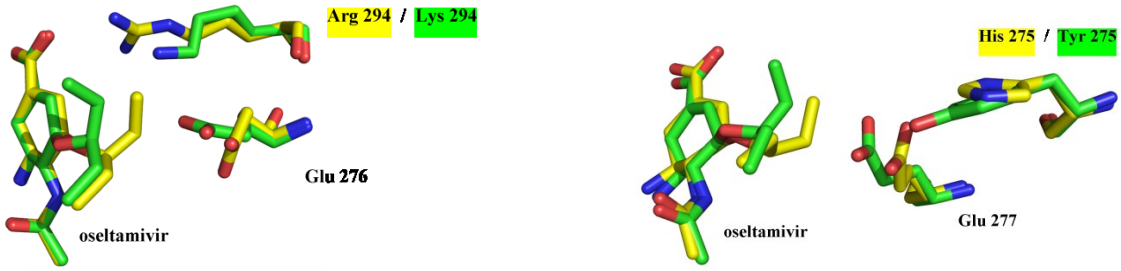


Figure 9. Left: The effect of the mutation H275Y on E277 and oseltamivir carboxylate **2** in the wild-type N1 NA (yellow) and mutant N1 NA (green; right: The effect of the mutation R294K on E276 and oseltamivir carboxylate **2** in the wild-type N9 NA (yellow) and mutant N9 NA(green).[28]

Despite the original success of oseltamivir and its textbook drug design the resistance towards oseltamivir frequently appears. The resistance towards NA inhibitors is not that frequent as in the case of M2 ion channel inhibitors (amantadine, rimantadine) but some strains of the virus are showing low sensitivity towards oseltamivir. Mutation causing viral resistance against influenza inhibitors appears often in patients undergoing the treatment. Influenza viruses with resistance towards neuraminidase inhibitors typically contain mutations that directly or indirectly change the shape of neuraminidase catalytic site, thus reducing the inhibitor affinity. Mutation H275Y has been associated with oseltamivir resistance development in patients.[29] Histidine (H) in a position 275 is changed for bulkier tyrosine (Y). Tyrosine 275 pushes the carboxyl group of Glu227 into the lipophilic pocket and causing a significant change in the conformation of the pentyloxy substituent of oseltamivir (Figure 9 left).[28] Mutation R294K causes a high level of resistance in N9 NA of avian virus H7N9. Structural analysis of crystallographic data revealed that Lys294 forms a salt bridge with Glu276 and pushes the pentyloxy group out of the active site (Figure 9 right).[30] There is urgent need to overcome the oseltamivir resistance. One of the ways may be targeting the exo-site cavity called 150-cavity.

1.5. Cavity-150

The two groups of Influenza A neuraminidases are structurally distinct and the main difference is the presence of so-called 150-cavity adjacent to the active site of NA in group 1. The flexible part of the protein called the 150 loop (residues 147-152, N2 numbering) can adopt different conformations that can result in a formation of 150-cavity or not. The open conformation results in a presence of 150-cavity, that is 10 Å long, 5 Å wide, 5 Å deep and located next to the enzyme catalytic site (Figure 10).

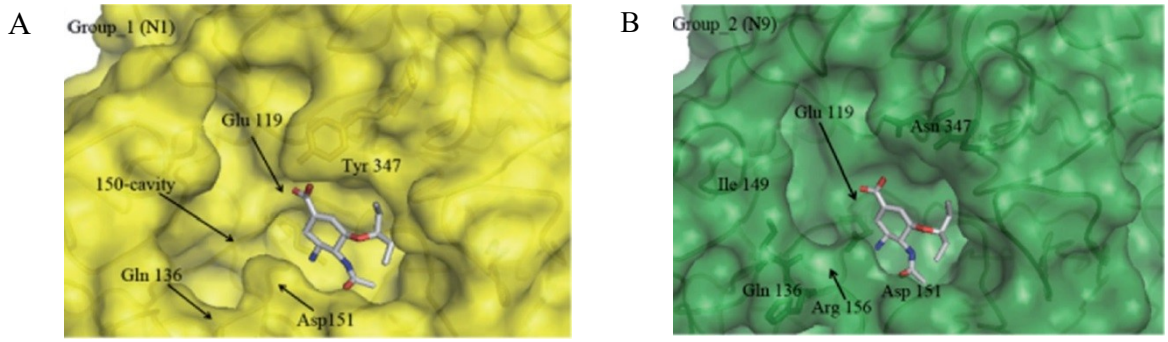


Figure 10. Two binding modes of oseltamivir. A) oseltamivir **3** binds to N1 NA from group 1; B) oseltamivir binds to N9 NA from group 2; 150-cavity is highlighted.[31]

Due to the proximity of the 150-cavity and the enzyme active-site it is possible to design small molecules that can target both sites. The closed conformation results in active site lacking the 150-cavity. Molecular dynamics studies independently suggest that all NAs are able to adopt both conformations resulting in an equilibrium that varies between the subtypes. Neuraminidase from the 2009 influenza pandemic (H1N1) lacks the 150-cavity which does not correspond with the fact that this N1 subtype originally belongs to the group 1.[32] Crystallographic studies and MS simulations showed that the formation of the cavity is controlled by formation of a salt bridge between D147-H/R150 in both group 1 and group 2 neuraminidases.[33] Both groups are therefore able to adopt flexible loop conformation and this makes the design, synthesis and evaluation of dual site binders more attractive. Inhibitors of active site and 150-cavity can be used for all influenza subtypes.

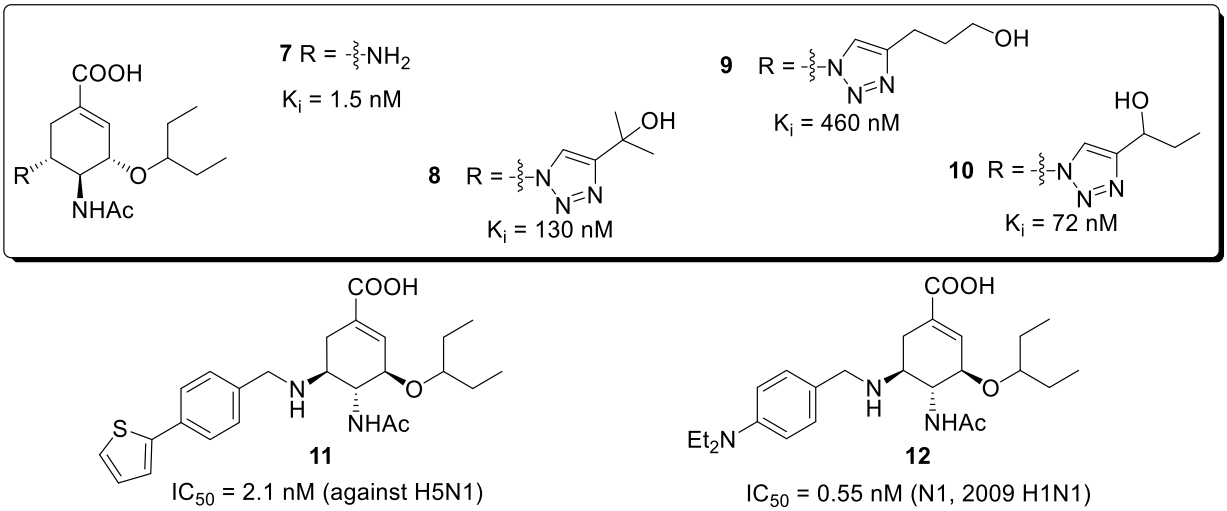


Figure 11. Neuraminidase inhibitors equipped with binders of 150-cavity.[34][35]

Several groups reported dual binders of 150-cavity and the active site of neuraminidase. Exploitation of new interactions in the 150-cavity was achieved by different approaches. CuAAC reactions of iso-oseltamivir **7** with various alkynes furnished compounds **8-10**.[34] Unfortunately, these compounds did not showed improved activity compared to the original iso-oseltamivir **7**. Introduction of several lipophilic moieties by reductive amination to oseltamivir furnished compounds **11** and **12**. These compounds (Figure 11) are promising neuraminidase inhibitors with thiophene derivative showing increased activity (IC_{50} = 0.16 μM) against mutation H275Y when compared to oseltamivir (IC_{50} = 2.1 μM).[35] 150-Cavity in therefore a viable target when combating resistance.

The first part of this thesis is focused on synthesis and biological activities of oseltamivir derivatives equipped with binders of 150-cavity. In the next part, the Thesis is focused on preparation of PA endonuclease inhibitors, and PA-PB1 interaction inhibitors. The two mentioned enzymes PA and PB1 are part of influenza RNA-dependent RNA polymerase. In the next chapter, the function and the subunits of the RNA-dependent RNA polymerase will be described.

1.6. RNA-dependent RNA polymerase (RdRp)

Influenza A RNA-dependent RNA polymerase belongs to a large family of template-dependent nucleic acid polymerases. Unlike others RNA RdRp, Influenza A virus polymerase consist of three protein subunits called polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA) proteins. The complete structure of polymerase of human-infecting strain of influenza A virus is yet to be determined; on the other hand most of the structure of bat influenza A polymerase in complex with vRNA has been solved.[36]

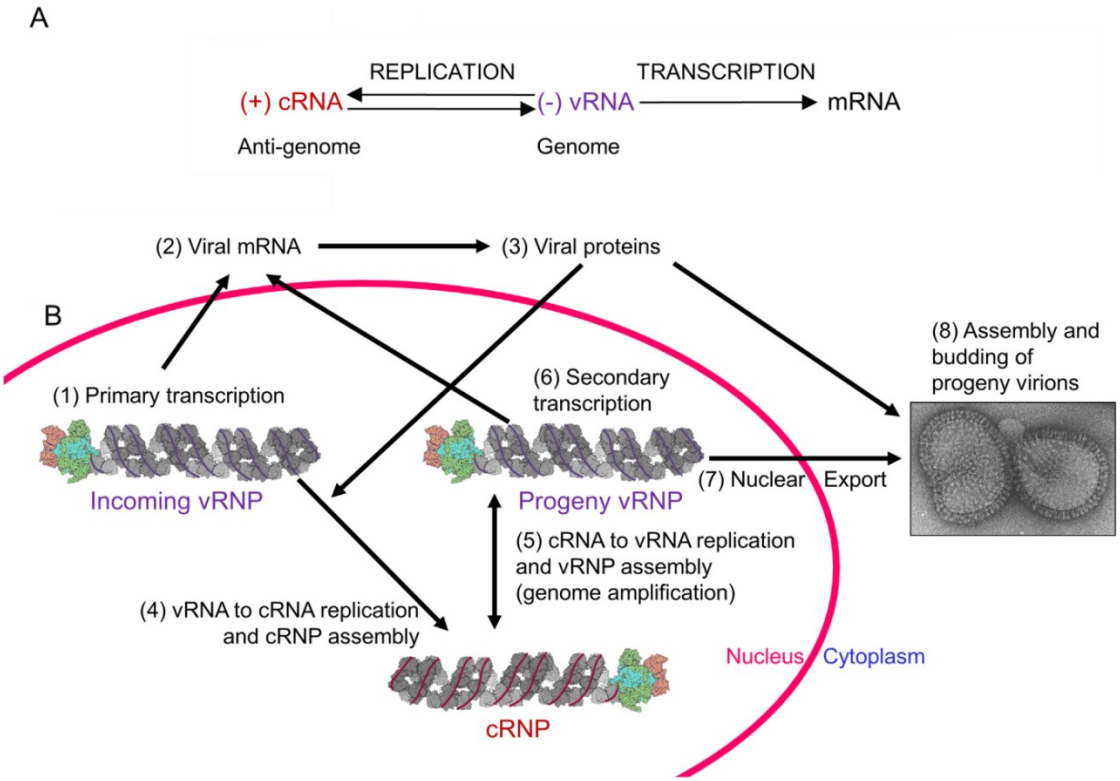


Figure 12. (A) Schematic of RNA synthesis performed by influenza polymerase. (B) Simplified life-cycle of influenza showing successive steps in the transcription and replication of RNPs.[37]

Each individual RNA segment is associated with a protein complex. The 3' and 5' ends are associated with the RdRp and the rest is coated with numerous copies of nucleoprotein. This complex protein is called viral ribonucleoprotein (vRNP) and upon infection of the host cells vRNP is transported into nucleus where both transcription and replication of each vRNA is carried out by the RdRp (Figure 12 A). Even though replication is a primer-independent process the synthesis of the viral mRNA is dependent on short, capped primers derived from the host RNA polymerase II transcripts.[38] During a unique process called cap-snatching (Figure 13), the viral RdRp interacts with the C-terminal domain of the host RNA polymerase II and cleaves

the newly synthesized transcript 10-13 nucleotides downstream of the 5' end 7-methylguanosine (m7G) cap structure. More precisely the PB2 subunit is involved in the binding of the 5' end cap (m7G) and subsequently, the PA subunit featuring endonuclease activity cleaves the RNA strain. This 5' end capped oligonucleotide primer is than used to initiate the transcription of the vRNA template. [39] The m7G protection of 5' end of the RNA transcript facilitates transport from the nucleus into cytoplasm and recognition in ribosomes.[40]

The replication is a primer independent process in which the negative sense vRNA is copied into a full-length copy called complementary RNA (cRNA), (Figure 12 A). The cRNA is used as a template for the production of more vRNA. The vRNAs than forms a vRNPs with NP and RdRp. Newly form vRNPs are then transported out of the nucleus to the cell membrane where they form new virions (Figure 12 B).

RdRp is crucial for influenza viral life cycle plus it is also involved in the evolution of the virus. Influenza virus RdRp polymerase lack proofreading mechanism and therefore wrong nucleotides are often integrated during the viral replication process with the rate of $\approx 10^{-4}$ per site per year.[41] RNA-dependent RNA polymerase of Influenza A virus is highly conserved among influenza A strains and does not appear in mammalian cells. This makes RNA-dependent RNA polymerase a good target for the drug development. The two of the aims of this thesis are to design and synthesize inhibitors of PA endonuclease subunit and also to design PA-PB1 interaction inhibitors. The next two chapters will briefly comment on PA endonuclease subunit and the PA-PB1 protein-protein interaction.

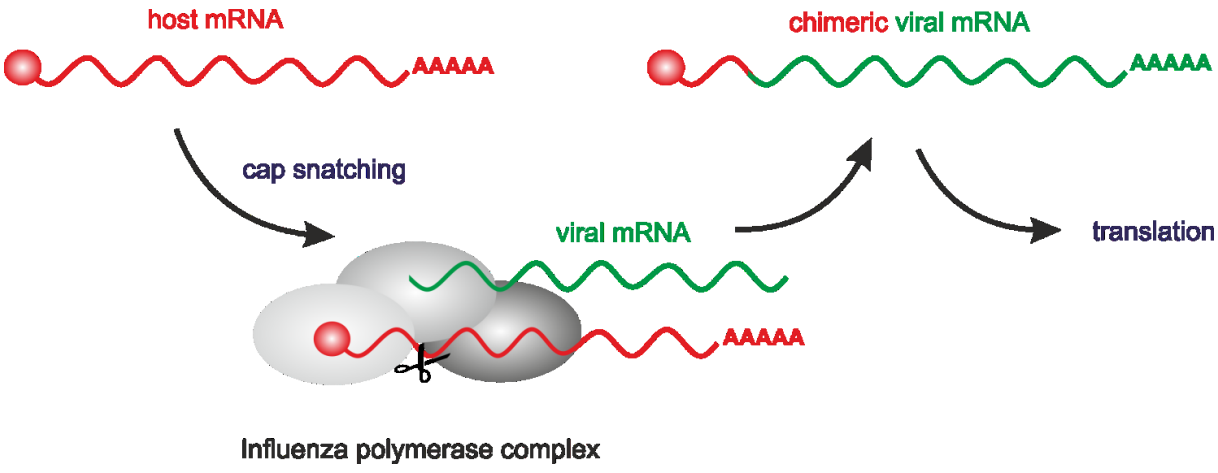


Figure 13. Schematic representation of influenza polymerase cap stanching.

1.6.1 PA endonuclease

PA subunit is a bridged dinuclear metalloenzyme consisting of C-terminal domain bridged by a long peptide chain with the N-terminal domain. The PA N-terminal domain contains the endonuclease active site whereas the PA C-terminal domain takes part in a protein-protein interaction (PPI) with PB1 subunit. This PPI is discussed in the next chapter. As was mention above the PA N-terminal domain contains the active site that consist of His41, Lys134 and three acidic residues Glu80, Asp108 and Glu119 which bind with Mg^{2+} or Mn^{2+} ions.[42] The two ions are positioned deep in the PA N-terminal domain active site and are necessary for the enzyme function. Any potential inhibitor must be able to chelate these two ions efficiently. PA subunit is an ideal drug target because of the following reasons: a) it is vital for the influenza virus life cycle; b) its active site is highly conserved among influenza types A, B and C; c) it does not appear in human cells.

1.6.1.1. PA endonuclease inhibitors

Extensive effort was put into finding PA N-terminal domain inhibitors. These inhibitors can be sorted into following groups: Baloxavir acid-like compounds **13**, diketo acid derivatives **L-742,001**,[43] flutimide congeners **14**,[44] hydroxylated heterocycles **15**,[45] catechol derivatives **16**,**17**[46] and other scaffolds (Figure 14).[47]

The screening of metal chelating compounds by enzymatic assay revealed the structure of **13**. Subsequent optimization resulted in baloxavir marboxil featuring an enhanced oral absorption compared to baloxavir acid.[48] The drug has a butterfly-like structure with pyridotriazin-dione moiety engaged in metal binding and lipophilic part facilitating favorable van der Waals interaction with certain amino acids. The baloxavir acid has a potent inhibitory activity against endonuclease activity of various RdRp from influenza A and B viruses (including neuraminidase inhibitor-resistant strains) with IC_{50} values 1.4-3.1 nM and 4.5-8.9 nM.[49]

Compound called **L-742,001** is a typical example of α,γ-diketo acid class of PA inhibitors. The compound possesses the α,γ-diketo moiety essential for metal binding and a piperidinyl moiety bearing two benzyl substituent that occupies two different hydrophobic pockets. A structural analysis of PA N-terminal domain occupied with **L-742,001** revealed that one metal ion is coordinated by carboxylate and oxygen atom at α-position and the second ion is coordinated by oxygen atom in α and γ position. **L-741,001** has a potency in a submicromolar

range (IC_{50} = 0.43 μM, EC_{50} = 0.35 μM) making it one of the most potent inhibitor in the entire class. Nitrogen atom in the piperidinyl moiety can undergo different substitutions that do not change the inhibitory potency. This makes this compound an ideal candidate for further modification.

Another class of PA N-terminal domain are catechol derivatives mainly (-)-epigallocatechin gallate **16**, a major component of a green tea.[50][51] The compound **16** showed inhibition of endonuclease activity in a plaque assay using MDCK cells (EC_{50} = 28.4 μM). The galloyl moiety was identified as crucial for anti-influenza activity. X-ray analysis of complex of PA N-terminal domain with compound **16** clearly shows the Mn^{2+} ions coordinated with two hydroxyl groups of the galloyl moiety.[42]

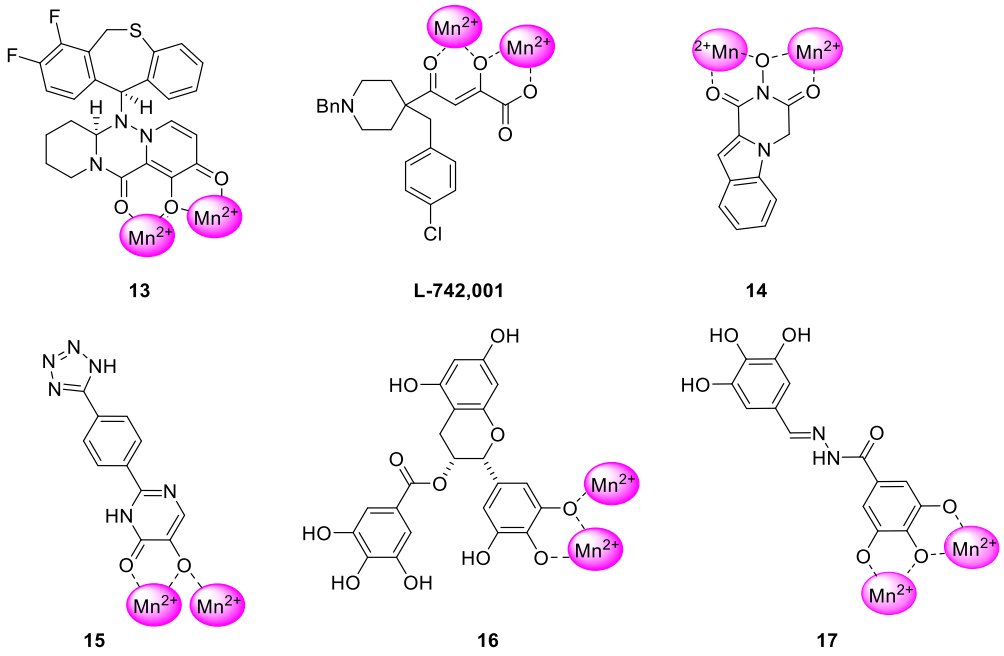


Figure 14. Binding modes of baloxavir acid (BA) **13**, compound **L-742,001**, flutimide congener **14**,[44] hydroxylated heterocycle **15**,[45] epigallocatechin gallate **16,** catechol derivative **17**.[46][47]

Flavonoids are polyphenolic phytochemicals representing important category in pharmacognosy. They are a natural part of human diet and their daily dose is approximately 1 gram (flavonoids or their glycosides). Many studies have suggested that consumption of flavonoids may be beneficial because it may prevent bone loss,[52] cancer[53] or age-related diseases.[54] Several publications from the past decades have also documented that flavonoids may possess anti-inflammatory, anti-bacterial[55] and anti-viral activity[56] with one flavonoid in particular: quercetin **18**.

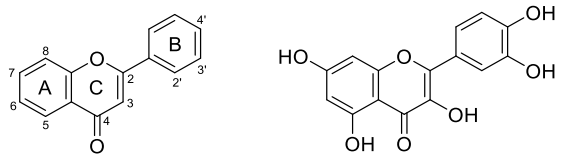


Figure 15. Left: numbering of flavonoids; Right: quercetin **18**.

Quercetin **18** (3,3',4',5,7-pentahydroxyflavone, Figure 15) is one of the most common flavones in the nature. Quercetin can be found in various berries, green tea, onions, caper, pomegranates or in apples.[57] Quercetin is also available as a dietary supplement. Intake of quercetin or other supplements such as vitamin C and E protects body from the oxidative stress.[58,59] Quercetin **18** was reported to have anti-inflammatory,[60] anti-bacterial[61] and anti-cancer effect.[62] There are also reports claiming anti-influenza activity of quercetin[63–65] or its glycoside.[66–68] *In vivo* experiments with mice shows that treatment with quercetin or its glycosides leads to reduction in influenza-induced cytopathic effect and reduces lung damage induced by influenza virus infection.[69] The exact mode of action of quercetin is yet to be determined. Several reports link the anti-influenza effect of quercetin to inhibition of influenza surface proteins hemagglutinin and neuraminidase and one report suggest interaction with PB2 subunit of the RdRp.

- Hemagglutinin: HA-quercetin interaction is yet to be confirmed by a structural analysis)[70]
- Neuraminidase: Reported IC_{50} values ranges from 20 to 90 μM[63] at this concentration it is possible that flavonoids interfere with the used MUNANA assay since they have quenching effect on the fluorogenic product 4-methylumbelliferone.[71,72]
- PB2 inhibitor: quercetin and its glycoside were proposed to block the RNA polymerase via occupying the binding site of 7-methylguanosine-5'-triphosphate. Time-dependent incubation assay was used to study the antiviral effect of quercetin-7-*O*-glycoside. The influenza RNA replication inhibition effect of quercetin-7-*O*-glycoside was proved by quantitative RT-PCR method. The blocking of the PB2 subunit was supported by molecular docking studies using the AutoDock Vina program. The anti-influenza activity of quercetine-7-*O*-glycoside in MDCK cells was reported to be 3-8 better than that of

oseltamivir. The study also suggests that quercetin-glycoside do not inhibit hemagglutinin and neuraminidase.[73]

The anti-influenza activity of flavonoids may be attributed to their ability to bind metal ions. In this thesis it is proposed that the possible mode of action of quercetin is the inhibition of PA endonuclease subunit. There are several quercetin/metal complexes reported in a literature: Quercetin coordinates metals such as Cu(II),[74] Fe(II),[75] Fe(III),[76] Gd(III),[77] Mg(II),[78] and Mn(II).[79]

Two binding modes of quercetin in an active site of PA N-terminal domain are suggested in Figure 16. Binding mode A is based on previously mentioned inhibitor **16** (Figure 14) where the galloyl moiety is coordinating Mn^{2+} ion. Mode B is inspired by [Mn(quercetin)$(H_2O)_2$][80] complex in which the 3,5-hydroxyflavone moiety is engaged in a metal binding rather than the 3',4'-dihydroxyphenyl moiety of the B ring. Nevertheless, these are only prediction based on previously published coordination complexes of dissociated ions and quercetin. Two proposed binding modes of quercetin do not take into account the complex binding interactions typical for the enzyme active site.

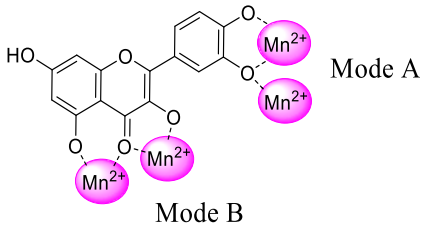


Figure 16. Two proposed binding modes of quercetin.

Quercetin derivatives with activity against hepatitis C virus (HCV) were recently discovered.[81] In case of the HCV, the function of RNA-dependent RNA polymerase is performed by non-structural protein 5B (NS5B). The active site of NS5B contains three aspartic acid residues that coordinate two ions in the active site (Mn^{2+} or Mg^{2+}). The presence of these two ions is crucial for the enzyme function. The two ions are responsible for the ligation of ribonucleoside triphosphate precursors of RNA. Quercetin **18** showed moderate activities against hepatitis C virus with EC_{50} = 19.8 μM (HCV infection/replication system in the human hepatoma cell lines, Huh7) with low cytotoxic activities (CC_{50} > 50 μM).[82] The docking studies presented in the publication suggested that the quercetin derivatives coordinate the divalent ions via binding mode B. This discovery supports the hypothesis that the anti-influenza

activity of flavonoids may be attributed to their metal-binding abilities and that the mechanism of action may be the inhibition of the PA subunit.

1.6.2. PA-PB1 protein-protein interaction

Recombinant PA subunit can be cleaved by trypsin digestion into two fragments: the 25 kDa PA N-terminal domain and 55 kDa PA C-terminal domain. The N-terminal domain is understood to be sufficient for the ribonuclease activity and RNA synthesis activity of the polymerase whereas the C-terminal domain is responsible for the binding to the PB1 resulting in complex formation and nuclear transport.[83] In 2005 it was proposed that PA and PB1 forms a heterodimer in the cytoplasm and then this heterodimeric complex is transported into the nucleus.[84] This was concluded from the observation that PB1 accumulate in the nucleus only if co-expressed with the PA[85] whereas individually expressed PB2 efficiently accumulate in the nucleus, which was in agreement with previous reports that PB2 contains nuclear localization signal.[86,87] The PA-PB1 heterodimer is transported into the nucleus by import factor RanBP5[88] and once in the nucleus, the PA, PB1 and PB2 subunit can constitute active RdRp (Figure 17).

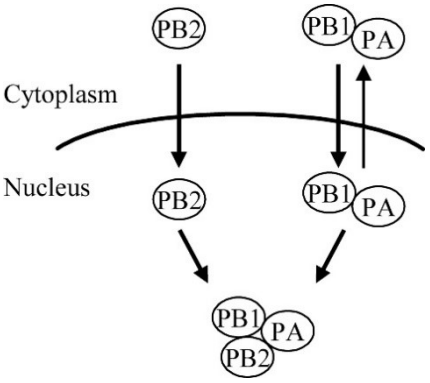


Figure 17. Model of nuclear import and assembly of RdRp. PA-PB1 heterodimer is imported separately from the PB2 and once in nucleus subunits assemble the polymerase.[89]

In 2008 there were two independent publications with X-ray structures of PA C-terminal domain in a complex with PB1 N-terminal domain. As was mentioned above, the PPIs usually involve large surface areas lacking grooves or pocket. This PPI is of distinct character with only 15 amino acids residues[90] of PB1 N-terminal domain involved in a deep and hydrophobic pocket of PA C-terminal domain (Figure 18).[91]

This pocket was found to be a valid target for therapeutic intervention since disrupting PB1 and PA assembly inhibits the transport of PA-PB1 heterodimer into the nucleus of infected cell. Peptide consisting of 25 amino acids of PB1 N-terminal domain was found to block the polymerase activity. HEK293T cells were transfected with plasmids expressing PB1(1-25)-GFP (green fluorescent protein). Viral replication was measured by quantifying nucleoprotein levels. Approximately 24 h later, the cultures were infected with influenza A virus. After 12 h analysis revealed 70% decrease in nucleoprotein expression.[92] In a follow-up work the authors showed 15 AAs of PB1-derived peptide, that can inhibit polymerase activity and spread of influenza viruses A and B.[93] PA-PB1 protein-protein interaction was validated as a drug target. Peptides are generally not a good candidate for the development of antivirals because of their low proteolytic stability and inability to enter cells. Several groups reported low molecular weight compounds as PA-PB1 interaction inhibitors. These compounds are commented on in the next chapter.

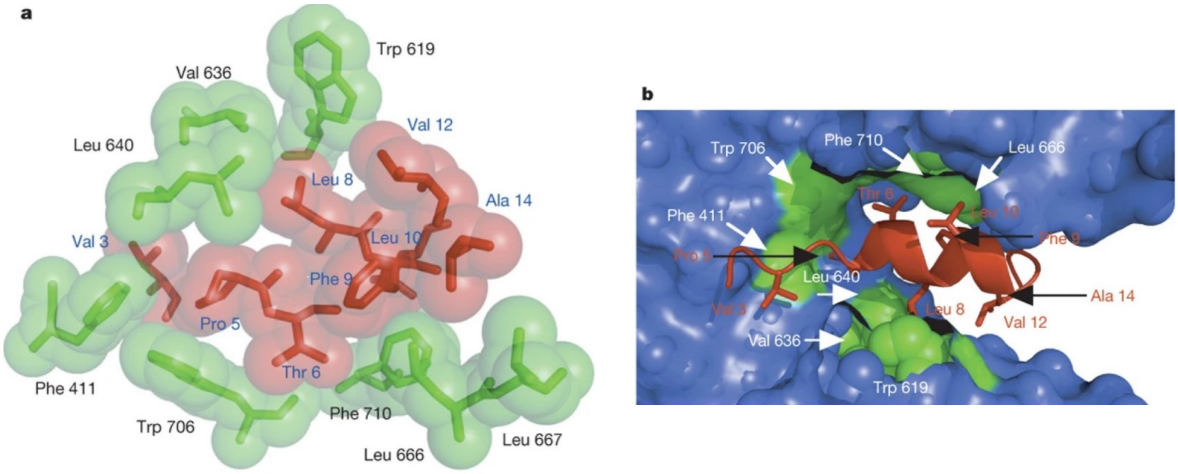


Figure 18. a) Space-filling representation, with PA residues shown in green and PB1(1-15) residues in red. **b)** Cut-away diagram showing the binding site. Hydrophobic residues of PA binding PB1(1-15) are colored green and the rest is blue. PB1(1-15) is colored red.[91]

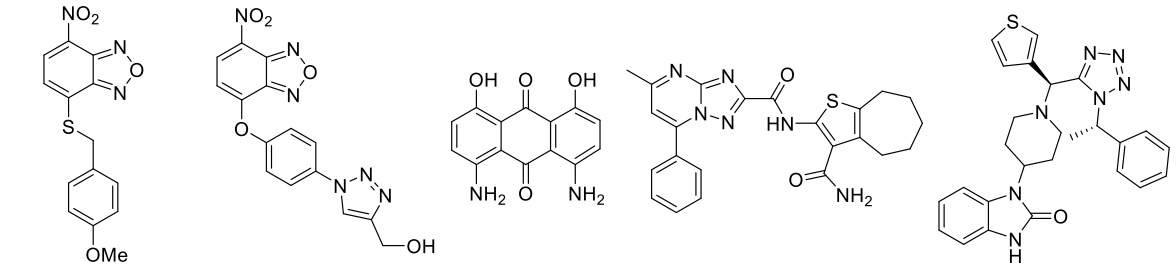
1.6.3. Low-molecular-weight inhibitors of PA-PB1 complex formation

In 2013 Kessler at al. published several PA-PB1 interaction inhibitors identified by high-throughput-screening approach.[94] Around 15 000 compounds were tested with ELISA assay for PA subunit binding. Plaque reduction assay on A/WSN/33-strain-infected MDCK cells identified benzofurazan **19**. Compound **19** inhibited viral replication at a micromolar level with EC_{50} = 5 μM and cytotoxicity CC_{50} = 20 μM.[95] Subsequent study by the same authors revealed compound **20** as PA-PB1 interaction inhibitor with IC_{50} = 35 μM, anti-influenza activity EC_{50} = 1 μM and cytotoxicity of 40 μM (Figure 19).[96]

Compound **21** was serendipitously identified as a PA-PB1 interaction inhibitor with IC_{50} = 20 μM determined by ELISA. The compound inhibited influenza polymerase activity with EC_{50} = 16 μM determined by minireplicon assay. Plaque reduction assay on MDCK cells revealed inhibition of replication with EC_{50} = 14 and 8.3 μM for influenza A and B strains respectively. The compound **21** did not show any cytotoxic affect in MDCK cells up to 250 μM (Figure 19).[97]

In 2015 compound **22** was identified as PA-PB1 interaction inhibitor with IC_{50} = 1.1 μM determined by ELISA. It is known that the formation of PA-PB1 heterodimer is crucial for the nuclear import of the subunits. In order to confirm the mechanism of action PA-PB1-PB2 co-expressing HEK293T cells were treated with compound **22**. The treatment resulted in a reduced nuclear localization of PA subunit. The compound **22** showed inhibition of polymerase activity with EC_{50} = 12 μM determined by minireplicon assay. The compound **22** showed inhibition of influenza A and B growth with EC_{50} values from 7-25 μM determined by plaque reduction assay on MDCK cells, and cytotoxicity CC_{50} = 250 μM (Figure 19).[94]

Zhang et al. discovered compound **23** in 2018 as a potent PA-PB1 interaction inhibitors with IC_{50} = 7.6 μM. Compound **23** showed cytotoxicity CC_{50} = 150 μM and 98 μM towards MDCK and A539 cells respectively (Figure 19).[98]



19 IC_{50} = 5 μM **20** IC_{50} = 35 μM **21** IC_{50} = 20 μM **22** IC_{50} = 1.1 μM **23** IC_{50} = 7.6 μM

Figure 19. Most potent inhibitors of PA-PB1 interaction published up to now.

To find inhibitors of PA-PB1 protein-protein interaction many different approaches were used. In the next chapters these approaches are briefly introduced with examples from the literature. My own motivation why to use such methods will be discussed in the chapter Results and Discussion.

1.7. Peptidomimetics

Protein-protein interactions (PPI) are involved in vast area of cellular processes. Dysregulating PPI is therefore a viable and an attractive target for the treatment of various diseases. PPIs are underdeveloped field in medicinal chemistry because it is generally hard to target these interactions with suitable low-molecular-weight inhibitor. PPIs take place on large surface areas (varying from 1600 to 4500 $Å^2$) lacking pockets and grooves. Surface areas involved in PPIs are solvent exposed when unbound, with binding domains arranged in a noncontiguous manner. Despite these challenges, PPIs are becoming target for a development of therapeutics. In spite of large areas involved in PPIs, most of the free energy of binding is attributed to "hot spots", which are smaller domains within PPIs that can be used for inhibitor rational design. "Hot spots" can be revealed by alanine scan. Each amino acid of peptide inhibitor is one by one systematically and individually replaced for alanine. If the AA substitution for alanine leads to decreased activity, this AA is marked as "hot spots".[99] Around two thirds of all PPIs found in Protein Data Bank (PDB), are mediated by α-helices where are the key hot spots projected from the helix in an organized fashion. It is worth mentioning that roughly half of the α-helix mediated PPI use only one side of the helix for the projection.

1.7.1. Helix mimetics

The rational design of helix mimics aims to project functional groups from a scaffold of organic molecule in an orientation, which is similar to that of the native helix, and avoiding the laborious synthesis of peptides. Peptidomimetic approach can be highly advantageous because it allows for a design of metabolically stable compounds with similar binding mode compared to the original protein. Peptidomimetics should be able to penetrate cell membrane more efficiently than the native peptide. Moreover, organic synthesis provides us with a wide range of structural motifs that can be used to project different array of groups with different steric and electronic properties (Figure 20).

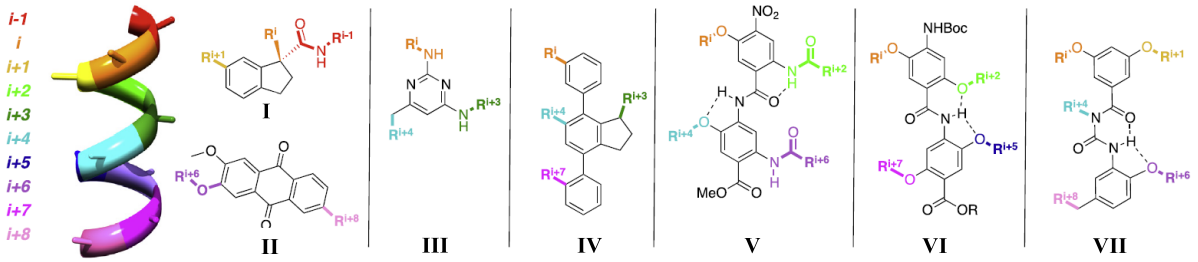


Figure 20. Schematic representation of an α-helix alongside double-sided mimics: indane **I**, anthracene **II**, pyrimidine **III**, diphenylindane **IV**, oligobenzamides **V** and **VI**, and benzoylurea **VII**.[100]

α-Helical sequence *i*, *i* + 4 and *i* + 7 is projected from a single face of the α-helix. Therefore residues *i*, *i* + 4 and *i* + 7 are often chosen to be mimicked (Figure 21).

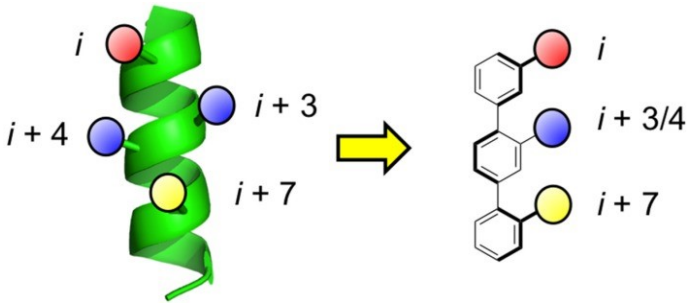


Figure 21. An α-helix (**left**) and a terphenyl-based α-helix mimetic (**right**).

It is worth mentioning the work done by Yin et al. The authors designed peptidomimetics based on a para-terphenyl scaffold **IV** that were supposed to inhibit p53/HDM2 interaction. The lead compound showed inhibition *in vitro* with K_i of 180 nM compared to truncated native peptide with K_i of 3.50 μM.[101] The planar benzamides were also successfully used by Ravindranathan et al. as a potent peptidomimetics with IC_{50} equal to 40 nM inhibition of androgen receptor/PELP1 co-regulator PPI. *In vivo* mouse studies and cell-based assay highlighted the aqueous solubility and cell permeability of these compounds.[102]

1.7.2. Properties of 3_{10}-helix and α-helix

The main difference between 3_{10}-helix and α-helix is how the hydrogen bonding is established (Figure 22). In α-helices the carbonyl group of residue *i* interacts with the hydrogen attached to the nitrogen atom from the amide group in residue *i* + 4. Canonical α-helices have 3.6 residues per turn. Two consecutive residues make an angle of 100° around the helical axis. The height of one complete helix turn (helix pitch) is 5.4 Å making a helical rise per residue 1.5 Å. In comparison the 3_{10}-helix carbonyl group in residue *i* is bonded to the hydrogen attached to the nitrogen atom of the amide group in the residue *i* + 3. Canonical 3_{10}-helices have three residues per turn, with an angle of 120° between consecutive residues. Helical pitch is 5.8-6 Å. For the same number of residues it is possible to generalize that 3_{10}-helix is more unstable, thinner and longer than α-helix.[103]

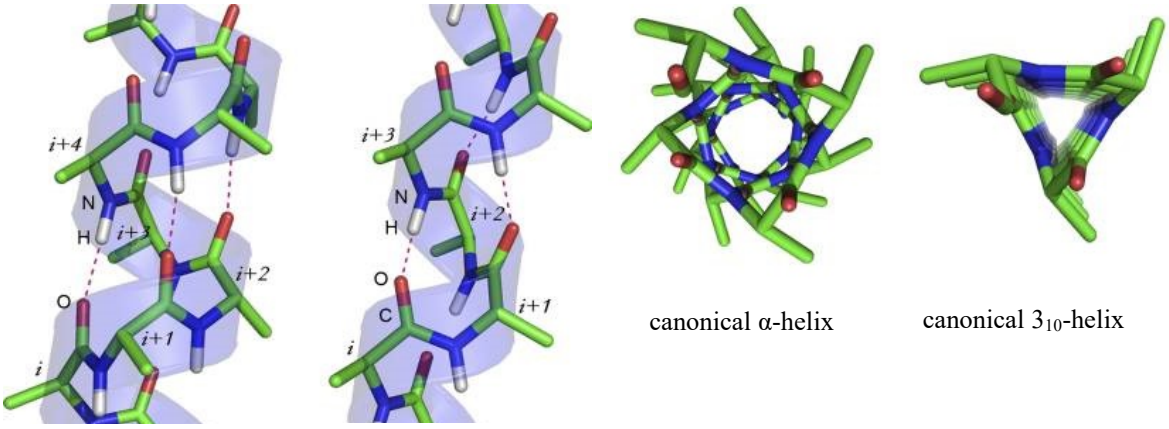


Figure 22. Canonical helical conformations. Hydrogen bonding indicated by dotted line. Representation of alanine containing peptides with canonical α-helix and 3_{10}-helix secondary structure. Amino acids are in stick representation. Color coding: carbon, green; nitrogen, blue; oxygen, red; hydrogen, white. Hydrogen bonding is represented by dotted line.

Since the introduction of α-helical and the 3_{10}-helical conformations as protein structural motifs it was suggested that 3_{10}-helix is most likely unstable due to its distorted hydrogen bond network.[104] In a comparative study of 57 protein structures it was shown that 32% of the total number residues were involved in the formation of α-helices with the average length of 10 residues and the longest had up to 27 residues. Only 3.4% of the total number of residues was presented in 3_{10}-helices with the mean length of 3.3 residue and only one helix with 6 residues.[105] After α-helices, β-sheets and reverse turns, 3_{10}-helix is the fourth most common type of secondary structure in proteins (approx. 15-20% of all helices are 3_{10}-helices). 3_{10}-

Helices are less common and less stable than α-helices nevertheless their formation can be induced by the presence of α-aminoisobutyric acid in the peptide chain.[106]

1.7.3. Peptide stapling

Majority of PPIs are mediated by α-helices as was stated previously. The native peptides are a clear starting point for the development of therapeutics. On the other hand, the isolated corresponding short peptides do not retain the native conformation and binding abilities. This is attributed to the lack of the structural reinforcement provided by the rest of the peptide. The peptides are also not very efficient in penetrating the cell membrane and are susceptible to the proteolytic degradation. One method how to possibly overcome the aforementioned difficulties is the peptide stapling. To stabilize helicity two amino acids from one side of the native helix are chosen. These amino acids are substituted with amino-acids possessing side-chains that can be covalently joined (stapled) and thus a macrocycle is formed. If the residues (i, i + 3/4, i + 7, etc.) are chosen correctly the stapling leads to stabilization of helicity, improved binding ability and better proteolytic stability.

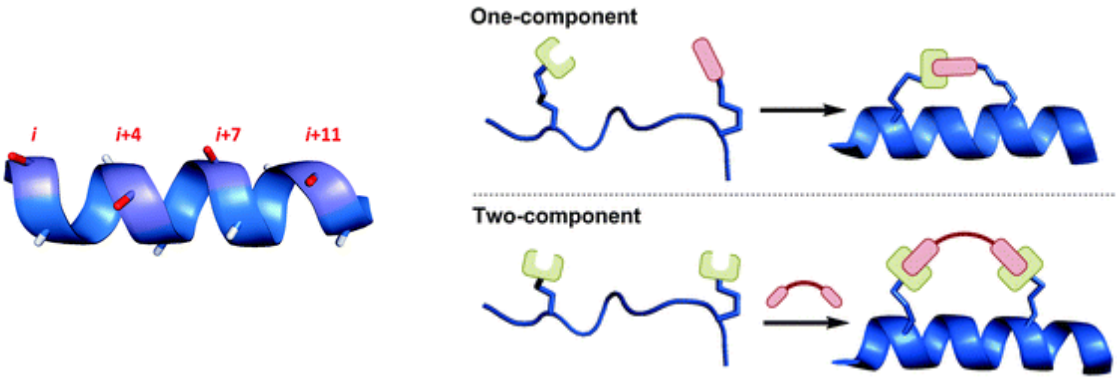


Figure 23. Left: Representation of α-helix and its residues; Right: One-component and two-component stapling techniques.[107]

Apparently, choosing the optimal residues and the optimal linker is crucial. The right approach varies from case to case and there are many different techniques available. The term "stapled peptide" was firstly mentioned by Verdine[108] who continued the work done by Blackwell and Grubbs on ring-closing metathesis (RCM) of olefin bearing amino acids.[109] RCM made the largest impact on the field so far and nowadays there are many other techniques

available.[107] These techniques can be divided to two main groups: one-component and two-component stapling (Figure 23). One component stapling technique uses two unnatural amino acid residues with complimentary side chains that can be covalently joined together. Two-component stapling techniques usually involve bifunctional linker that connects two amino-acid residues in a peptide. Two-component reactions are usually done in a solution because of possible site-isolation on resin. Common approaches for two-component stapling are alkylation, CuAAC (copper catalyzed alkyne-azide cycloaddition), amidation etc. (Figure 24).

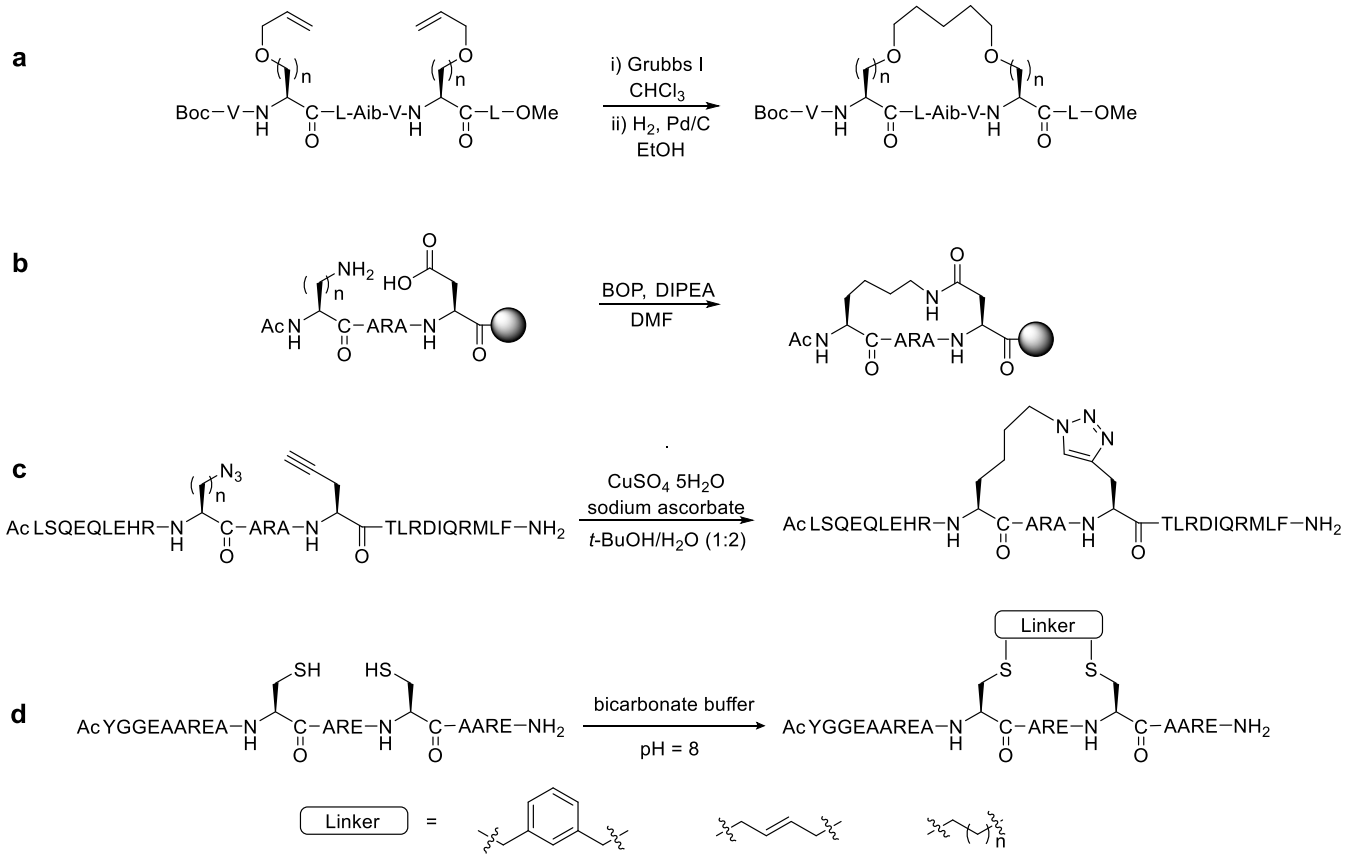


Figure 24. One component stapling techniques a) ring-closing metathesis; b) amidation; c) CuAAC; two-component stapling; d) alkylation.[107]

1.7.4. Cell-penetrating peptides (CPPs)

Compared to low-molecular-weight inhibitors, peptides are highly selective, effective and at the same time non-toxic option. One of the many applications of peptides is their use as inhibitors of protein-protein interactions (PPIs), which remain a challenging target for small molecules. There is an increased interest in proteins in pharmaceutical research and development. In 2015, there were 140 peptides therapeutics being evaluated in clinical trials.[110] In 2017, there were 286 US-FDA approved therapeutic peptides and proteins.[111] However, peptides are susceptible to proteolytic degradation and cannot penetrate cell membrane. This limits their application to extracellular targets.

Cell-penetrating peptides (CPPs) are short (up to 40 AAs) cationic and/or amphipathic peptides, which are capable of transporting a wide variety of cargo molecules across the eukaryotic cell membrane. The first discovered CPP was the TAT peptide (YGRKKRRQRRR) from the HIV trans-activator of transcription. Since the initial discovery of TAT peptide there were many other CPPs reported. All CPPs can be divided into two groups according to their sequence: amphipathic helical peptides (Transportan) with lysine as a main contributor to their positive charge and arginine-rich peptides (TAT_{47-57} peptide).[112] The mechanism of cellular uptake is subject of debate and likely varies with the cargo molecule and CPP sequence. On the other hand there is a growing consensus that at lower concentrations (< 10 μM) cationic CPP (TAT_{47-57}, nonaarginine) enter cells through endocytic mechanism.[113] Most of these CPPs are inefficient in escaping the endosome (especially when linked to a bulky cargo) and this results in a low cytosolic delivery.[114]

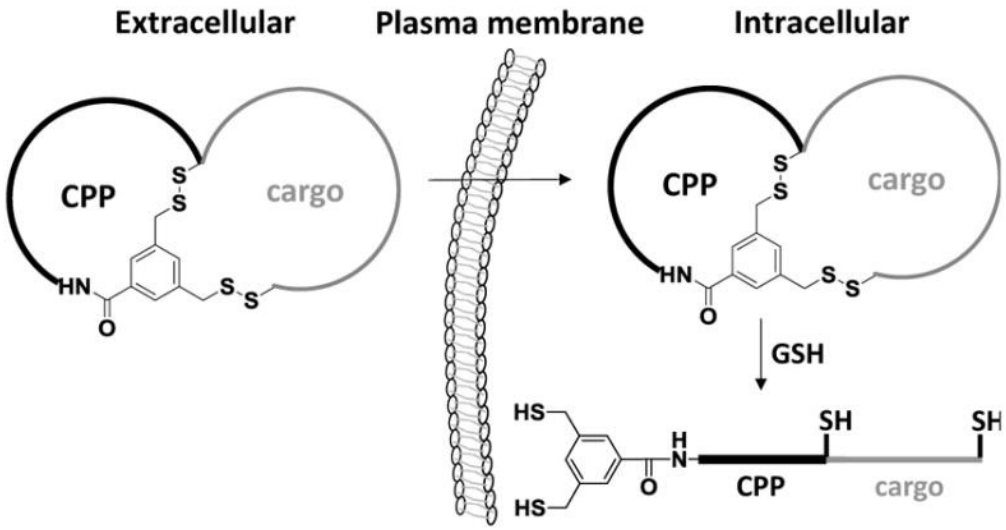


Figure 25. A reversible peptide bicyclization strategy. Bicyclic peptide with cell penetrating sequence and cargo peptide enter the intracellular space. The disulfide bridges are reduced by glutathione and the peptide becomes linear.[115]

In 2017, Qian et al. published cyclic peptide sequence containing FØRRRR or RRRRØF (CPP), where Ø is 3-(2-naphthyl)-*L*-alanine.[116] This sequence was found to bind directly to membrane phospholipids, enters the cell via endocytosis and on the top of that it escapes from early endosome into cytoplasm. Different cargos (SASAS and ALDWSWLQ) were delivered to cell via endocyclic or bicyclic approaches (Figure 25). The overall delivery efficiency of these peptides into plasma and nucleus was found to be 4-12 times higher than that of nonaarginine, TAT and penetratin. In extension of the original study, Qian et al. proposed the enhancement of cell permeability and serum stability of peptidyl drugs by reversible bicyclization. The bicyclic peptide enters the cell by endocytosis and escapes the early endosome into cytoplasm. In the cytoplasm, the disulfide bridges are reduced by glutathione (GSH) and become linear. This approach also allows to change the positions of the CARGO and CPP in the bicycle.

1.8. Methods for screening of inhibitors

In this Thesis the IC_{50} values are determined by AlphaScreen technology and K_i values are determined by DIANA technology. These methods are briefly introduced in this chapter.

1.8.1. DNA-linked inhibitor antibody assay (DIANA)

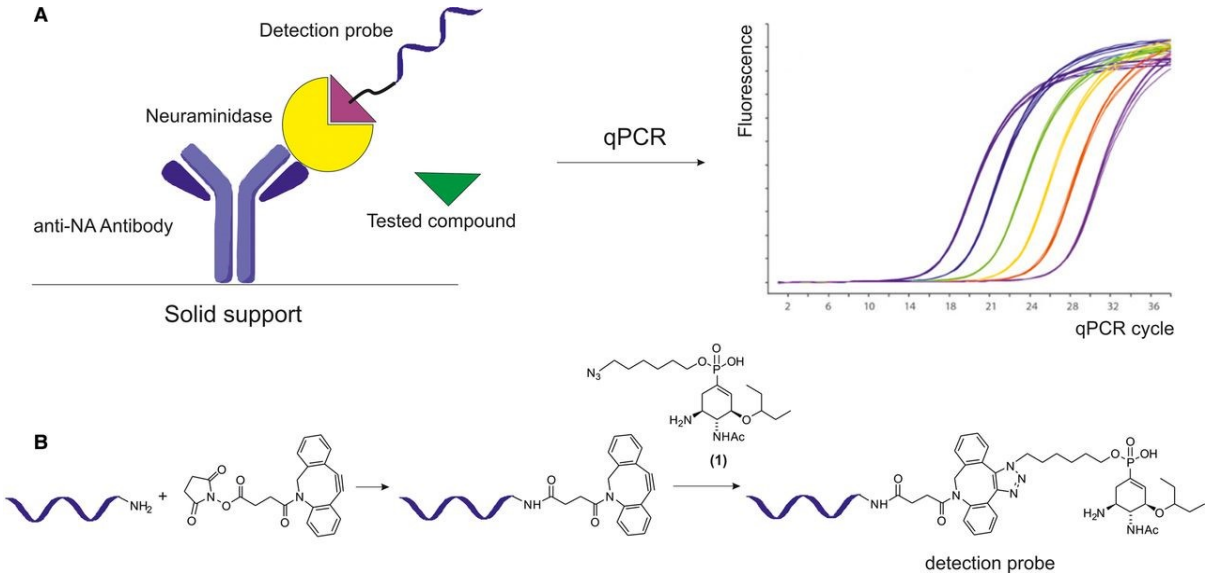


Figure 26. DNA-linked inhibitor antibody assay.[117]

DIANA is a multi-well plate-based assay, which is highly sensitive method that can quantify femtomolar concentrations of enzymes (Figure 26). DIANA can be also used for high-throughput enzyme inhibitor screening. The inhibition constant can be obtained from a single inhibitor concentration in one well. DIANA has a several advantages such as a wide range of concentrations it covers, high selectivity (the use of antibody), high sensitivity (the use of PCR) and it does not require the use of purified enzymes.

A single well is coated with antibody that captures the enzyme of interest. The enzyme is bound to a known inhibitor that is covalently attached to a short DNA segment. The amount of DNA in a well is than detected by quantitative PCR. In my Thesis I am presenting inhibition values obtain by DIANA for neuraminidase inhibitors.[117]

1.8.2. AlphaScreen for PA endonuclease

For the evaluation of N-terminal PA inhibitors a new assay was developed by Dr. Milan Kožíšek at IOCB. The method is based on the luminescent oxygen channeling immune assay system (AlphaScreen), which is a method for monitoring formation of particles pairs (beads) by chemiluminescence.[118] In this assay a biotinylated congener of compound **L-742,001** (synthesis described in the thesis of Dr. Carlos Berenguer)[119] is attached to streptavidin coated donor bead and N-terminal PA with GST tag is connected to glutathione-coated acceptor bead. The interaction of biotinylated compound **L-742,001** and N-terminal PA brings the two beads in a close proximity and upon excitation of the donor bead with laser at 680 nm the singlet oxygen is generated. Singlet oxygen can migrate around 200 nm during its half-life. If the donor and acceptor bead are within this 200 nm perimeter the singlet oxygen mediate energy transfer to acceptor bead and emission signal can be detected (Figure 27). If there is inhibitor present in the system, the beads will be far away from each other and decrease in fluorescence should be observed.

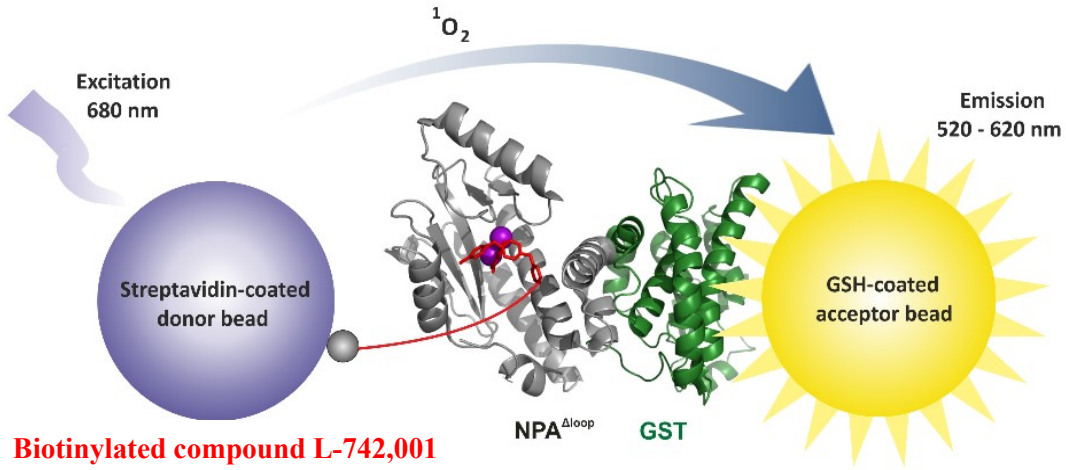


Figure 27. Depiction of AlphaScreen assay: Biotinylated compound **L-742,001** is connected to streptavidin-coated donor bead. N-Terminal PA with GST tag is connected to GSH-coated acceptor bead. Upon excitation by laser at 680 nm, the donor bead releases singlet oxygen. If there is binding between the PA N-terminal subunit and biotinylated compound **L-742,001** the singlet oxygen will travel to the acceptor bead and trigger emission of light. If there is an inhibitor present in the solution, the two beads will not be in a proximity and the singlet oxygen will go back to its ground state. Metal-binding motif of **L-742,001** was decorated with biotinylated flexible linker. This constructed adduct was then noncovalently bound to donor beads coated with streptavidin. The PA N-terminal domain was expressed in E.coli with an N-terminal GST-tag, purified and bound to acceptor beads coated with glutathione.

1.8.3. AlphaScreen for PA-PB1 interaction

For evaluation of PA C-terminal subunit interaction with N-terminal PB1 subunit AlphaScreen assay was developed by Dr. Milan Kožíšek at IOCB (Figure 28). Biotinylated PB1-derived peptide: MDVNPTLLFLKIPAQNAISTTFPYTGGSK-biotin is attached to the streptavidin coated donor bead via streptavidin-biotin interaction. C-terminal PA subunit with the GST tag is connected to the glutathione-coated donor bead. The protein-protein interaction between C-terminal PA with N-terminal PB1 subunits brings the beads in a close proximity. Upon excitation by laser at 680 nm, the donor bead releases singlet oxygen. If there is interaction between the subunits, the singlet oxygen is able to reach the acceptor bead and create an emission of light. If there is a potent inhibitor of the PA-PB1 interaction, the two beads will be far away from each other and the singlet oxygen will go back to its ground state and there will be the decrease of emission (Alpha Counts).

Every hit compound we had was also checked for false positivity. The false positivity could be revealed by CounterScreen. In the CounterScreen the donor and the acceptor beads are connected by biotinylated-GST linker. If the compound of interest will interfere with the biotin-streptavidin or GST-GSH connection, it will be revealed by the CounterScreen. If the compound shows false positivity it only means that it interferes with the assay. It does not mean that it does not inhibit the PA-PB1 interaction.

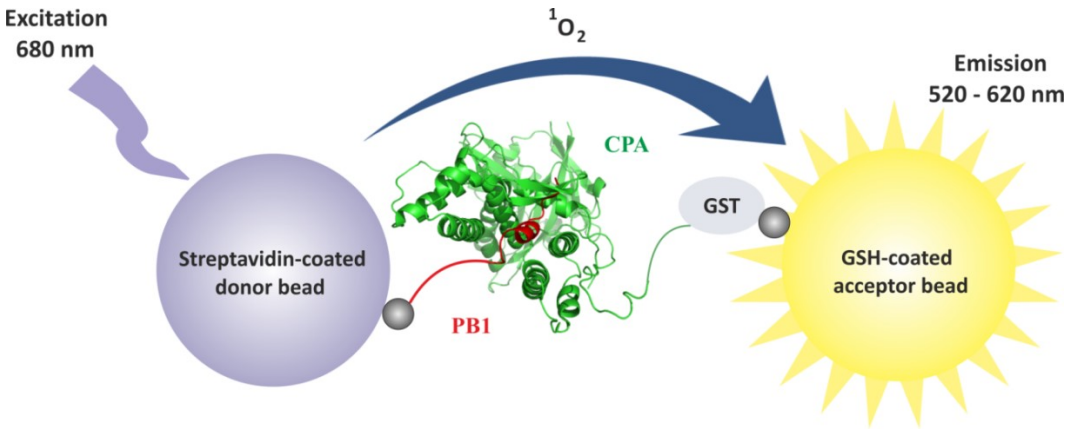


Figure 28. Depiction of AlphaScreen assay for PA-PB1 protein-protein interaction: Biotinylated PB1 derived peptide is attached to the streptavidin coated donor bead. PA C-terminal domain with GST tag is connected to the GSH-coated acceptor bead. Upon excitation by laser at 680 nm, the donor bead releases singlet oxygen. If there is binding between the PB1 derived peptide and PA C-terminal domain the singlet oxygen will travel to the acceptor bead and trigger emission of light. If there is inhibitor present in the solution, the two beads will not be in proximity and the singlet oxygen will go back to its ground state.

2. Objectives of the Thesis

- Design and synthesize a set of oseltamivir derivatives with modification at C-5 with binders of 150-cavity.
- Synthesize flavonoid derivatives to experimentally validate their biological activity as influenza PA endonuclease inhibitors.
- Optimize hit compounds from library screen against PA-PB1 protein-protein interaction and prepare the most potent PA-PB1 interaction inhibitors according to the literature.
- Prepare peptidomimetics designed to disrupt PA-PB1 protein-protein interaction.
- Identify and optimize hot spots of PA-PB1 protein-protein interaction. Prepare hexa- and decapeptides with optimized hotspots via solid-phase peptide synthesis and solution-phase peptide synthesis.
- Design and synthesize stapled peptides with the goal of stabilizing their helicity and improving their binding capacities to PA subunit.
- Increase the cell permeability and metabolic stability of decapeptide inhibitor of PA-PB1 protein-protein interaction by reversible bicyclization.

3. Results and Discussion

The work reported here is subdivided into three sections. The first part deals with the synthesis of C-5 modified oseltamivir with binders of 150-cavity. This was accomplished by CuAAC reaction of easily accessible 5-azidooseltamivir **24** with several propargylated anilines or other terminal alkynes.

The second part is focused on the synthesis of different flavonoids and similar compounds to ascertain and confirm their mechanism of action against influenza virus.

The third part is a complex endeavor to design PA-PB1 interaction inhibitors. At the beginning the project was focused on the synthesis of low-molecular-weight inhibitors (optimizing hits from AlphaScreen assay and synthesis of published compounds). Based on the X-ray crystal structure (PA C-terminal subunit with PB1 N-terminal derived decapeptide) peptidomimetics were designed to mimic hot spots of the peptidic nanomolar inhibitors. Optimization of the hexa/decapeptides hot spots was accomplished. To improve binding and proteolytic stability peptide stapling was performed. To improve cell penetrating ability and proteolytic stability of the peptides decoration with cell-penetrating peptide and bicyclization was performed.

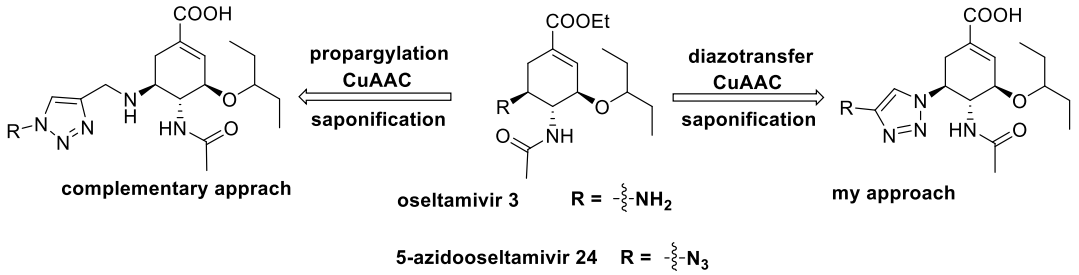
3.1. Synthesis of oseltamivir derivatives

This chapter is focused on design and synthesis of oseltamivir derivatives equipped with binders of 150-cavity. Neuraminidase inhibitors are the most used drugs for treatment of influenza. The oseltamivir is of particular importance since it can be administered orally. The emergence of oseltamivir-resistant strains (mutation H275Y, R294K) only highlights the need to develop new low-molecular-weight inhibitors. It was shown that it is possible to modify oseltamivir at C-5 position with different binders of 150-cavity. Compounds targeting both the active site and the 150-cavity were published with very potent IC_{50} values (Figure 11).

3.1.1 Synthesis

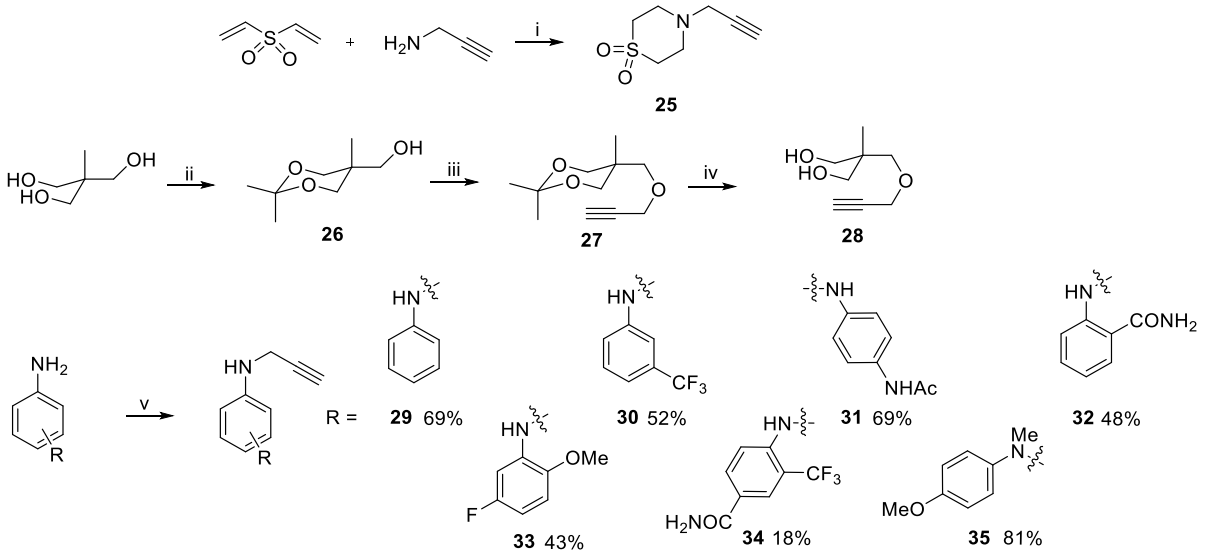
The oseltamivir **3** structure was extended at C-5 position by introducing various side-chains connected to the triazole linker. Synthetic approach was based on CuAAC of 5-azidooseltamivir **24** with the alkynes that are mainly lipophilic in nature. The second approach was based on the propargylation of C-5 amine of oseltamivir **3** and was described in the thesis

of Dr. Carlos Berenguer (Scheme 2).[119] I contributed to this approach only with the synthesis of the corresponding azides.



Scheme 2. Two approaches in preparation of binders of 150-cavity. Left: described in the Thesis of Dr. Carlos Berenguer. My contribution to this approach was the synthesis of azides; Right: Approach based on C-5 azidation of oseltamivir **3** described in this Thesis.

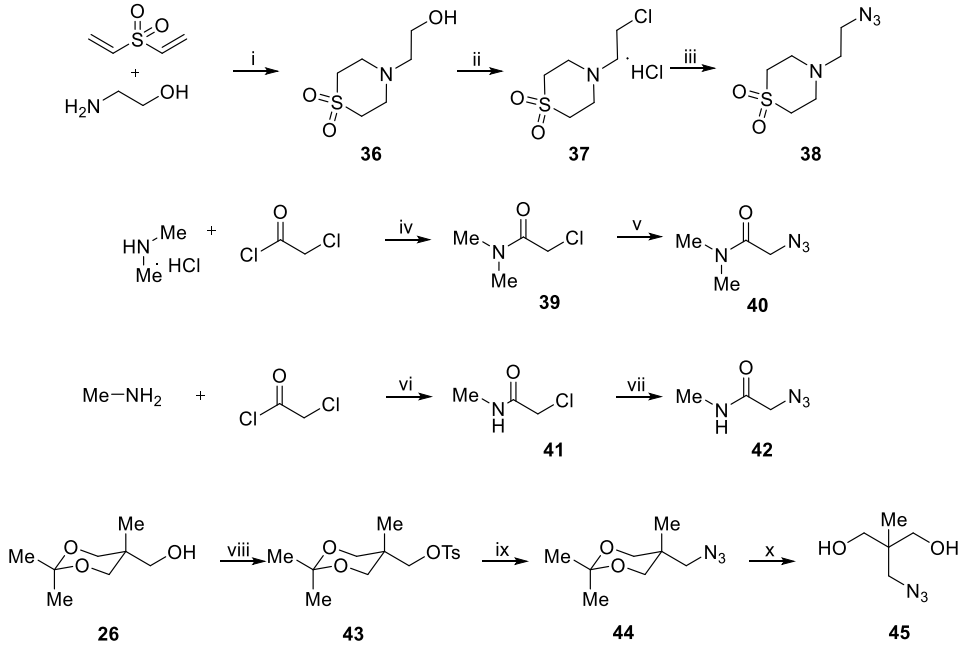
To probe the cavity-150 different azides and propargyl bearing compounds were prepared. Double Michael addition of divinyl sulfone with propargylamine furnished the 1,1-thiomorpholine dioxide **25** (Scheme 3). Acetonide **26** was prepared from 2-hydroxymethyl-1,3-propanediol in acetone with catalytic amount of *para*-toluenesulfonic acid. The free alcohol **26** was propargylated with propargyl bromide and sodium hydride. The acetonide **27** was deprotected using an aqueous solution of HCl. The 150-cavity is hydrophobic in nature therefore a set of differently substituted anilines **29-35** was alkylated with propargyl bromide in a presence of potassium carbonate.



Scheme 3. *Reagents and conditions*. (i) Ethanol, reflux, 5h, 70% yield; (ii) acetone, *para*-toluenesulfonic acid, overnight, 77% yield; (iii) propargyl bromide, NaH, THF, overnight, r.t.,87% yield; (iv) 1M HCl (aq.), MeOH, r.t., 60% yield; (v) potassium carbonate, propargyl bromide, acetonitrile.

To suppress the formation of bis-alkylated products the excess of aniline was used. The molar ratio was 4 equivalents of aniline, 2 equivalents of potassium carbonate and 1 equivalent of propargyl bromide. The monoalkylation proceeded with acceptable yields (Scheme 3).

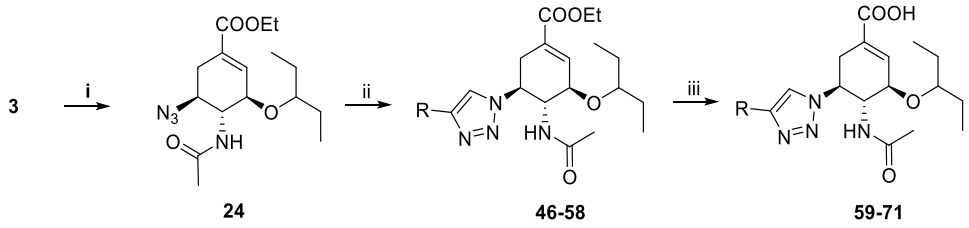
Set of azides was prepared for the CuAAC reaction with propargylated oseltamivir (Scheme 4). Double Michael addition of divinyl sulfone with ethanolamine furnished the alcohol **36**. Alcohol was converted to its chloride hydrochloride **37** using thionyl chloride in refluxing 1,2-DCE. Nucleophilic substitution of chloride with azide in DMF furnished compound **38**. Both reactions afforded the products in excellent yields. Reaction of chloracetyl chloride with dimethylamine hydrochloride in a presence of triethylamine led to compound **39**. Nucleophilic substitution at elevated temperature provided the azide **40**. Reaction of chloroacetyl chloride with methylamine in a presence of NaOH furnished the compound **41**. Nucleophilic substitution at elevated temperature with sodium azide furnished the azide **42**. Tosylation of **26** in pyridine gave compound **43** in acceptable yield and subsequent substitution with sodium azide provided compound **44**. The last step in this sequence was acid hydrolysis of acetonide that gave the compound **45**.



Scheme 4. *Reagents and conditions.* (i) propan-2-ol, reflux 5h, 88%; (ii) thionyl chloride, 1,2-DCE, reflux; 93% yield; (iii) NaN_3, H_2O, 3h, 60 °C, 89% yield; (iv) TEA, DCM; (v) NaN_3, H_2O, 2h, 60 °C; 94% yield; (vi) NaOH, DCM, H_2O, yield 25%; (vii) NaN_3, H_2O, 75 °C, 65% yield; (viii) *para*-toluenesulfonyl chloride, pyridine, 100°C, 53% yield; (ix) NaN_3, DMF/H_2O, overnight, 75 °C, 82% yield; (x) 37% HCl(aq.), THF, H_2O, 1h, 92% yield.

5-Azidooseltamivir **24** was prepared from commercially available oseltamivir phosphate **3** through diazotransfer reaction. Treatment of **3** with imidazole-1-sulfonyl azide

hydrochloride (prepared according to the published procedure)[120] furnished **24** in 80% yield. **24** was then coupled with thirteen different alkynes in the presence of Cu(I)-catalyst (Scheme 5). The corresponding alkynes were either prepared or purchased if possible. CuAAC was performed using standard reaction conditions of copper(II) sulfate and sodium *L*-ascorbate as the reducing agent. The CuAAC reaction furnished series of ethyl ester derivatives in good yields (Table 1). These ethyl esters were subsequently subjected to saponification and final products were purified by preparative HPLC.

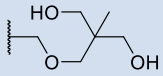
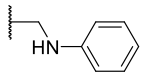
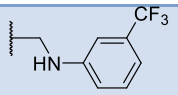
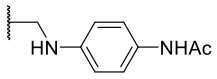
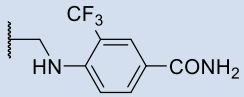
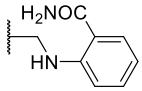
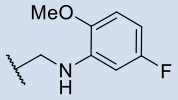
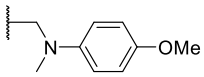
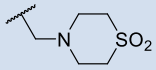
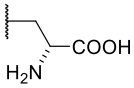
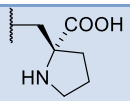


Scheme 5. *Reagents and conditions.* (i) imidazole-1-sulfonyl azide, $CuSO_4 \cdot 5H_2O$, K_2CO_3, EtOH, r.t., 2h, 80% yield; (ii) $CuSO_4 \cdot 5H_2O$, sodium *L*-ascorbate, THF/H_2O; (iii) NaOH, H_2O, MeOH, 1,4-dioxane, yields are reported in Table 1.

3.1.2. Biological evaluation

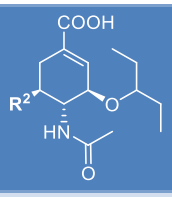
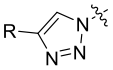
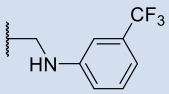
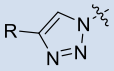
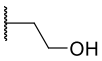
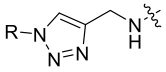
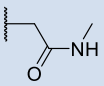
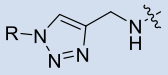
To evaluate the inhibitory potency of our compounds H1N1 A/California/04/09 and H5N1 A/chicken/Nakorn-Patom/Thailand/CU-K2-2004 as N1 subtypes were chosen. California 09N1 has a neuraminidase with the unclear location of the 150-loop, on the other hand Thailand 04N1 has a textbook neuraminidase with 150-cavity. The results based on DIANA assay are shown in Table 1. It is obvious that the 150-cavity does not tolerate hydrophilic moieties such as the diol **59**, thiomorpholine 1,1-dioxide **67** or acids **70-71**. The activities of alkylated anilines are similar and the inhibitory potency of the series is quite weak. It is obvious that the presence of C-5 basic amine is crucial for the inhibitor potency. The presence of an adjacent amino group is not enough to compensate for the missing C-5 basic function. Every compound shows superior activity towards neuraminidase H5N1 which is in agreement with the clear presence of 150-cavity in NA 2004.

Table 1. K_i determined by DIANA assay; * yields for CuAAC (in brackets) followed by yields for saponification; ** over two steps; *** synthesis described in the thesis of Dr. Carlos Berenguer.[119] K_i determined by Mgr. Kateřina Čermáková.

Compound	R	NA H1N1/K_i [μM]	NA H5N1/K_i [μM]	Yields* [%]
59		180±52	49±9.2	(68) 50
60		19±5.0	5.0±0.5	(47) 35
61		4.2±0.5	1.9±0.5	(50) 31
62		9.0±3.6	5.6±1.4	(60) 57
63		14±2.6	5.0±0.4	(45) 30
64		13±1.0	5.0±1.4	(38) 57
65		22±4.3	11±2.1	(60) 50
66		19±7.7	8.1±0.8	(94) 28
67		340±7.0	94±11	(49) 55
68		16±5.2	4.0±0.5	(91) 26
69		20±4.3	7.1±0.5	(43) 40
70		64±15	25±1.8	(38) 22**
71		98±28	49±7.5	(72) 25**
72		12±4.0	0.5±0.1	***

The inhibitory potency of compounds **72** and **61** determined at physiological pH by DIANA is significantly lower (by one order of magnitude) as opposed to the values determined by MUNANA assay at pH 6.15 (see Table 2). It is evident that the K_i values of neuraminidase inhibitors without the C-5 basic functionality are profoundly influenced by the experimental conditions of the assay. If the C-5 basic group is preserved the K_i values determined by DIANA and MUNANA are in agreement despite the difference in pH (see Table 2, compounds **4**, **73** and **74**).

Table 2. Comparison of K_i values obtained from DIANA (pH = 7.40) and MUNANA (pH = 6.14) assay for NA2009, * synthesis described in the thesis of Dr. Carlos Berenguer,[119] K_i determined by Mgr. Kateřina Čermáková. I contributed to the synthesis of compound **73** and **74** by synthesis of the corresponding azides.

Compound	R	DIANA/K_i [μM]	MUNANA/K_i [μM]	 R^2 =
4	n/a	0.031 ±0.006	0.024±0.004	
72*		12.0±4.10	0.47±0.14	
61		4.20±0.50	0.47±0.03	
73*		0.75±0.35	0.65±0.05	
74*		1.2±0.4	0.87±0.07	

3.1.3. Summary

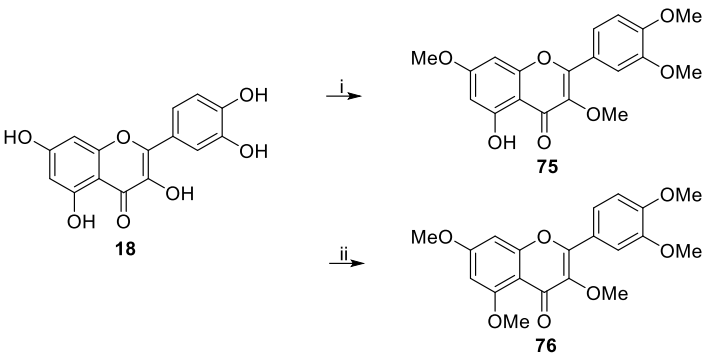
Oseltamivir was successfully modified on carbon C-5. Series of various azide and alkyne fragments for CuAAC reaction were prepared. The CuAAC reaction and subsequent saponification furnished the desired compounds in good yields. The K_i values determined by DIANA clearly show that the inhibition potency of compounds **59-72** is better against H5N1 A/chicken/Nakorn-Patom/Thailand/CU-K2-2004. This is in agreement with the clear location of the 150-cavity in H5N1. Unfortunately, the overall inhibitory potency of this series was weak

when compared to oseltamivir. Comparison of the Ki values determined by MUNANA and DIANA were well comparable when the C-5 amino moiety was present (see Table 1, compounds: **4**, **73**, **74)**. On the other hand inhibitors with triazole moiety on the C-5 carbon (see Table 1, compounds: **72**, **61**) showed one magnitude higher K_i when determined by MUNANA. This difference can be attributed to the different experimental condition of MUNANA (pH = 6.15) and DIANA (pH = 7.40) assays.

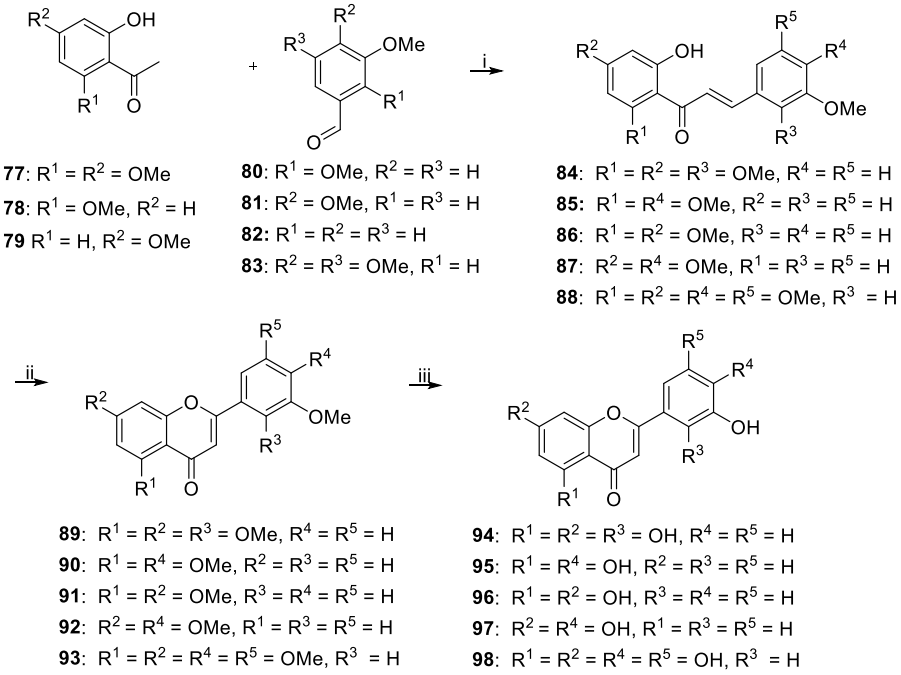
3.2. PA subunit inhibitors

3.2.1. Synthesis

With AlphaScreen assay in our hands the SAR study of flavonoids and related compounds has been performed. The simplest way to access quercetin derivatives was methylation. Efficient way how to selectively methylate quercetin was not yet described, therefore only tetra and penta-methylation was performed to obtain the derivatives **75** and **76**, respectively (Scheme 6).[121] Many flavonoids were commercially available. To access particular flavones with carefully selected hydroxyl groups a three-step synthesis had to be carried out (see Scheme 7). Well documented strategy in the literature was followed.[122] At first the hydroxyacetophenones **77-79**[123],[124],[125] were prepared by selective methylation with dimethyl sulfate in refluxing acetone. The aldol condensation of *ortho*-hydroxyacetophenones **77-79** with *meta*-methoxybenzaldehydes **80-83** under basic condition gave the corresponding 2'-hydroxychalcones **84-88**. Their cyclisation and oxidation was accomplished with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) at elevated temperature overnight in a sealed tube.[126] Methoxyflavones **89-93** were obtained by this *one-pot* procedure in acceptable yields. The treatment of flavones **89-93** with concentrated hydrobromic acid under reflux furnished the O-demethylated products **94-98.**



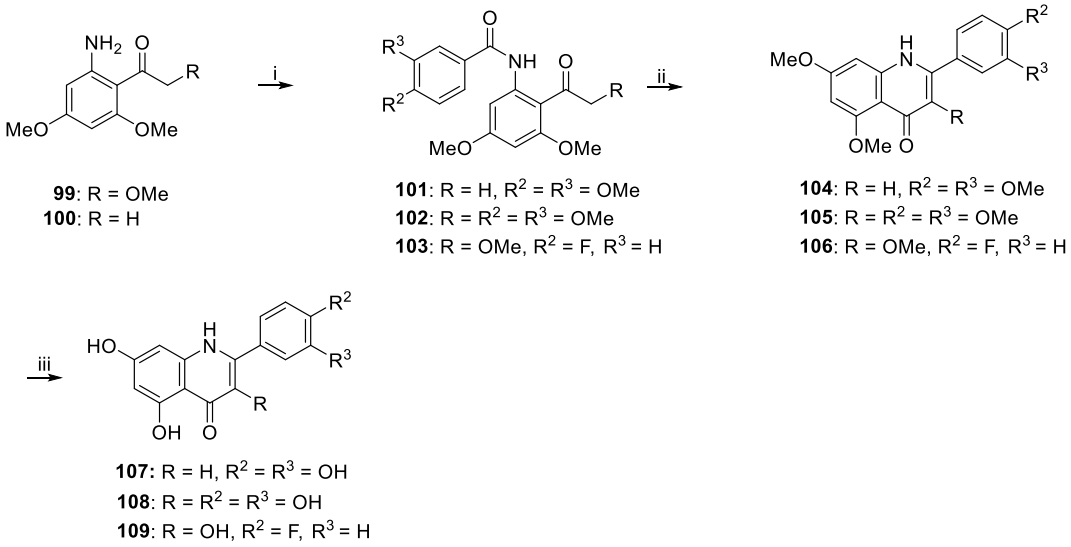
Scheme 6. *Reagents and conditions.* (i) methyl iodide, K_2CO_3, DMF, 35 °C, 9% yield; (ii) methyl iodide, K_2CO_3, DMF, 40 °C, 6% yield.



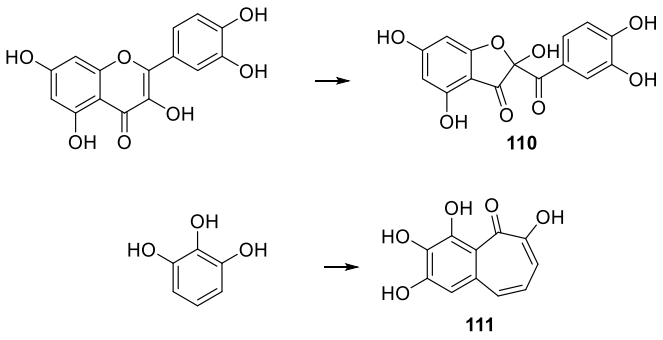
Scheme 7. Preparation of flavonoids. *Reagents and conditions.* (i) KOH, 24 h, r.t.; (ii) 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, 1,4-dioxane, 12h, 80 °C; (iii) 40% HBr in H_2O, 4-12h, reflux.

This study provided us with the opportunity to study the chemical synthesis and biological activity of aza-analogues of flavonoids. The synthesis of three aza-analogues of flavonoid is shown in a Scheme 8. The aza-analogues were prepared following previously published procedure with some modifications.[127] Preparation of compound **99** was done by Friedel-Crafts acylation of 3,5-dimethoxyaniline with methoxyacetonitrile in a presence of boron trichloride and titanium tetrachloride with the low 18% yield. This reaction provided specific *ortho* substituted anilines. Dilution of the reaction mixture reaction as well as the usage of aluminium trichloride was reported to improve the yield of the reaction.[128] Compound **100** was prepared using boron trichloride and aluminium trichloride in a larger dilution with 34%

yield. No further optimization was needed. The subsequent acylation with 3,4-dimethoxybenzoyl chloride and 4-fluorobenzoyl chloride required elevated temperatures and furnished the quinolones **101-103** in satisfactory yields. The acyl derivatives were cyclized using sodium ethoxide. Cyclization under the reported conditions suffered from low yields therefore, ethoxide was replaced with potassium *tert*-butoxide. The cyclization with potassium *tert*-butoxide in *tert*-butanol at elevated temperature in a sealed tube led to significantly improved yields.[129] In the last step, intermediates **104-106** were subjected to O-demethylation in refluxing hydrobromic acid that afforded the desired compounds **107-109**.

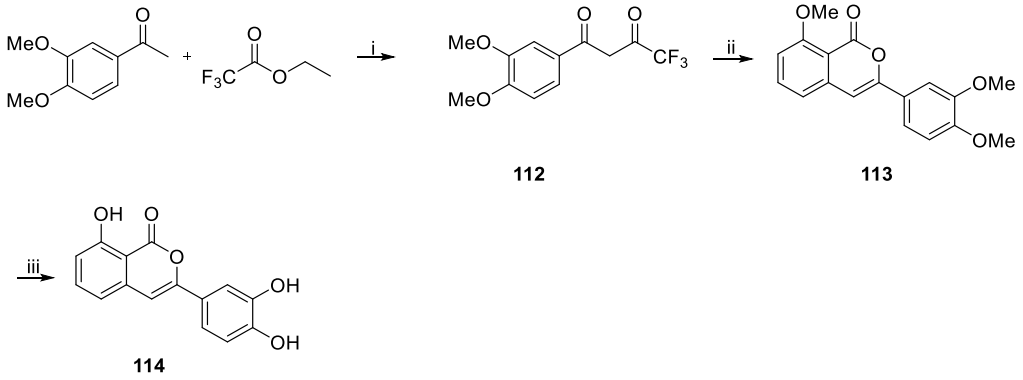


Scheme 8. Preparation of quinolones (aza-flavones). *Reagents and conditions.* (i) 3,4-dimethoxybenzoyl chloride/ 4-fluorobenzoyl chloride, DIPEA, DMAP, toluene, 12h, 80 °C; (ii) potassium *tert*-butoxide, *t*-BuOH, 16h, 80 °C, sealed tube; (iii) 40% HBr in H_2O, 4-12h, reflux.



Scheme 9. *Reagents and conditions.* (i) $CuCl_2 \cdot 2H_2O$, CH_3CN, 0.24M HCl (aq.), 40 °C, 19% yield; (ii) $K_3[Fe(CN)_6]$, $NaHCO_3$, 5 °C to r.t., 6% yield.

A copper(II) mediated rearrangement of quercetin furnished polyol **110** with a contracted C-ring (Scheme 9).[130] Purpurogallin **111** was prepared by oxidation of pyrogallol with potassium ferricyanide in basic conditions (Scheme 9).[131]



Scheme 10. *Reagents and conditions.* (i) THF, NaH, 0 °C, r.t., 5h; (ii) 3-methoxy-2-(trimethylsilyl)phenyl trifluoromethanesulfonate, CsF, CH_3CN, reflux, 4h; (iii) BBr_3 1M in DCM, 2h. [132]

Thunberginol A (**114**) was prepared according to the published procedure (Scheme 10).[132] Isocumarine **114** partially resembles the compound **95**. The only difference is the position of the oxygen and the double bond in the ring C. Other compounds were purchased.

3.2.2. SAR of flavonoids as PA N-terminal domain inhibitors

All the synthesized compounds were tested using the AlphaScreen assay. The IC_{50} values (Table 3) were determined by our collaborators from at the biochemistry department. For the sake of more meaningful SAR study, the activities of purchased compounds are also reported.

Quercetin **18** was found to be active in the AlphaScreen assay with IC_{50} equal to 0.67 μM. Tetra- and pentamethylated quercetin congeners **75** and **76** showed complete loss of activity. Diosmetine **115**, which is a 4'-monomethylated quercetin congener lacking the C3 hydroxyl group, did not show any activity below 50 μM. It is therefore reasonable to assume that unsubstituted 3',4'-dihydroxyphenyl motif or the presence of a hydroxyl group at C3 are critical for the inhibition activity. 3',4'-Dihydroxyflavone **116** showed moderate activity indicating the 3',4'-dihydroxyphenyl motif as critical for the activity. Luteolin **117** was found to be superior inhibitor to the other tested compounds with IC_{50} = 0.072 μM. Luteolin **117**

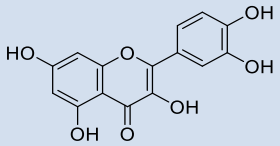
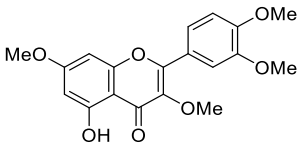
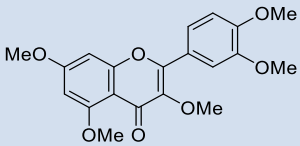
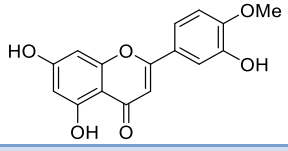
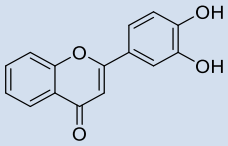
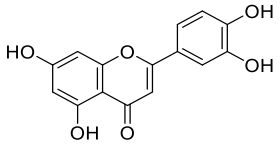
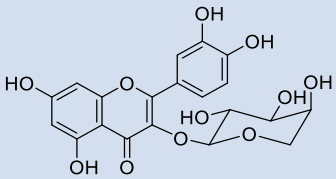
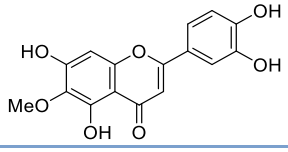
confirmed the importance of 3',4'-dihydroxyphenyl and clearly demonstrates the positive effect of the two additional hydroxyl groups at C5 and C7 on the inhibitory potency. Quercetin 3-*O*-glycoside, guaijaverin **118** had more than two orders of magnitude weaker binding potency compare to luteolin. Nepetin **119** (C6-methoxy luteolin) showed comparable activity to quercetin. Introduction of bulky glucose residue to C-6 position resulted in a complete loss of activity (isoorientin **120**). Cynaroside **121**, a luteolin 7-*O*-β-D-glucoside showed weak inhibitory potency with IC_{50} = 32 μM. This may indicate that C7 position is not as sensitive to modification as is position C6, nevertheless the decrease of potency is still significant. Surprisingly orientin **122** showed better inhibition activity compare to luteolin. Extending the structure of luteolin at C8 provided opportunity for further chemical modifications.

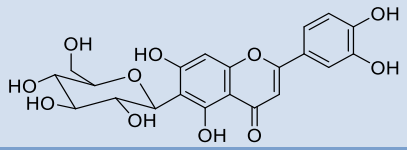
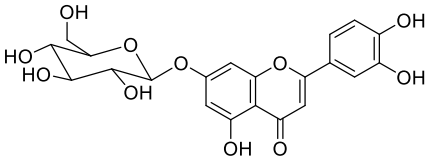
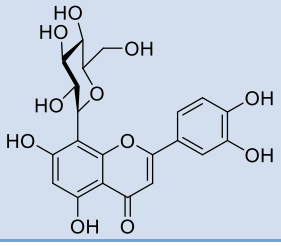
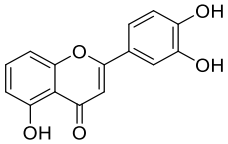
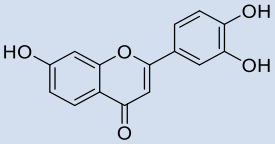
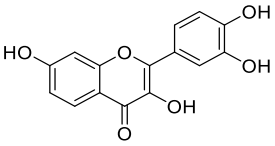
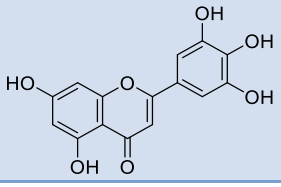
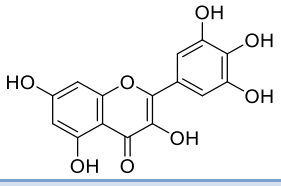
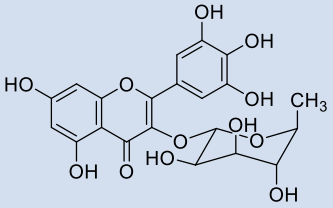
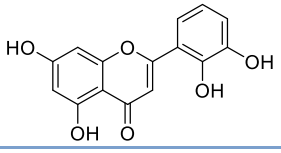
Constitutional isomers **95** and **97** along with fisetin **123** do not underline the presence of hydroxyl group in position C5 or C7 as crucial for inhibition however, the importance of 3',4'-dihydroxyphenyl motif is clear. On the other hand, the presence of two free hydroxyl group at positions C5 and C7 on the 3',4'-dihydroxyflavone scaffold gave the most active compound.

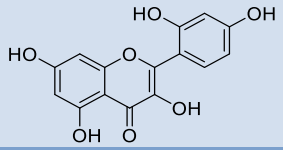
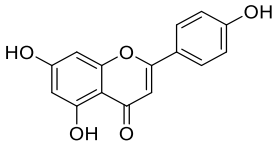
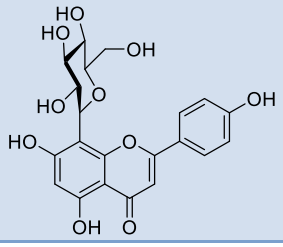
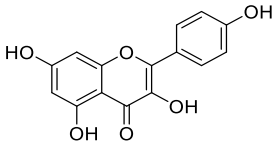
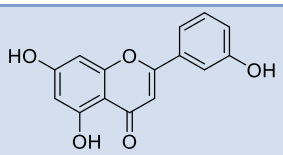
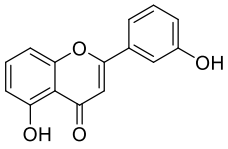
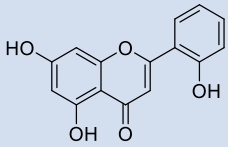
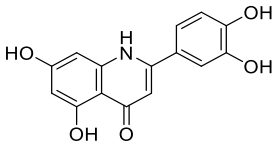
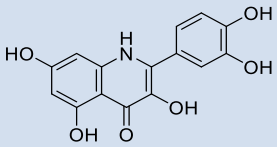
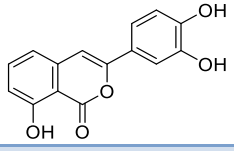
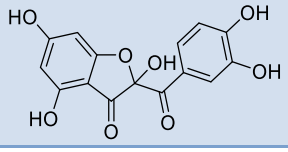
The IC_{50} values for compound **98** and its C3 congener myricetin **124** supports the importance of 3',4'-dihydroxyphenyl motif with C5 and C7 hydroxy groups. Congener **98** is slightly more active than quercetin congener **124**, which is a nice analogy to the similar trend between quercetin **18** and luteolin **117**. A glycosidation of myricetin at C3 furnished myricitrin **125**, and lead to a similar decrease of activity as in the case of quercetin **18** and its glycoside guaijaverin **118**. Compounds **94** and **126** carry two hydroxyl groups on the B ring in a different pattern than the 3',4'-dihydroxy. Both compounds showed significant decrease in activity when compared to luteolin. The presence of only one hydroxyl group on the B ring is not compensated by the presence of C5 and C7 hydroxy groups as can be seen in lack of activity of apigenin **127**. Interestingly the presence of 8-*C*-glucoside moiety on apigenin results in moderate activity of vitexin **128**: IC_{50} = 1.9 μM. Similar effect of the 8-*C*-glucoside was also observed in the case of orientin **122**. The positive effect of C3 hydroxy group on a substrate lacking the 3',4'-dihydroxy motif is shown by the activity of kaempferol **129** with IC_{50} = 16 μM. Compound **98**, **130** and **131** are congeners of apigenine monohydroxylated on a ring B and do not exhibit any activity. The aza-analogs of luteolin quercetin and **107** and **108** share the common structure as well as inhibitory potency. Isocumarine thunberginol A **114** showed very similar activity to flavone **95** (IC_{50} = 1.5 μM). A copper(II) mediated rearrangement of quercetin furnished the

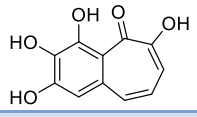
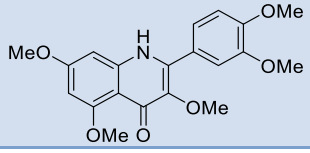
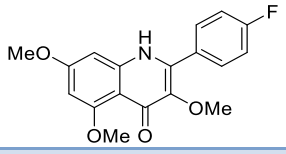
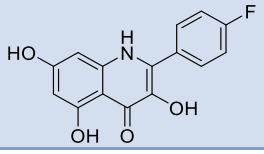
polyol **110** with a contracted C-ring. The polyol **110** exhibit moderate activity against PA endonuclease with IC_{50} = 9.8 ± 0.5. Purpurogallin **111** is a textbook example of efficient metal-binding aromatic polyol with IC_{50} = 1.5 µM. Methylated compounds **104-106** did not show an inhibition activity. Introducing 2'-fluorophenyl instead of 3',4'-dihydroxyphenyl motif resulted in no inhibition activity below 50 µM.

Table 3. Inhibition activities determined by Dr. Milan Kožíšek.

Compound	Structure	Trivial name	IC_{50} (µM) (AlphaScreen)
18		quercetin	0.67±0.02
75		N/A	> 50
76		N/A	> 50
115		diosmetin	> 50
116		3',4'-dihydroxy-flavone	2.4±0.3
117		luteolin	0.072±0.002
118		guaijaverin	17±2
119		nepetin	0.93±0.03

120		isoorientin	> 50
121		cynaroside	32±3
122		orientin	0.043±0.002
95		N/A	1.8±0.1
97		N/A	1.3±0.1
123		fisetin	0.96±0.05
98		N/A	0.16±0.02
124		myricetin	0.76±0.03
125		myricitrin	1.8±0.1
94		N/A	17±2

126		morin	9±1
127		apigenin	> 50
128		vitexin	1.9±0.1
129		kaempferol	16±3
96		N/A	> 50
130		N/A	> 50
131		N/A	> 50
107		azaluteolin	0.068±0.002
108		azaquercetin	0.2±0.02
114		thunberginol A	1.4 ± 0.2
110		N/A	9.8 ± 0.5

111		purpurogallin	1.5 ± 0.04
104		N/A	> 50 µM
105		N/A	> 50 µM
106		N/A	> 50 µM

3.2.2. Structural analysis of luteolin and myricitine in complex with PA-Nter

In our collaboration laboratory, luteolin **117** and myricetin **124** were soaked into empty protein crystals. The structure of the PA N-terminal subunit in complex with **117** was refined to 2.0 Å resolution. In the case of myricetin's complex with PA N-terminal domain, the electron density of the flavonol was of poor quality and its positioning in the active site was ambiguous. With the help of molecular modelling, the binding mode B was suggested (Figure 29).

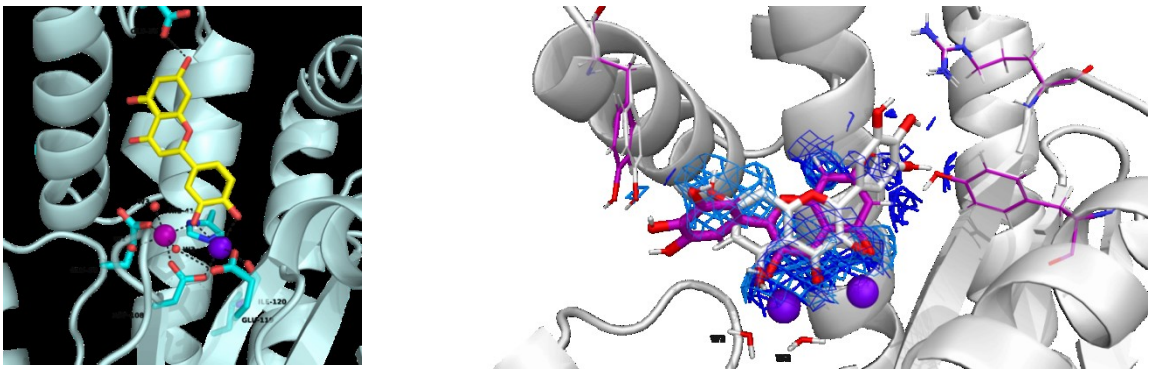


Figure 29. Left: Luteolin **117** in a complex with PA N-terminal domain. Luteolin coordinates metal ions with 3',4'-dihydroxyphenyl moiety and resembles binding mode A. Additionally, hydroxyl groups at C7 creates hydrogen bond with Glu-26. Right: Two optimized quantum mechanics/molecular mechanics structures and their comparison (work of Dr. Jindřich Fanfrlík). Carbon atoms of the myricetin **124** in the native binding mode are shown in white. The carbon atoms of the hypothetical complex with overturned myricetin are shown in purple. Color coding: nitrogen (blue), oxygen (red), manganese (purple blue).

3.2.3. Summary

Both the bidentate mode A (luteolin complex), and tridentate binding mode B (myricetin complex - supported by molecular modeling and crystallographic data), have been observed for flavonoids (Figure 29).

It was determined that 3',4'-dihydroxyflavone motif is important for the inhibitory activity. The presence of both C5 and C7 hydroxy groups is also highly important for the binding of the inhibitor to the PA N-terminal domain active site. This SAR study also revealed the importance of the C3 hydroxy group. Data suggest that the presence of C3 hydroxy group is crucial only when the inhibitor in question lack the 3'-hydroxyl group. Apigenin **127** and related compound are not active unless the C3 hydroxyl group in introduced. This introduction leads to only moderate inhibitory activity (see inhibitors **126** and **129**). X-ray crystallographic data combined with molecular modeling revealed that flavones with 3,5-dihydroxy motif are able to bind the PA N-terminal subunit via binding mode B. On the other hand the introduction of a C3 hydroxyl group in 3',4'-dihydroxyflavone derivatives and in their aza-analogues either do not dramatically influence their activity (see comparison between **97**/**123**), or it leads to decreased activity (see comparison between **117**/**18**, **107**/**108**, **98**/**124**). The substitution in C8 position is well tolerated and when *C*-glycoside is introduced (orientin **122**), it can lead to increase of inhibitory potency.

3.3. PA-PB1 interaction

AlphaScreen assay for evaluation of PA-PB1 interaction inhibitors was developed by Dr. Milan Kožíšek from Jan Konvalinka's laboratory. With this assay available, a preliminary screening of our group's library of compounds was done. This particular library was originally focused on a disruption of a different protein-protein interaction essential for HIV virus capsid assembly.[133] The screening of the library provided two classes of hits (Figure 30). The first class featured 4-nitrobenzene-1,2-diamine moiety **132-133** and the second class is represented by variously substituted ureas **134-135**.

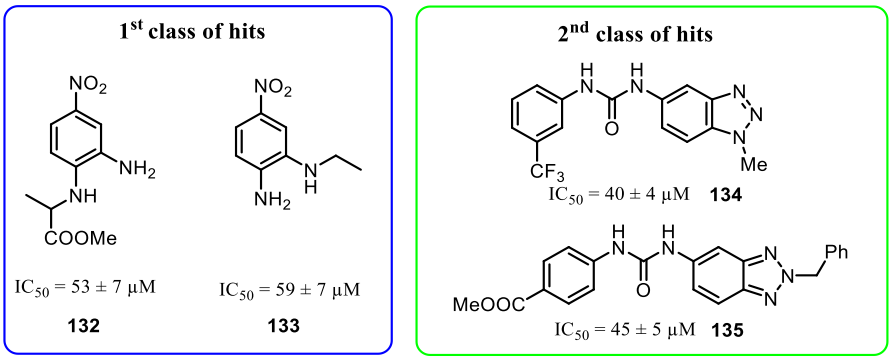
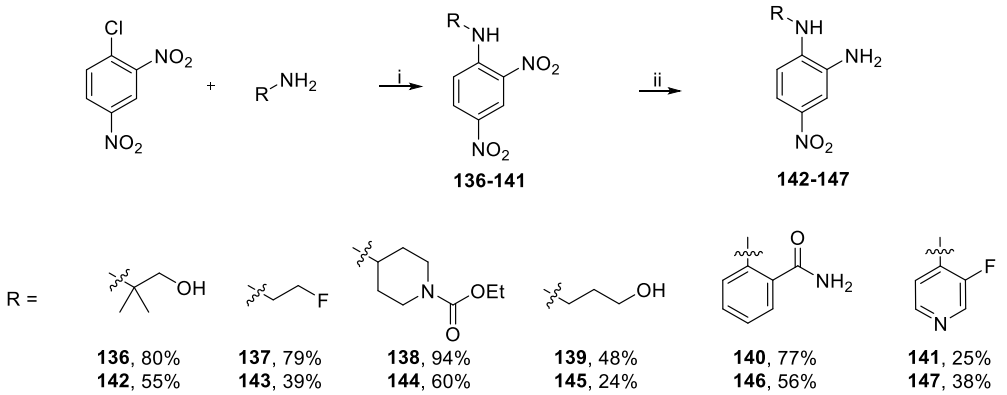


Figure 30. First and second class of hits emerged from the library screen.

Compounds related to the first structure were accessible by a nucleophilic substitution of 2,4-dinitrochlorobenzene with various amines. The regioselective reduction with disulfide generated *in situ* furnished desired nitrobenzenes **142-147** (see Scheme 11).

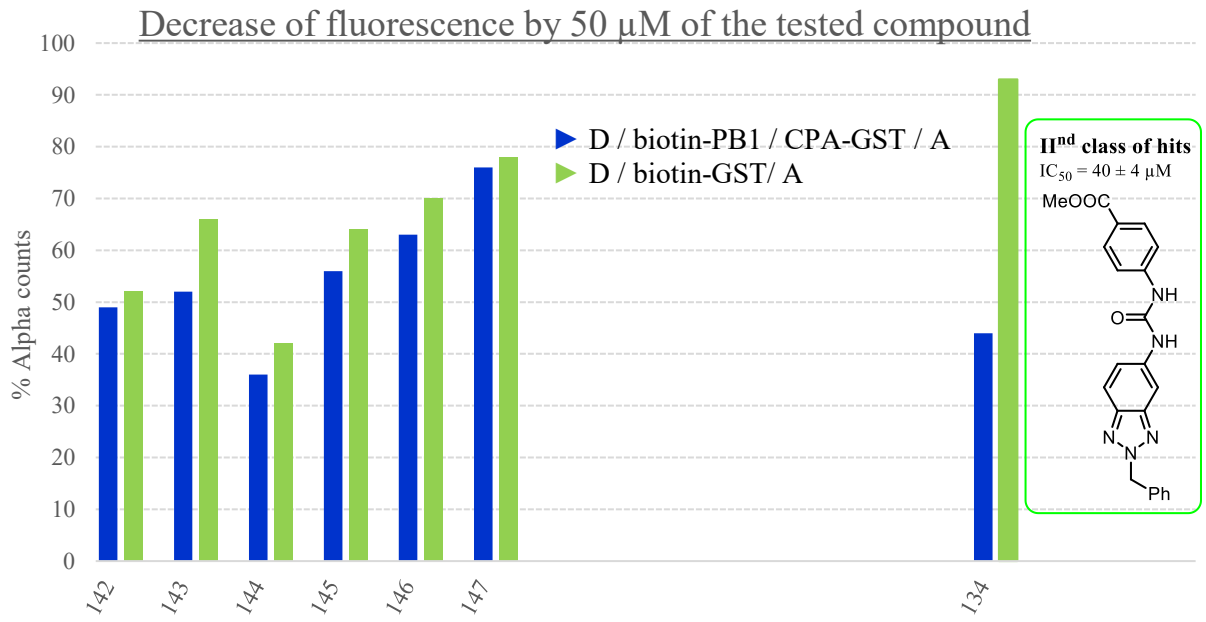


Scheme 11. Preparation of the alkyl-*N*,*N*-diaminonitrobenzenes. *Regents and conditions.* (i) TEA, DMF, 1,4-dioxane, 100 °C, sealed tube, overnight; (ii) a) sulphur, sodium sulfide nonahydrate, H_2O/EtOH, 60 °C, argon; b) ice/water.

These compounds showed weak inhibitory potency in the AlphaScreen assay but, unfortunately, the CounterScreen revealed that these compounds were false positives. The

difference between actual hit and the false positive hit is shown below (Graph 1). When the compound shows activity in the AlphaScreen assay (the decrease of fluorescence is observed) the compound is additionally measured in the CounterScreen assay. When there is no decrease in fluorescence observed in the CounterScreen the compound is an inhibitor. The inactivity in the CounterScreen assay means that the compound is not interfering with the streptavidin-biotin (streptavidin-coated donor bead) or GST-GSH (GSH-coated acceptor bead) connections.

Graph 1. AlphaScreen and CounterScreen data for **142-147** and **134**. Determined by Dr. Milan Kožíšek. Blue slope represents decrease of fluorescence in AlphaScreen for PA-PB1 interaction. Green slope represents decrease of fluorescence in CounterScreen. D = donor bead, A = acceptor bead. Compound **134** showed activity in the AlphaScreen in blue. The CounterScreen did not reveal any significant decrease in the fluorescence. Therefore, compound **134** is an inhibitor. On the other hand, compounds **142-147** showed activity in the AlphaScreen, but the CounterScreen showed similar decrease in the fluorescence as in the AlphaScreen. That meant that the compounds were interfering with the streptavidin-biotin (streptavidin-coated donor bead) or GST-GSH (GSH-coated acceptor bead) connections. It should be noted that the compounds **142-147** could still have been inhibiting the PA-PB1 interaction, but the activity can only be determined by a different assay.



The conclusion from this series was that these compounds are interfering with the assay so it was decided to pursue the second class of hits.

The next series of compounds was prepared by reaction of corresponding isocyanates/isothiocyanates with corresponding substituted benzotriazole in THF. In this series, the *N*-methyl of the benzotriazole **134** was changed for cyclopropyl. The urea moiety was substituted for thiourea and chloro, fluoro, trifluoromethyl substituents were introduced to the other aromatic core (Figure 31). Unfortunately, compounds **148-159** were inactive below 50

μM. The previous series of compounds may have been unsuccessful because of their low solubility so in the next series, the 3-trifluoromethylphenyl moiety was preserved and the other phenyl moiety was equipped with polar groups. The enhanced solubility of compounds **160-164** did not improve the activity.

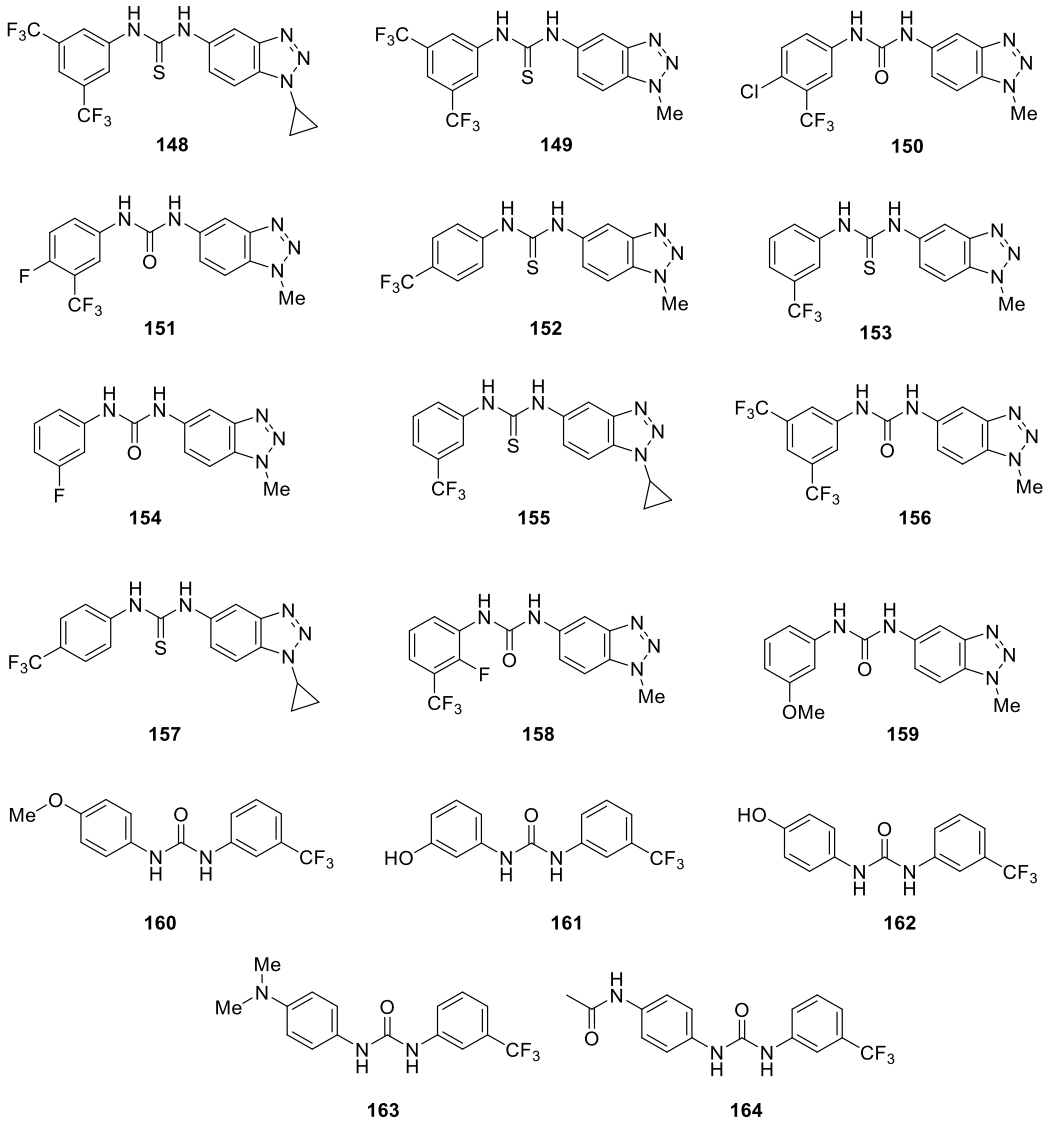
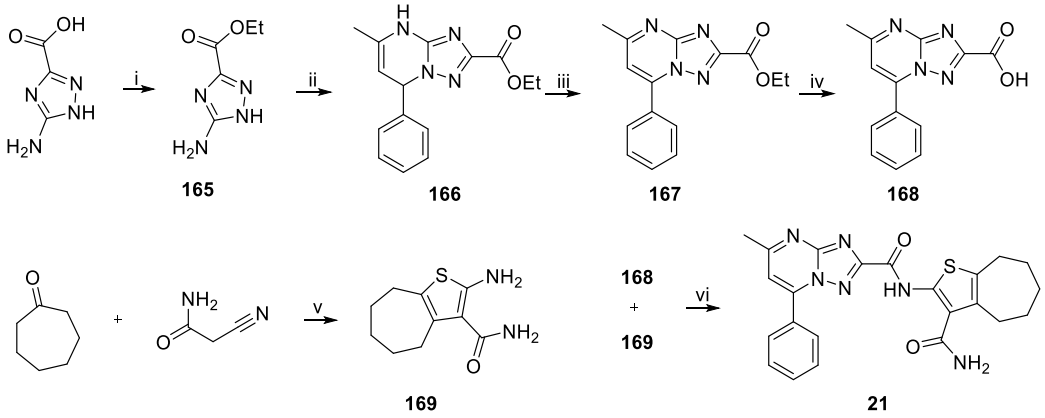


Figure 31. Urea and thiourea products.

3.3.1. Synthesis of published inhibitors

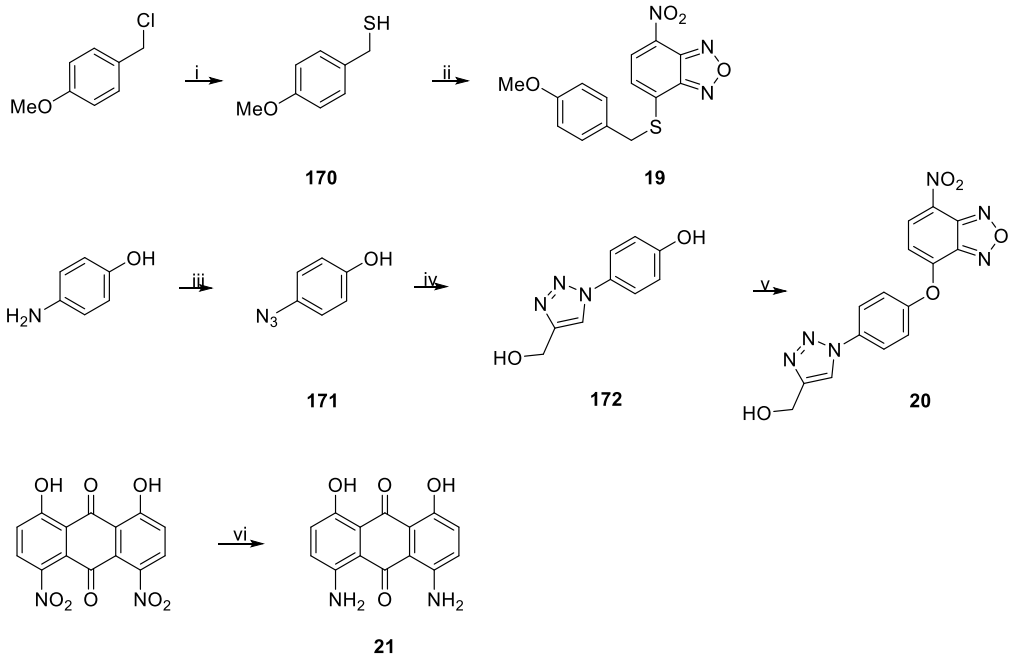
To validate the AlphaScreen assay developed for PA-PB1 interaction five most potent inhibitors according to the literature were prepared. The synthesis and biological activities are commented on in this chapter.

The 5-amino-1*H*-1,2,4-triazole-3-carboxylic acid was converted to ethyl ester **165** with thionyl chloride in ethanol (Scheme 12). Condensation of **165** with benzylideneacetone in DMF furnished the compound **166**. Aromatization of **166** with DDQ provided compound **167**. The successful saponification furnished the free acid **168**. The Gewald reaction[134] of cycloheptanone, cyanoacetamide and sulphur in the presence of diethylamine furnished the amine **169**.[135] The amine **169** and acid **168** were coupled together using TBTU in a presence of DIPEA in DMF. The final compound **21** was crystallized from methanol.



Scheme 12. *Reagents and conditions.* (i) EtOH, $SOCl_2$, reflux, 6h, 80% yield; (ii) benzylideneacetone, DMF, reflux, 4h, 55% yield; (iii) DDQ, 1,4-dioxane, reflux, 1h, 78% yield; (iv) NaOH, MeOH, reflux, 2h, 70% yield; (v) Et_2NH, MeOH, overnight, 17% yield; (vi) TBTU, DMF, TEA, 4h, r.t., 31% yield.

The 4-methoxybenzyl chloride was converted to thiol **170** (Scheme 13). Nucleophilic substitution furnished compound **19**. The 4-aminophenol was converted to azide **171** by diazotization followed by azidation reaction. The azide **171** underwent CuAAC reaction with propargyl alcohol to furnish compound **172**. Nucleophilic aromatic substitution furnished compound **20**. Reduction of 1,8-dihydroxy-4,5-dinitroanthracene-9,10-dione using tin chloride dihydrate furnished the diaminoderivative **21**.



Scheme 13. *Reagents and conditions.* (i) a) thiourea, EtOH, 7h reflux b) NH_4OH, reflux 4h, 75% yield over two steps; (ii) 4-chloro-7-nitrobenzofurazane, pyridine, EtOH, reflux, 1h, 29% yield; (iii) $NaNO_2$, NaN_3, 2M HCl, 0 °C, 2h, 51% yield; (iv) $[Cu(CH_3CN)_4PF_6]$, *t*BuOH/H_2O 1:1, overnight, r.t., 82% yield; (v) 4-chloro-7-nitrobenzofurazane, pyridine, EtOH, r.t. 83% yield; (vi) $SnCl_2 \cdot 2H_2O$, EtOH, 38% yield.

Compound **23** (see page 150) was prepared by Ugi-azide four-component reaction methodology described in the Experimental part. Two diastereoisomers were prepared and separated.

Both nitrobenzofurazane derivatives **19-20** showed false positivity in the CounterScreen assay. Compound **21** interfered with the AlphaScreen assay because it stuck to the beads. The fact that these compounds are showing false positivity in the AlphaScreen only means that these compounds are interfering with the assay and still, they may be inhibitors of PA-PB1 heterodimer formation. Diastereoisomers **23a-b** were inactive below 50 μM. This result indicated limited ability of low-molecular-weight inhibitors to disrupt interactions within the PA-PB1 interface and thus we focused on more conservative approach based on peptides.

3.3.2. Truncation of PB1-derived peptide

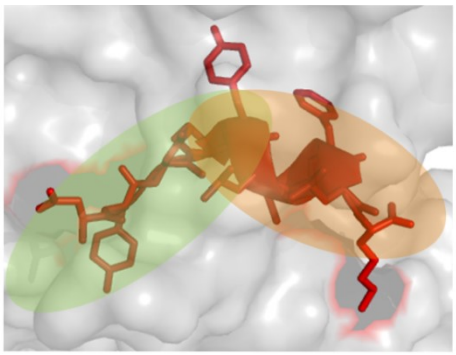
Since all efforts to obtain low-molecular-weight inhibitors were unsuccessful, the strategy had to be changed. The new strategy was to truncate the PB1-derived peptide (**PB1-0**)

and to get a crystal structure of its complex with PA C-terminal domain. The truncation of the original peptide led to a loss of activity but after the introduction of the affinity enhancing mutations (two tyrosines instead of valine and threonine)[136] our collaborators at the biochemistry department were able to get decapeptide **PB1-11** (Table 4) with 13 nM half maximal inhibitory concentration (IC_{50}). Our collaborators were also able to solve X-ray crystal structure of **PB1-11** binding the PA C-terminal domain. Further truncation of the decapeptide is shown in Figure 32. It is clear that further truncation of **PB1-11** led to a major decrease of activity.

Table 4. Truncation of PB1 derived peptide and inhibitory potency from AlphaScreen determined by Milan Kožíšek. Peptides were prepared on PS2 machine by Mirka Blechová and Alexandra Prichodko. Part of the peptide resembling 3_{10}-helix is in grey.

		IC_{50}
PB1-0 :	MDVNPTLLFLKIPA	6 ± 1 nM
PB1-1 :	MDVNPTLLFLKIP	22 ± 1 nM
PB1-2 :	MDVNPTLLFLKI	39 ± 4 nM
PB1-3 :	MDVNPTLLFLK	130 ± 20 nM
PB1-4 :	MDVNPTLLFL	440 ± 40 nM
PB1-5 :	MDVNPTLLF	7,200 ± 1,000 nM
PB1-6 :	DVNPTLLFLKIPA	33 ± 3 nM
PB1-7 :	VNPTLLFLKIPA	390 ± 50 nM
PB1-8 :	NPTLLFLKIPA	20,200 ± 1,700 nM
PB1-9 :	PTLLFLKIPA	36,000 ± 4,000 nM
PB1-10 :	DVNPTLLFLK	3,400 ± 300 nM
PB1-11 :	DYNPYLLFLK	13 ± 1 nM

PB1-		IC_{50} [μM]
11	DYNPYLLFLK	0.013
12	DYNPYLLFL	0.05
13	DYNPYLLF	7.4
14	DYNPYLL	~100
15	DYNPYL	>100
16	DYNPY	>100



IC_{50} [μM]		PB1-
0.013	DYNPYLLFLK	11
0.14	YNPYLLFLK	17
32	NPYLLFLK	18
19	PYLLFLK	19
>100	YLLFLK	20
18	Ac-YLLFLK	21

Figure 32. Further truncation of **PB1-11** from the C-terminus in green, from the N-terminus in red. Inhibition activity determined by Milan Kožíšek. Peptides were prepared on PS2 machine by Mirka Blechová and Alexandra Prichodko (servis staff from Pavel Majer group).

3.3.3. Peptidomimetic approach

X-ray crystal structure of **PB1-11** binding the PA C-terminal domain provided many important findings. Part of the peptide (7 residues) had a 3_{10}-helix secondary structure, which was in agreement with the literature.[90] It was apparent that the mutated tyrosine hot spots were engaged in a formation of net bonds with the surrounding residues (Figure 33). Tyrosine and phenylalanine were projected from the 3_{10}-helix from one face. In protein-protein interaction most of the free energy of binding can be attributed to hot spots. Two tyrosines and phenylalanine were identified as hot spots since the introduction of the two tyrosine residues led to nanomolar activity of the peptide **PB1-11** (see above Table 4).

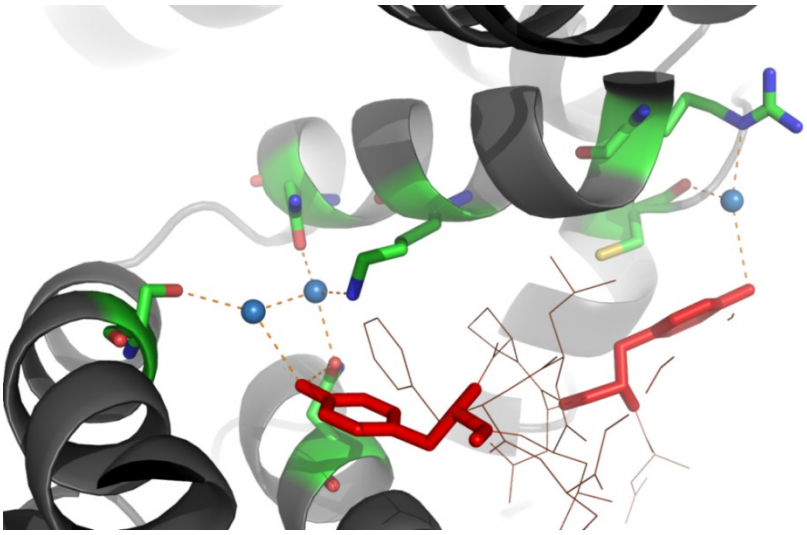


Figure 33. X-ray crystal structure of **PB1-11** in a complex with PA C-terminal domain. Depiction of mutated tyrosines of **PB1-11** (in red, stick representation) engaged in the net bonds (dotted lines) with PA C-terminal domain (black/green, cartoon representation).

Therefore, it was decided to design 3_{10}-helix peptidomimetics and project moieties representing tyrosine and phenylalanine residues. In the literature, the oligobenzamide scaffold is used to mimic α-helix with projection of *i* and *i* + 4 residues from one face. This approach can be used for 3_{10}-helix residues *i*, *i* + 3 as well since the 3_{10}-helix pitch is longer than α-helix pitch. The distance between α-helix residues *i*, *i* + 4 and 3_{10}-helix residue *i*, *i* + 3 is very similar.

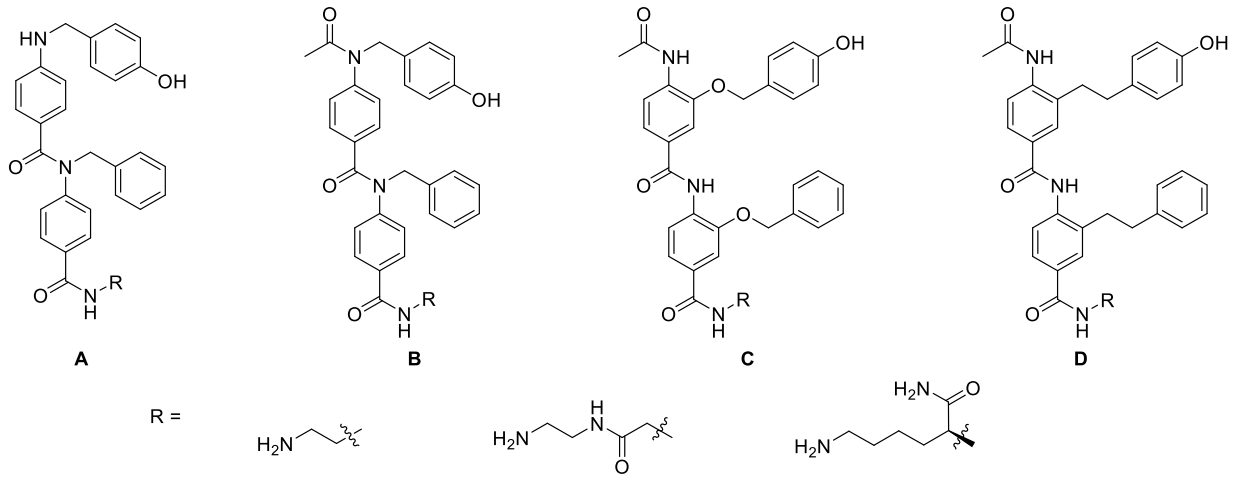


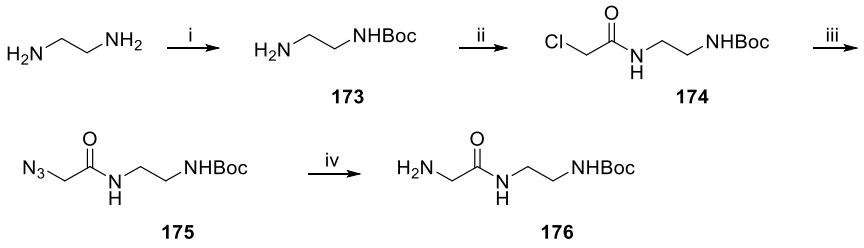
Figure 34. Designed oligobenzamides.

Benzyl and 4-hydroxybenzyl moieties were chosen to mimic phenylalanine and tyrosine residues. To mimic amine in the lysine side-chain various polar moieties were chosen to be projected from the C-end of the benzamide scaffold (Figure 34). The goal was to mimic two hot spots of the hexapeptide and to get at least close to its 18 μM half maximal inhibitory concentration (IC_{50}). Solved X-ray crystal structure of oligobenzamide inhibitor binding the PA C-terminal domain would be a starting point in proposing more potent inhibitors of non-peptidic character.

3.3.3.1. Synthesis

To mimic lysine side chains two different ethylene diamines derivatives were prepared. Mono Boc-protected ethylenediamine **173** was easily prepared from ethylenediamine and Boc-

anhydride (Scheme 14). Amine **173** was extended with chloroacetyl chloride. The nucleophilic substitution of **174** and subsequent reduction furnished the compound **176** in an excellent yield.



Scheme 14. *Reagents and conditions*. (i) Boc_2O, $CHCl_3$, 2h, 0 °C → 3h, r.t., 94% yield; (ii) chloroacetyl chloride, DIPEA, THF, -5 °C, 30 min. 65% yield; (iii) sodium azide, H_2O/EtOH, 60 °C, overnight, 95% yield; (iv) 10% Pd/C, H_2, r.t., 4h, 89% yield.

Retrosynthetic analysis of oligobenzamide **B** is shown in Figure 35. The two logical disconnections were made at two amide bonds of the oligobenzamide scaffold. After connecting synthon **A** and **B** the scaffold can be decorated with different chains mimicking lysine.

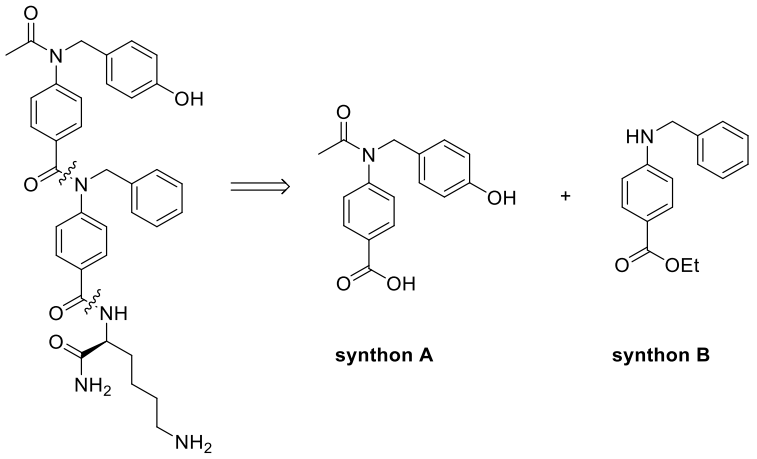
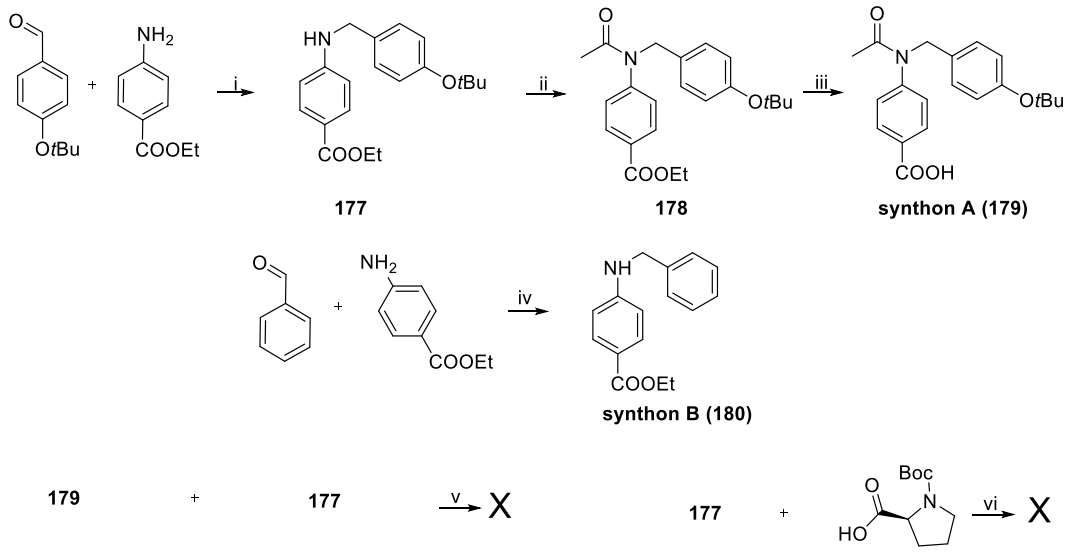


Figure 35. Retrosynthetic analysis of oligobenzamide B.

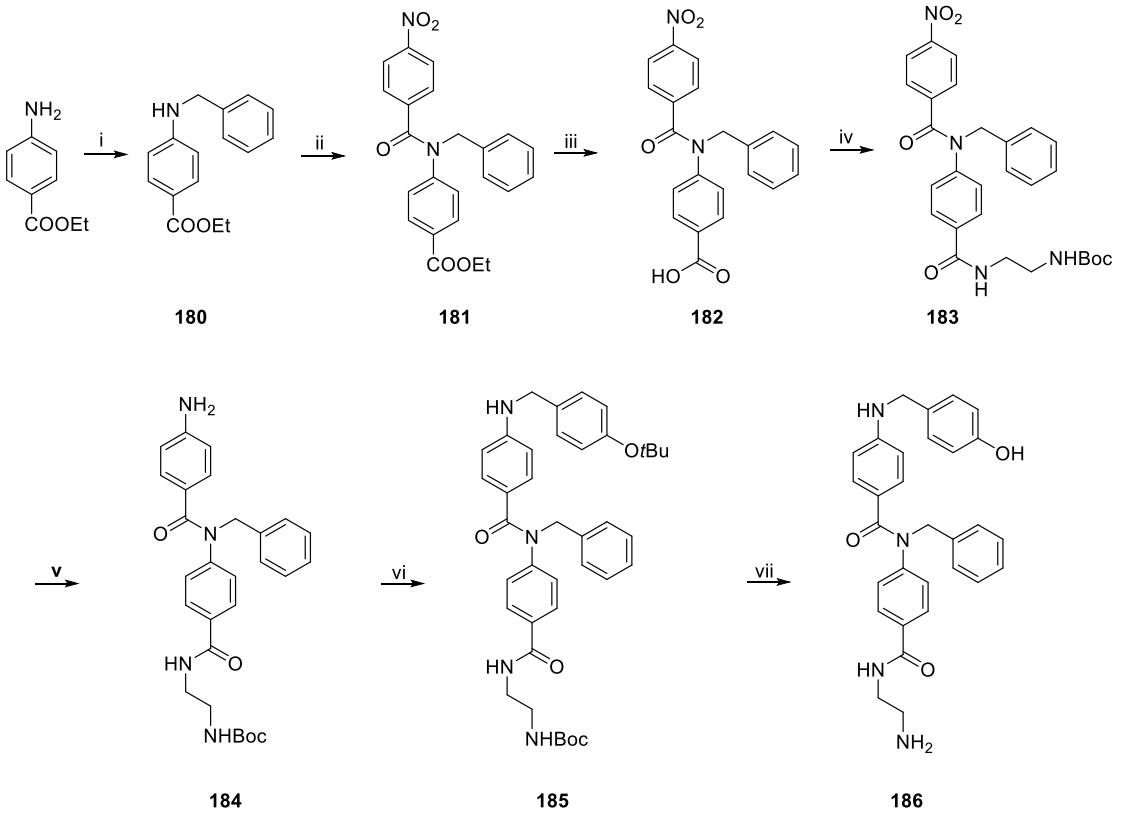
Preparation of synthon A started with reductive amination of benzocaine with 4-(*tert*-butoxy)benzaldehyde (Scheme 15). Use of sodium cyanoborohydride in 2,2,2-trifluoroethanol resulted in formation of **177** in only 35% yield. The best condition for the reductive amination turned out to be borane in a complex with 2-picoline in methanol. Using this condition, the secondary amine **177** was obtained in a satisfactory 87% yield. Following acetylation and saponification proceeded with quantitative yields. Synthon B (**180**) was prepared using similar conditions with 75% yield. Connection of synthon **A** and **B** prove to be problematic due to the steric hindrance and electronic deactivation of the secondary amine in Synthon **B**. Use of classical amide coupling resulted only in a recovery of starting material. Conversion of the free

acid to its corresponding chloride or azide *in situ* with PPh_3Cl_2 or DPPA was also ineffective. Low nucleophilicity of similar secondary amines, as in the case of synthon B, was described in the literature.[137] For similar cases Ghosez's reagent was found to be efficient reagent for conversion of carboxylic acids to its chlorides in a neutral environment.[138] Model reaction of synthon B and Boc-Pro-OH was chosen. The reaction had also a very low yield. Therefore, it was decided to build the oligobenzamide scaffold step by step.



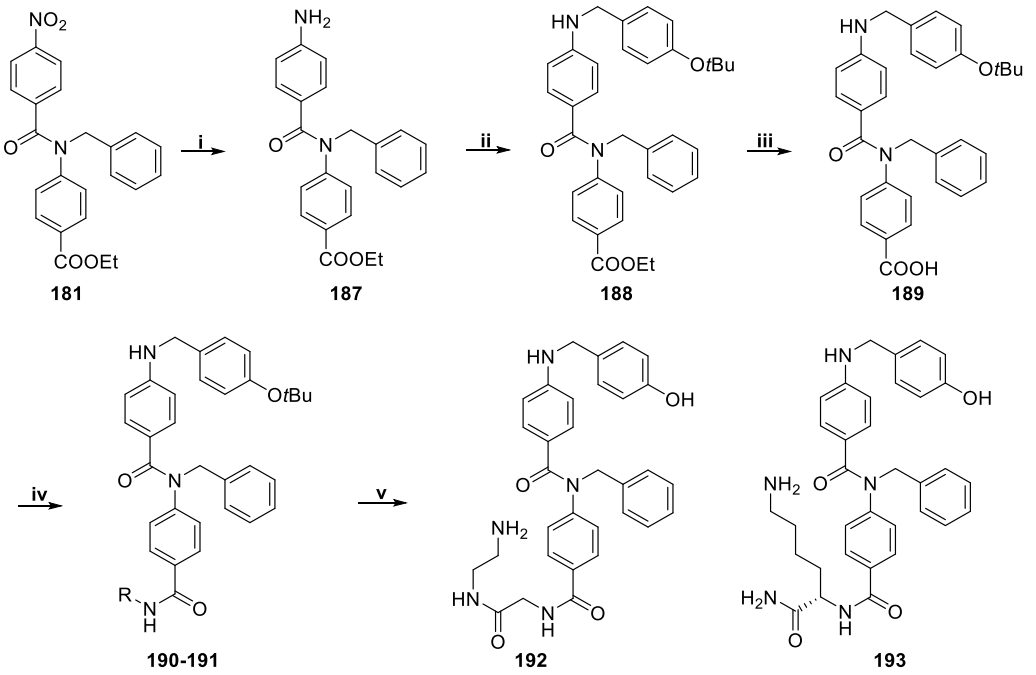
Scheme 15. Synthesis of synthon A and B. (i) 2-Pic BH_3, MeOH, r.t., overnight, 87% yield; (ii) acetyl chloride, DMAP, DIPEA, DCM; (iii) NaOH, 1,4-dioxane/MeOH/H_2O, 86% yield; (iv) 2-Pic BH_3, MeOH, r.t. overnight, 75% yield; (v) Cl_2PPh_3, DCM; (vi) 1-chloro-*N*,*N*,2-trimethyl-1-propenylamine.

The reaction of **180** with 4-nitrobenzoyl chloride required elevated temperature and provided the compound **181** in an excellent yield (Scheme 16). Saponification of ester **181** gave free acid **182** that underwent classical amide coupling with *N*-Boc-ethylenediamine **173**. The following reduction furnished the free amine **184** in only 21% yield. The reductive amination with 2-Pic-BH_3 complex worked well with 81% yield. The compound **185** was deprotected with the solution of TFA/DCM and subsequent HPLC purification provided the final compound **186** in a satisfactory 43% yield. With this synthetic route in hand it was possible to decorate the oligobenzamide scaffold with different moieties mimicking tyrosine residue and preserve the phenylalanine residue and ethylenediamine side-chain. Modular way of preparing the oligobenzamide peptidomimetics with different side chains at the C-end of the scaffold was needed.



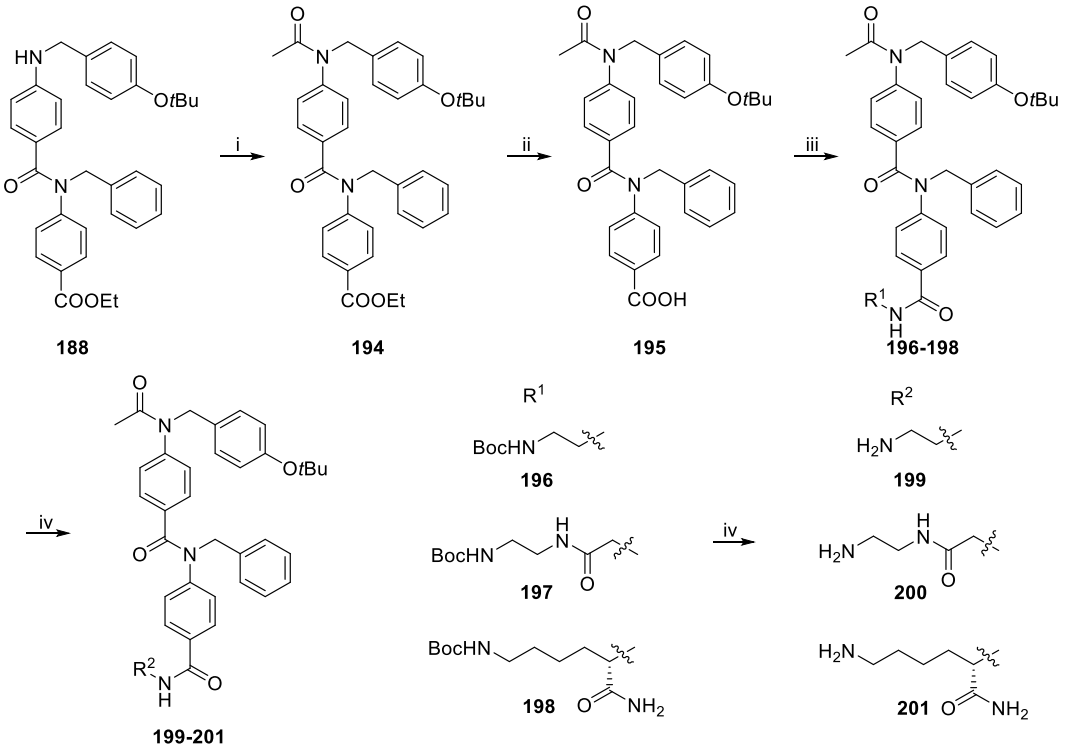
Scheme 16. *Reagents and conditions.* (i) benzaldehyde, 2-Pic-BH_3, MeOH, r.t., overnight, 78% yield; (ii) 4-nitrobenzoyl chloride, DIPEA, toluene, 4h, 90 °C, sealed tube, 90% yield; (iii) NaOH, THF/MeOH 1:1, 60 °C, 2.5h, 85% yield; (iv) TBTU, DMF, TEA, r.t., *tert*-butyl (2-aminoethyl)carbamate, 86% yield; (v) tin(II) chloride dihydrate, MeOH/EtOAc 1:2, overnight, 60 °C, 21% yield; (vi) 4-(*tert*-butoxy)benzaldehyde, 2-Pic-BH_3, MeOH, overnight, r.t., 81% yield; (vii) TFA, DCM, r.t., 2h, 43% yield.

After the poor performance of tin chloride dihydrate in the previous reduction, activated zinc in a presence of ammonium chloride was used to reduce the nitro moiety in compound **181** (Scheme 17). Reduction provided the amine **187** with the 74% yield. The reductive amination took place with satisfactory 54% yield. The saponification furnished the free acid **189** that underwent amide coupling with two different amines. The amides **190-191** were deprotected using TFA/DCM and subsequent preparative HPLC provided the two compounds **192-193** for testing.



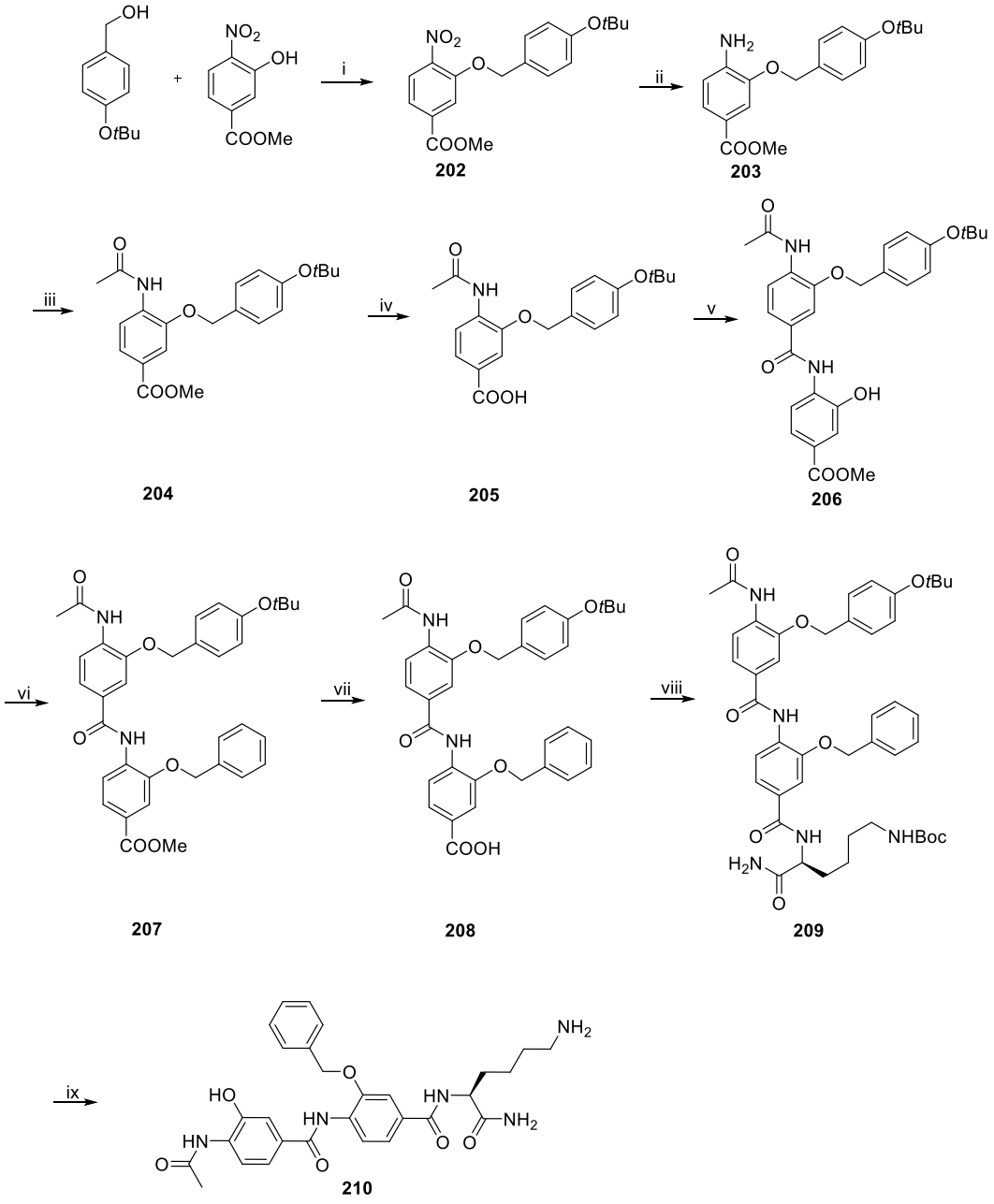
Scheme 17. *Reagents and conditions.* (i) Zn (activated powder), EtOAc/MeOH 1:1, NH_4Cl, overnight, r.t., 74% yield; (ii) 4-(*tert*-butoxy)benzaldehyde, 2-Pic-BH_3, MeOH, overnight, r.t., 54% yield; (iii) NaOH, THF/MeOH 1:1, reflux, 2.5h, 91% yield; (iv) TBTU, DMF, TEA, r.t.; yields **190**: *tert*-butyl (2-(2-aminoacetamido)ethyl)carbamate, 64%,**191** N^{ε}-Boc-*L*-lysine amide hydrochloride, 77%, (v) DCM, TFA, r.t., 2h. yields: **192**: 58%, **193**: 54%.

In the next series (Scheme 18) it was decided to prepare acetylated analogs of the former series because the active hexapeptide **PB1-21** that was used during the design of peptidomimetics was *N*-acetylated. The synthesis started from the compound **188**. Because of the low nucleophilicity of the sterically hindered amine elevated temperature and acetyl chloride in a presence of DMAP were chosen. These conditions furnished the compound **194** in an excellent 98% yield. The following saponification furnished the acid **195** that underwent three different amide couplings with TBTU. Three amides **196-198** were deprotected using TFA/DCM and purified by preparative HPLC to obtain three amines for testing **199-201**.



Scheme 18. *Reagents and conditions.* (i) acetyl chloride, DMAP, DCM, reflux, 4h, 98% yield; (ii) NaOH, THF/MeOH 1:1, reflux, 5h, 97% yield; (iii) TBTU, DMF, TEA, r.t., **196**: *tert*-butyl (2-aminoethyl)carbamate, 60% yield, **197**: *tert*-butyl (2-(2-aminoacetamido)ethyl)carbamate, 42% yield, **198**: *N*$^{\varepsilon}$-Boc-*L*-lysine amide hydrochloride, 57% yield; (iv) DCM, TFA, r.t., 2h, yields: **199**: 46%, **200**: 87%, **201**: 82%.

In the next series of compounds, the moieties mimicking the amino acid residues of tyrosine and phenylalanine were projected through oxygen linkage (Figure 34 C). The two alcohols were combined together using Mitsunobu reaction (Scheme 19). The Mitsunobu reaction furnished **202** with 75% yield. The reduction provided the free amine **203** in 80% yield. The following acetylation a saponification both had close to quantitative yields.



Scheme 19. *Reagents and conditions.* (i) DIAD, PPh_3, THF, 0 °C → r.t., overnight, 75% yield; (ii) tin(II) chloride dihydrate, EtOAc/MeOH 1:1, overnight, r.t., 80% yield; (iii) acetyl chloride, DIPEA, DCM, r.t., 40 min., 98% yield; (iv) NaOH, THF/MeOH 1:1, reflux, 1h, 95% yield; (v) methyl 4-amino-3-hydroxybenzoate, TBTU, DMF, TEA, r.t., 34% yield; (vi) benzyl bromide, DMF, 50°C, 34% yield; (vii) NaOH, THF/MeOH 1:1, reflux, 3h, 88% yield; DCM, TFA, r.t., 2h; (viii) N^{ε}-Boc-*L*-lysine amide hydrochloride, TBTU, DMF, TEA, r.t., 73% yield; (ix) TFA, DCM, 2h, r.t., 56% yield.

The primary amine **203** was sterically hindered, therefore, it was decided to use unsubstituted methyl 4-amino-3-hydroxybenzoate for the amide coupling and that was also what the literature suggested.[139] The amide coupling provided the compound **206** with only 34% yield and the benzylation with benzylbromide gave the compound **207**. Saponification furnished the acid **208** that underwent amide coupling with N^{ε}-Boc-*L*-lysine amide

hydrochloride. The deprotection of compound **209** with TFA/DCM unfortunately provided the compound **210** lacking tyrosine-like moiety (namely 4-hydroxybenzyl). Removal of *tert*-butoxy(benzyl) group in acidic environment of TFA is not described in the literature, however the 4-hydroxybenzyl deprotection has been reported.[140] Here is the proposed mechanism (see Figure 36).

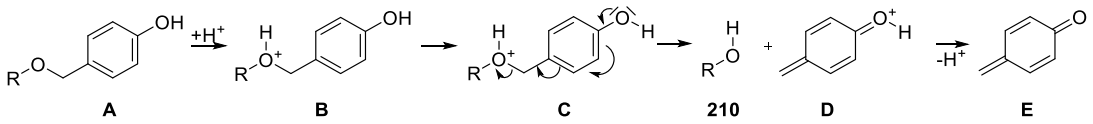


Figure 36. Suggested mechanism for the deprotection of 4-hydroxybenzyl moiety. Protonation of ether oxygen atom in **A** is followed by the formation of alcohol **210**, presumably by the loss of methylene quinone **E** that is intercepted in the reaction media.

After the unsuccessful attempt to synthesize peptidomimetic based on oligobenzamide C (Figure 34) the two congeners were proposed (Figure 37). The synthesis of the congener **A** started with the Wittig reaction to get methyl enol ether **211** in 52% yield (Scheme 20). Because of the *tert*-butoxy moiety present the classical acidic condition for the enol ether hydrolysis could not be used. Mercury acetate was used for the hydrolysis followed by reductive work up. Mercury acetate mediated hydrolysis furnished the phenethyl alcohol **212** in 69% yield over two steps.

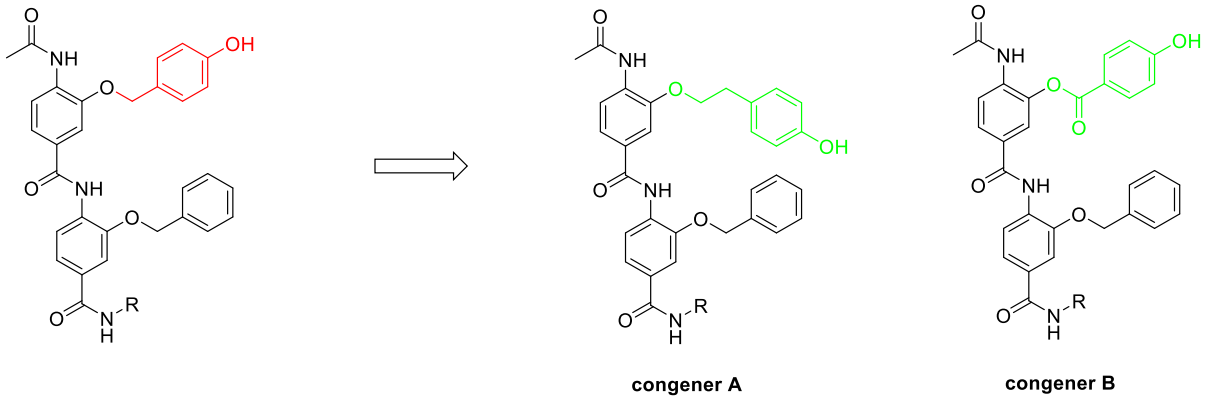
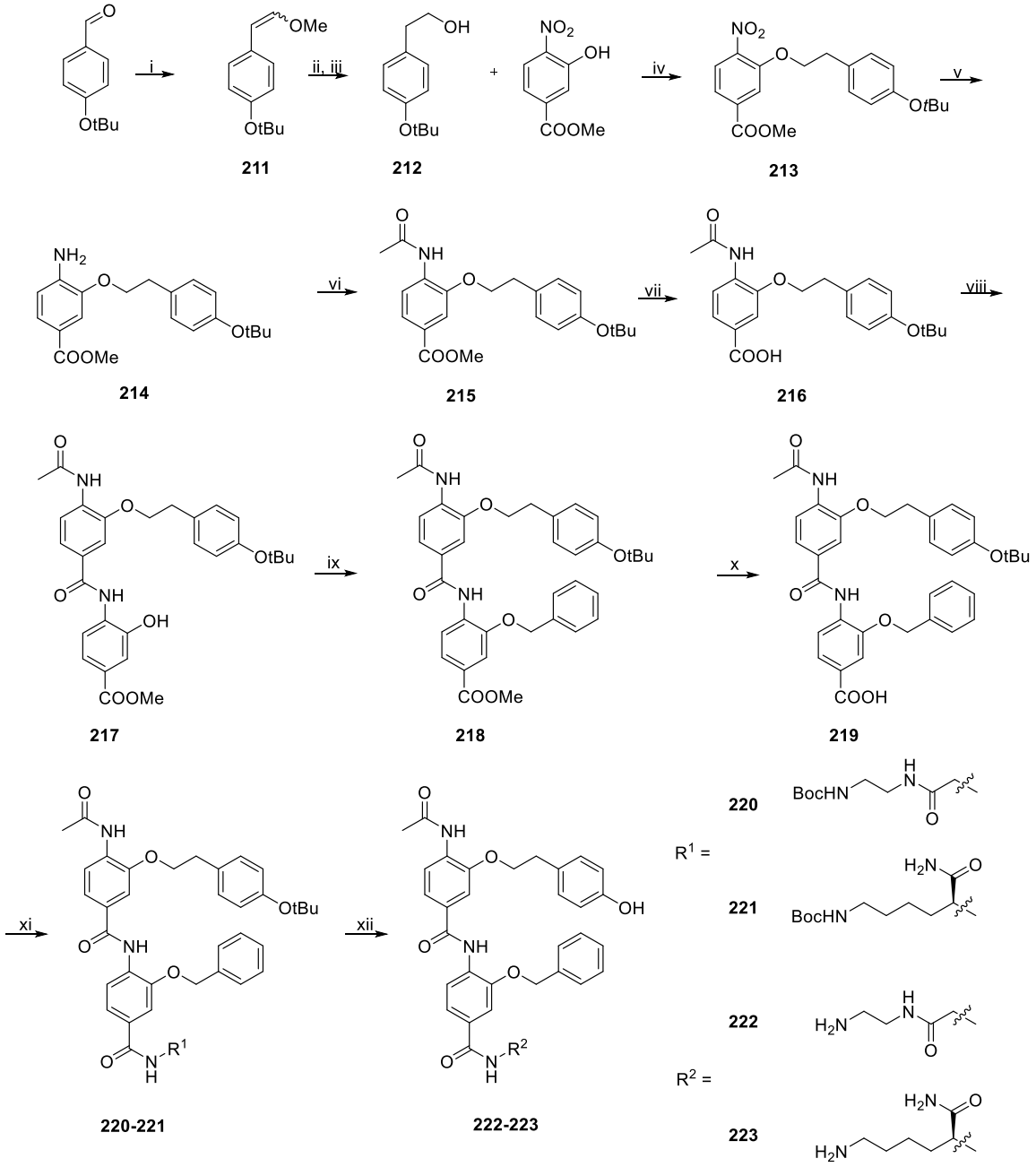


Figure 37. Proposed congeners.

Mitsunobu reaction furnished product **213** in 75% yield. The following steps were reduction, acetylation and hydrolysis. All three reactions worked with excellent yields. Amide coupling of the free acid **216** and the subsequent alkylation were again problematic. Saponification gave product **217** in 25% yield and the alkylation in DMF with benzyl bromide led to **218** with 34% yield. Hydrolysis of the ester **218** provided the acid **219** with excellent

yield. The acid **219** underwent two different amide couplings and the amides **220**-**221** were deprotected with DCM/TFA to give the final compounds **222** and **223**.



Scheme 20. *Reagents and conditions.* (i) (methoxymethyl)triphenylphosphonium chloride, BuLi, THF, -50 °C → r.t., 3h, 52% yield; (ii) mercury(II) acetate, THF/H_2O 1:1, 30 min.; (iii) $NaBH_4$, K_2CO_3, 69% yield; (iv) DIAD, PPh_3, THF, 0 °C → r.t., overnight, 75% yield; (v) Pd/C 10%, EtOH, 2h, r.t., 85% yield; (vi) acetyl chloride, DIPEA, DCM, r.t., 40 min. 99% yield; (vii) NaOH, THF/MeOH 1:1, reflux, 1h, 94% yield; (viii) TBTU, DMF, TEA, r.t. 25% yield; (ix) benzyl bromide, DMF, 50°C, 34% yield; (x) NaOH, THF/MeOH 1:1, 60 °C, 2h, 98% yield; (xi) TBTU, DMF, TEA, r.t. **220**: *tert*-butyl (2-(2-aminoacetamido)ethyl)carbamate, 77% yield **221**: N^{ε}-Boc-*L*-lysine amide hydrochloride, 75% yield; (xii) TFA/DCM 2/1, 2h, r.t., **222**: 84% yield **223**: 90% yield.

3.3.3.2. Conclusion from the peptidomimetic approach

All the oligobenzamides showed good solubility but, unfortunately, there was no inhibition observed below 50 μM. This project revolving around peptidomimetic approach in inhibition of protein-protein interactions (PPI) was from its beginning very risky and eventually none of the massive effort paid off in the form of publishable results. Doubtlessly, inhibition of protein-protein interaction by low-molecular-weight inhibitors represents one of the biggest challenges in medicinal chemistry of 21st century and these "risky projects with a low chance of success" have to be undertaken to make the break through.

3.3.4. Optimization of the hexapeptides

At this point, it was decided to find out more about the three hot spots of the decapeptide **PB1-11** (**DYNPYLLFLK, DANPBLLCLK**) and two hot spots of the hexapeptide **PB1-21** (Figure 38); **AcYLLFLK, AcBLLCLK**). Key hot spots are underlined and each hot spot is designated with a letter A, B and C. Therefore hot spot C corresponds to the same amino acid in a hexapeptide and in a decapeptide. A series of different hexapeptides were prepared using SPPS or liquid phase chemistry. In the next chapter, the synthesis and biological activities of these compounds are discussed. The goal of this optimization was to increase solubility and to maintain or to improve the activity of the peptides. Optimized peptide should have been a new candidate for the crystallization of the peptide with PA C-terminal domain. The new X-ray crystal structure of optimized peptide with PA C-terminal domain should provide crucial structural information for the development of low-molecular-weight inhibitors for PA-PB1 interaction of non-peptidic character.

PB1-21 **AcBLLCLK**

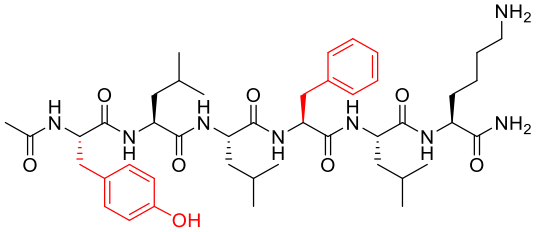
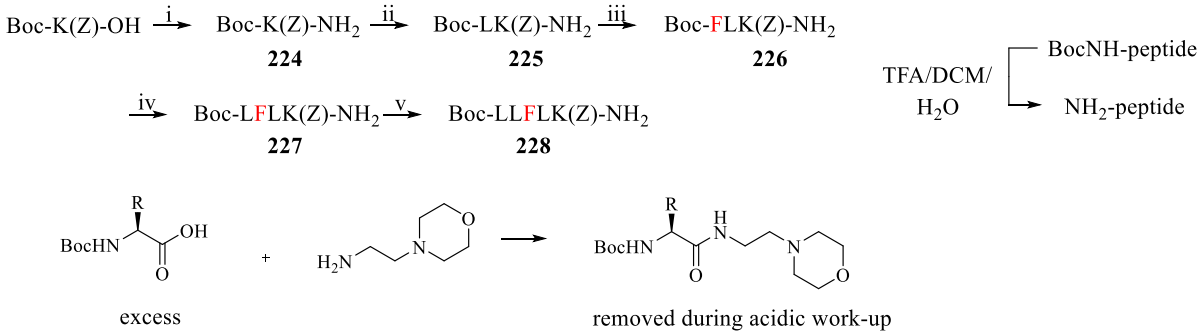


Figure 38. Hexapeptide with hot spots B for tyrosine, C for phenylalanine.

3.3.4.1 Synthesis

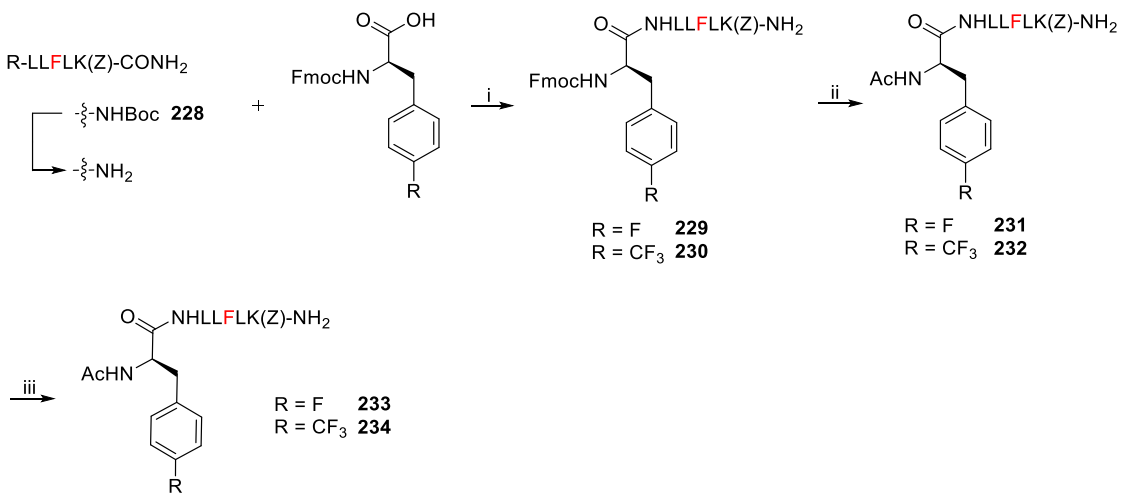
Hexapeptides were synthetically more accessible. First round of optimization focused on the hot spots B and C (Figure 38). To prepare a series with different B substitution pentapeptide, Boc-LLFLK(Z)NH_2 (**228**) was prepared in a solution. Because of the lipophilic

nature of the amino acid residues in peptide the amide couplings required only acidic and basic work ups and the pure peptides were precipitated with *tert*-butyl methyl ether. Protected amino acids were used in small excess of 1.1 equivalent. To get rid of any remaining amino acid in the solution 0.3 equivalent of 2-aminoethyl morpholine was added to the reaction after 2.5 h. Remaining amino acid was converted to corresponding water-soluble amide and it was eliminated during acidic work up. Prior to each amide coupling the Boc-protected peptide was deprotected. The deprotection was achieved with TFA/DCM 1:1 solution with few drops of water. This robust method allowed for preparation of the desired pentapeptide **228** on a gram scale (Scheme 21).



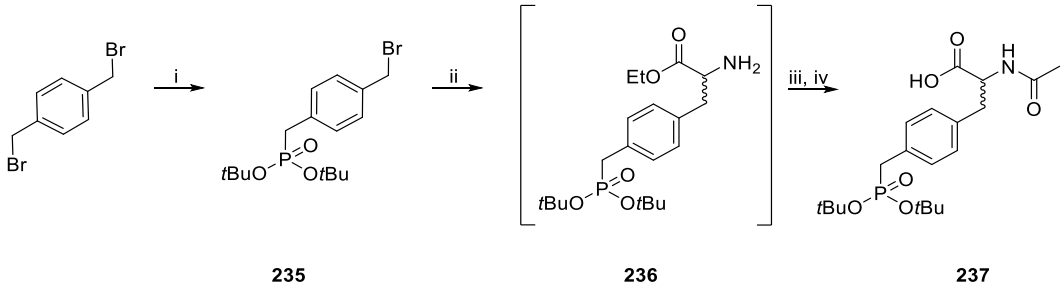
Scheme 21. *Reagents and conditions.* (i) TBTU, DIPEA, DMF, NH_4Cl, 3h, 78% yield; (ii) Boc-Leu-OH, TBTU, DIPEA, DMF, 3h, 65% yield; (iii) Boc-Phe-OH, TBTU, DIPEA, DMF, 3h, 83% yield; (iv) Boc-Leu-OH, TBTU, DIPEA, DMF, 3h, 77% yield; (v) Boc-Leu-OH, TBTU, DIPEA, DMF, 3h, 86% yield.

To evaluate the importance of tyrosine hydroxyl group a substitution for fluoro and trifluoromethyl groups was done. Fmoc-4-fluorophenylalanine and fmoc-4-trifluoromethylphenylalanine were coupled with the deprotected pentapeptide **228** to obtain peptides **229-230** (Scheme 22). Deprotection of the fmoc groups and acetylation were carried out simultaneously to obtain **231-232**. Fmoc group was slowly deprotected using DMAP and in the presence of acetanhydride the free amine was acetylated. The only drawback of this reaction was the long reaction time needed for the fmoc deprotection (2-3 days). The carboxybenzyl protected amine in lysine was deprotected using Pd/C in ethanol to obtain hexapeptides **233** and **234 (**Scheme 22**)**.



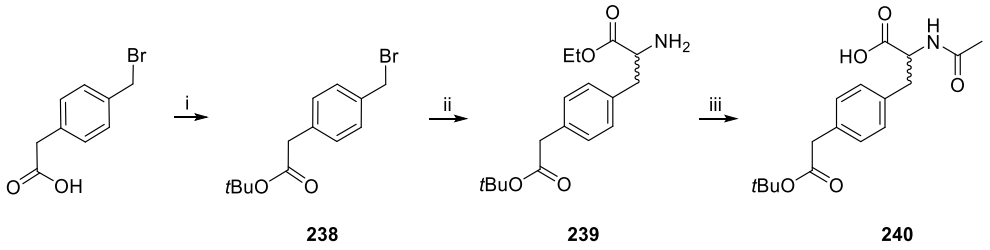
Scheme 22. *Reagents and conditions.* (i) TBTU, DIPEA, DMF, 3h, yields **229**: 78%, **230**: 60%; (ii) DMAP, Acetanhydride, DMF, 3 days, yields **231**: 56%, **232** 50%; (iii) Pd/C, H_2, EtOH/CH_3COOH 10/1, 3h, yields **233**: 35%, **234:** 30%.

According to the crystal structure of **PB1-11** with PA C-terminal domain the tyrosine hot spot B is engaged in a formation of net bonds with the surrounding PA C-terminal subunit (Figure 33). To exploit this interaction it was decided to extend the hot spot B with carboxylic and phosphonic acid residues. These two amino acids were not commercially available and had to be prepared as racemates. Modified procedure from the literature was followed.[141] Nucleophilic substitution of the 1,4-bis(bromomethyl)benzene with di-*tert*-butylphosphite furnished the benzylbromide **235** in 23% yield with the rest of the dibromide being recovered from the reaction mixture (Scheme 23). The alkylation of the *N*-(diphenylmethylene)glycine ethyl ester with **235** in a presence of benzyltrimethylammonium hydroxide was followed by acid catalyzed hydrolysis to furnish the compound **236**. Saponification and acetylation of **236** furnished the amino acid **237**.



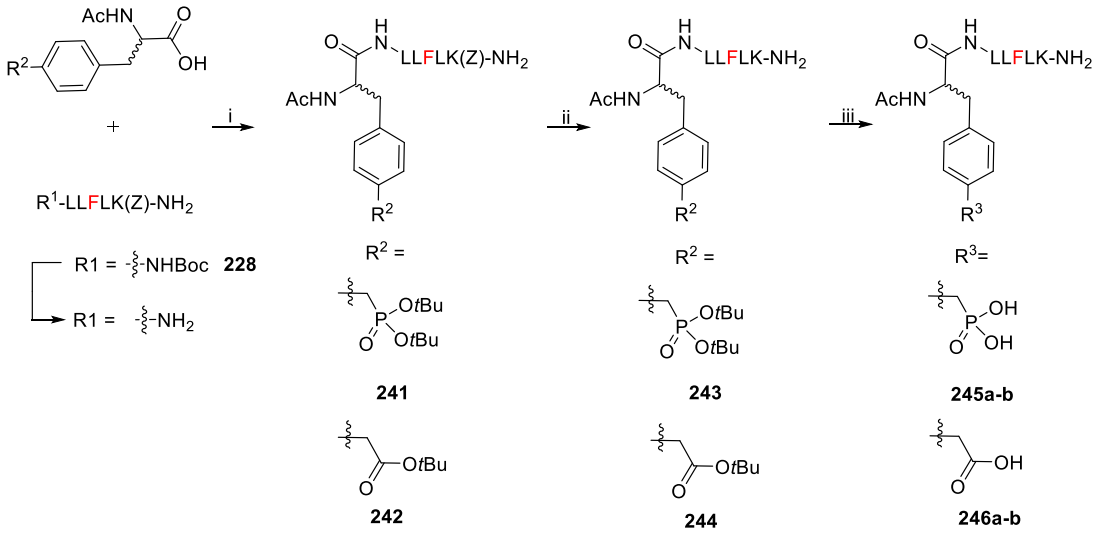
Scheme 23. *Reagents and conditions.* (i) di-*tert*-butyl phosphite, THF, NaH, sealed tube, 23% yield; (ii) *N*-(diphenylmethylene)glycine ethyl ester, KI, 1,4-dioxane, benzyltrimethylammonium hydroxide, 45% yield; (iii) NaOH, 1,4-dioxane, MeOH; (iv) *N*-acetoxysuccinimide, DIPEA 32% yield over two steps.

For preparation of another building block *tert*-butylester was introduced by *tert*-butyl 2,2,2-trichloroimidate in a presence of boron trifluoride diethyl etherate to furnish compound **238** in 80% yield (Scheme 24). Compound **238** was used to alkylate *N*-(diphenylmethylene)glycine ethyl ester followed by mild acidic hydrolysis that gave the compound **239**. Saponification and acetylation of **239** afforded compound **240**.



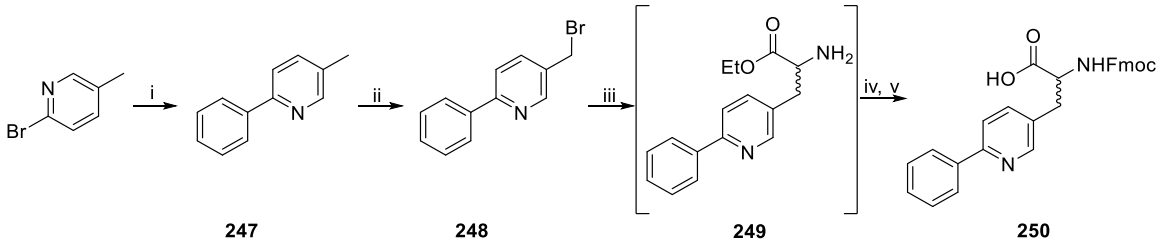
Scheme 24. *Reagents and conditions.* (i) *tert*-Butyl 2,2,2-trichloroacetimidate, $BF_3 \cdot OEt_2$, DCM, 2h, r.t., 85%; (ii) *N*-(diphenylmethylene)glycine ethyl ester, KI, 1,4-dioxane, benzyltrimethylammonium hydroxide, 48% yield; (iii) a) NaOH, H_2O, 1,4-dioxane, MeOH (b) *N*-acetoxysuccinimide, DIPEA 72% yield over two steps.

Compounds **237** and **240** were coupled with deprotected pentapeptide **228** using standard amide coupling conditions to furnish peptides **241** and **242** (Scheme 25). The carboxybenzyl (Z) group was deprotected with hydrogenation on Pd/C to obtain **243-244** the *tert*-butyl groups were deprotected with the solution of TFA/DCM to furnish the hexapeptides **245** and **246** as racemates. The two diastereoisomers were separated and their biological activities were measured separately.



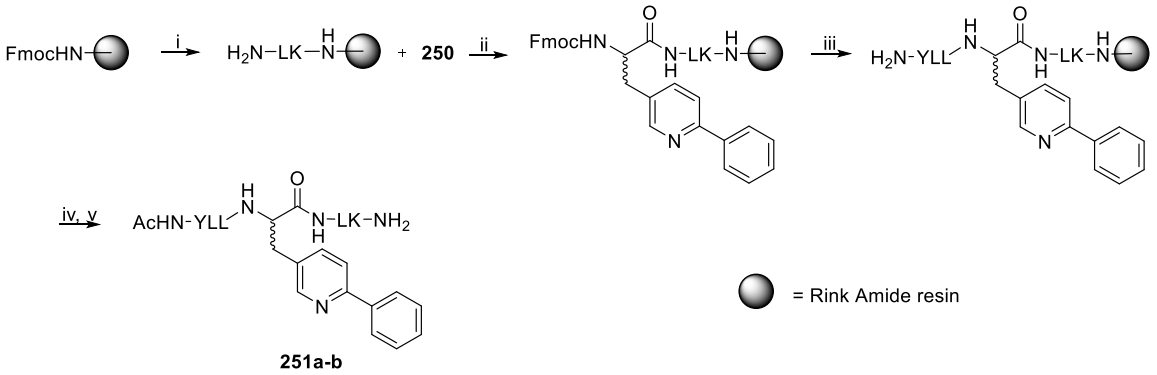
Scheme 25. *Reagents and conditions.* (i) TBTU, DIPEA, DMF, 3h, yield **241**: 94%, **242**: 840%; (ii) DMAP, acetanhydride, DMF, 3 days, yield **243**: 96%, **244**: 97%; (iii) Pd/C, H_2, EtOH/CH_3COOH 10/1, 3h, yield **245**: a) 9% b) 9%, **246** a) 9% b) 9%.

Optimization of the hot spot C was also done by Bc. Miroslava Palacková and is described in her bachelor thesis.[142] Her work revealed that the hot spot C was able to accommodate biphenylalanine (IC_{50} = 2 μM, maximal solubility: 10 μM) and naphthylalanine (IC_{50} = 5.5 μM, maximal solubility: 10 μM) residues. These hexapeptides showed improved activity but the solubility was lowered. Deteriorating solubility was a big issue since the aim of this optimization was to get a peptide suitable for crystallization with PA C-terminal subunit. To improve the solubility it was decided to synthesize Fmoc-4-phenyl-3-pyridylalanine (Scheme 26). Suzuki coupling of 2-bromo-5-methylpyridine and phenylboronic acid furnished the compound **247**. Radical bromination furnished benzyl bromide **248**. The alkylation of the *N*-(diphenylmethylene)glycine ethyl ester with **248** was followed by acid catalyzed hydrolysis to furnished amine intermediate **249**. Saponification and fmoc protection of the amine intermediate **249** furnished the amino acid **250**.



Scheme 26. *Reagents and conditions.* (i) phenylboronic acid, H_2O, EtOH, toluene, Na_2CO_3, $Pd(PPh_3)_4$, 80 °C, 92% yield; (ii) NBS, benzoyl peroxide, $CHCl_3$, reflux, 3h, 63% yield; (iii) *N*-(diphenylmethylene)glycine ethyl ester, KI, 1,4-dioxane, benzyltrimethylammonium hydroxide; (iv) NaOH, 1,4-dioxane, MeOH (v) Fmoc *N*-hydroxysuccinimide ester, DIPEA, 66% yield over three steps.

The amino acid **250** was used for a SPPS of peptide **251** (Scheme 27). First amino acids were introduced by SPPS on PS3 machine. Amino acid **257** was introduced by SPPS in a syringe and the rest of the peptide was synthesized using PS3. Acetylation was carried out in a syringe and the final deprotection followed by preparative HPLC purification furnished the two diastereoisomers **251a-b**.



Scheme 27. *Reagents and conditions.* (i) SPPS on PS3; (ii) HBTU, DIPEA, DMF, in a syringe; (iii) SPPS on PS3; (iv) CH_3COOH, DIC, HOBT, DMF, in a syringe; (v) TFA/TIS/H_2O 95/2.5/2.5, 1h.

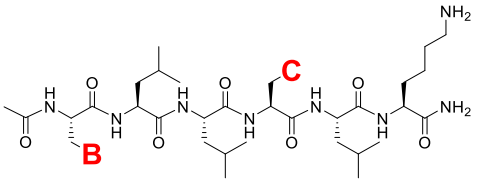
The other peptides were prepared using SPPS in a syringe or on PS3.

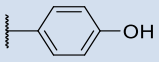
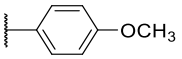
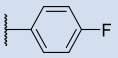
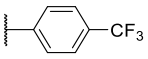
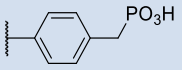
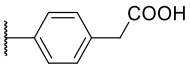
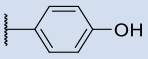
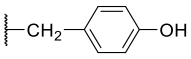
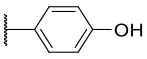
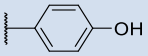
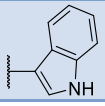
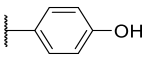
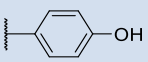
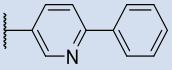
3.3.4.2. Biological activities

Biological activities of the hexapeptides are shown in a Table 5. Compounds **240**, **241** and **252** showed that the tyrosine hydroxyl moiety is crucial for the activity and solubility of the hexapeptides. The activity of these compounds is influenced by the low solubility. This confirmed that the tyrosine residue is involved in important hydrogen bonding with PA C-terminal subunit. Phosphonate and carboxylate containing hexapeptides were prepared as racemates (Scheme 25). The diastereoisomers were separated and their activity was measured separately. The activity of compounds **245a,b** and **246a,b** was also influenced by the solubility. Presence of 3-pyridyl moiety in a position C of hexapeptide **253** led to a significantly higher solubility and loss of activity. Homotyrosine in position B and tyrosine in position C led to a loss of activity of peptide **254**. Tryptophan in a position C led to improved activity and also solubility of the peptide **255**. Compound **255** was so far the best choice for crystallization with PA C-terminal domain. Histidine in a position C was not tolerated and the peptide **256** lost its inhibitory potency. Peptide **251** was designed to combine the biphenyl moiety responsible for the increase in the activity and the 3-pyridyl moiety to improve the solubility, however, the activity of compound **251** was influenced by the solubility.

Table 5. Optimization of hexapeptide hot spots B and C. Hexapeptides with activity being affected by solubility in red. Hexapeptide with enhanced activity and solubility in green.*only one diastereoisomer showed any activity.

AcBLLCLK



Compound	B	C	IC_{50} AlphaScreen	max. solubility
PB1-21			18 µM	35 µM
252			> 9 µM	9 µM
240			> 6 µM	6 µM
241			> 7 µM	7 µM
245a,b			> 18 µM, n.d.	18 µM, < 1 µM
246a,b			> 43 µM, > 21 µM	43 µM, 21 µM
253			> 100 µM	> 100 µM
254			> 50 µM	40 µM
255			**5.1 µM**	**43 µM**
256			> 100 µM	96 µM
251*			9.7 µM	10 µM

To probe the importance of leucine residues in the structures of tested peptides the alanine scan was performed. Leucines were one by one substituted for alanine in this scan. Compound **255** was chosen for the scan because of its superior activity and solubility. Alanine scan showed that all three leucines are important for the peptide binding to the PA C-terminal domain (peptides **257-259**). This may explain why the peptidomimetic approach (see Chapter 3.3.3.) was not successful. Only two residues were projected from the oligobenzamide scaffold

without the consideration for the opposite side of the helix. To successfully mimic the hexapeptide **PB1-22** leucine residues are obviously also crucial, therefore projecting only two residues corresponding to tyrosine and phenylalanine may have not been enough. Hexapeptides **260**, **261** were also prepared to improve solubility and to maintain or to improve the activity (Table 6). The employed residues were chosen according to the publication that identified high-affinity PB1 derived peptides with enhanced affinity to the PA C-terminal domain.[136]

It was reported that α-aminoisobutyric acid (Aib) containing peptides are more likely to adopt helical secondary structure in solution.[106] Aib scan was done on peptide **255**. Peptides **262-264** were prepared on PS3 machine and there was no activity below 50 μM (Table 6). In further step, N-terminus was extended with glycine (peptide **265)** to improve solubility and activity, but peptide **255** remained the best choice for crystallization with PA C-terminal domain.

Table 6. Alanine scan and Aib scan.

Compound	Sequence	IC_{50} (AlphaScreen)
257	AcY**A**LWLK-NH_2	> 50 μM
258	AcYL**A**WLK-NH_2	> 50 μM
259	AcYLLW**A**K-NH_2	~ 32 μM
260	AcY**Y**LWLK-NH_2	~ 44 μM
261	AcYLLW**T**K-NH_2	19 μM
262	AcY**Aib**LWLK-NH_2	> 50 μM
263	AcYL**Aib**WLK-NH_2	~ 50 μM
264	AcYLLW**Aib**K-NH_2	> 50 μM
265	**G**YLLWLK-NH_2	~ 35 μM, ~ 60 μM

3.3.5. Optimization of the decapeptide

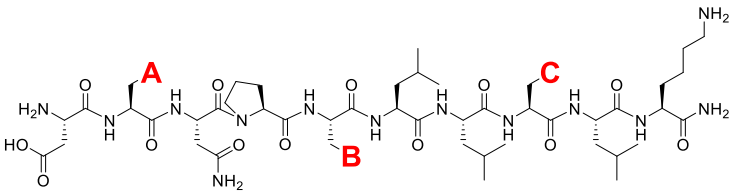
What was learned from the optimization of the hexapeptides was later applied to the decapeptides series (Table 7). Homotyrosine and *p*-carboxyphenylalanine in position B led to decrease of the activity of **266** and **268**. Introducing homotyrosine and *p*-carboxyphenylalanine in position A led to improved activity of peptides **271**, **272**, **274**. Introducing the biphenylalanine **267** and the naphthylalanine **270** to the position C gave the desired effect of

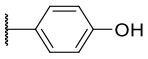
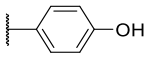
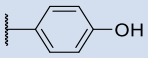
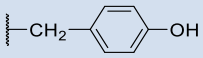
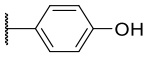
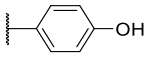
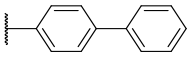
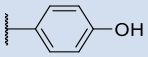
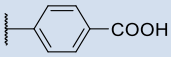
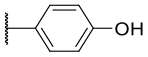
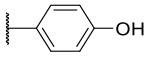
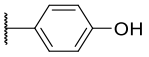
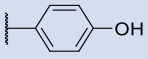
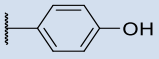
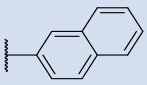
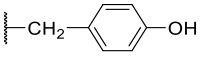
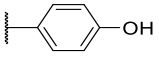
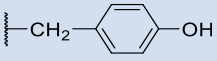
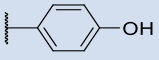
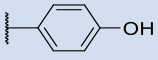
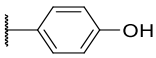
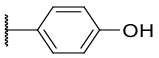
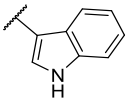
increased activity. Decapeptide **267** with the biphenylalanine amino acid in position C is our most potent inhibitor so far. Tryptophan in position C also led to improved activity of the peptide **273**. Introduction of tyrosine to the position C led to improved activity and to very good solubility of the peptide **269**. All the decapeptides were synthesized using SPPS in a syringe, PS3 or a combination of both.

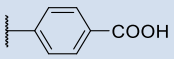
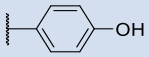
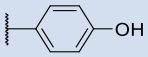
Table 7. Decapeptides with optimized hot spots with their activity and solubility.

PB1-11

DANPBLLCLK



Compound	A	B	C	IC_{50} AlphaScreen	Maximal solubility
PB1-10				3 400 nM	25 µM
PB1-11				13 nM	66 µM
266				54 nM	n.d.
267				4 nM	n.d.
268				450 nM	n.d.
269				**5.5 nM**	**> 200 µM**
270				4.8 nM	n.d.
271				6.8 nM	n.d.
272				6.2 nM	n.d.
273				7.8 nM	n.d.

274				5.7 nM	n.d.

The excellent solubility and activity of decapeptide **269** allowed for measurement of the thermodynamics of binding of the decapeptide **269** to the PA C-terminal domain via isothermal titration calorimetry (ITC).[143] This method performed by Dr. Milan Kožíšek confirmed the stoichiometry of binding as 1:1 and determined K_d = 1.1 nM. More importantly, the enthalpy $_{\Delta}H°$ and entropy $-T_{\Delta}S°$ contributions to the Gibbs free energy $_{\Delta}G°$ of binding were determined (Figure 39). The formation of the complex is enthalpy driven with $_{\Delta}H° = -18.89$ kcal/mol. To get better free energy of binding the enthalpic contribution can be increased or the entropic penalization can be decreased. The analysis of a large set of thermodynamic data of protein-ligand interaction determined by ITC suggests that the enthalpy contribution has its limits around -23 kcal/mol.[144] Enthalpy contribution of -18.89 kcal/mol is already very high and it would be unlikely to get even higher. Therefore, it was decided that to get better energy of binding the entropic penalization had to be decreased. Entropic penalization is a complex phenomenon caused by many dynamic processes. One particular process that can be influenced is the formation of the 3_{10}-helix. One possible way how to induce helical secondary structure in a solution is to perform peptide stapling. Furthermore, our collaborators from the biochemistry department were able to solve X-ray crystal structure of **269** binding the PA C-terminal domain (Figure 40).

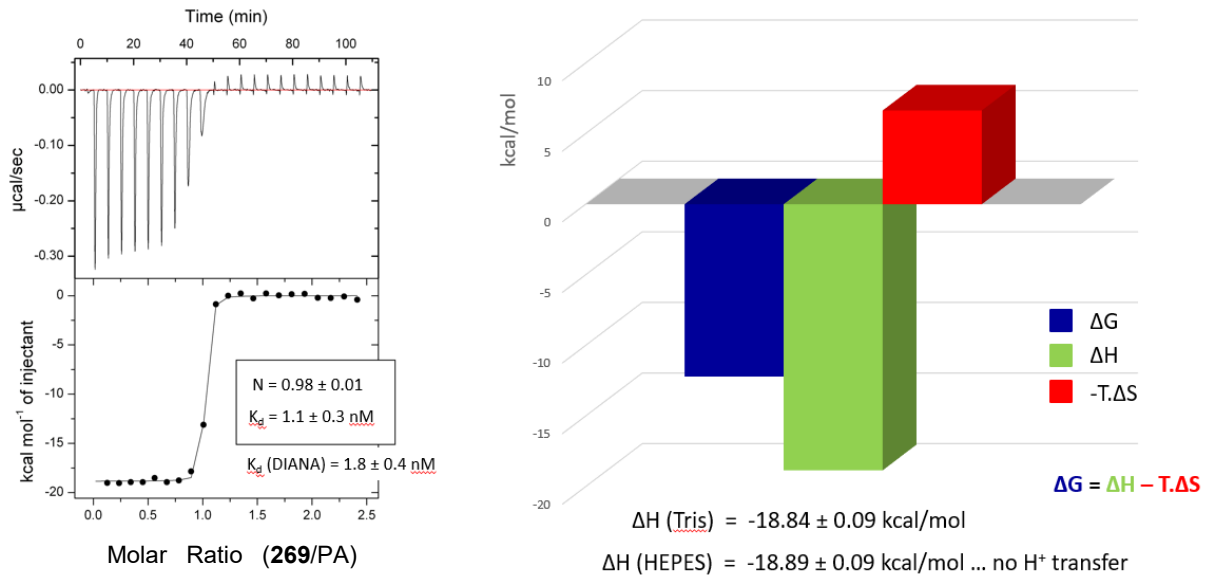


Figure 39. Thermodynamics of binding of peptide **269** to PA C-terminal domain determined by Dr. Milan Kožíšek. The enthalpy $_{\Delta}H°$ (in green), the entropy $-T_{\Delta}S°$ (in red), the Gibb's free energy $_{\Delta}G°$ (in blue).

3.3.6. Peptide stapling

Part of the entropic penalization can be attributed to the formation of the 3_{10}-helix upon binding of the decapeptide to the PA C-terminal domain. To improve the thermodynamics of binding it was decided to perform peptide stapling and stabilize the helical secondary structure of the decapeptide. Two leucines *i* and *i* + 3 were chosen. The two leucines are on the same side of the helix and represent one turn of the helix (Figure 40).

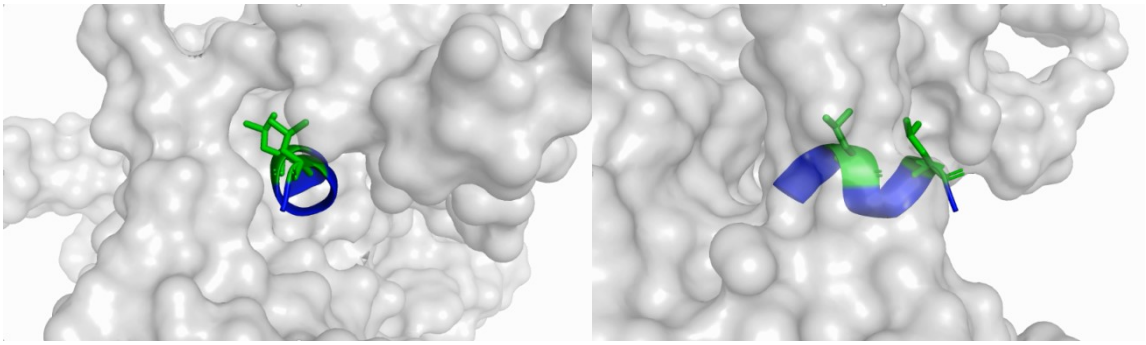
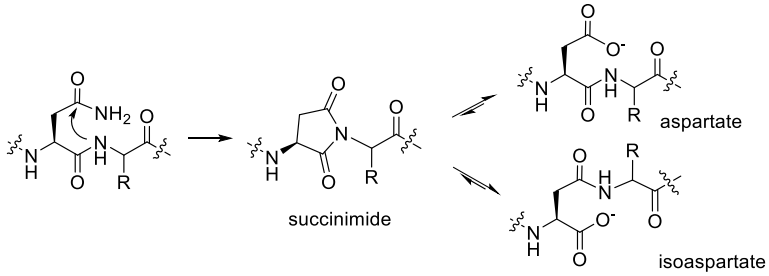


Figure 40. Representation of the 3_{10}-helix part (in blue) of the decapeptide **DYNPYLLYLK** in a complex with PA C-terminal domain (in gray) with the depiction of the two leucines residues (in green), that are suitable for the peptide stapling. Work of Kateřina Radilová.

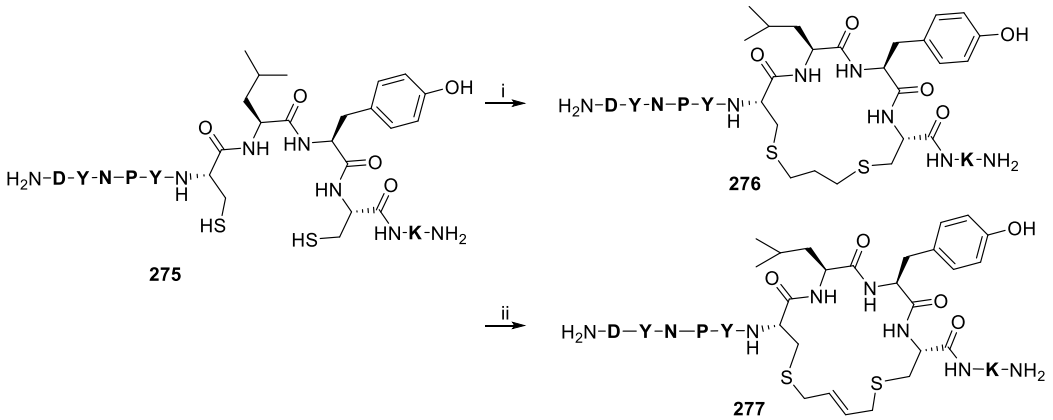
The two leucines were at first substituted with cysteine and major decrease in activity was observed (IC_{50} in table 8). The peptide **275** was then stapled. For the successful bis-alkylation of the two cysteine residues in a presence of other reactive groups, ammonium

carbonate buffer was found to be ideal.[145] At the pH ≈ 8 the carboxylate of aspartic acid is deprotonated and the N-terminal amine of the peptide and the lysine side chain amine are protonated. Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride was used to prevent unwanted formation of disulfide-bridges. Ideal combination of solvents was found to be degassed CH_3CN/H_2O 1:1. To prevent intermolecular reaction of two peptides a high dilution was applied (0.80 μM). TCEP hydrochloride could also be alkylated, therefore, 3 equivalents of dibromopropane were used. The reaction was stirred at 60 °C overnight in a sealed tube. The HPLC-MS analysis of the reaction revealed many side-products including the product of deamidation of asparagine (Scheme 28), starting material with disulfide bridges but also the stapled peptide was present.



Scheme 28. Degradation pathway of asparagine containing peptides via succinimide intermediate. The intermediate is unstable and is hydrolyzed to peptides containing aspartic acid or isoaspartic acid residues.[146]

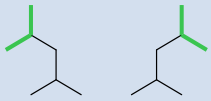
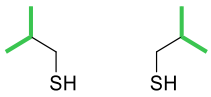
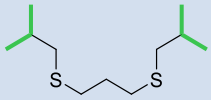
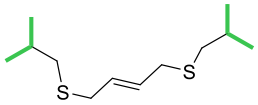
The deamidation could have been avoided by letting the reaction stir at room temperature. Bis-alkylation took three days and provided the compound **276** in an acceptable yield (Scheme 29). The next attempt was performed with 1,4-dibromobut-2-en. Same conditions were used and the reaction was quantitative in 1h according to the HPLC analysis.



Scheme 29. *Reagents and conditions.* (i) **275** (0.80 μM in CH_3CN/H_2O 1:1), ammonium bicarbonate, 3 eq. 1,3-dibrompropan in THF (0.3 M), tris(2-carboxyethyl)phosphine hydrochloride, r.t., 3 days, 20% yield; (ii) **275** (0.78 μM in CH_3CN/H_2O), ammonium bicarbonate, 3 eq. 1,4-dibrombutane in THF (0.3 M), tris(2-carboxyethyl)phosphine hydrochloride, r.t., 1 h, 63% yield.

Peptides **276** and **277** had nanomolar IC_{50} values (Table 8). Analogous cyclizations with dibromomethane and dibromoethane were also attempted using the aforementioned conditions but with no product formed. MS analysis revealed that the alkylation occurred at the two cysteine residues. MS precursors were monitored and then selected for following MS2/MS3/MS4 fragmentation by collision-induced dissociation (CID). Assignment of the ions to their structure is detailed in the Attachment.

Table 8. Peptide stapling of the **275 DYNPYCLYCK**

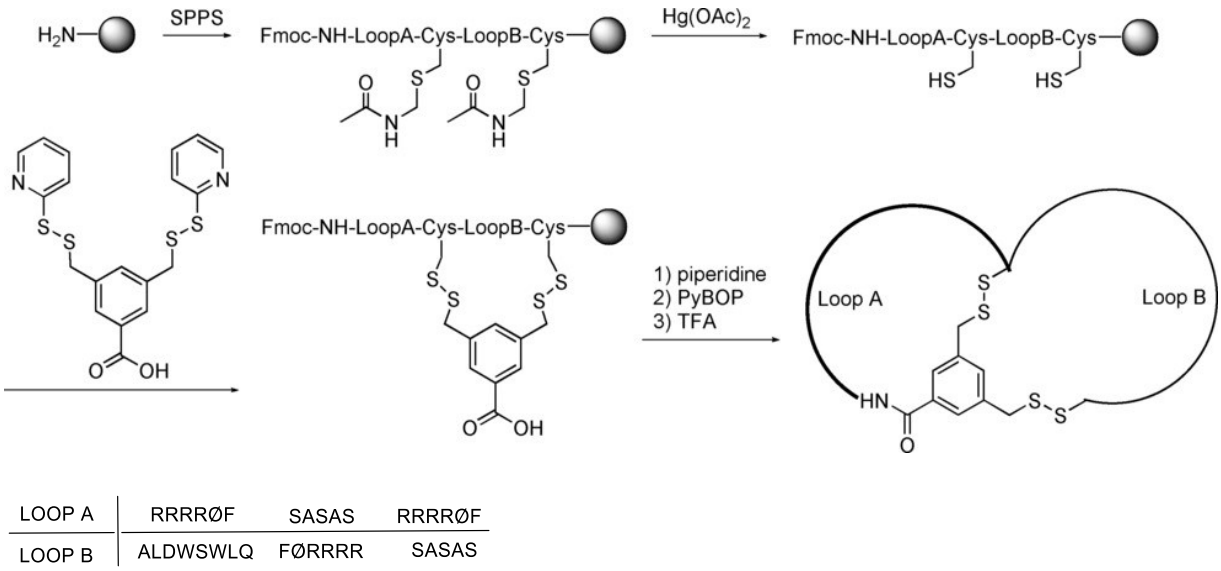
Compound	Sequence	IC_{50} (AlphaScreen)
269		5.5 nM
275		14 000 nM
276		60 nM
277		25 nM

3.3.7. Cell-penetrating peptides

Our most potent inhibitors of PA-PB1 interaction were so far peptides. In general, peptides are not very efficient in penetrating cell membranes and are readily degraded by proteolysis. In this case, the inability of peptides to enter cells was a big issue since the purpose of our peptides was to disrupt the formation of PA-PB1 heterodimer that is present in the infected cells in the nucleus and in the cytoplasm. Thus, any efficient way how to deliver our peptides through the cell membrane was needed.

The procedure for reversible bicyclization published by Qian in 2017 (Scheme 30) was followed.[115] This strategy was supposed to enhance both the proteolytic stability and cell permeability of our peptidic inhibitors. Using the SPPS the peptide of interest was extended with cell-penetrating sequence (RRRRØF/ FØRRRR, Ø- 3-(2-naphthyl-*L*-alanine) and Acm

protected cysteines. The first "LOOP" is created by introduction of the central fragment through the formation of disulfide bond. The second "LOOP" is then cyclized through amide coupling.



Scheme 30. Preparation of the bicyclic peptide.[115]

The corresponding peptide was prepared using SPPS (*t*Bu/Fmoc protection strategy). The cysteines were deprotected using mercury(II) acetate followed by reduction of potentially formed disulfide-bridges with 2-mercaptoethanol. The peptide was right away cyclized around the central fragment. The introduction of the central fragment was done on a solid phase by pyridinethiol-activated 3,5-bis(mercaptomethyl)-benzoic acid. The last step of the synthesis involved fmoc-deprotection and amide coupling using PyBOP. Finally, the peptide was deprotected and cleaved from the resin using TFA and purified. This approach allowed for two different topologies of LOOP A and B. Such bicyclic peptides were supposed to efficiently enter the cell. Moreover, the cytosolic delivery was supposed to be superior when compared to the TAT extended peptides. In the cell the disulfide bridges were reduced by glutathione and the linear peptide was then released in the cytoplasm (Figure 41).

Anti-influenza effect of these compounds cannot be determined by enzymatic AlphaScreen assay. The bicyclic peptides will be tested via cell based assays, because it needs to be checked whether the compounds are efficient in entering the cells. The anti-influenza activity will be determined by the ability of the peptides to reduce virus-induced cytopathic effect (CPE). The effect of the peptides on polymerase activity will be determined by minireplicon assay.[147]

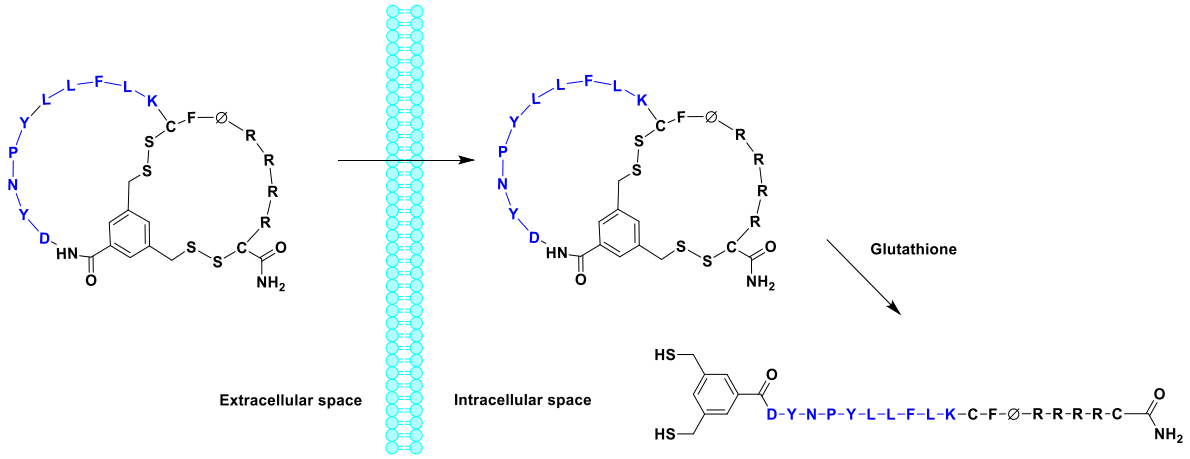


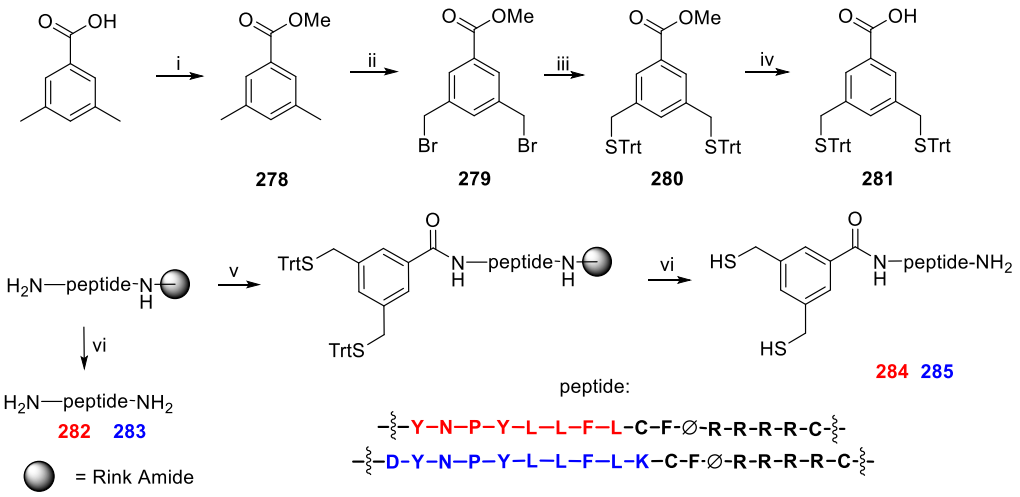
Figure 41. Representation of the cell penetration of the bicyclic peptide and subsequent reduction of the disulfide bridges with glutathione.

3.3.7.1. The effect of CPP sequence on peptide inhibitors

In the original publication the peptides of interest were pentapeptide SASAS and octapeptide ALDWSWLQ.[115] Therefore, it was decided to cyclize the most potent inhibitor **PB1-11** and also shorter octapeptide. The shortening of the decapeptide from only C or N terminus by two amino acids led to IC_{50} 7.4 µM and 32 µM, respectively (Figure 32). The best option was to remove one amino acid from N-terminus and one amino acid from C-terminus of the original decapeptide. Such octapeptide (**PB1-22,** prepared on PS2 machine by Miroslava Blechová and Alexandra Prichodko) had IC_{50} = 910 nM.

Upon entering the cell the bicyclic peptides are likely reduced by glutathione and should become linear. Therefore, to measure the bicyclic peptides by enzymatic AlphaScreen assay would not be productive. On the other hand, it is possible to determine the effect of the CPP on the decapeptide **PB1-11** and octapeptide **PB1-22** by synthesizing the corresponding linear peptides and measure their activity by AlphaScreen.

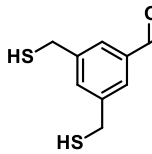
To see the effect of the decoration of the octapeptide/decapeptide with the CPP sequence six peptides were synthesized on PS2 machine and then were cleaved from resin and purified. To see the effect of the 3,5-bismethylthiobenzamide at the N-terminus of the peptide **282** and **283**, the 3,5-bis((tritylthio)methyl)benzoic acid **281** had to be synthesized and introduced to the peptide using SPPS (Scheme 31).



Scheme 31. *Reagents and conditions.* (i) H_2SO_4, MeOH, reflux, overnight, 97% yield; (ii) NBS, benzoyl peroxide, $CHCl_3$, reflux, 3h, 44% yield; (iii) triphenylmethanethiol, DIPEA, DMF, 4h, 97% yield; (iv) NaOH, 1,4-dioxane, 60 °C, 67% yield; (v) **281**, HBTU, DIPEA, DMF; (vi) TFA/thioanisole/anisole/1,2-ethanedithiol 9/0.5/0.2/0.3, 1h.

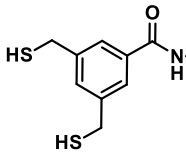
Esterification of 3,5-dimethylbenzoic acid furnished the methyl ester derivative **278** in an excellent yield. The radical bromination with NBS and benzoyl peroxide as initiator provided dibromo derivative **279** in a good yield. The nucleophilic substitution in DMF in the presence of DIPEA provided the compound **280**. The subsequent saponification furnished the free acid **281** (Scheme 31). The protected peptide was prepared on the PS2 machine, after fmoc deprotection the peptide was coupled with the free acid **281** using standard amide coupling conditions. The results of CPP decoration for octapeptide and decapeptide are summarized in Table 9 and Table 10, respectively.

Table 9. The effect of CPP on the C- and N-terminus of the octapeptide. The effect of the 3,5-bis(methylthio)benzamide at the N-terminus of the octapeptide (AlphaScreen).

	CPP	Octapeptide	CPP	IC_{50} [nM]
PB1-22		H_2N—Y–N–P–Y–L–L–F–L–NH_2		910
282		H_2N—Y–N–P–Y–L–L–F–L–C–F–∅–R–R–R–R–C–NH_2		38
284		N-Y–N–P–Y–L–L–F–L–C–F–∅–R–R–R–R–C–NH_2		360
286		H_2N—R–R–R–R–∅–F–C–Y–N–P–Y–L–L–F–L–C–NH_2		60

Octapeptide equipped with CPP sequence on C-/N-terminus led to increased activity in all the cases. The effect of the central fragment at the N-terminus of peptide **282** led to decreased activity when compared to the original peptide **282** but still better than the **PB1-22.**

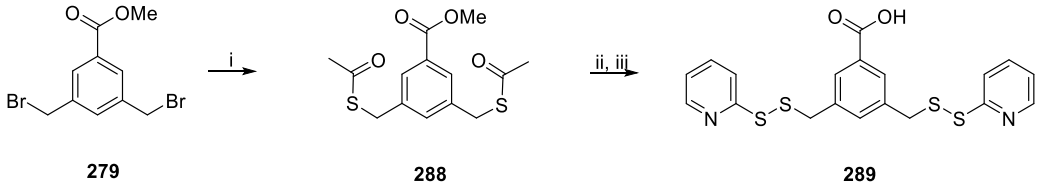
Table 10. The effect of CPP sequence attached to the C- and N-terminus of the octapeptide. The effect of the 3,5-bis(methylthio)benzoic acid at the N-terminus of the decapeptide (AlphaScreen).

	CPP	Decapeptide	CPP	IC_{50} [nM]
PB1-11		H_2N—D-Y-N-P-Y-L-L-F-L-K–NH_2		12
283		H_2N—D-Y-N-P-Y-L-L-F-L-K-C-F-∅-R-R-R-R-C–NH_2		10
285		N-D-Y-N-P-Y-L-L-F-L-K-C-F-∅-R-R-R-R-C–NH_2		80
287	H_2N—R-R-R-R-∅-F-C-D-Y-N-P-Y-L-L-F-L-K-C–NH_2			12

The presence of the CPP sequence on the C- or N-terminus of **PB1-11** had a little effect on the activity (Table 10, peptides **283, 287**). Similarly to the octapeptides, the introduction of the 3,5-bis(methylthio)benzamide led to a decrease in the activity of the peptide **285**. The peptides **286** and **287** were not equipped with the 3,5-bis(methylthio)benzamide fragment because the fragment would be far away from the active peptide sequence and thus irrelevant.

3.3.7.2. Central fragment synthesis

The nucleophilic substitution of **279** with potassium thioacetate in refluxing acetone afforded the compound **288** (Scheme 32). For the next step to be successful, the reaction mixture had to be oxygen free. The methanolic solution of **288** was bubbled with argon and the aqueous solution of sodium hydroxide was bubbled as well. Compound **288** was hydrolyzed by 7M solution of NaOH overnight under inert atmosphere. The disappearance of the starting material was monitored by the TLC analysis and the reaction mixture was subsequently acidified with acetic acid. 2,2'-Dithiopyridine was added and the reaction mixture was stirred for 3h. The isolated yields of **289** were around 18-30% over two steps.



279 288 289

Scheme 32. *Reagents and conditions.* (i) potassium thioacetate, acetone, reflux, 4h, 77% yield; (iv) NaOH, H_2O, MeOH, argon, overnight; (ii) 2,2'-dithiopyridine; (iii) CH_3COOH, 2h, 18% yield over two steps.

3.3.7.3. Bicyclization of PB1-11 equipped with CPP on the C terminus

The topology of the first bicyclic peptide is shown here (see Figure 42). Peptide **290** was prepared on a PS2 machine using Rink amide resin (RAM). Amino acids side chains were protected with various protecting groups (*t*Bu/Boc/Trt/Pbf).

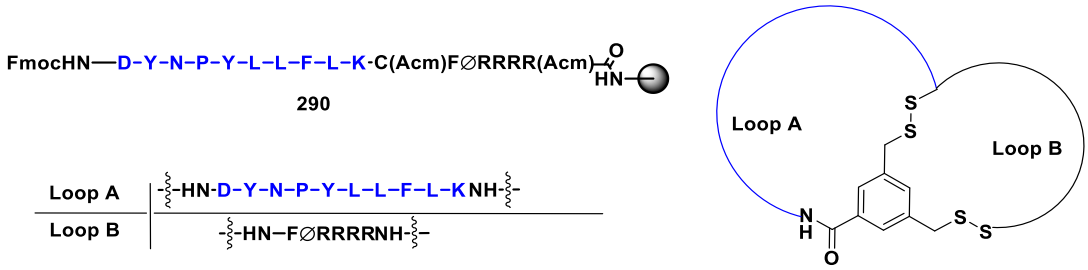
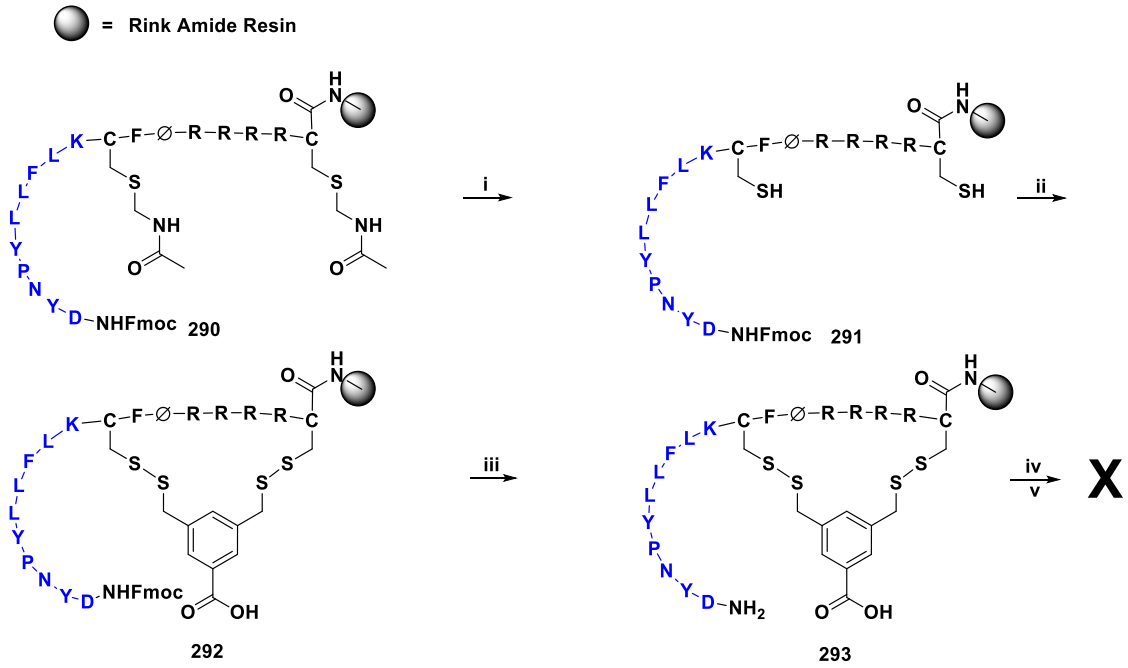


Figure 42. Left: Decapeptide (**PB1-11**) extended with CPP sequence (FØRRRR), Ø: 3-(2-naphthyl-*L*-alanine); Right: Representation of the bicycle topology.

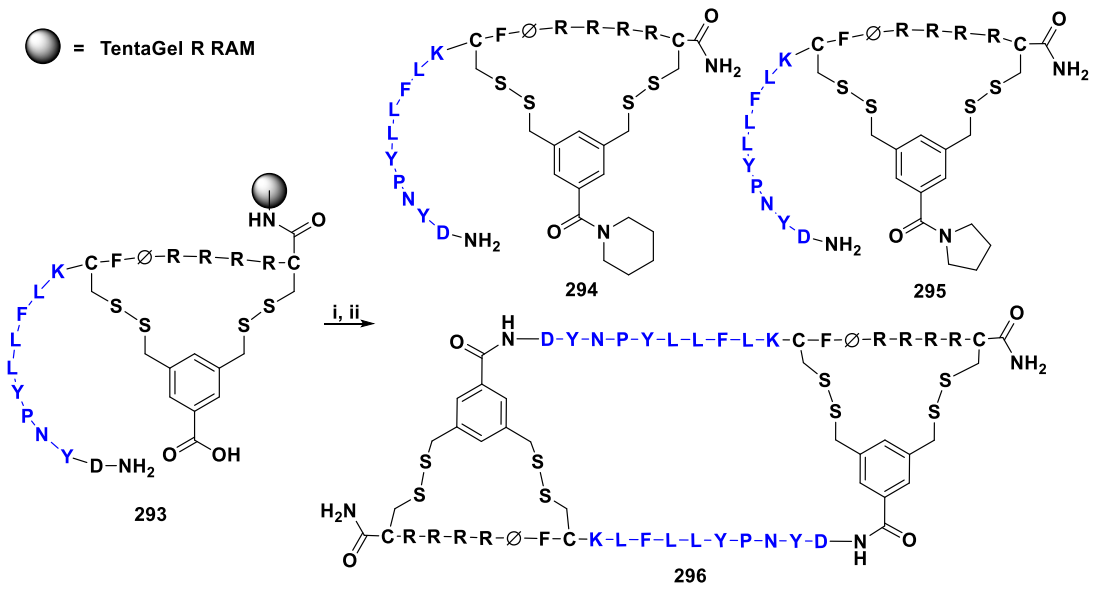
The resin with protected peptide was transferred to the syringe. The *S*-acetamidomethyl (Acm) groups were deprotected using saturated solution of mercury(II) acetate in DMF under argon atmosphere overnight (Scheme 33). The resin was washed with DMF several times and then it was incubated with 20% 2-mercaptoethanol in DMF twice for 2h. The resin was washed several times with DMF to get rid of the reducing agent. The resin with peptide **291** was incubated overnight with 1 equivalent of compound **289** in DMF with 1% CH_3COOH. The resin was then washed with DMF and a small portion of the peptide was cleaved using TFA/TIS/H_2O (95/2.5/2.5) solution. Because of the presence of four Pbf-protected arginines the resin had to be deprotected for 2.5 h. The HPLC analysis confirmed the presence of the peptide **292**. The fmoc deprotection was successful but the subsequent bicyclization of **293** mediated by PyBOP did not work. HPLC-MS analysis did not show the starting material of the

last reaction (deprotected **292**) or the product. This reaction was tried many times and always with the same result.



Scheme 33. *Reagents and conditions.* (i) a) $Hg(OAc)_2$, DMF, argon, incubation overnight, subsequent DMF wash; b) 20% 2-mercaptoethanol in DMF, incubation 2×2h, subsequent DMF wash; (ii) **289**, DMF 1% CH_3COOH overnight, subsequent DMF wash; (iii) 20% piperidine in DMF incubation 2×10 min., DMF wash; (iv) PyBOP, DIPEA, DMF; (v) TFA, TIS, H_2O 95/2.5/2.5, 2.5h, Ø: 3-(2-naphthyl-*L*-alanine).

For the next attempt, TentaGel R RAM was used instead of the original Rink Amide resin (RAM). TentaGel R RAM resin is grafted copolymer consisting of low cross-linked polystyrene matrix with grafted ethylene glycol. TentaGel R RAM contains 50 – 70 % PEG (w/w). The properties of TentaGel are dominated by the properties of PEG and no longer by the properties of polystyrene matrix.[148] TentaGel is usually used for sequences that are more complicated. The PyBOP mediated cyclisation resulted in a formation of side products **294** and **295** (Scheme 34). Dimer **296** was also detected and purified. The formation of **294** can be explained by insufficient washing of the piperidine and the formation of side product **295** can be attributed to the presence of pyrrolidine as contaminant in PyBOP batch (0.5% w/w).[149] Even such a small amount can be significant since 10 equivalents of PyBOP were used. The formation of the side-product **294** was suppressed by incubation of the resin with 1M HOBT in DMF. However, with less equivalents of PyBOP (3 eq.) the formation of the product **295** was not suppressed and only traces of **296** were detected.



Scheme 34. Bicyclization of decapeptide-CPP sequence on TentaGel RAM resin. *Reagents and conditions.* (i) PyBOP, DIPEA, DMF; (ii) TFA, TIS, H_2O 95/2.5/2.5, 2.5h, Ø: 3-(2-naphthyl-*L*-alanine).

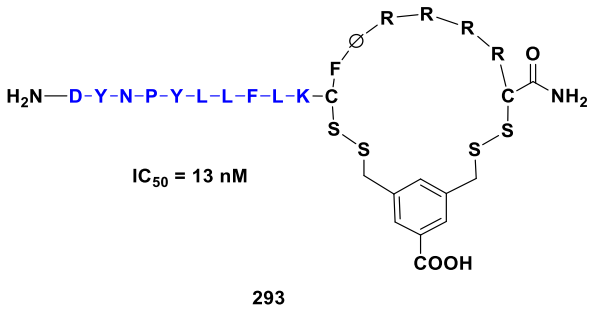
It was decided to use another type of resin for the bicyclization. ChemMatrix resin is all poly(ethylene glycol) (PEG)-based resin. ChemMatrix resin is highly chemically stable because it is made from primary ether bonds. It is suitable for complex, highly structured, poly-arginines and hydrophobic peptides. For these peptides ChemMatrix performs excellently when compared to polystyrene-based resins.[150] ChemMatrix resin was therefore an ideal candidate for the peptide synthesis. ChemMatrix resin substitution had to be established. The Fmoc-Cys(Acm)-OH was introduced to the resin by standard amide coupling (HBTU/DIPEA/DMF). Protected cysteine was used in 10 equivalents excess to the highest possible advertised substitution. The resin was washed with DMF and MeOH and dried on vacuum overnight. Five milligrams of the resin were washed twice with the 40% piperidine in DMF (0.50 mL) to deprotect the fmoc group of the attached amino acid. The filtrates were transferred to the 25 mL volumetric flask and filled with MeOH. Absorbance of the sample was measured at 301 nm. The absorbance A was substituted into the Equation 1. And the substitution S was established as 0.31 mmol/g.

$$S = \frac{A \times V\ (ml)}{7.8 \times m\ (mg)}$$

Equation 1.[151]

With the substitution established, the corresponding linear peptide **290** was prepared on PS2 machine. Every attempt to cyclize peptide **293** led to the formation of the dimer **296** and a small amount of **295**. It seems that the intramolecular bicyclization of peptide **293** is not

favorable and that it is more likely that the peptide cyclizes in an intermolecular fashion. The peptide **293** was successfully prepared on RAM, TentaGel R RAM, and ChemMatrix resins using the aforementioned conditions. The peptide **293** was successfully cleaved from the resin and IC_{50} = 13 nM was established (Figure 43).



293

Figure 43. Peptides submitted for testing in AlphaScreen.

Peptides with protected arginines (Pbf) and lysines (Boc) are making hydrogen bonds with carboxylic acids, making them less reactive for the amide coupling. Protected arginines and lysines are also making hydrogen bonds among themselves (Figure 44). Peptides with hydrogen bond network do not allow the coupling reagent to enter the resin. For this reason it was decided to use chaotropic agent that should suppress the formation of hydrogen bonds. The usage of reported ideal mixture for cyclisation (DMF/DCM/NMP 3:2:2 with 1% Triton X-100)[152] only resulted in a very small amount of the desired product which could not be purified in a sufficient amount for the cell based assay.

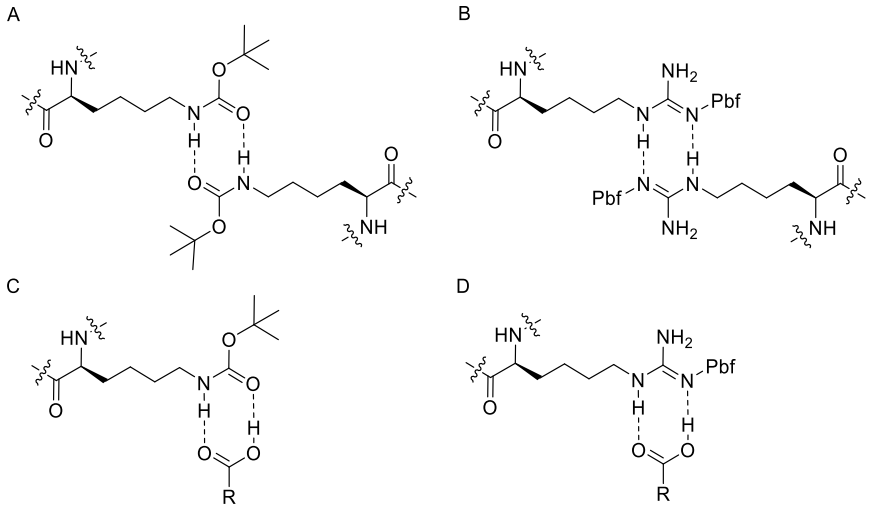


Figure 44. Hydrogen bonding interaction between A) Boc protected lysine side chains; B) Pbf protected arginine side chains; R substituted carboxyl group with C) Boc protected lysine side chains; D) Pbf protected arginine side chains. [152]

3.3.7.4. Bicyclization of PB1-11 equipped with CPP on the N terminus

To study bicyclic peptide with the opposite topology to peptide described in Chapter 3.3.7.3 the peptide **297** was prepared on the PS2 peptide synthesizer using TentaGel RAM resin (Figure 45).

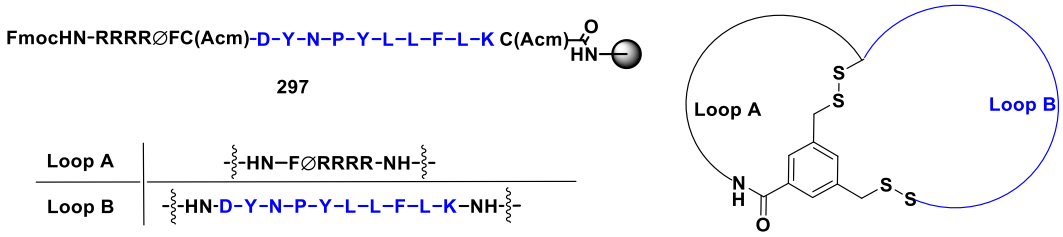
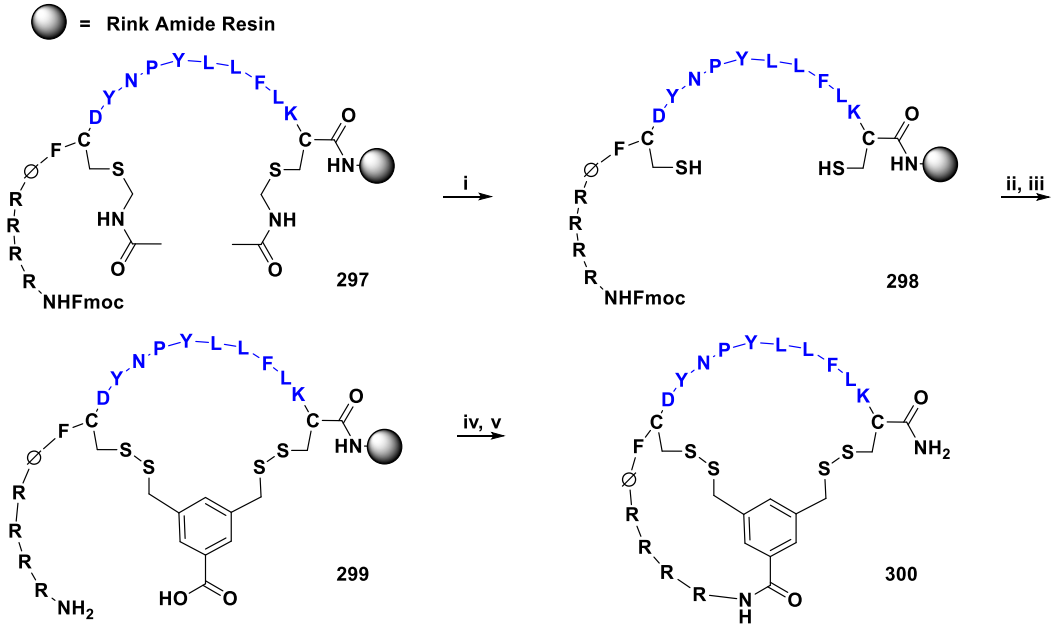


Figure 45. Left: Decapeptide (**PB1-11**) extended with CPP sequence (RRRRØF), Ø: 3-(2-naphthyl-*L*-alanine); Right: Representation of the bicycle topology.

Introduction of the central fragment and fmoc-deprotection provided the compound **299** (Scheme 35). Prior to the amide coupling the resin was incubated with 1M HOBT in DMF. The PyBOP mediated bicyclization worked well and the HPLC analysis showed the desired product **300**. The following HPLC purification proved to be challenging. Therefore, it was decided to use ChemMatrix resin. The PyBOP mediated bicyclization on ChemMatrix and the following preparative HPLC provided enough peptide **300** for the cell based assays.



Scheme 35. *Reagents and conditions.* (i) a) $Hg(OAc)_2$, DMF, argon, incubation overnight, subsequent DMF wash; b) 20% 2-mercaptoethanol in DMF, incubation 2×2h, subsequent DMF wash; (ii) compound **256**, DMF 1% CH_3COOH overnight, subsequent DMF wash; (iii) 20% piperidine in DMF incubation 2×10 min., DMF wash; (iv) 1M HOBT in DMF incubation, PyBOP, DIPEA, DMF; (v) TFA, TIS, H_2O 95/2.5/2.5, 2.5h, Ø: 3-(2-naphthyl-*L*-alanine).

3.3.7.5. Bicyclization of PB1-22 equipped with CPP on the C terminus

AlphaScreen data in Table 9 suggested that the octapeptides extended with CPP sequence were potent inhibitors of PA-PB1 protein-protein interaction. Therefore, it was decided to cyclize the CPP extended octapeptides. This also provided the opportunity to study the influence of the peptide length on cyclizations.

The peptide **301** was prepared on PS2 peptide synthesizer. The sequence and the topology of the bicyclic peptide are shown here in Figure 46.

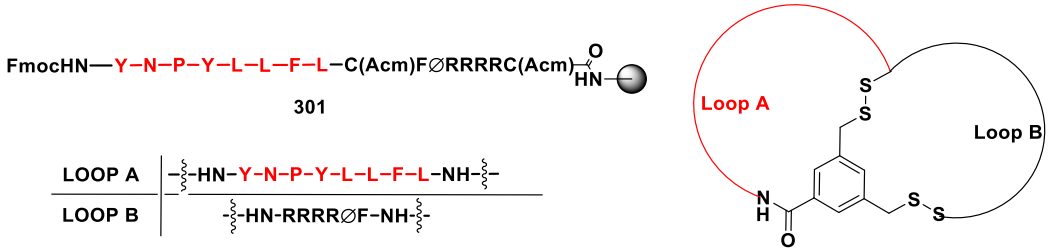
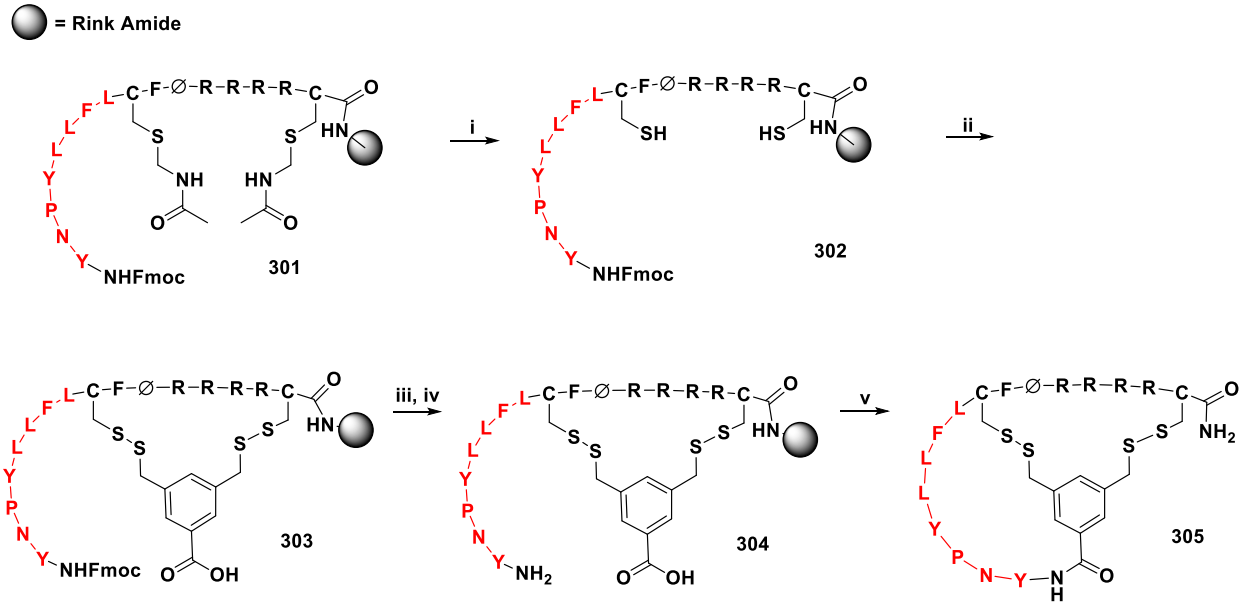


Figure 46. Left: Octapeptide (**PB1-22**) extended with CPP sequence (FØRRRR), Ø: 3-(2-naphthyl-*L*-alanine); Right: Representation of the bicycle topology.

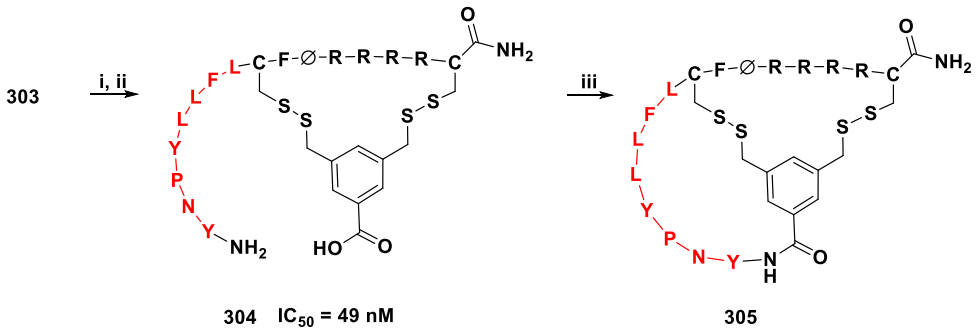
Intermediate **304** was prepared on the RAM resin as described in

Scheme 36. The bicyclization of peptide **304** worked well on the Rink Amide resin but during the following extensive purification significant amount of the peptide **305** was lost.



Scheme 36. *Reagents and conditions.* (i) a) $Hg(OAc)_2$, DMF, argon, incubation overnight, subsequent DMF wash; b) 20% 2-mercaptoethanol in DMF, incubation 2×2h, subsequent DMF wash; (ii) compound **256**, DMF 1% CH_3COOH overnight, subsequent DMF wash; (iii) 20% piperidine in DMF incubation 2×10 min., DMF wash; (iv) PyBOP, DIPEA, DMF; (v) TFA, TIS, H_2O 95/2.5/2.5, 2.5h, Ø: 3-(2-naphthyl-*L*-alanine).

The amino acids of the octapeptides do not have any side chain functionalities that could interfere with the bicyclization in the solution. Therefore, after the cleavage from the resin, the CPP-equipped octapeptide **304** can be bicyclized in the solution. This approach was not possible for the CPP-equipped decapeptide which possess aspartic acid and lysine. Peptide **304** was prepared and cleaved from the resin in a sufficient amount (IC_{50} = 49 nM was determined by the AlphaScreen assay) and the bicyclization was carried out in the solution phase (Scheme 37). To avoid side-reaction such as intermolecular reaction the crucial cyclization was carried out in high dilution (10^{-3}M). Solution of the peptide **304** in DMF was dropped over 8h into the solution of the PyBOP and DIPEA in DMF. The HPLC analysis revealed the formation of the desired product. The reaction mixture was lyophilized and purified by preparative HPLC to get the bicyclic peptide **305**. The reaction provided sufficient amount of the peptide **305** for cell based assays.



Scheme 37. Deprotection and cleavage of the peptide from the resin. *Reagents and conditions.* (i) a) 20% piperidine in DMF incubation 2×10 min, b) subsequent DMF wash; (ii) TFA, TIS, H_2O 95/2.5/2.5, 2.5h; (iii) PyBOP, DIPEA, DMF, 2h.

3.3.7.6. Bicyclization of PB1-22 equipped with CPP on the N terminus

Peptide **306** was prepared on PS2 synthesizer on Rink Amide resin (RAM). The complementary topology to the peptide from the chapter: 3.3.7.5. is depicted in Figure 47.

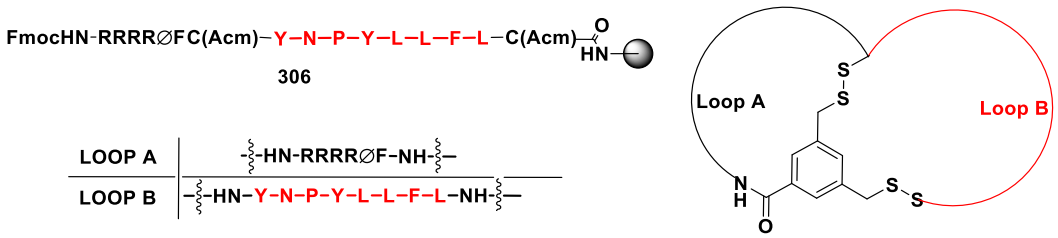
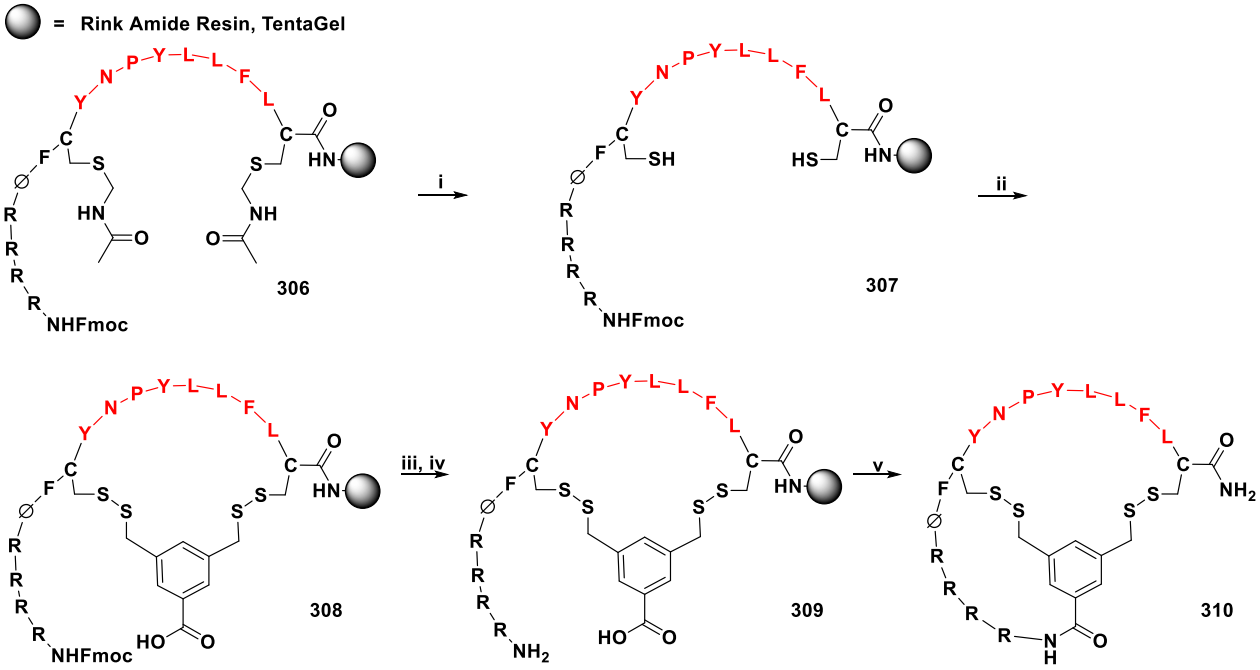


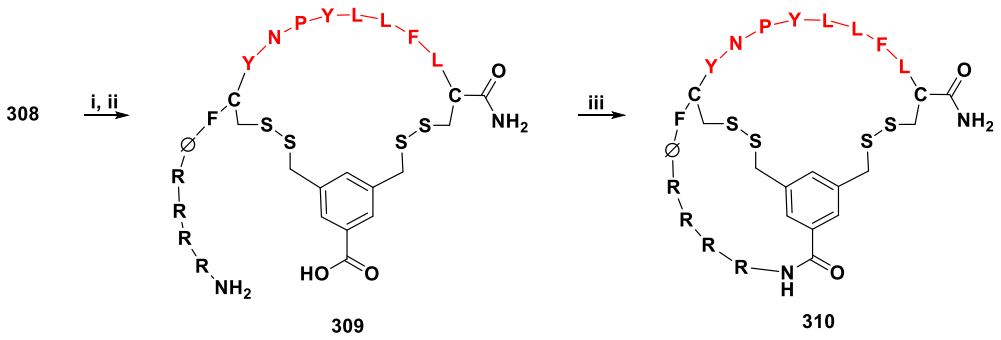
Figure 47. Left: Octapeptide (**PB1-22**) extended with CPP sequence (RRRRØF), Ø: 3-(2-naphthyl-*L*-alanine); Right: Representation of the bicycle topology.

The introduction of the central fragment and the bicyclization was successful on a solid phase (Scheme 38). However, during the preparative HPLC most of the product **310** was lost. The same procedure was repeated on TentaGel R RAM resin and the subsequent preparative HPLC provided enough peptide **310** for the cell based assay.



Scheme 38. *Reagents and conditions.* (i) a) $Hg(OAc)_2$, DMF, argon, incubation overnight, subsequent DMF wash; b) 20% 2-mercaptoethanol in DMF, incubation 2×2h, subsequent DMF wash; (ii) compound **256**, DMF 1% CH_3COOH overnight, subsequent DMF wash; (iii) 20% piperidine in DMF incubation 2×10 min., DMF wash; (iv) PyBOP, DIPEA, DMF; (v) TFA, TIS, H_2O 95/2.5/2.5, 2.5h.

The bicyclization was also tried in the solution. Peptide **308** was fmoc-deprotected and cleaved from the resin (Scheme 39). The peptide **309** was bicyclized in the same fashion as the peptide **304**. The solution of peptide **309** was dropped over 8h to a solution of PyBOP and DIPEA in DMF. The subsequent purification provided peptide **310** with sufficient purity.



Scheme 39. *Reagents and conditions.* (i) a) 20% piperidine in DMF incubation 2×10 min, b) subsequent DMF wash; (ii) TFA, TIS, H_2O 95/2.5/2.5, 2.5h. (iii) PyBOP, DIPEA, DMF, 2h.

3.3.8. Biological testing

Peptides were evaluated at the virology department of IOCB. Peptides were tested using CPE assay (virus induced cytopathic assay), influenza minireplicon assay and cytotoxicity in MDCK/HEK293T cells (Table 11). Compound **PB1-22** did not show any activity in both assays. The rest of the compounds show anti-influenza effect in minireplicon assay. The result of minireplicon assay for compound **285** may be affected by the cytotoxicity on HEK293T cells. Compounds **283, 286, 304, 305, 309, 310,** showed activity in minireplicon assay comparable with favipiravir and no toxicity up to 50 µM. Unfortunately these compounds did not reduce the cytopathic effect in CPE assay. Compound **300** shows anti-influenza effect in both minireplicon and CPE assays.

Table 11. CPE assay (virus induced cytopathic assay), influenza minireplicon assay, cytotoxicity in MDCK/HEK293T cells. Work of Jan Hodek.

Comp.	EC_{50} CPE [µM]	95% CI of EC_{50}	EC50 minireplicon [µM]	95% CI of EC_{50}	CC50 (MDCK cells)	95% CI of CC_{50}	CC_{50} (HEK293T cells)	95% CI of CC_{50}
PB1-22	>50	NA	>50	NA	>50	NA	>50	NA
286	>50	NA	27.7	17.7 to 43.4	>50	NA	>50 (~200)	wide
283	>50	NA	19.2	16.8 to 21.9	>50	NA	>50	NA
285	>50	NA	>50 (62)	22 to 180	>50	NA	~ 52	Very wide
304	>50	NA	25.5	18.4 to 35.4	>50	NA	>50	NA
309	>50	NA	18.8	15.9 to 22.3	>50	NA	>50	NA
310	>50	NA	12.8	10.0 to 16.5	>50	NA	>50	NA
305	>50	NA	42.6	22.0 to 82	>50	NA	>50	NA

300	**24.8**	**20.5 to 29.9**	**20.7**	**16.0 to 26.7**	**>50**	**NA**	**>50**	**NA**
Favipiravir	15.9	11.1 to 22.8	15.3	12.1 to 19.3	>50	NA	>50	NA
Baloxavir Marboxil	0.019	0.013 to 0.029	0.016	0.015 to 0.017	34.38	22.09 to 53.51	>50	NA

3.3.9. Summary

Most potent low-molecular-weight inhibitors of the PA-PB1 interaction reported in the literature were prepared and evaluated. A series of oligobenzamide peptidomimetic were designed and synthesized based on the crystal structure of PA C-terminal domain with PB1 N-terminal domain derived decapeptide.

Tyrosine and phenylalanine hot spots were optimized in the series of hexapeptides. Tyrosine was identified as crucial in maintaining the solubility and activity of the hexapeptides. Alanine and α-aminoisobutyric acid (Aib) scans revealed that each leucine residue is important for the peptide binding to the PA C-terminal domain. Decapeptide series was prepared. Optimized decapeptide showed improved activity and solubility. The improved solubility of peptide **269** allowed for the measurement of the Isothermal Titration Calorimetry (ITC). Moreover, our collaborators at the biochemistry department were able to solve new X-ray structure of PA C-terminal domain binding peptide **269**.

An attempt to decrease the entropic penalization peptide stapling was performed on the decapeptide **275.** The stapling was successfully accomplished with two linkers. The stapled peptides **276** and **277** showed nanomolar activities in the AlphaScreen assay. The structure of **276** and **277** was confirmed by CID MS^4 experiments.

The influence of the attached CPP sequence on C-/N-terminus of deca/octapeptides was determined via AlphaScreen assay. Series of CPP equipped peptides was successfully synthesized. The activity of these peptides was determined at virology department of IOCB. The only peptide that showed activity in both experiments was compound **300** (CPP equipped decapeptide). Activity of **300** is comparable with another FDA approved drug favipiravir.

4. Conclusion and outlook

Oseltamivir was successfully extended at C-5 position with different binders of 150-cavity. K_i values determined by DIANA and MUNANA assay were compared and discussed. If the C-5 amine functionality is present the K_i values determined by both assays are in a good agreement. The presence of triazole moiety at C-5 results in a one order of magnitude difference between K_i values determined by MUNANA (pH = 6.15) and DIANA (pH = 7.40) assay. This can be attributed to the different experimental conditions of the two assays. This work was successfully published in *Bioorganic & Medicinal Chemistry* journal.

In another project a series of flavonoids and related compounds were prepared. This series served to elucidate the mechanism of action of quercetin and other flavonoids as influenza PA endonuclease inhibitors. Submicromolar inhibitors were prepared. This series of compounds contributed to the extensive structure-activity relationship study of different classes of compounds as PA endonuclease inhibitors. Manuscript covering these results was published in *European Journal of Medicinal Chemistry*.

Optimization of original hit compounds obtained from our library and discovered by screening against PA-PB1 interaction did not resulted in a superior inhibitor. In addition to that the most potent published inhibitors of PA-PB1 interaction were prepared and all were falsely positive in the AlphaScreen assay.

Peptidomimetics based on oligobenzamide scaffold were designed based on a crystal structure of decapeptide PB1-11 and PA C-terminal domain. Peptidomimetics showed a good solubility but did not show any inhibition below 50 μM.

Hexapeptides inhibitors of PA-PB1 interaction inhibitor were optimized to be more soluble and therefore more suitable for crystallization with PA C-terminal domain. Hot spots were optimized and the knowledge gathered was applied for the decapeptides. Nanomolar inhibitors of PA-PB1 interaction were prepared. The good solubility of decapeptide **269** allowed for the measurement of isothermal titration calorimetry (ITC). X-ray structure of PA C-terminal domain with the decapeptide **269** was obtained.

The X-ray structure and ITC guided me to carry out peptide stapling. Two stapled peptides **276** and **277** were prepared and they showed nanomolar activities. Collision induced

dissociation spectra (CID) confirmed the structure and these peptides are now ready to be co-crystalized with PA C-terminal domain.

A series of CPP equipped bicyclic peptides was successfully synthesized and their antiviral activity was determined. The antiviral activity of bicyclic peptide **300** in both minireplicon and CPE assays is conclusively confirmed and this bicyclic peptide will become a subject of successive research.

5. Experimental section

The solvents used for reactions were purchased from Lachner, Sigma Aldrich, Penta or Fluorochem. THF was dried over CaH_2, filtered and distilled from Na/benzophenone under inter atmosphere. Other solvents were purchased already dry. Chromatography was performed using Fluka silica gel 60 (0.040–0.063 mm) or Merck silica gel 60 RP-18 F_{254} -coated aluminum sheets were used. The spots were detected in UV or by the solution of $Ce(SO_4)_2 \cdot 4H_2O$ (1%) and $H_3P(Mo_3O_{10})_4$ (2%) in 10% sulfuric acid (10%), $KMnO_4$ (1%) K_2CO_3 (5%) in 15 mM NaOH or ninhydrin (19%) in *n*-butanol. All starting materials were used as purchased (Sigma Aldrich, Alfa Aesar, TCI), unless otherwise indicated. Oseltamivir phosphate was purchased from Santiago. All tested inhibitors were purified using preparative HPLC of Jasco brand (flow rate 10 mL/min; gradient 2–100% ACN in 50 min), with column Waters SunFire C18 OBD Prep Column, 5 μm, 19 × 150 mm. The purity of compounds was tested on analytical Jasco PU-1580 HPLC (flow rate 1 mL/min, invariable gradient 2–100% ACN in 30 min) with column Watrex C18 Analytical Column, 5 μm, length 250 mm. The final inhibitors were all at least of 95% purity. The ^{1}H NMR spectra were recorded on Bruker instruments at 300, 400 or 600 MHz, the ^{13}C NMR spectra at were recorded at 75, 100 or 150 MHz, respectively. Tetramethylsilane was used as an internal standard. The chemical shifts are given in δ-scale, coupling constants J are given in Hz. The EI mass spectra were determined at an ionizing voltage of 70 eV, the m/z values are given alone with their relative intensities (%). The ESI mass spectra were recorded using ZQ micromass mass spectrometer (Waters) equipped with an ESCi multimode ion source and controlled by MassLynx software. Methanol was used as a solvent. MS and MSMS spectra of the peptides were measured by Orbitrap Fusion LUMOS mass spectrometer (Thermo Scientific). The peptides were introduced into the instrument by Triversa Nanomate (Advion) direct infusion. First, MS precursors were monitored and then selected for following MS2, MS3 fragmentation by CID. Solid-phase peptide synthesis was carried out either in syringe or on two peptide synthesizers. Long peptides were synthesize on a Liberty Blue™ Automated Microwave Peptide Synthesizer (PS2) using Oxyma/DIC for activation and 20% piperidine in DMF for Fmoc-deprotection. Shorter peptides were prepared on Gyros Protein Technologies 3-channel peptide synthesizer (PS3). ChemMatrix resin was purchased from Sigma Aldrich as H-Rink amide ChemMatrix® resin 35-100 mesh particle size (wet sieved). PS2 synthesizer was operated only by Miroslava Blechová and Alexandra Prichodko.

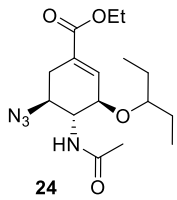
General procedure for saponification of the ethyl esters

To a solution of the ethyl ester (1 eq.) in 1,4-dioxane (0.14 M) was added an aqueous solution of NaOH (2 eq., 0.5 M) and the reaction mixture was stirred from 2 h to 24 h at room temperature. After the disappearance of the starting material (monitored by TLC), the pH of the reaction was adjusted to neutral by addition of Amberlite IR 120. The Amberlite beads were filtered out and the collected beads were rinsed with MeOH several times; the filtrate was then evaporated to dryness and the residue was purified by either preparative HPLC or flash chromatography to afford the corresponding free acid.

General procedure for CuAAC reaction between alkynes and azido-oseltamivir (24)

Compound **24** (1 eq.) was dissolved in a THF/water mixture (2:1, 2 mL/0.1 mmol of **24**), the corresponding alkyne (1.3 eq.), sodium *L*-ascorbate (5 eq.) and copper(II) sulfate pentahydrate (2 eq.) were added and the reaction was stirred from 2 h to 3 h. Water (10 mL) was added and the aqueous layer was extracted with EtOAc (3 × 10 mL), the combined organic layers were washed with brine (10 mL), dried with $MgSO_4$ and evaporated to dryness under reduced pressure. The products were purified by column chromatography.

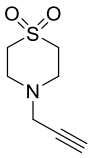
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(ethylpropoxy)-1-cyclohexene-1-carboxylate (24)



24

Oseltamivir phosphate (1.0 g, 2.4 mmol) was dissolved in a solution of potassium carbonate (1.5 g, 10.9 mmol) and copper(II) sulfate pentahydrate (0.002 g, 0.024 mmol) in methanol (20 mL). The mixture was stirred for 10 min followed by addition of imidazole-1-sulfonyl azide hydrochloride (0.6 g, 2.9 mmol) and further stirring for 2 h. The reaction mixture was quenched by addition of H_2O and extracted with DCM (3 × 30 mL), the combined organic phases were washed with H_2O and brine, dried over Na_2SO_4 and evaporated to dryness under reduced pressure. Purification of crude product by flash column chromatography (eluent Hexane/EtOAc 1:1) furnished the azide **15** (0.66 g, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.79 (d, J = 2.4 Hz, 1H), 5.73 (bs, 1H), 4.60 (d, J = 8.9 Hz, 1H), 4.34 (td, J = 10.6, 5.7 Hz, 1H), 4.22 (q, J = 7.1 Hz, 2H), 3.38–3.18 (m, 2H), 2.87 (dd, J = 17.7, 5.8 Hz, 1H), 2.30–2.12 (m, 1H), 2.04 (s, 3H), 1.60–1.43 (m, 4H), 1.30 (t, J = 7.1 Hz, 3H), 0.91 (td, J = 7.4, 3.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 165.9, 138.1, 128.3, 82.1, 73.4, 61.2, 58.4, 57.2, 30.7, 26.4, 25.7, 23.7, 14.3, 9.7, 9.5.

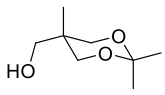
4-(Prop-2-yn-1-yl)thiomorpholine 1,1-dioxide (25)



25

Divinyl sulfone (0.10 g, 0.84 mmol) was added to a solution of propargylamine (0.05 g, 0.93 mmol) in ethanol (10 mL). Solution was refluxed and monitored by TLC (DCM/MeOH 25:1). After 5h reaction mixture was evaporated and purified by column chromatography (DCM → EtOAc) to yield the title compound as a colorless oil (0.10 g, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.42 (d, *J* = 2.4 Hz, 2H), 3.09 (s, 8H), 2.33 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 77.6, 74.5, 51.6, 50.2, 46.8. HRMS (TOF EI) calcd for $C_7H_{12}NO_2S$ $[M+H]^+$ 174.0589, found 174.0590.

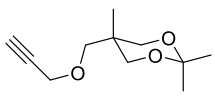
(2,2,5-Trimethyl-1,3-dioxan-5-yl)methanol (26)



26

2-(Hydroxymethyl)-2-methylpropane-1,3-diol (5.00 g, 41.00 mmol) and *p*-toluenesulfonic acid monohydrate (0.05 g, 0.26 mmol) were dissolved in acetone (50 mL, pre-dried over 4Å sieves 10 % w/v) and the reaction mixture was stirred overnight. Solid K_2CO_3 (0.20 g, 1.45 mmol) was added and the suspension was filtered. The filtrate was concentrated down and the residue was purified by distillation under reduced pressure to afford a transparent oil as a product (5.14 g, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70-3.68 (d, *J* = 5.6 Hz, 2H), 3.65 (d, *J* = 12.3 Hz, 2H), 3.60 (d, *J* = 11.0 Hz, 2H), 2.0 – 1.87 (m, 1H), 1.44-1.39 (d, *J* = 19.1 Hz, 6H), 0.82 (s, 3H).

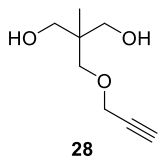
2,2,5-Trimethyl-5-((prop-2-yn-1-yloxy)methyl)-1,3-dioxane (27)



27

(2,2,5-Trimethyl-1,3-dioxan-5-yl)methanol (0.20 g, 1.25 mmol) sodium hydride (60 % dispersion in mineral oil, 0.06 g, 2.50 mmol) were suspended in THF under inert atmosphere and the suspension was stirred for 1 h. Propargyl bromide (80 % in toluene, 0.21 mL, 1.87 mmol) was added dropwise and the reaction mixture was stirred overnight. The reaction was quenched with water (15 mL) and extracted with EtOAc (2 × 15 mL). The combined organic phase was washed with brine and co-distilled with toluene (3 × 50 mL). The residue was dissolved in DCM and filtered over celite to afford the crude product (0.22 g, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.16 (d, *J* = 2.4 Hz, 2H), 3.71 (d, *J* = 12.1 Hz, 4H), 3.55 (d, *J* = 12, 2H) 3.53 (s, 2H), 2.41 (t, *J* = 2.4 Hz, 1H), 1.43 (s, 3H), 1.40 (s, 3H), 0.88 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 98.0, 80.1, 74.3, 73.1, 66.6, 58.9, 34.3, 26.6, 21.2, 18.3.

2-Methyl-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diol (28)



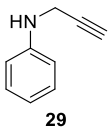
28

2,2,5-Trimethyl-5-((prop-2-yn-1-yloxy)methyl)-1,3-dioxane (0.21 g, 1.04 mmol) was dissolved in MeOH (4 mL) and an HCl solution (1M, 4 mL) was added, the resulting reaction was stirred for 2 h at room temperature. A solution of NaOH (1M, 4 mL) was added and MeOH was evaporated under reduced pressure. The aqueous layer was extracted with EtOAc (3 × 10 mL), the combined organic phase was washed with brine (10 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The product was purified by column chromatography (DCM/MeOH 15:1) to furnish the propargyl derivative (0.10 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 – 4.14 (d, *J* = 2.4 Hz, 2H), 3.66 (dd, *J* = 11.2 Hz, 2H), 3.55 (d, *J* = 10.2 Hz, 2H), 3.52 (s, 2H), 2.51 (bs, 2H), 2.45 – 2.44 (t, *J* = 2.4 Hz, 1H), 0.83 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 79.7, 74.9, 74.8, 67.9, 58.9, 40.9, 17.2.

General procedure for propargylation of anilines 29-34.

The corresponding aniline (4 eq.) and K_2CO_3 (2 eq.) were suspended in acetonitrile (1 mL per 2 mmol of aniline) followed by dropwise addition of propargyl bromide (80% in toluene, 1 eq.), the resulting solution was stirred overnight at room temperature. Water (10 mL) was added to the reaction mixture and the aqueous layer was extracted with EtOAc (3 × 10 mL), the combined organic phase was washed with brine (10 mL), dried with MgSO4 and evaporated to dryness under reduced pressure. The corresponding products were purified by column chromatography.

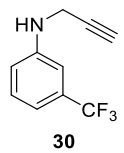
***N*-(Prop-2-yn-1-yl)aniline (29)**



29

Aniline hydrochloride (1.00 g, 8.00 mmol) was alkylated using the conditions described in the general procedure for propargylation of anilines. The purification was done by column chromatography (hexane/EtOAc, 4:1) to furnish the propargyl derivative (0.18 g, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 (t, 2H), 6.81 (t, 1H), 6.70 (d, *J* = 9.6 Hz, 2H), 3.94 (d, *J* = 2.4 Hz, 2H), 3.87 (s, 1H), 2.22 (t, *J* = 2.2 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 147.0, 129.4, 118.8, 113.6, 81.1, 71.4, 33.8. HRMS (EI) *m/z* calcd for $C_9H_{10}N_2$ $[M+H]^+$ 132.0813, found: 132.0814.

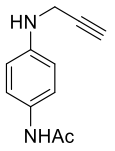
_N_-(Prop-2-yn-1-yl)-3-(trifluoromethyl)aniline (30)



30

3-(Trifluoromethyl)aniline (1.30 g, 8.00 mmol) was alkylated using the conditions described in the general procedure for propargylation of anilines. The purification was done by column chromatography (hexane/EtOAc, 4:1) to furnish the propargyl derivative (0.21 g, 52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (t, J = 7.9 Hz, 1H), 7.03 (d, J = 7.0 Hz, 1H), 6.89 (s, 1H), 6.83 (dd, J = 8.2, 2.3 Hz, 1H), 4.09 (bs, 1H), 3.97 (dd, J = 5.7, 2.4 Hz, 2H), 2.25 (t, J = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 147.1, 131.7 (q, $^{2}J_{C\text{-}F}$ = 31.9 Hz), 129.8, 124.4 (q, $^{1}J_{C\text{-}F}$ = 272.3 Hz), 116.5, 115.1 (q, $^{3}J_{C\text{-}F}$ = 3.9 Hz), 109.9 (q, $^{3}J_{C\text{-}F}$ = 3.9 Hz), 80.3, 71.9, 33.6. ^{19}F NMR (375 MHz, $CDCl_3$) δ -62.9. HRMS (CI) *m/z* calcd for $C_{10}H_9NF_3$ $[M+H]^+$ 200.0687, found: 200.0686.

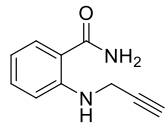
_N_-(4-(Prop-2-yn-1-ylamino)phenyl)acetamide (31)



31

N-(4-Aminophenyl)acetamide (1.20 g, 8.00 mmol) was alkylated using the conditions described in the general procedure for propargylation of anilines. The purification was done by column chromatography (hexane/EtOAc 1:1) to furnish the propargyl derivative (0.26 g, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40-7.20 (bs, 1H), 7.33 (d, J = 8.7 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 3.96 (s, 1H), 3.93 (d, J = 2.2 Hz, 2H), 2.23 (t, J = 2.4 Hz, 1H), 2.15 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.4, 144.1, 129.4, 122.3, 114.0, 81.1, 71.5, 34.0, 24.4. HRMS (EI) *m/z* calcd for $C_{11}H_{12}N_2O$ $[M]^{+\bullet}$ 188.0950, found: 188.0954.

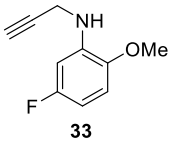
2-(Prop-2-yn-1-ylamino)benzamide (32)



32

2-Aminobenzamide (1.09 g, 8.00 mmol) was alkylated using the conditions described in the general procedure for propargylation of anilines. The purification was done by column chromatography (hexane/EtOAc 1:1) to furnish the propargyl derivative (0.17 g, 48%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.58 (dd, J = 7.9, 1.5 Hz, 1H), 7.34 (t, 1H), 6.82 (d, J = 8.4 Hz, 1H), 6.71 – 6.61 (m, 1H), 3.98 (d, J = 2.4 Hz, 2H), 2.55 (t, J = 2.5 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ 174.7, 150.2, 134.0, 129.9, 116.8, 116.4, 113.3, 81.8, 72.1, 33.0. HRMS (ESI) *m/z* calcd for $C_{10}H_{11}N_2O$ $[M+H]^+$ 175.0865, found: 175.0865.

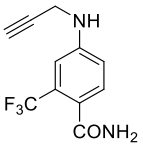
5-Fluoro-2-methoxy-*N*-(prop-2-yn-1-yl)aniline (33)



33

5-Fluoro-2-methoxyaniline (0.30 g, 2.10 mmol) was alkylated using the conditions described in the general procedure for propargylation of anilines. The purification was done by column chromatography (hexane/EtOAc 4:1) to furnish the propargyl derivative (0.04 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (dd, *J* = 8.7, 5.0 Hz, 1H), 6.44 (dd, *J* = 10.5, 2.9 Hz, 1H), 6.38 (td, *J* = 8.6, 2.9 Hz, 1H), 4.58 (s, 1H), 3.93 (d, *J* = 2.4 Hz, 2H), 3.82 (s, 3H), 2.23 (t, *J* = 2.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.3 (d, $^{1}J_{C\text{-}F}$ = 235.9 Hz), 143.4, 138.2, 109.9 (d, $^{3}J_{C\text{-}F}$ = 10.0 Hz), 102.4 (d, $^{2}J_{C\text{-}F}$ = 23.0 Hz), 98.7 (d, $^{2}J_{C\text{-}F}$ = 28.3 Hz), 80.5, 71.6, 56.1, 33.2. ^{19}F NMR (375 MHz, $CDCl_3$) δ -122.0. HRMS (CI) *m/z* calcd for $C_{10}H_{11}NOF$ $[M+H]^+$ 180.0825, found: 180.0829.

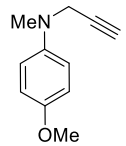
4-(Prop-2-yn-1-ylamino)-2-(trifluoromethyl) (34)



34

4-Amino-2-(trifluoromethyl)benzamide (1.63 g, 8.00 mmol) was alkylated using the conditions described in the general procedure for propargylation of anilines. The purification was done by column chromatography (hexane/EtOAc 1:1) to furnish the propargyl derivative (0.088 g, 18%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 1H), 7.50 (dd, *J* = 8.2, 1.0 Hz, 1H), 7.09 – 6.99 (m, 1H), 6.90 (ddd, *J* = 8.2, 1.7, 0.7 Hz, 1H), 5.82 (s, 2H), 4.03 (dd, *J* = 5.8, 2.5 Hz, 2H), 2.24 (t, *J* = 2.5 Hz, 1H), 1.66 – 1.53 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 149.1, 135.1 (q, *J* = 32.1 Hz), 128.8, 123.8 (q, $^{1}J_{C\text{-}F}$ = 272.9 Hz), 116.8, 112.1, 109.2, 79.7, 71.8, 32.6. ^{19}F NMR (375 MHz, $CDCl_3$) δ -63.7. HRMS (CI) *m/z* calcd for $C_{11}H_{10}N_2OF_3$ $[M+H]^+$ 243.0745, found: 243.0757.

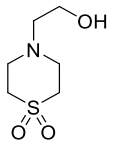
4-Methoxy-*N*-methyl-*N*-(prop-2-yn-1-yl)aniline (35)



35

4-Methoxy-*N*-methylaniline (0.30 g, 2.18 mmol) was dissolved in acetone (4 mL) followed by addition of K_2CO_3 (0.60 g, 4.37 mmol) and propargyl bromide (80% in toluene, 0.20 mL, 2.18 mmol), the resulting reaction was stirred overnight. The reaction mixture was quenched with water (12 mL) and the aqueous layer was extracted with EtOAc (3 × 20 mL), the combined organic phase was washed with brine (10 mL), dried with $MgSO_4$ and evaporated to dryness under reduced pressure. The products were purified by column chromatography (DCM) to furnish the propargyl derivative (0.32 g, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.87 (s, 4H), 3.98 (d, *J* = 2.3 Hz, 2H), 3.77 (s, 3H), 2.90 (s, 3H), 2.18 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.2, 143.8, 117.0, 114.6, 79.4, 72.4, 55.7, 43.9, 39.5. HRMS (EI) *m/z* calcd for $C_{11}H_{13}NO$ $[M]^{+\bullet}$ 175.0997, found: 175.0996.

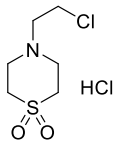
4-(2-Hydroxyethyl)thiomorpholine 1,1-dioxide (36)



36

Divinyl sulfone (3.50 g, 29.00 mmol) and ethanolamine (1.99 g, 32.00 mmol) were refluxed in propan-2-ol (42 mL) for 5h. The reaction mixture was concentrated under reduced pressure and purified by column chromatography (DCM/MeOH 10:1) to afford the alcohol (4.70 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.67-3.64 (q, J = 4.7 Hz, 2H), 3.08 (s, 8H), 2.73 – 2.70 (m, 2H), 2.27 – 2.25 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 58.7, 58.2, 51.4, 51.0.

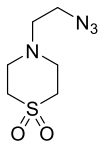
4-(2-Chloroethyl)thiomorpholine 1,1-dioxide hydrochloride (37)



37

4-(2-Hydroxyethyl)thiomorpholine 1,1-dioxide (3.36 g, 18.00 mmol) was dissolved in 1,2-DCE (90 mL) and $SOCl_2$ (11.16 g, 93.00 mmol) was added dropwise under argon, the resulting reaction was refluxed overnight. The reaction mixture was carefully quenched with water (9.5 mL) and the aqueous solution was stirred for further 30 min. The reaction mixture was dried under reduced pressure to afford a solid product (4.10 g, 93%) which was used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.07 – 4.03 (t, J = 6.9 Hz, 2H), 3.80 – 3.69 (m, 8H), 3.66 – 3.61 (t, J = 6.9 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 55.4, 50.4, 47.7, 37.3.

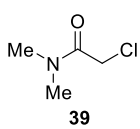
4-(2-Azidoethyl)thiomorpholine 1,1-dioxide (38)



38

4-(2-Chloroethyl)thiomorpholine 1,1-dioxide hydrochloride (0.30 g, 1.28 mmol) and sodium azide (0.25 g, 3.86 mmol) were dissolved in water (10 mL) and the resulting reaction mixture was stirred at 60 °C for 3 h. Solid KOH (0.14 g, 2.57 mmol) was added and the reaction mixture was extracted with DCM (3 × 15 mL), the organic phase was extracted with brine (10 mL) and dried over $MgSO_4$. The reaction mixture was concentrated under reduced pressure to afford the product as a colorless oil (0.24 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.35 – 3.32 (m, 2H), 3.08 (s, 8H), 2.79-2.76 (dd, J = 6.2, 5.2 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 56.1, 51.5, 51.0, 48.6.

2-Chloro-*N,N*-dimethylacetamide (39)

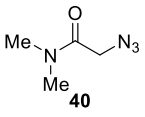


39

Dimethylamine hydrochloride (2.00 g, 27.70 mmol), chloroacetyl chloride (2 mL, 25.00 mmol) and triethylamine (7 mL, 50.00 mmol) were dissolved in DCM (40 mL) and the reaction mixture was stirred for 2 h. The reaction was washed with water (2 × 15 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$, filtered and

evaporated down to afford product (1.45 g, 50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.07 (s, 2H), 3.08-2.98 (d, *J* = 32.9 Hz, 6H).

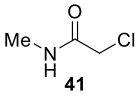
2-Azido-*N*,*N*-dimethylacetamide (40)



40

2-Chloro-*N*,*N*-dimethylacetamide (1.45 g, 12.00 mmol) and sodium azide (2.33 g, 36.00 mmol) were dissolved in water (25 mL) and the reaction mixture was stirred for 2 h at 60 °C. The reaction was cooled down and extracted with EtOAc (3 × 12 mL), the organic phase was washed with brine, dried over $MgSO_4$ and concentrated down. The residue was purified by column chromatography (DCM/MeOH 20:1) to afford the product (1.51 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.91 (s, 1H), 2.98 (d, *J* = 11.3 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.2, 50.6, 36.5, 35.8.

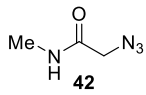
2-Chloro-*N*-methylacetamide (41)



41

To a solution of methylamine (1.55 g, 40 % w/v, 20.00 mmol) in DCM (8 mL) was added NaOH (0.80 g, 20.00 mmol) and the resulting mixture was cooled down to -10 °C. Chloroacetyl chloride (2.50 g, 22.52 mmol) was added and the reaction mixture was stirred further 20 min at -10 °C. The reaction mixture was warmed up to r.t. and water (0.5 mL) was added. The reaction mixture was extracted with DCM (3 × 12 mL), the organic phase was washed with HCl (5 %, 10 mL), water (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure to afford the product (0.61 g, 25%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.61 (bs, 1H), 4.05 (s, 2H), 2.89-2.88 (d, *J* = 4.9 Hz, 3H).

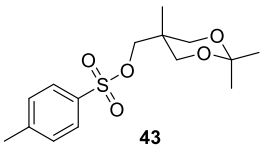
2-Azido-*N*-methylacetamide (42)



42

2-Chloro-*N*-methylacetamide (0.61 g, 5.73 mmol) and sodium azide (1.50 g, 23.00 mmol) were dissolved in water (15 mL) and the reaction mixture was stirred overnight at 75 °C. The reaction was cooled down and extracted with DCM (3 × 12 mL), the organic phase was dried over $MgSO_4$ and concentrated down. The residue was purified by column chromatography (DCM/MeOH 20:1) to afford the product (0.43 g, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.36 (bs, 1H), 3.98 (s, 2H), 2.86-2.85 (d, *J* = 4.9 Hz, 3H).

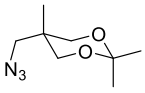
(2,2,5-Trimethyl-1,3-dioxan-5-yl)methyl 4-methylbenzenesulfonate (43)



43

A solution of **26** (1.99 g, 12.43 mmol) in pyridine (6.6 mL) was mixed with a solution of *p*-toluenesulfonyl chloride (2.37 g, 12.47 mmol) in pyridine (3.3 mL). The reaction mixture was stirred at 100 °C for 2 h. The reaction was poured onto ice/H_2O (200 mL) and was left in the fridge overnight. The precipitate was formed, filtered, and dried under vacuum to afford the product (2.07 g, 53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82-7.79 (dd, *J* = 8.3, 1.7 Hz, 2H), 7.36-7.33 (d, *J* = 7.9 Hz, 2H), 4.08 (d, *J* = 1.7 Hz, 2H), 3.55 (s, 4H), 2.44 (s, 3H), 1.37 (s, 3H), 1.22 (s, 3H), 0.82 (d, *J* = 1.7 Hz, 3H).

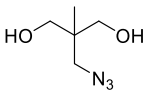
5-(Azidomethyl)-2,2,5-trimethyl-1,3-dioxane (44)



44

Compound **43** (2.06 g, 6.58 mmol) and sodium azide (1.28 g, 19.76 mmol) were dissolved in DMF/H_2O (10:1, 12 mL) and the reaction mixture was stirred overnight at 70 °C. Water (30 mL) was added and the mixture was extracted with Et_2O (3 × 35 mL). The organic phase was concentrated under reduced pressure and purified using column chromatography (hexane/ EtOAc, 1:1) to afford the product (0.99 g, 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.59 (s, 4H), 3.52 (s, 2H), 1.42 (s, 3H), 1.39 (s, 3H), 0.82 (s, 3H).

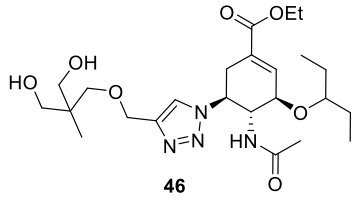
2-(Azidomethyl)-2-methylpropane-1,3-diol (45)



45

Compound **44** (0.07 g, 0.37 mmol) was dissolved in THF/H_2O (5:1, 3 mL) and HCl (37% in water; 0.1 mL) was added dropwise, the reaction mixture was stirred for 1 h. The reaction mixture was concentrated under reduced pressure and purified using column chromatography (DCM/MeOH 20:1) to afford the product (0.05 g, 92%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.60 (s, 4H), 3.44 (s, 2H), 2.26 (bs, 2H), 0.86 (d, *J* = 1.6 Hz, 3H).

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-((3-hydroxy-2-(hydroxymethyl)-2-methylpropoxy) methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate (46)

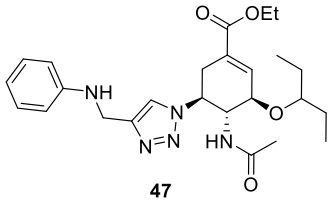


46

Compound **28** (0.042 g, 0.27 mmol) and compound **15** (0.070 g, 0.20 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (DCM/MeOH 20:1) to furnish the ethyl ester derivative (0.070 g, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.78 (s, 1H), 6.88 (s,

1H), 6.63 (d, J = 6.8 Hz, 1H), 5.27 (s, 1H), 4.71–4.45 (m, 4H), 4.22 (q, J = 7.1 Hz, 2H), 3.73–3.64 (m, 3H), 3.42–3.30 (m, 2H), 3.00 (dq, J = 27.7, 17.0, 14.8 Hz, 4H), 1.75 (s, 3H), 1.51 (dddd, J = 13.4, 7.5, 5.8, 1.9 Hz, 4H), 1.29 (t, J = 7.1 Hz, 3H), 0.91 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 7.4 Hz, 4H), 0.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 165.7, 138.6, 128.2, 82.6, 76.8, 74.6, 74.2, 70.9, 70.2, 68.0, 64.4, 61.4, 58.3, 56.4, 50.8, 40.9, 31.8, 26.4, 25.7, 17.4, 14.3, 9.7, 9.3. HRMS (ESI) *m/z* calcd for $C_{24}H_{41}O_7N_4$ $[M+H]^+$ 497.2969, found 497.2970.

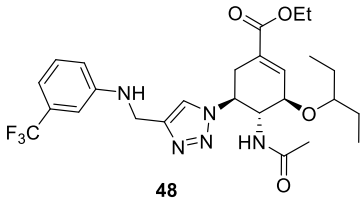
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(4-((phenylamino)methyl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylate (47)



47

Compound **29** (0.025 g, 0.19 mmol) and compound **15** (0.050 g, 0.15 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (cyclohexane/EtOAc 1:1) to furnish the ethyl ester derivative (0.033 g, 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (s, 1H), 7.17–7.13 (m, 2H), 6.86 (s, 1H), 6.71 (t, J = 7.3 Hz, 1H), 6.65 (d, J = 7.8 Hz, 2H), 6.27 (d, J = 7.7 Hz, 1H), 5.31 (td, J = 11.0, 6.1 Hz, 1H), 4.66 (d, J = 8.6 Hz, 1H), 4.41 (s, 2H), 4.21 (q, J = 7.1 Hz, 2H), 3.93 (dt, J = 11.6, 8.6 Hz, 1H), 3.35 (p, J = 5.6 Hz, 1H), 3.03–2.89 (m, 2H), 1.63 (s, 3H), 1.58–1.41 (m, 4H), 1.28 (t, J = 7.1 Hz, 3H), 0.91 (t, J = 7.4 Hz, 3H), 0.86 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 165.7, 147.3, 145.5, 138.6, 129.4, 128.3, 122.4, 118.3, 113.4, 82.5, 74.0, 61.3, 57.3, 39.9, 31.9, 29.8, 26.4, 25.7, 23.1, 14.3, 9.8, 9.3. HRMS (ESI) *m/z* calcd for $C_{25}H_{35}O_4N_5Na$ $[M+Na]^+$ 492.2581, found 492.2578.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(4-(((3-(trifluoromethyl)phenyl)amino) methyl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylate (48)

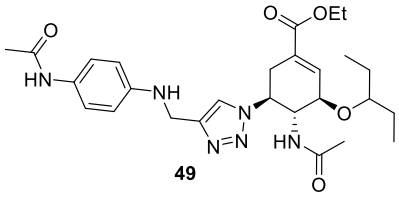


48

Compound **30** (0.038 g, 0.19 mmol) and compound **15** (0.050 g, 0.15 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (cyclohexane/EtOAc 1:1) to furnish the ethyl ester derivative (0.040 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (bs, 1H), 7.24 (t, J = 7.7 Hz, 1H) 6.93 (d, J = 7.5 Hz, 1H), 6.86 (s, 2H), 6.8 (s, 1H), 6.83 (d, J = 7.9 Hz, 1H), 6.78 (d, J = 7.9 Hz, 1H), 6.25 (d, J = 7.7 Hz, 1H), 5.33 (q, J = 10.3 Hz, 1H), 4.64 (d, J = 8.8 Hz, 1H), 4.44 (bs, 2H), 4.20 (q, J = 7.1 Hz, 2H), 3.95 (dt, J = 11.3, 8.6 Hz, 1H), 3.34 (p,

J = 5.6 Hz, 1H), 3.05–2.89 (m, 2H), 1.61 (s, 3H), 1.56–1.41 (m, 4H), 1.28 (t, J = 7.1 Hz, 3H), 0.91 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 165.6, 147.7, 144.9, 138.5, 131.7 (q, J = 31.5 Hz), 129.9, 128.3, 124.3 (q, J = 274.6 Hz), 122.4, 116.1, 114.5, 109.5, 82.5, 74.1, 61.3, 57.2, 39.5, 31.9, 29.8, 26.4, 25.7, 23.1, 14.3, 9.7, 9.3. ^{19}F NMR (375 MHz, $CDCl_3$) δ −62.9. HRMS (ESI) *m/z* calcd for $C_{26}H_{34}O_4N_5F_3Na$ $[M+Na]^+$ 560.2455, found 560.2454.

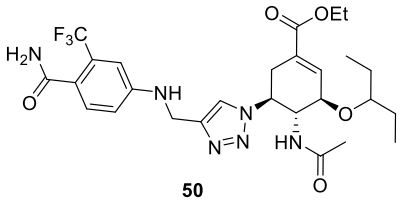
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-(((4-acetamidophenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate (49)



49

Compound **31** (0.036 g, 0.20 mmol) and compound **15** (0.050 g, 0.15 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (DCM/MeOH 20:1) to furnish the ethyl ester derivative (0.047 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.58 (s, 1H), 7.16 (d, J = 8.1 Hz, 2H), 6.84 (s, 1H), 6.79 (s, 1H), 6.51 (d, J = 7.9 Hz, 2H), 5.15 (q, J = 10.3 Hz, 1H), 4.56 (m 1H), 4.31 (s, 2H), 4.20 (q, J = 7.0 Hz, 2H), 4.02 (q, J = 9.2 Hz, 1H), 3.34 (p, J = 5.6 Hz, 1H), 3.06–2.80 (m, 2H), 2.08 (s, 3H), 1.58 (s, 3H), 1.54–1.40 (m, 4H), 1.27 (t, J = 7.1 Hz, 3H), 0.89 (t, J = 7.4 Hz, 3H), 0.82 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 168.8, 165.8, 145.5, 144.7, 138.7, 128.8, 128.2, 122.8, 122.4, 113.5, 82.5, 74.4, 61.3, 57.7, 56.5, 40.0, 31.8, 26.4, 25.7, 24.2, 23.0, 14.3, 9.7, 9.3. HRMS (ESI) *m/z* calcd for $C_{27}H_{39}N_5O_6$ $[M+H]^+$ 527.2976, found 527.2976.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-(((4-carbamoyl-3-(trifluoromethyl)phenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate (50)

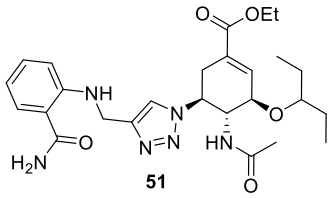


50

Compound **34** (0.016 g, 0.060 mmol) and compound **15** (0.017 g, 0.049 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (EtOAc) to furnish the ethyl ester derivative (0.012 g, 45%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (t, J = 5.5 Hz, 1H), 8.04 (s, 1H), 7.96 (s, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.77 (d, J = 8.1 Hz, 1H), 7.44 (s, 1H), 7.04 (s, 1H), 6.84 (d, J = 8.1 Hz, 1H), 6.74 (s, 1H), 4.90 (td, J = 11.1, 5.8 Hz, 1H), 4.44 (d, J = 5.2 Hz, 2H), 4.31 (d, J = 8.7 Hz, 1H), 4.20–4.08 (m, 3H), 3.37 (p, J = 5.6 Hz,

1H), 2.98–2.87 (m, 1H), 2.83 (dd, J = 17.1, 5.8 Hz, 1H), 1.48 (s, 3H), 1.46–1.31 (m, 4H), 1.22 (t, J = 7.1 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H), 0.75 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.4, 169.0, 165.1, 149.2, 143.9, 138.4, 133.7, 132.3 (q, J = 32.5 Hz), 129.9, 127.8, 123.9 (q, J = 273.1 Hz), 122.0, 117.5, 110.2, 107.6, 81.1, 74.8, 60.6, 57.9, 53.5, 37.9, 31.0, 25.6, 25.0, 22.3, 14.0, 9.5, 8.8. HRMS (ESI) *m/z* calcd for $C_{27}H_{35}O_5N_6F_3Na$ $[M+Na]^+$ 603.2513, found 603.2507.

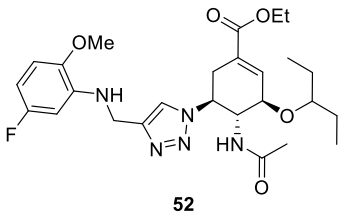
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-(((2-carbamoylphenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate (51)



51

Compound **32** (0.033 g, 0.20 mmol) and compound **15** (0.050 g, 0.15 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (DCM/MeOH 20:1) to furnish the ethyl ester derivative (0.029 g, 38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.39 (d, J = 7.8 Hz, 1H), 7.26 (d, J = 16.9 Hz, 1H), 6.85 (s, 1H), 6.71 (d, J = 8.4 Hz, 1H), 6.58 (t, J = 7.5 Hz, 1H), 6.53 (d, J = 7.5 Hz, 1H), 6.19 (s, 2H), 5.35–5.20 (m, 1H), 4.67 (d, J = 8.7 Hz, 1H), 4.47 (s, 2H), 4.20 (q, J = 7.1 Hz, 2H), 3.98–3.82 (m, 1H), 3.34 (p, J = 5.7 Hz, 1H), 2.96 (d, J = 7.1 Hz, 3H), 1.64 (s, 3H), 1.54–1.39 (m, 4H), 1.27 (t, J = 7.1 Hz, 3H), 0.90 (t, J = 7.4 Hz, 3H), 0.84 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.7, 172.4, 171.4, 165.7, 149.4, 145.5, 138.6, 133.5, 128.5, 128.1, 122.3, 115.4, 113.9, 112.0, 82.3, 73.9, 61.1, 57.1, 38.9, 31.7, 29.7, 26.3, 25.5, 23.0, 20.9, 14.2, 9.6, 9.2. HRMS (ESI) *m/z* calcd for $C_{24}H_{28}N_6O_5$ $[M+Na]^+$ 535.2639, found 535.2639.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-(((5-fluoro-2-methoxyphenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate (52)

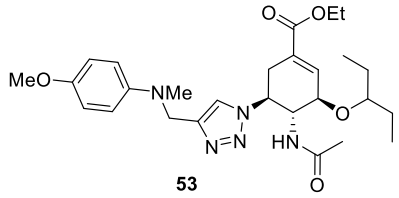


52

Compound **33** (0.036 g, 0.20 mmol) and compound **15** (0.061 g, 0.18 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (cyclohexane/EtOAc 1:1) to furnish the ethyl ester derivative (0.056 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (s, 1H), 6.87–6.82 (m, 1H), 6.61 (ddd, J = 7.6, 5.0, 1.1 Hz, 1H), 6.35–6.23 (m, 3H), 5.34 (ddd, J = 11.7, 9.6, 7.0 Hz, 1H), 4.69 (d, J = 8.7, 2.3 Hz, 1H), 4.40 (s, 2H), 4.20 (q, J = 7.1 Hz, 2H), 4.00–3.85 (m, 1H), 3.78 (s, 3H), 3.34 (p, J = 5.7 Hz, 1H), 2.98 (m, 2H),

1.28 (t, J = 7.1 Hz, 3H), 0.88 (dt, J = 20.5, 7.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5, 165.7, 158.2 (d, $^{1}J_{C\text{-}F}$ = 235.9), 157.1, 145.2, 143.2, 138.6, 128.2, 122.5, 109.8 (d, $^{3}J_{C\text{-}F}$ = 10.0 Hz), 101.8 (d, $^{2}J_{C\text{-}F}$ = 23.0 Hz), 98.1 (d, $^{2}J_{C\text{-}F}$ = 28.2 Hz), 82.5, 73.9, 61.3, 57.4, 57.2, 56.0, 39.5, 31.9, 26.4, 25.7, 23.1, 14.3, 9.7, 9.4. ^{19}F NMR (375 MHz, $CDCl_3$) δ −121.8. HRMS (ESI) *m/z* calcd for $C_{26}H_{37}O_5N_4F$ $[M+H]^+$ 518.2773, found 518.2773.

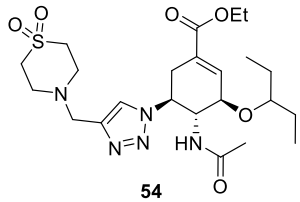
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-(((4-methoxyphenyl)(methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate (53)



53

Compound **34** (0.033 g, 0.18 mmol) and compound **15** (0.050 g, 0.15 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (DCM/MeOH 20:1) to furnish the ethyl ester derivative (0.072 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (s, 1H), 6.84 (s, 1H), 6.77 (s, 2H), 6.55 (d, J = 8.0 Hz, 1H), 5.26 (td, J = 11.4, 6.0 Hz, 2H), 4.63 (d, J = 8.8 Hz, 1H), 4.45 (d, J = 4.6 Hz, 2H), 4.19 (q, J = 7.2 Hz, 3H), 4.02–3.89 (m, 2H), 3.76–3.73 (m, 1H), 3.71 (s, 3H), 3.34 (p, J = 5.7 Hz, 2H), 3.04–2.91 (m, 2H), 2.85 (s, 3H), 1.61 (s, 3H), 1.49 (ddd, J = 13.1, 8.6, 3.8 Hz, 5H), 1.27 (t, J = 7.1 Hz, 3H), 0.89 (t, J = 7.4 Hz, 4H), 0.85 (t, J = 7.4 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 165.7, 152.4, 144.0, 138.7, 128.1, 116.9, 115.6, 114.8, 82.5, 74.1, 61.2, 57.3, 56.9, 55.7, 49.7, 43.9, 39.3, 31.8, 26.3, 25.7, 23.0, 14.3, 9.7, 9.3. HRMS (ESI) *m/z* calcd for $C_{27}H_{39}O_5N_5Na$ $[M+Na]^+$ 536.2843, found 536.2843.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-((1,1-dioxidothiomorpholino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate (54)

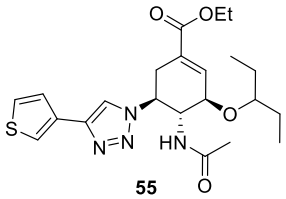


54

Compound **25** (0.033 g, 0.19 mmol) and compound **15** (0.050 g, 0.15 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (DCM/MeOH 10:1) to furnish the ethyl ester derivative (0.037 g, 49%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (s, 1H), 6.88 (s, 1H), 6.04 (d, J = 8.2 Hz, 1H), 5.26 (td, J = 11.4, 5.6 Hz, 1H), 4.55 (d, J = 9.0 Hz, 1H), 4.22 (q, J = 7.1 Hz, 2H), 4.10 (dt, J = 11.7, 8.4 Hz, 1H), 3.81 (s, 2H), 3.36 (p, J = 5.6 Hz, 1H), 3.09–2.92 (m, 8H), 1.74 (s, 3H), 1.57–1.44 (m, 4H), 1.29 (t, J = 7.1 Hz, 4H), 0.91 (t, J = 7.4 Hz, 3H), 0.86 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.1, 165.6, 142.9, 138.5, 128.3, 123.2, 82.6,

74.5, 61.4, 57.8, 56.6, 52.1, 51.5, 50.5, 32.1, 26.4, 25.7, 23.3, 14.3, 9.7, 9.4. HRMS (ESI) *m/z* calcd for $C_{23}H_{37}N_5O_6NaS$ $[M+Na]^+$ 534.2356, found 534.2356.

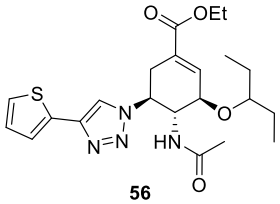
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(4-(thiophen-3-yl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylate (55)



55

3-Ethynylthiophene (0.029 g, 0.27 mmol) and compound **15** (0.070 g, 0.21 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (cyclohexane/EtOAc 1:1) to furnish the ethyl ester derivative (0.084 g, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.62 (s, 1H), 7.40 (d, J = 4.9 Hz, 1H), 7.36–7.28 (m, 1H), 6.88 (d, J = 7.2 Hz, 2H), 5.33 (td, J = 11.2, 5.9 Hz, 1H), 4.67–4.60 (m, 1H), 4.20 (dt, J = 16.6, 8.4 Hz, 3H), 3.40 (p, J = 5.7 Hz, 1H), 3.12–2.86 (m, 2H), 1.69 (d, J = 1.4 Hz, 3H), 1.53 (m, J = 6.5 Hz, 4H), 1.28 (td, J = 7.1, 1.4 Hz, 3H), 0.97–0.80 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 165.7, 143.6, 138.7, 131.7, 128.2, 126.5, 125.9, 121.3, 120.0, 82.5, 74.6, 61.3, 57.7, 56.3, 32.1, 26.4, 25.7, 23.2, 14.3, 9.8, 9.3. HRMS (ESI) *m/z* calcd for $C_{22}H_{30}O_4N_4NaS$ $[M+Na]^+$ 469.1880, found 469.1880.

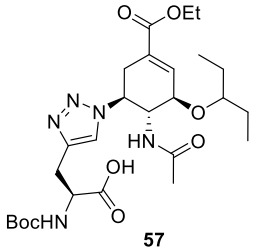
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(4-(thiophen-2-yl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylate (56)



56

2-Ethynylthiophene (0.029 g, 0.27 mmol) and compound **15** (0.070 g, 0.21 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (cyclohexane/EtOAc 1:1) to furnish the ethyl ester derivative (0.040 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.38–7.32 (m, 1H), 7.28 (d, J = 5.0 Hz, 1H), 7.05 (dd, J = 5.0, 3.1 Hz, 1H), 6.91 (t, J = 2.1 Hz, 1H), 6.41 (d, J = 7.7 Hz, 1H), 4.22 (q, J = 7.1 Hz, 2H), 4.14–3.95 (m, 1H), 3.41 (p, J = 5.7 Hz, 1H), 3.04 (qd, J = 17.4, 9.7 Hz, 2H), 1.74 (s, 3H), 1.60–1.47 (m, 3H), 1.29 (t, J = 7.1 Hz, 3H), 0.91 (dt, J = 19.1, 7.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5, 165.7, 138.7, 132.7, 128.2, 127.8, 125.3, 124.5, 82.5, 74.1, 61.3, 57.6, 57.0, 32.1, 29.8, 27.1, 26.4, 25.7, 23.4, 14.3, 9.8, 9.4. HRMS (ESI) *m/z* calcd for $C_{22}H_{31}O_4N_4S$ $[M+H]^+$ 447.2060, found 447.2060.

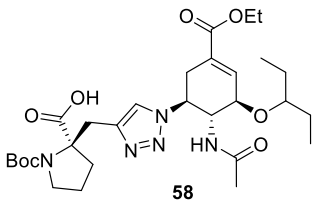
(*S*)-3-(1-((1*S*,5*R*,6*R*)-6-Acetamido-3-(ethoxycarbonyl)-5-(pentan-3-yloxy)cyclohex-3-en-1-yl)-1*H*-1,2,3-triazol-4-yl)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (57)



57

(*S*)-2-((*tert*-Butoxycarbonyl)amino)pent-4-ynoic acid (0.030 g, 0.14 mmol) and compound **15** (0.040 g, 0.12 mmol) were coupled under the modified conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (DCM/MeOH 10:1) to furnish the ethyl ester derivative (0.026 g, 38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (s, 1H), 7.65 (s, 1H), 7.23 (s, 1H), 6.88 (s, 1H), 5.78 (s, 1H), 5.29 (s, 1H), 4.69 (s, 1H), 4.59 (s, 1H), 4.29–4.19 (q, J = 6.7 Hz, 2H), 4.14 (s, 1H), 3.40 (s, 2H), 3.15 (dd, J = 14.4, 4.8 Hz, 1H), 3.01 (s, 2H), 1.86 (s, 3H), 1.52 (s, 5H), 1.37 (s, 9H), 1.28 (t, J = 7.1 Hz, 3H), 0.92 (t, J = 7.5 Hz, 3H), 0.86 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.5, 172.7, 165.5, 155.9, 141.8, 138.3, 128.2, 123.4, 82.6, 79.8, 74.3, 61.2, 57.5, 56.4, 53.0, 31.5, 28.3, 26.2, 25.5, 22.8, 14.2, 14.2, 9.7, 9.1. HRMS (ESI) *m/z* calcd for $C_{26}H_{41}O_8N_5Na$ $[M+Na]^+$ 574.2847, found 574.2845.

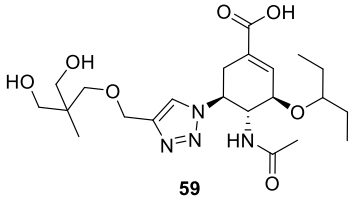
(*R*)-2-((1-((1*S*,5*R*,6*R*)-6-Acetamido-3-(ethoxycarbonyl)-5-(pentan-3-yloxy)cyclohex-3-en-1-yl)-1*H*-1,2,3-triazol-4-yl)methyl)-1-(tert-butoxycarbonyl)pyrrolidine-2-carboxylic acid (58)



58

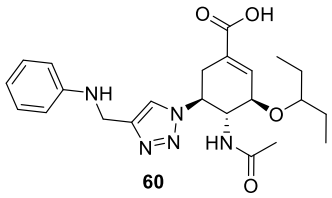
(*R*)-1-(*tert*-Butoxycarbonyl)-2-(prop-2-yn-1-yl)pyrrolidine-2-carboxylic acid (0.068 g, 0.27 mmol) and compound **15** (0.070 g, 0.21 mmol) were coupled under the conditions described in the general procedure for the CuAAC reaction. The purification was done by column chromatography (DCM/MeOH 10:1) to furnish the ethyl ester derivative (0.088 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, J = 12.1 Hz, 1H), 6.86 (s, 1H), 6.48 (dd, J = 45.1, 7.0 Hz, 1H), 5.53–5.28 (m, 1H), 4.75 (dd, J = 19.7, 7.9 Hz, 1H), 4.20 (q, J = 7.1 Hz, 3H), 4.00–3.78 (m, 1H), 3.69–3.55 (m, 1H), 3.56–3.28 (m, 5H), 3.14 (q, J = 7.5 Hz, 1H), 3.10–2.87 (m, 3H), 2.30–2.07 (m, 3H), 1.77 (d, J = 4.1 Hz, 4H), 1.55–1.41 (m, 16H), 1.27 (t, J = 7.1 Hz, 3H), 0.87 (dt, J = 21.0, 7.3 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.7, 176.0, 171.5, 165.6, 155.5, 153.8, 142.8, 142.4, 138.6, 128.1, 82.4, 81.0, 80.8, 73.7, 73.4, 68.5, 67.2, 61.1, 57.7, 57.4, 57.0, 56.8, 48.9, 48.4, 37.0, 35.4, 31.8, 31.1, 30.1, 29.7, 28.5, 26.2, 25.5, 23.1, 22.6, 22.4, 14.2, 9.6, 9.3. HRMS (ESI) *m/z* calcd for $C_{29}H_{45}N_5O_8$ $[M+H]^+$ 592.3340, found 592.3342.

(3*R*,4*R*,5*S*)-4-Acetamido-5-(4-((3-hydroxy-2-(hydroxymethyl)-2-methylpropoxy)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (59)



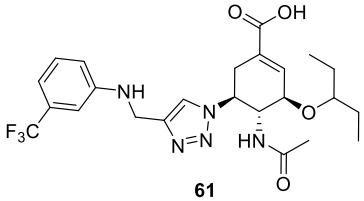
Ethyl ester **46** (0.066 g, 0.13 mmol) was hydrolyzed under the conditions described in the general procedure for saponification of esters (0.031 g, 50%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (s, 1H), 6.90 (s, 1H), 5.03 (ddd, J = 11.7, 9.8, 6.8 Hz, 1H), 4.57 (s, 2H), 4.41 (dd, J = 9.0, 2.5 Hz, 1H), 4.34–4.18 (m, 1H), 3.49–3.38 (m, 5H), 3.36 (s, 2H), 3.11–2.90 (m, 2H), 1.72 (s, 3H), 1.58–1.46 (m, 4H), 0.95 (t, J = 7.4 Hz, 3H), 0.91–0.83 (m, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.3, 168.8, 139.4, 129.7, 124.2, 83.8, 76.6, 74.0, 66.2, 66.2, 65.2, 60.1, 56.2, 42.6, 32.3, 27.3, 26.7, 22.7, 17.1, 9.9, 9.5. HRMS (ESI) *m/z* calcd for $C_{22}H_{36}N_4O_7Na$ $[M+Na]^+$ 491.2476, found 491.2475.

(3*R*,4*R*,5*S*)-4-Acetamido-3-(pentan-3-yloxy)-5-(4-((phenylamino)methyl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylic acid (60)



Ethyl ester **47** (0.050 g, 0.10 mmol) was hydrolyzed under the conditions described in the general procedure for saponification of esters (0.017 g, 35%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.96 (s, 1H), 7.41–7.28 (m, 2H), 7.17–6.98 (m, 3H), 6.89 (s, 1H), 5.07–4.94 (m, 1H), 4.55 (s, 2H), 4.45–4.33 (m, 1H), 4.27 (dd, J = 11.4, 9.2 Hz, 1H), 3.41 (s, 1H), 3.09–2.84 (m, 2H), 1.64 (s, 3H), 1.61–1.39 (m, 4H), 0.94 (t, J = 7.4 Hz, 3H), 0.86 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.5, 168.7, 143.5, 139.4, 130.8, 129.6, 124.5, 119.0, 115.8, 98.0, 83.8, 76.6, 60.2, 56.1, 43.7, 32.4, 27.2, 26.7, 22.6, 9.9, 9.5. IR (KBr): υ = 1206, 1605, 1680, 1698, 2968, 3435 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{23}H_{32}N_5O_4$ $[M+H]^+$ 442.2448, found 442.2449.

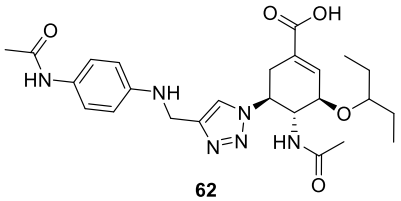
(3*R*,4*R*,5*S*)-4-Acetamido-3-(pentan-3-yloxy)-5-(4-(((3-(trifluoromethyl)phenyl)amino) methyl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylic acid (61)



Ethyl ester **48** (0.082 g, 0.16 mmol) was hydrolyzed under the conditions described in the general procedure for saponification of esters (0.025 g, 31%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.88 (s, 1H), 7.26 (t, J = 7.9 Hz, 1H), 6.95–6.82 (m, 4H), 5.00 (m, 1H), 4.43 (s, 2H), 4.41–4.34 (m, 1H), 4.23 (dd, J = 11.8, 8.9 Hz, 1H), 3.40 (p, J = 5.6 Hz, 1H), 3.06–

2.89 (m, 2H), 1.57 (s, 3H), 1.56–1.44 (m, 4H), 0.93 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.4, 168.8, 149.9, 147.1, 139.4, 132.2 (q, $^{2}J_{C\text{-}F}$ = 31.4 Hz), 130.8, 129.7, 126.0 (q, $^{1}J_{C\text{-}F}$ = 271.6 Hz), 123.3, 116.7, 114.2, 110.3, 83.8, 76.6, 60.0, 56.2, 49.4, 39.8, 32.5, 27.2, 26.7, 22.5, 9.9, 9.5. ^{19}F NMR (375 MHz, CD_3OD) δ −64.34. IR (KBr): υ = 1617, 1660, 1699, 2939, 2969, 3422 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{24}H_{31}N_5O_4F_3$ $[M+H]^+$ 510.2322, found 510.2322.

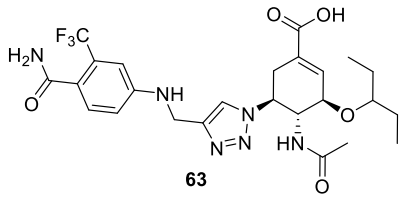
(3*R*,4*R*,5S)-4-Acetamido-5-(4-(((4-acetamidophenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (62)



62

Ethyl ester **49** (0.047 g, 0.89 mmol) was hydrolyzed under the conditions used are described in the general procedure for saponification of esters (0.025 g, 57%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.97 (s, 1H), 7.52 (d, J = 8.9 Hz, 2H), 7.02 (d, J = 8.7 Hz, 2H), 6.89 (s, 1H), 4.98 (dt, J = 10.8, 5.2 Hz, 1H), 4.55 (s, 2H), 4.37 (d, J = 8.8 Hz, 1H), 4.27 (dd, J = 11.4, 9.1 Hz, 1H), 3.41 (p, J = 5.6 Hz, 1H), 3.05–2.82 (m, 2H), 2.10 (s, 3H), 1.65 (s, 3H), 1.62–1.39 (m, 4H), 0.94 (t, J = 7.4 Hz, 3H), 0.86 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 170.5, 168.6, 165.8, 139.9, 136.4, 135.2, 133.4, 126.6, 121.8, 119.7, 116.9, 80.8, 73.7, 57.3, 53.1, 41.2, 29.5, 24.3, 23.7, 20.7, 19.7, 7.0, 6.6. IR (KBr): υ = 1515, 1548, 1637, 2879, 2968, 3437 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{25}H_{33}N_6O_5$ $[M+H]^+$ 497.2517, found 497.2509.

(3*R*,4*R*,5*S*)-4-Acetamido-5-(4-(((4-carbamoyl-3-(trifluoromethyl)phenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (63)

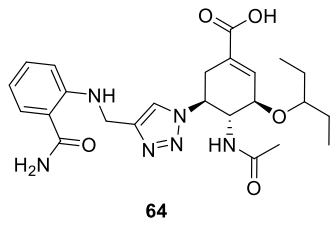


63

Ethyl ester **50** (0.070 g, 0.12 mmol) was hydrolyzed using the conditions described in the general procedure for saponification of esters (0.021 g, 30%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.94 (s, 1H), 7.71 (dd, J = 8.1, 0.9 Hz, 1H), 7.00 (d, J = 1.7 Hz, 1H), 6.94–6.78 (m, 2H), 5.11–4.98 (m, 1H), 4.52 (s, 2H), 4.46–4.37 (m, 1H), 4.23 (dd, J = 11.8, 8.9 Hz, 1H), 3.40 (p, J = 5.6 Hz, 1H), 3.08–2.88 (m, 2H), 1.62 (s, 3H), 1.57–1.40 (m, 4H), 0.93 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.4, 168.8, 150.4, 146.4, 139.5, 135.3 (q, J = 31.8 Hz), 130.9, 129.7, 125.4 (q, J = 271.9 Hz) 123.5, 119.3, 112.4, 112.3, 109.0, 108.9, 83.8, 76.5, 60.0, 56.2, 39.3, 32.5, 27.2, 26.7, 22.5, 9.9, 9.5.

^{19}F NMR (375 MHz, CD_3OD) δ −66.48. IR (KBr): υ = 1129, 1527, 1586, 1657, 2942, 3432 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{25}H_{31}N_6O_5F_3Na$ $[M+Na]^+$ 575.2200, found 575.2199.

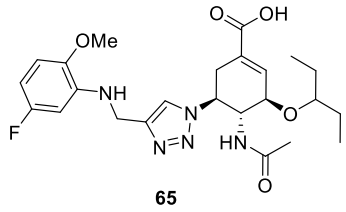
(3*R*,4*R*,5*S*)-4-Acetamido-5-(4-(((2-carbamoylphenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (64)



64

Ethyl ester **51** (0.028 g, 0.05 mmol) was hydrolyzed using the conditions described in the general procedure for saponification of esters (0.015 g, 57%). ^{1}H NMR (400 MHz, CD_3OD) *δ* 7.91 (s, 1H), 7.60 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.40–7.27 (m, 1H), 6.88 (q, *J* = 1.9 Hz, 1H), 6.82 (dd, *J* = 8.4, 1.1 Hz, 1H), 6.70 (t, *J* = 8.2, 7.3, 1.1 Hz, 1H), 5.08–4.96 (m, 1H), 4.51 (s, 2H), 4.41 (dd, *J* = 8.0, 2.5 Hz, 1H), 4.20 (dd, *J* = 11.8, 8.9 Hz, 1H), 3.40 (p, *J* = 5.6 Hz, 1H), 2.99 (dt, *J* = 9.0, 2.4 Hz, 2H), 1.61 (s, 3H), 1.59–1.40 (m, 5H), 0.93 (t, *J* = 7.4 Hz, 3H), 0.85 (t, *J* = 7.4 Hz, 4H). ^{13}C NMR (100 MHz, CD_3OD) *δ* 174.4, 173.4, 168.8, 149.5, 146.6, 139.4, 134.2, 130.1, 129.7, 129.5, 129.2, 123.7, 117.6, 116.9, 113.8, 83.7, 76.5, 59.9, 56.4, 47.8, 40.0, 32.4, 28.1, 27.2, 26.7, 22.6, 9.9, 9.5. IR (KBr): υ = 1649, 1698, 2878, 2939, 2968, 3435 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{24}H_{31}N_6O_5$ $[M-H]^-$ 483.2361, found 483.2352.

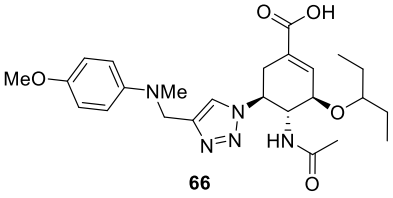
(3*R*,4*R*,5*S*)-4-Acetamido-5-(4-(((5-fluoro-2-methoxyphenyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (65)



65

Ethyl ester **52** (0.056 g, 0.11 mmol) was hydrolyzed using the conditions described in the general procedure for saponification of esters (0.026 g, 50%). ^{1}H NMR (400 MHz, CD_3OD) *δ* 7.88 (s, 1H), 6.88 (t, *J* = 2.0 Hz, 1H), 6.79 (dd, *J* = 8.7, 5.0 Hz, 1H), 6.49–6.31 (m, 2H), 5.01 (ddd, *J* = 11.7, 9.6, 6.9 Hz, 1H), 4.45 (s, 2H), 4.39 (dd, *J* = 8.9, 2.6 Hz, 1H), 4.21 (dd, *J* = 11.8, 9.0 Hz, 1H), 3.83 (s, 3H), 3.39 (q, *J* = 5.6 Hz, 1H), 3.04–2.90 (m, 2H), 1.61 (s, 3H), 1.58–1.44 (m, 4H), 0.93 (t, *J* = 7.4 Hz, 3H), 0.85 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) *δ* 173.4, 168.8, 159.3 (d, $^1J_{C-F}$ = 234.7), 146.1, 145.5, 139.4, 138.4, 129.7, 123.8, 111.6 (d, $^3J_{C-F}$ = 9.8 Hz), 104.2 (d, $^2J_{C-F}$ = 22.9 Hz), 100.4 (d, $^2J_{C-F}$ = 28.4 Hz), 83.8, 76.5, 60.0, 56.6, 56.3, 40.4, 32.4, 27.2, 26.7, 22.5, 9.9, 9.5. ^{19}F NMR (375 MHz, CD_3OD) δ −123.79. IR (KBr): *v* = 1218, 1522, 1682, 2879, 2938, 2967 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{24}H_{32}O_5N_5FNa$ $[M+Na]^+$ 512.2279, found 512.2279.

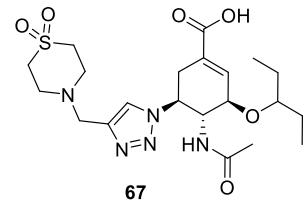
(3*R*,4*R*,5*S*)-4-Acetamido-5-(4-(((4-methoxyphenyl)(methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (66)



66

Ethyl ester **53** (0.072 g, 0.14 mmol) was hydrolyzed under the conditions described in the general procedure for saponification of esters (0.019 g, 28%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.97 (s, 1H), 7.30 (d, J = 9,0 Hz, 2H), 7.02 (d, J = 9.0 Hz, 2H), 6.90 (t, J = 1.7 Hz, 1H), 4.98 (td, J = 11.1, 5.9 Hz, 1H), 4.78 (s, 2H), 4.40–4.21 (m, 2H), 3.82 (s, 3H), 3.43 (p, J = 5.6 Hz, 1H), 3.08–2.79 (m, 2H), 1.68 (s, 3H), 1.61–1.42 (m, 4H), 0.91 (dt, J = 32.7, 7.4 Hz, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.3, 168.7, 160.3, 139.7, 139.4, 136.8, 129.5, 126.1, 122.3, 116.3, 83.8, 76.7, 60.4, 56.2, 55.8, 54.3, 43.7, 32.4, 27.3, 26.7, 22.7, 9.9, 9.5. IR (KBr): v = 1202, 1515, 1675, 1698, 2967, 3432 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{25}H_{34}N_5O_5$ $[M-H]^-$ 484.2565, found 484.2559.

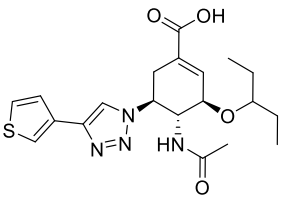
(3*S*,4*S*,5*R*)-4-Acetamido-5-(4-((1,1-dioxidothiomorpholino)methyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (67)



67

Ethyl ester **54** (0.037 g, 0.07 mmol) was hydrolyzed under the conditions described in the general procedure for saponification of esters (0.019 g, 55%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (s, 1H), 7.92 (d, J = 9.0 Hz, 2H), 6.71 (s, 1H), 4.84 (td, J = 11.1, 5.9 Hz, 1H), 4.28 (d, J = 8.2 Hz, 1H), 4.25–4.16 (m, 1H), 3.73 (s, 1H), 3.08 (s, 4H), 2.83 (s, 6H), 1.55 (s, 3H), 1.49–1.30 (m, 4H), 0.86 (t, J = 7.4 Hz, 3H), 0.76 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.5, 167.3, 143.0, 138.4, 128.9, 123.4, 81.7, 75.6, 58.7, 54.0, 51.4, 51.1, 50.2, 31.6, 26.1, 25.5, 22.9, 9.9, 9.3. IR (KBr): υ 1134, 1676, 1698, 2938, 2969, 3454 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{24}H_{28}N_6O_5$ $[M+H]^+$ 482.2045, found 482.2053.

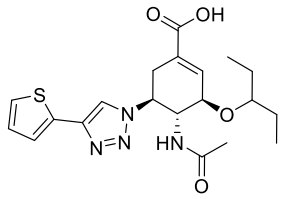
(3*R*,4*R*,5*S*)-4-Acetamido-3-(pentan-3-yloxy)-5-(4-(thiophen-3-yl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylic acid (68)



Ethyl ester **55** (0.083 g, 0.18 mmol) was hydrolyzed under the described in the general procedure for saponification of esters (0.020 g, 26%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.29 (s, 1H), 7.74 (dd, J = 2.9, 1.4 Hz, 1H), 7.59–7.39 (m, 2H), 6.92 (s, 1H), 5.17–4.97 (m, 1H), 4.49–4.38 (m, 1H), 4.33 (dd, J = 11.8, 8.9 Hz, 1H), 3.43 (p, J = 5.6 Hz, 1H), 3.15–2.91 (m, 2H), 1.71

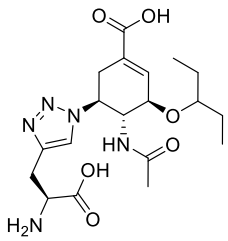
(s, 3H), 1.63–1.43 (m, 4H), 0.95 (t, J = 7.4 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.4, 168.8, 144.9, 139.4, 132.8, 129.7, 127.6, 126.7, 122.2, 121.1, 83.8, 76.6, 60.2, 56.2, 32.4, 27.3, 26.7, 22.6, 9.9, 9.5. IR (KBr): υ = 1657, 1701, 2878, 2937, 2967, 3435 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{20}H_{26}N_4O_4SNa$ $[M+Na]^+$ 441.1567, found 441.1567.

(3*R*,4*R*,5*S*)-4-Acetamido-3-(pentan-3-yloxy)-5-(4-(thiophen-2-yl)-1*H*-1,2,3-triazol-1-yl)cyclohex-1-ene-1-carboxylic acid (69)



Ethyl ester **56** (0.037 g, 0.08 mmol) was hydrolyzed under the conditions described in the general procedure for saponification of esters (0.014 g, 40%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.29 (s, 1H), 7.47–7.34 (m, 2H), 7.10 (dd, J = 4.9, 3.7 Hz, 1H), 6.92 (q, J = 1.9 Hz, 1H), 5.15–4.99 (m, 1H), 4.44 (dd, J = 8.8, 2.5 Hz, 1H), 4.32 (dd, J = 11.7, 8.8 Hz, 1H), 3.43 (p, J = 5.7 Hz, 1H), 3.10–2.98 (m, 2H), 1.73 (s, 3H), 1.64–1.44 (m, 4H), 0.95 (t, J = 7.4 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.4, 168.8, 143.8, 139.4, 139.4, 133.7, 129.7, 128.7, 126.3, 125.6, 120.8, 83.9, 76.6, 60.2, 56.2, 32.3, 27.3, 26.7, 22.6, 9.9, 9.5. IR (KBr): υ = 1212, 1660, 1702, 2878, 2939, 2966 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{20}H_{26}N_4O_4NaS$ $[M+Na]^+$ 441.1567, found 441.1567.

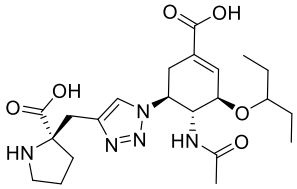
(3*R*,4*R*,5*S*)-4-Acetamido-5-(4-((*S*)-2-amino-2-carboxyethyl)-1*H*-1,2,3-triazol-1-yl)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid (70)



Ethyl ester **57** (0.093 g, 16 mmol) was dissolved in DCM (2 mL) and TFA (2 mL). Deprotection was monitored by HPLC. After 3 h the reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in 1,4-dioxane/methanol 1:1 (3 mL) and an aqueous solution of NaOH (2 equivalents, 0.125 M) was added. The reaction was monitored by HPLC. Once the reaction was complete, the pH of the reaction was adjusted to neutral by addition of Amberlite IR 120. The Amberlite beads were filtered out and the collected beads were rinsed with MeOH several times, the filtrate was then evaporated to dryness under reduced pressure to afford the desired free acid. The residue was purified by preparative HPLC (0.015 g, 22% over two steps). ^{1}H NMR (400 MHz, CD_3OD) δ 8.03 (s, 1H), 6.90 (d, J = 2.1 Hz, 1H), 4.92 (s, 1H), 4.34 (m, 3H), 3.44 (s, 1H), 3.37 (d, J = 5.6 Hz, 2H), 3.02 (s, 2H), 1.75 (s, 3H), 1.62–1.38 (m, 4H), 0.94 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.9, 170.8, 168.8, 139.3, 129.6, 124.4, 84.0, 76.7, 60.7, 56.3, 53.8, 31.8, 30.7,

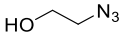
27.4, 27.2, 26.8, 22.6, 9.9, 9.6. HRMS (ESI) *m/z* calcd for $C_{19}H_{28}N_5O_6$ $[M-H]^-$ 422.2045, found 422.2050.

(*R*)-2-((1-((1*S*,5*R*,6*R*)-6-Acetamido-3-carboxy-5-(pentan-3-yloxy)cyclohex-3-en-1-yl)-1*H*-1,2,3-triazol-4-yl)methyl)pyrrolidine-2-carboxylic acid (71)



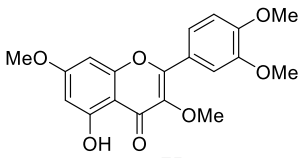
To a solution of ethyl ester **58** (0.088 g, 0.15 mmol) in 1,4-dioxane (4 mL) was added an aqueous solution of NaOH (2 equivalents, 0.125 M), the reaction mixture was stirred from 3 h at room temperature. The reaction was monitored by HPLC. Once the reaction was complete, the pH of the reaction was adjusted to neutral by addition of Amberlite IR 120. The Amberlite beads were filtered out and the collected beads were rinsed with MeOH several times, the filtrate was then evaporated to dryness under reduced pressure to afford the desired free acid. Boc-protected carboxylic acid was dissolved in DCM (2 mL) and TFA (2 mL). Deprotection was monitored by HPLC. After one hour the reaction mixture was evaporated to dryness under reduced pressure. The residue was purified by preparative HPLC (0.020 g, 25% over two steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.80 (s, 1H), 7.97 (s, 1H), 6.73 (s, 1H), 4.98–4.79 (m, 1H), 4.30 (d, J = 5.0, 2.4 Hz, 1H), 4.21 (q, J = 11.6, 8.9 Hz, 1H), 3.20 (d, J = 15.5 Hz, 2H), 2.91–2.77 (m, 2H), 2.38–2.26 (m, 1H), 2.17–1.76 (m, 3H), 1.58 (s, 3H), 1.50–1.30 (m, 4H), 0.87 (t, J = 7.4 Hz, 3H), 0.77 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.3, 169.2, 166.8, 139.9, 137.9, 128.3, 122.6, 81.2, 75.0, 71.1, 58.3, 58.2, 53.3, 45.6, 34.3, 31.3, 30.6, 25.6, 25.0, 22.4, 22.1, 9.4, 8.7. IR (KBr): ν = 1199, 1663, 1699, 2880, 2940, 3074 cm^{-1}. HRMS (ESI) *m/z* calcd for $C_{22}H_{32}N_5O_6$ $[M-H]^-$ 462.2358, found 462.2353.

2-Azidoethan-1-ol (72)



2-Bromoethan-1-ol (0.50 g, 4.00 mmol) and sodium azide (0.39 g, 6.00 mmol) were dissolved in water (10 mL) and the reaction mixture was stirred for 2 h at 100 °C. The reaction mixture was extracted with DCM (3 × 10 mL) and the combined organic phase was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (DCM/MeOH 20:1) to afford product (0.35 g, 98%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.77 (q, J = 5.2 Hz, 2H), 3.45 – 3.42 (m, 2H), 2.2 – 2.0 (m, 1H).

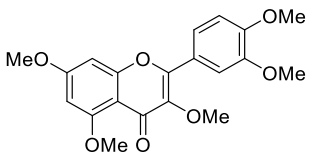
2-(3,4-Dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4*H*-chromen-4-one (75)



75

Methyl iodide (1.00 g, 7.10 mmol) was added to a solution of quercetin **18** (0.30 g, 1.00 mmol) and potassium carbonate (0.83 g, 7.20 mmol) in DMF (2.5 mL). The reaction mixture was stirred at 35 °C overnight under inert atmosphere. The reaction mixture was quenched by addition of 5% HCl aqueous solution (30 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (20 mL), dried over $MgSO_4$ and evaporate to dryness under reduced pressure. The residue was purified by column chromatography (EtOAc). Subsequent crystallization (ethanol) afforded yellow solid (0.035 g, 9%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.67 (s, 1H), 7.76 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.72 (d, *J* = 2.1 Hz, 1H), 7.02 (d, *J* = 8.6 Hz, 1H), 6.47 (d, *J* = 2.3 Hz, 1H), 6.39 (d, *J* = 2.2 Hz, 1H), 3.97 (s, 3H), 3.96 (s, 3H), 3.88 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.9, 165.6, 162.2, 156.9, 156.0, 151.5, 148.9, 139.1, 123.1, 122.3, 111.4, 111.0, 106.2, 98.0, 92.4, 60.4, 56.2, 56.1, 56.0. HRMS (ESI) *m/z* calcd for $C_{19}H_{19}O_7$ $[M+H]^+$ 359.1125, found 359.1124.

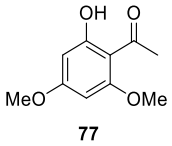
2-(3,4-Dimethoxyphenyl)-3,5,7-trimethoxy-4*H*-chromen-4-one (76)



76

Methyl iodide (1.80 g, 12.70 mmol) was added to a solution of quercetin **18** (0.30, 1.00 mmol) and potassium carbonate (1.00 g, 7.20 mmol) in DMF (6 mL). The reaction mixture was stirred at 40 °C overnight under inert atmosphere. The reaction mixture was quenched by addition of aqueous solution of HCl 5% (30 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$ and evaporate to dryness under reduced pressure. The residue was purified by column chromatography (EtOAc). Subsequent crystallization (ethanol) afforded yellow solid (0.050 g, 14%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 – 7.69 (m, 2H), 7.00 (d, *J* = 8.9 Hz, 1H), 6.53 (d, *J* = 2.2 Hz, 1H), 6.37 (d, *J* = 2.3 Hz, 1H), 3.99 (s, 9H), 3.93 (s, 3H), 3.90 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 164.0, 161.1, 158.9, 152.7, 150.9, 148.8, 141.3, 123.5, 121.7, 111.4, 110.9, 109.6, 95.9, 92.6, 60.1, 56.5, 56.2, 56.1, 55.9. HRMS (ESI) *m/z* calcd for $C_{20}H_{21}O_7$ $[M+H]^+$ 373.1281, found 373.1280.

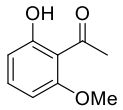
1-(2-Hydroxy-4,6-dimethoxyphenyl)ethan-1-one (**77**)



77

1-(2,4,6-Trihydroxyphenyl)ethan-1-one (0.50 g, 3.00 mmol), potassium carbonate (0.86 g, 6.25 mmol) were suspended in dry acetone (7 mL) and dimethyl sulfate (0.60 mL, 6.00 mmol) was added. The reaction mixture was refluxed for 4 h under inert atmosphere. The reaction mixture was quenched by addition of 5% HCl aqueous solution (40 mL) and extracted with diethyl ether (3 × 20 mL). The combined organic layers were washed with brine (20 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (DCM) to furnished the alcohol derivative **77** (0.56 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 14.03 (s, 1H), 6.06 (d, *J* = 2.4 Hz, 1H), 5.92 (d, *J* = 2.4 Hz, 1H), 3.85 (s, 3H), 3.82 (s, 3H), 2.61 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.3, 167.8, 166.2, 163.1, 106.2, 93.6, 90.9, 55.7(2x), 33.1. HRMS (EI) *m/z* calcd for $C_{10}H_{12}O_4$ $[M]^{+\bullet}$ 196.0736, found 196.0734.

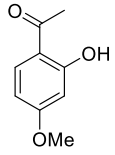
1-(2-Hydroxy-6-methoxyphenyl)ethan-1-one (**78**)



78

1-(2,6-Dihydroxyphenyl)ethan-1-one (1.00 g, 6.57 mmol) was dissolved in acetone (14 mL) and potassium carbonate (2.73 g, 7.23 mmol) and dimethyl sulfate (0.70 mL, 7.23 mmol) were added. The reaction was refluxed for 2 h under inert atmosphere. The reaction mixture was quenched by addition of 2% HCl (50 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layer was extracted with brine (20 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (DCM) to obtain the alcohol derivative **78** (1.05 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.25 (s, 1H), 7.34 (t, *J* = 8.4 Hz, 1H), 6.57 (dd, *J* = 8.4, 1.0 Hz, 1H), 6.39 (dd, *J* = 8.3, 1.0 Hz, 1H), 3.90 (s, 3H), 2.67 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.3, 164.8, 161.7, 136.2, 111.4, 110.9, 101.2, 55.8, 33.8. HRMS (EI) *m/z* calcd for $C_9H_{10}O_3$ $[M]^{+\bullet}$ 166.0630, found 166.0627.

1-(2-Hydroxy-4-methoxyphenyl)ethan-1-one (**79**)



79

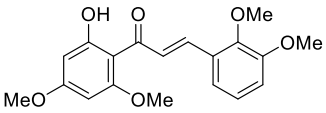
1-(2,4-Dihydroxyphenyl)ethan-1-one (2.00 g, 13.10 mmol), potassium carbonate (5.45 g, 39.40 mmol) were suspended in acetone (26 mL) and dimethyl sulfate (1.37 mL, 14.50 mmol) was added. The reaction mixture was refluxed for 1 h. The reaction mixture was quenched by addition of 5% HCl aqueous solution (40 mL) and extracted with diethyl ether (3 × 20 mL). The combined organic layers were washed with brine (20 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The

residue was purified by column chromatography (DCM) to furnish the alcohol derivative **79** (1.60 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.75 (s, 1H), 7.63 (d, *J* = 8.7 Hz, 1H), 6.51 – 6.38 (m, 2H), 3.84 (s, 3H), 2.55 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.7, 166.2, 165.4, 132.4, 114.0, 107.8, 101.0, 55.7, 26.4. HRMS (EI) *m/z* calcd for $C_9H_{10}O_3$ $[M]^{+\bullet}$ 166.0630, found 166.0627.

General procedure I: preparation of chalcone

To a solution of aldehyde (1.2-1.4 eq.) and ketone (1 eq.) in ethanol (7 mL) was added potassium hydroxide (3 eq.) at 0 °C and the reaction mixture was stirred overnight at room temperature under inert atmosphere. The reaction mixture was acidified by addition of aqueous solution of hydrogen chloride (5%) and extracted with DCM (3 × 12 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The products were purified by column chromatography and crystalized if needed.

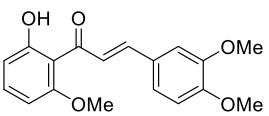
(*E*)-3-(2,3-Dimethoxyphenyl)-1-(2-hydroxy-4,6-dimethoxyphenyl)prop-2-en-1-one (84)



84

The title compound was synthesized from 1-(2-hydroxy-4,6-dimethoxyphenyl)ethan-1-one (0.20 g, 1.00 mmol) and 2,3-dimethoxybenzaldehyde (0.20 g, 1.20 mmol) following the general procedure I. The product was purified by column chromatography (cyclohexane/EtOAc 2:1) to furnish the chalcone **84** (0.26 g, 75%). 1H NMR (400 MHz, CDCl3) δ 12.91 (s, 1H), 6.66 (d, *J* = 15.8 Hz, 1H), 6.54 (d, *J* = 15.8 Hz, 1H), 5.81 (dd, *J* = 8.0, 1.4 Hz, 1H), 5.66 (t, *J* = 8.0 Hz, 1H), 5.53 (dd, *J* = 8.1, 1.5 Hz, 1H), 4.69 (d, *J* = 2.3 Hz, 1H), 4.54 (d, *J* = 2.4 Hz, 1H), 2.49 (s, 3H), 2.47 (s, 6H), 2.42 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.0, 168.5, 166.3, 162.7, 153.4, 149.0, 137.3, 129.9, 129.0, 124.3, 119.9, 113.8, 106.6, 93.9, 91.4, 61.4, 56.0, 56.0, 55.7. HRMS (ESI) *m/z* calcd for $C_{19}H_{21}O_6$ $[M+H]^+$ 345.1332, found 345.1333.

(*E*)-3-(3,4-Dimethoxyphenyl)-1-(2-hydroxy-6-methoxyphenyl)prop-2-en-1-one (**85**).

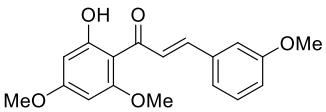


85

The title compound was synthesized from 1-(2-hydroxy-6-methoxyphenyl)ethan-1-one (0.50 g, 3.00 mmol) and 3,4-dimethoxybenzaldehyde (0.70 g, 4.20 mmol) following the general procedure I. The product was purified by column chromatography (cyclohexane/EtOAc 2:1)

and crystallized from ethanol to furnish the chalcone **85** (0.70 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.20 (s, 1H), 7.88 – 7.68 (m, 2H), 7.35 (t, *J* = 8.3 Hz, 1H), 7.24 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.14 (d, *J* = 2.0 Hz, 1H), 6.91 (d, *J* = 8.3 Hz, 1H), 6.62 (dd, *J* = 8.4, 1.0 Hz, 1H), 6.43 (dd, *J* = 8.3, 1.0 Hz, 1H), 3.95 (s, 3H), 3.94 (s, 3H), 3.94 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.3, 165.0, 161.0, 151.4, 149.3, 143.4, 135.8, 128.5, 125.6, 123.0, 112.2, 111.3, 111.2, 110.6, 101.7, 56.2, 56.1, 56.0. HRMS (ESI) *m/z* calcd for $C_{18}H_{18}O_5Na$ $[M+Na]^+$ 337.1046, found 337.1047.

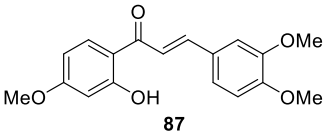
(*E*)-1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (**86**).



86

The title compound was synthesized from 1-(2-hydroxy-4,6-dimethoxyphenyl)ethan-1-one (0.32 g, 1.60 mmol) and 3-methoxybenzaldehyde (0.285 g, 2.10 mmol) following the general procedure I. The product was purified by column chromatography (toluene) to obtain the chalcone **86** (0.20 g, 38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 14.27 (s, 1H), 7.88 (d, *J* = 15.6 Hz, 1H), 7.74 (d, *J* = 15.6 Hz, 1H), 7.32 (t, *J* = 7.9 Hz, 1H), 7.23 – 7.19 (m, 1H), 7.12 (dd, *J* = 2.6, 1.6 Hz, 1H), 6.94 (ddd, *J* = 8.2, 2.6, 1.0 Hz, 1H), 6.11 (d, *J* = 2.4 Hz, 1H), 5.96 (d, *J* = 2.4 Hz, 1H), 3.92 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.7, 168.5, 166.4, 162.6, 160.0, 142.3, 137.1, 130.0, 128.0, 121.1, 115.7, 113.8, 106.5, 93.9, 91.4, 56.0, 55.8, 55.4. HRMS (ESI) *m/z* calcd for $C_{18}H_{19}O_5$ $[M+H]^+$ 315.1227, found 315.1228.

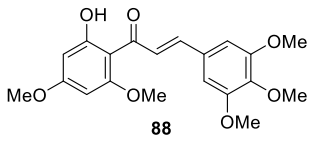
(*E*)-3-(3,4-Dimethoxyphenyl)-1-(2-hydroxy-4-methoxyphenyl)prop-2-en-1-one (**87**)



87

The title compound was synthesized from 1-(2-hydroxy-4-methoxyphenyl)ethan-1-one (0.75 g, 4.50 mmol) and 3,4-dimethoxybenzaldehyde (0.97 g. 5.90 mmol) following the general procedure I. The product was purified by column chromatography (cyclohexane/EtOAc 2:1) and crystallized from ethanol to furnish the chalcone **87** (0.18 g, 13%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.53 (s, 1H), 7.89 – 7.85 (m, 1H), 7.83 (s, 1H), 7.44 (dd, *J* = 15.3, 0.5 Hz, 1H), 7.26 (dd, *J* = 8.2, 2.2 Hz, 1H), 7.16 (d, *J* = 2.0 Hz, 1H), 6.91 (d, *J* = 8.3 Hz, 1H), 6.52 – 6.46 (m, 2H), 3.97 (s, 3H), 3.94 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 166.8, 166.2, 151.7, 149.4, 144.7, 131.3, 127.9, 123.5, 118.2, 114.3, 111.3, 110.4, 107.8, 101.2, 56.2, 56.2, 55.7. HRMS (ESI) *m/z* calcd for $C_{18}H_{18}O_5Na$ $[M+Na]^+$ 337.1046, found 337.1047.

(*E*)-1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (88)



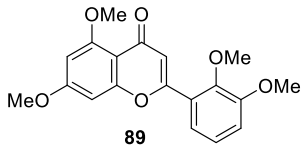
88

The title compound was synthesized from 3,4,5-trimethoxybenzaldehyde (0.57 g, 2.90 mmol) and 1-(2-hydroxy-4,6-dimethoxyphenyl)ethan-1-one (0.48 g, 2.40 mmol) following the general procedure I. The product was purified by column chromatography (cyclohexane/EtOAc from 3:1 to 1:1) to furnish the chalcone **88** (0.35 g, 38%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.32 (s, 1H), 7.81 (d, *J* = 15.5 Hz, 1H), 7.75 – 7.66 (m, 1H), 6.84 (d, *J* = 0.4 Hz, 2H), 6.12 (d, *J* = 2.4 Hz, 1H), 5.97 (d, *J* = 2.4 Hz, 1H), 3.91 (s, 6H), 3.91 (s, 3H), 3.90 (s, 3H), 3.84 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.5, 168.6, 166.3, 162.5, 153.5, 142.5, 131.3, 127.1, 106.4, 105.7, 100.1, 94.0, 91.5, 61.2, 56.3, 55.9, 55.8. HRMS (ESI) *m/z* calcd for $C_{20}H_{22}O_7Na$ $[M+Na]^+$ 397.1257, found 397.1258.

General procedure II: cyclization of chalcones

To a solution of chalcone (1.0 eq.) in dry 1,4-dioxane (0.1 M) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (2.0 eq.). The solution was stirred in a sealed tube at 80 °C for 12 h. The reaction mixture was quenched by addition of water or saturated sodium bicarbonate solution (10 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The products were purified by column chromatography.

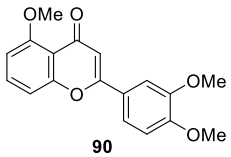
2-(2,3-Dimethoxyphenyl)-5,7-dimethoxy-4*H*-chromen-4-one (89).



89

Compound **84** (0.061 g, 0.17 mmol) was cyclized using the general procedure II. The column chromatography (DCM/MeOH 20:1) of the residue furnished the flavone **89** (0.058 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.17 (t, *J* = 8.0 Hz, 1H), 7.05 (dd, *J* = 8.2, 1.6 Hz, 1H), 6.85 (s, 1H), 6.52 (d, *J* = 2.3 Hz, 1H), 6.38 (d, *J* = 2.3 Hz, 1H), 3.95 (s, 3H), 3.92 (s, 3H), 3.90 (s, 3H), 3.88 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.2, 164.3, 161.1, 160.3, 159.4, 153.5, 127.7, 126.4, 124.3, 120.8, 115.0, 113.7, 96.3, 92.9, 67.2, 61.1, 56.5, 56.2, 55.9. HRMS (ESI) *m/z* calcd for $C_{19}H_{19}O_6$ $[M+H]^+$ 343.1176, found 343.1178.

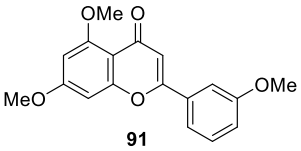
2-(3,4-Dimethoxyphenyl)-5-methoxy-4*H*-chromen-4-one (**90**).



90

Compound **85** (0.20 g, 0.63 mmol) was cyclized using the general procedure II. The column chromatography (DCM/MeOH 20:1) of the residue furnished the flavone **90** (0.13 g, 66%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.47 (m, 2H), 7.32 (d, *J* = 2.1 Hz, 1H), 7.11 (dd, *J* = 8.4, 1.0 Hz, 1H), 6.95 (d, *J* = 8.5 Hz, 1H), 6.80 (dd, *J* = 8.3, 0.9 Hz, 1H), 6.65 (s, 1H), 3.98 (s, 3H), 3.95 (s, 3H), 3.93 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.4, 161.1, 159.8, 158.3, 151.9, 149.3, 133.7, 124.0, 119.8, 114.6, 111.2, 110.2, 108.7, 108.1, 106.5, 56.6, 56.2 (2x). HRMS (ESI) *m/z* calcd for $C_{18}H_{17}O_5$ $[M+H]^+$ 313.1070, found 313.1071.

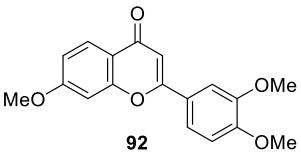
5,7-Dimethoxy-2-(3-methoxyphenyl)-4*H*-chromen-4-one (**91**).



91

Compound **86** (0.10 g, 0.32 mmol) was cyclized using the general procedure II. The column chromatography (DCM/MeOH 20:1) of the residue furnished the flavone **91** (0.095 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (dt, *J* = 7.8, 1.3 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.37 (dd, *J* = 2.5, 1.5 Hz, 1H), 7.03 (ddd, *J* = 8.0, 2.6, 1.1 Hz, 1H), 6.66 (s, 1H), 6.56 (d, *J* = 2.3 Hz, 1H), 6.37 (d, *J* = 2.3 Hz, 1H), 3.95 (s, 3H), 3.90 (s, 3H), 3.87 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.8, 164.2, 161.0, 160.6, 160.1, 160.0, 133.0, 130.1, 118.5, 117.0, 111.4, 109.4, 109.4, 96.3, 92.9, 56.6, 55.9, 55.6. HRMS (ESI) *m/z* calcd for $C_{18}H_{16}O_5Na$ $[M+Na]^+$ 335.0889, found 335.0890.

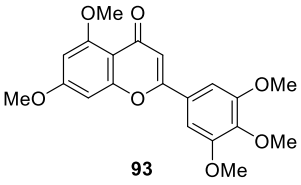
2-(3,4-Dimethoxyphenyl)-7-methoxy-4*H*-chromen-4-one (**92**).



92

Compound **87** (0.18 g, 0.57 mmol) was cyclized using the general procedure II. The column chromatography (cyclohexane/EtOAc 1:1) of the residue furnished the flavone **92** (0.074 g, 41%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (dd, *J* = 8.7, 0.5 Hz, 1H), 7.57 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.40 (d, *J* = 2.2 Hz, 1H), 7.03 (d, *J* = 2.6 Hz, 1H), 7.00 (q, *J* = 2.4 Hz, 2H), 6.73 (s, 1H), 4.01 (s, 3H), 3.99 (s, 3H), 3.97 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.0, 164.2, 163.2, 158.1, 152.1, 149.4, 127.2, 124.5, 120.0, 118.0, 114.4, 111.3, 108.9, 106.6, 100.6, 56.3, 56.2, 56.0. HRMS (ESI) *m/z* calcd for $C_{18}H_{17}O_5$ $[M+H]^+$ 313.1070, found 313.1071.

4.4.5 5,7-Dimethoxy-2-(3,4,5-trimethoxyphenyl)-4*H*-chromen-4-one (93).



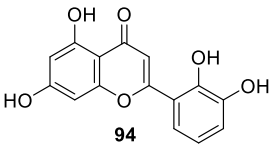
93

Compound **88** (0.37 g, 0.94 mmol) was cyclized using the general procedure II. The column chromatography (EtOAc/MeOH 50:1) of the residue furnished the flavone **93** (0.21 g, 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.07 (s, 2H), 6.63 (s, 1H), 6.56 (d, *J* = 2.2 Hz, 1H), 6.39 (d, *J* = 2.3 Hz, 1H), 3.97 (s, 3H), 3.95 (m, 6H), 3.93 – 3.90 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.8, 164.4, 161.1, 160.9, 160.0, 153.7, 141.1, 126.8, 109.2, 108.8, 103.6, 96.4, 93.0, 61.2, 56.6, 56.5, 56.0. HRMS (ESI) *m/z* calcd for $C_{20}H_{21}O_7$ $[M+H]^+$ 373.1281, found 373.1282.

General procedure III: *O*-demethylation

An aqueous solution of hydrogen bromide (48%; 1 mL per 0.10 mmol of the substrate) was mixed with the flavone. The reaction mixture was refluxed for 6-9 h and during this time the reaction progress was monitored by UPLC-MS. After the disappearance of partially O-demethylated intermediates the reaction mixture was evaporated under reduced pressure.

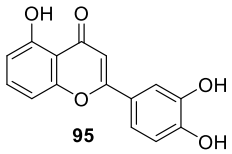
4.5.1 2-(2,3-Dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one (**94**).



94

Compound **94** was obtained from **89** (0.047 g, 0.14 mmol) using the general procedure III. The residue was purified by column chromatography (DCM/MeOH 10:1), and crystallized from chloroform/acetone to furnish the flavone **94** (0.012 g, 30%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.89 (s, 1H), 10.85 (s, 1H), 10.02 (s, 1H), 9.62 (s, 1H), 7.31 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.03 (s, 1H), 6.99 (dd, *J* = 7.8, 1.6 Hz, 1H), 6.82 (t, *J* = 8.0 Hz, 1H), 6.45 (d, *J* = 2.1 Hz, 1H), 6.20 (d, *J* = 2.1 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.9, 164.3, 161.7, 161.4, 157.5, 145.9, 145.5, 119.2, 118.4, 117.8, 117.7, 109.1, 103.7, 98.8, 93.9. HRMS (ESI) *m/z* calcd for $C_{15}H_{10}O_6Na$ $[M+Na]^+$ 309.0369, found 309.0367.

2-(3,4-Dihydroxyphenyl)-5-hydroxy-4*H*-chromen-4-one (95).

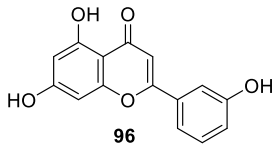


95

Compound **95** was obtained from **90** (0.13 g, 0.42 mmol) using the general procedure III. The residue was purified by column chromatography (DCM/MeOH 100:3) and crystallized from ethanol to obtain the flavone **95**

(0.040 g, 35%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.84 (s, 1H), 10.01 (bs, 1H), 9.44 (bs, 1H), 7.65 (t, J = 8.3 Hz, 1H), 7.48 (dd, J = 8.3, 2.3 Hz, 1H), 7.46 (d, J = 2.3 Hz, 1H), 7.14 (dd, J = 8.4, 0.9 Hz, 1H), 6.91 (d, J = 8.3 Hz, 1H), 6.83 (s, 1H), 6.80 (dd, J = 8.2, 0.9 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 182.9, 164.8, 159.9, 155.8, 150.1, 145.8, 135.7, 121.3, 119.4, 116.0, 113.6, 110.9, 109.9, 107.3, 103.4. HRMS (ESI) *m/z* calcd for $C_{15}H_{11}O_5$ $[M+H]^+$ 271.0601, found 271.0599.

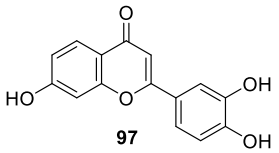
5,7-Dihydroxy-2-(3-hydroxyphenyl)-4*H*-chromen-4-one (96).



96

Compound **96** was obtained from **91** (0.10 g, 0.32 mmol) using the general procedure III. The residue was purified by preparative HPLC to obtain the flavone **96** (0.030 g, 34%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.84 (s, 1H), 10.92 (s, 1H), 9.90 (s, 1H), 7.53 – 7.47 (m, 1H), 7.42 – 7.33 (m, 2H), 7.01 (ddd, J = 8.3, 2.6, 1.0 Hz, 1H), 6.86 (s, 1H), 6.50 (d, J = 2.1 Hz, 1H), 6.23 (d, J = 2.1 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.8, 164.4, 163.3, 161.5, 157.9, 157.4, 132.0, 130.3, 119.1, 117.3, 112.8, 105.1, 104.0, 99.0, 94.0. HRMS (ESI) *m/z* calcd for $C_{15}H_{11}O_5$ $[M+H]^+$ 271.0601, found 271.0599.

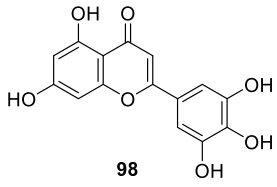
2-(3,4-Dihydroxyphenyl)-7-hydroxy-4*H*-chromen-4-one (97).



97

Compound **97** was obtained from **92** (0.074 g, 0.23 mmol) using the general procedure III. The residue was purified by preparative HPLC to obtain the flavone **97** (0.044 g, 68%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.75 (bs, 1H), 9.80 (bs, 1H), 9.38 (bs, 1H), 7.85 (d, J = 8.6 Hz, 1H), 7.43 – 7.35 (m, 2H), 6.93 – 6.86 (m, 3H), 6.60 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.2, 162.6, 162.5, 157.3, 149.1, 145.7, 126.5, 122.1, 118.5, 116.1, 116.0, 114.8, 113.2, 104.5, 102.4. HRMS (ESI) *m/z* calcd for $C_{15}H_{10}O_5Na$ $[M+Na]^+$ 293.0420, found 293.0418.

5,7-Dihydroxy-2-(3,4,5-trihydroxyphenyl)-4*H*-chromen-4-one (98)

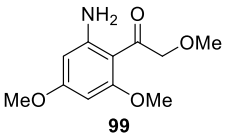


98

Compound **98** was obtained from **93** (0.21 g, 0.56 mmol) using the general procedure III. The residue was purified by preparative HPLC to obtain the flavone **98** (0.060 g, 35%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.96 (s, 2H), 6.51 (s, 1H), 6.39 (d, J = 2.1 Hz, 1H), 6.17 (d, J = 2.1 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 182.0, 164.9, 164.5, 161.9, 157.7, 146.8, 138.5,

120.7, 106.1, 104.0, 103.3, 99.3, 94.2. HRMS (ESI) *m/z* calcd for $C_{15}H_{11}O_7$ $[M+H]^+$ 303.0499, found 303.0498.

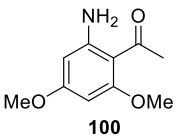
1-(2-Amino-4,6-dimethoxyphenyl)-2-methoxyethan-1-one (99).



99

3,5-Dimethoxyaniline (1.50 g, 9.80 mmol) was dissolved in dry benzene (1.5 mL) at 0 °C and 1M solution of boron trichloride (9.8 mL, 9.80 mmol) was added. The reaction mixture was stirred under nitrogen atmosphere for 1h. 1M solution of titanium tetrachloride (1.27 mL, 1.27 mmol) and acetonitrile (0.7 mL, 13.10 mmol) were added and the reaction mixture was stirred overnight at 80 °C in a sealed tube. The reaction mixture was acidified with an aqueous solution of HCl 2M (20 mL) and refluxed for 1h. The reaction mixture was quenched by addition of an aqueous solution of sodium hydroxide 2M (21 mL) and extracted with DCM (3 × 40 mL). The combined organic layer was washed with brine, dried with $MgSO_4$, evaporated to dryness under reduced pressure. The residue was purified by column chromatography (cyclohexane/EtOAc 1:1) to furnish the amine **99** (0.41 g, 18%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.61 (s, 2H), 5.74 (m, 2H), 4.55 (s, 2H), 3.84 (s, 3H), 3.80 (s, 3H), 3.50 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.7, 164.5, 163.6, 154.4, 103.6, 92.4, 88.3, 79.2, 59.3, 55.4, 55.4.

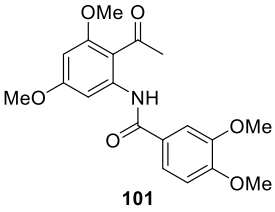
1-(2-Amino-4,6-dimethoxyphenyl)ethan-1-one (100).



100

3,5-Dimethoxyaniline (2.00 g, 13.10 mmol) was dissolved in dry 1,2-dichloroethane (15 mL) at 0 °C and 1M solution of boron trichloride (14 mL, 14.37 mmol) was added. The reaction mixture was stirred under nitrogen atmosphere for 1h. Aluminium chloride (1.91 g, 14.40 mmol) and acetonitrile (0.70 ml, 13.10 mmol) were added and the reaction mixture was stirred overnight at 80 °C in a sealed tube. The reaction mixture was acidified with 2M HCl (20 mL) and refluxed for 1h. The reaction mixture was quenched by addition of 2M sodium hydroxide (21 mL) and extracted with DCM (3 × 40 mL). The combined organic layer was washed with brine, dried with $MgSO_4$, evaporated to dryness under reduced pressure and crystallized from methanol to furnish the aniline **100** (0.87 g, 34%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.35 (bs, 1H), 5.76 (d, J = 2.3 Hz, 1H), 5.71 (d, J = 2.3 Hz, 1H), 3.82 (s, 3H), 3.77 (s, 3H), 2.53 (s, 3H).

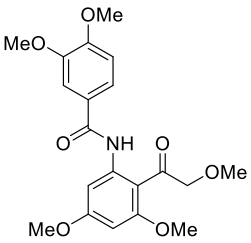
***N*-(2-Acetyl-3,5-dimethoxyphenyl)-3,4-dimethoxybenzamide (101).**



101

To a solution of **100** (0.38 g, 1.97 mmol) and 3,4-dimethoxybenzoyl chloride (0.44 g, 2.17 mmol) in toluene (3 mL) was added *N*,*N*-diisopropylethylamine (1.00 mL, 5.93 mmol) and 4-dimethylaminopyridine (0.024 g, 0.19 mmol). The reaction mixture was stirred overnight at 80 °C in a sealed tube. The reaction was quenched by addition of water and extracted with DCM (3 × 20 mL). The combined organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was crystallized from ethanol to obtain the amide **101** (0.70 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.88 (s, 1H), 8.25 (d, *J* = 2.4 Hz, 1H), 7.66 (dd, *J* = 8.4, 2.2 Hz, 1H), 7.63 (d, *J* = 2.1 Hz, 1H), 6.97 (d, *J* = 8.4 Hz, 1H), 6.22 (d, *J* = 2.4 Hz, 1H), 3.99 (s, 3H), 3.95 (s, 3H), 3.92 (s, 3H), 3.89 (s, 3H), 2.63 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.1, 166.1, 164.9, 162.9, 152.3, 149.2, 144.3, 127.6, 120.5, 110.8, 110.7, 108.7, 96.6, 94.4, 56.2, 56.2, 55.8, 55.7, 34.4. HRMS (ESI) *m/z* calcd for $C_{19}H_{21}O_6NNa$ $[M+Na]^+$ 382.1272, found 382.1267.

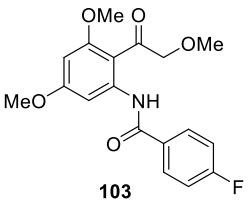
***N*-(3,5-Dimethoxy-2-(2-methoxyacetyl)phenyl)-3,4-dimethoxybenzamide (102).**



102

To a solution of **99** (0.40 g, 1.80 mmol) and 3,4-dimethoxybenzoyl chloride (0.38 g, 1.90 mmol) in DCM (4 mL) was added *N*,*N*-diisopropylethylamine (1.00 mL, 5.93 mmol) and 4-dimethylaminopyridine (0.044 g, 0.36 mmol). The reaction mixture was stirred overnight at 80 °C in a sealed tube. The reaction was quenched by addition of water and extracted with DCM (3 × 20 mL). The combined organic layers were dried over $MgSO_4$ and evaporated to dryness. The residue was crystallized from ethanol to obtain the amide **102** (0.50 g, 67%). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of two rotamers) δ 13.23 (bs, 1H), 8.37 (d, *J* = 2.4 Hz, 1H), 7.75 (dd, *J* = 8.4, 2.2 Hz, 1H), 7.65 (d, *J* = 2.1 Hz, 1H), 6.95 (d, *J* = 8.4 Hz, 1H), 6.21 (d, *J* = 2.4 Hz, 1H), 4.62 (s, 2H), 3.98 (s, 3H), 3.95 (s, 3H), 3.93 (s, 3H), 3.90 (s, 3H), 3.50 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.4, 166.2, 165.5, 163.0, 152.3, 149.1, 145.6, 127.3, 120.6, 110.8, 110.6, 110.5, 105.8, 96.7, 96.7, 94.0, 79.6, 59.3, 59.2, 56.1, 56.1, 56.0, 56.0, 55.8, 55.7, 55.7, 55.6. HRMS (ESI) *m/z* calcd for $C_{20}H_{23}O_7NNa$ $[M+Na]^+$ 412.1377, found 412.1379.

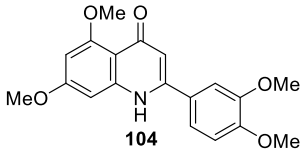
***N*-(3,5-Dimethoxy-2-(2-methoxyacetyl)phenyl)-4-fluorobenzamide (103).**



103

To a solution **99** (0.40 g, 1.78 mmol) and 4-fluorbenzoyl chloride (0.29 g, 1.87 mmol) in DCM (3 mL) was added DIPEA (1.00 mL, 5.90 mmol) and DMAP (0.043 g, 0.35 mmol). The reaction mixture was stirred overnight at 80 °C in a sealed tube. The reaction was quenched by addition of water and extracted with DCM (3 × 20 mL). The combined organic layer was dried with $MgSO_4$ and evaporated to dryness. The residue was crystalized from ethanol to obtain the amide **103** (0.31 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of two rotamers) δ 13.31 (s, 1H), 8.35 (d, *J* = 2.4 Hz, 1H), 8.12 (dd, *J* = 8.9, 5.2 Hz, 2H), 7.17 (dd, *J* = 8.9, 8.4 Hz, 3H), 6.22 (d, *J* = 2.4 Hz, 1H), 4.63 (s, 2H), 3.93 (s, 3H), 3.91 (s, 3H), 3.51 (s, 3H). ^{13}C NMR (100 MHz, CDCl3) δ 200.6, 166.5, 165.7, 164.39 (d, $^1J_{CF}$ = 231.7 Hz), 164.0, 145.6, 131.0 (d, $^4J_{CF}$ = 3.0 Hz), 130.2 (d, $^3J_{CF}$ = 9.0 Hz), 116.0 (d, $^2J_{CF}$ = 21.8 Hz), 105.9, 97.1, 97.1, 94.30(2×), 79.8, 59.3, 59.3, 55.8, 55.8, 55.7, 55.6.

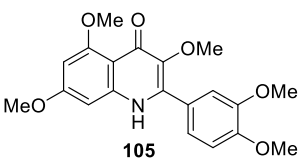
2-(3,4-Dimethoxyphenyl)-5,7-dimethoxyquinolin-4(1*H*)-one (104).



104

Compound **101** (0.074 g, 0.205 mmol) and potassium *tert*-butoxide (0.12 g, 1.03 mmol) were suspended in *tert*-butanol (4 mL) and the reaction mixture was stirred overnight at 80 °C in a sealed tube. The reaction mixture was quenched by addition of water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were extracted with brine (20 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The product was purified by column chromatography (DCM/MeOH 20:1) to furnish the flavone **104** (0.060 g, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 2.1 Hz, 1H), 7.55 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.02 (d, *J* = 2.2 Hz, 1H), 6.98 (s, 1H), 6.95 (d, *J* = 8.4 Hz, 1H), 6.42 (d, *J* = 2.2 Hz, 1H), 4.01 (s, 3H), 4.00 (s, 3H), 3.93 (s, 3H), 3.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.3, 161.2, 157.5, 156.2, 154.6, 150.4, 149.2, 149.2, 119.9, 111.0, 110.2, 106.5, 103.1, 97.0, 91.1, 56.3, 56.0, 56.0, 55.6. HRMS (ESI) *m/z* calcd for $C_{19}H_{20}O_5N$ $[M+H]^+$ 342.1336, found 342.1336.

2-(3,4-Dimethoxyphenyl)-3,5,7-trimethoxyquinolin-4(1*H*)-one (105).

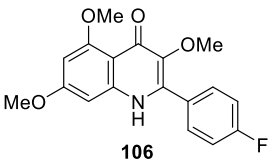


105

Compound **102** (0.20 g, 0.51 mmol) and potassium *terc*-butoxide (0.29 g, 2.60 mmol) were suspended in *tert*-butanol (4 mL). The reaction mixture was stirred overnight in a sealed tube at 80 °C. The reaction mixture was quenched by addition of saturated solution of ammonium chloride (25

mL) and extracted with DCM/propan-2-ol, 5:1 (3 × 15 mL). The combined organic layers were evaporated to dryness under reduced pressure. The residue was purified by column chromatography (DCM/MeOH 20:1) and crystallized from methanol to furnish the flavone **105** (0.15 g, 46%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.95 (bs, 1H), 7.28 – 7.02 (m, 3H), 6.67 (d, J = 2.3 Hz, 1H), 6.26 (d, J = 2.3 Hz, 1H), 3.83 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H), 3.78 (s, 3H), 3.60 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.8, 161.5, 160.8, 149.7, 148.2, 142.8, 139.7, 139.1, 124.3, 121.7, 112.5, 111.4, 111.0, 93.8, 91.2, 59.0, 55.6, 55.6, 55.2, 48.6. HRMS (ESI) *m/z* calcd for $C_{20}H_{22}O_6N$ $[M+H]^+$ 372.1441, found 372.1440.

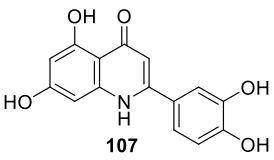
2-(4-Fluorophenyl)-3,5,7-trimethoxyquinolin-4(1*H*)-one (106).



106

Compound **103** (0.31 g, 0.88 mmol) and *t*BuOK (0.50 g, 4.42 mmol) were suspended in *tert*-butanol (3 mL). The reaction mixture was stirred overnight in a sealed tube at 80 °C. The reaction mixture was quenched by addition of saturated solution of NH_4Cl (30 mL) and extracted with DCM/iso-propanol; 5/1 (3 × 15 ml). The combined organic phase was evaporated to dryness under reduced pressure. The residue was purified by column chromatography (DCM/MeOH 20:1) and crystallized from ethanol to furnish the flavone **106** (0.13 g, 46%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.07 (bs, 1H), 7.69 (m, 2H), 7.47 – 7.26 (m, 2H), 6.65 (d, J = 2.3 Hz, 1H), 6.28 (d, J = 2.3 Hz, 1H), 3.80 (s, 3H), 3.78 (s, 3H) 3.59 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.7, 162.6 (d, $^{1}J_{C\text{-}F}$ = 246.8 Hz), 161.6, 160.8, 142.8, 139.7, 138.2, 131.3 (d, $^{3}J_{C\text{-}F}$ = 8.6 Hz), 128.4 (d, $^{4}J_{CF}$ = 3.2 Hz), 115.3 (d, $^{2}J_{C\text{-}F}$ = 21.7 Hz), 111.1, 93.9, 91.2, 59.0, 55.6, 55.2. HRMS (ESI) *m/z* calcd for $C_{18}H_{17}O_4NF$ $[M+H]^+$ 330.1136, found 330.1135.

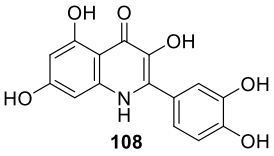
2-(3,4-Dihydroxyphenyl)-5,7-dihydroxyquinolin-4(1*H*)-one (107)



107

Compound **107** was obtained from **104** (0.074 g, 0.23 mmol) using the general procedure III. The residue was purified by preparative HPLC to obtain the flavone **107** (0.026 g, 51%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.55 (s, 1H), 10.20 (s, 1H), 9.35 (s, 1H), 7.15 (d, J = 2.3 Hz, 1H), 7.11 (dd, J = 8.2, 2.3 Hz, 1H), 6.89 (d, J = 8.2 Hz, 1H), 6.50 (d, J = 2.1 Hz, 1H), 6.05 (d, J = 1.7 Hz, 1H), 6.00 (d, J = 2.1 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.3, 162.9, 162.6, 151.6, 148.6, 146.1, 143.1, 125.0, 119.5, 116.3, 115.0, 106.8, 104.0, 97.7, 92.7. HRMS (ESI) *m/z* calcd for $C_{15}H_{12}O_5N$ $[M+H]^+$ 286.0710, found 286.0707.

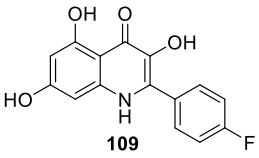
4.5.6 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxyquinolin-4(1*H*)-one (108)



108

Compound **108** was obtained from **105** (0.074 g, 0.23 mmol) using the general procedure III. After complete consumption of the starting material, the reaction mixture was neutralized with aqueous sodium bicarbonate and a precipitate appeared. The precipitate was collected, washed with water and crystallized from methanol to obtain the flavone **108** (0.030 g, 20 %). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 14.11 (s, 1H), 11.31 (s, 1H), 10.04 (s, 1H), 9.40 (s, 1H), 9.24 (s, 1H), 8.24 (s, 1H), 7.20 (d, *J* = 2.2 Hz, 1H), 7.05 (dd, *J* = 8.2, 2.2 Hz, 1H), 6.88 (d, *J* = 8.2 Hz, 1H), 6.44 (d, *J* = 2.1 Hz, 1H), 5.97 (d, *J* = 2.1 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 172.3, 161.2, 146.8, 144.6, 140.0, 134.6, 133.6, 122.8, 120.7, 116.7, 115.3, 105.5, 96.2, 91.1, 39.5. HRMS (ESI) *m/z* calcd for $C_{15}H_{12}O_6N$ $[M+H]^+$ 302.0659, found 302.0660.

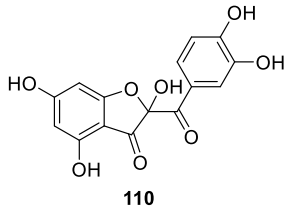
2-(4-Fluorophenyl)-3,5,7-trihydroxyquinolin-4(*1*H)-one (109)



109

Compound **106** (0.12 g, 0.36 mmol) was demethylated using the conditions described in the general procedure for demethylation of flavone. The reaction mixture was neutralized with $NaHCO_3$ and the precipitate appeared. The precipitate was filtered and the solid residue was purified using column chromatography (Cyclohexane/EtOAc 1:1). Crystallization from MeOH furnished the azaflavone derivative **109** (0.025 g, 24%). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 14.03 (s, 1H), 11.51 (s, 1H), 10.12 (s, 1H), 8.52 (s, 1H), 7.79 (ddd, *J* = 8.6, 5.4, 2.6 Hz, 2H), 7.44 – 7.33 (m, 2H), 6.41 (d, *J* = 2.1 Hz, 1H), 5.99 (d, *J* = 2.1 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 163.9, 161.9 (d, $^{1}J_{CF}$ = 247.0 Hz), 161.9, 161.7, 140.6, 135.5, 132.6, 132.1 (d, $^{3}J_{CF}$ = 8.6 Hz), 128.7 (d, $^{4}J_{CF}$ = 3.1 Hz), 115.7 (d, $^{2}J_{CF}$ = 21.7 Hz), 106.3, 96.9, 91.5. HRMS (ESI) *m/z* calcd for $C_{15}H_{11}O_4NF$ $[M+H]^+$ 288.0666, found 288.0664.

2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3(2*H*)-one (**110**)

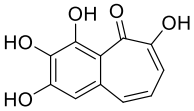


110

Quercetin **18** (0.10 g, 0.33 mmol) was dissolved in acetonitrile (60 mL) and aqueous solution of HCl (0.24 M; 20 mL) at 40 °C. Copper(II) chloride dihydrate (0.15 g, 0.88 mmol) was added and the reaction mixture was left stirred overnight at room temperature. Acetonitrile was evaporated under reduced pressure and the residue was extracted with EtOAc (3 × 20 mL). The combined organic layers were evaporated to dryness under reduced pressure and the residue was purified by preparative HPLC to obtain the

compound **110** (0.020 g, 19%). ^{1}H NMR (400 MHz, CD_3CN) δ 8.41 (bs, 1H), 8.16 (bs, 1H), 7.62 (bs, 1H), 7.47 – 7.37 (m, 2H), 7.17 (bs, 1H), 6.91 – 6.82 (m, 1H), 6.38 (bs, 1H), 6.11 (d, *J* = 1.8 Hz, 1H), 6.05 (d, *J* = 1.8 Hz, 1H). ^{13}C NMR (100 MHz, CD_3CN) δ 192.0, 190.3, 172.9, 170.0, 159.3, 152.2, 145.4, 125.8, 125.1, 117.3, 116.1, 103.7, 101.8, 98.2, 92.9. HRMS (ESI) *m/z* calcd for $C_{15}H_{10}O_8Na$ $[M+Na]^+$ 341.0264, found 341.0267.

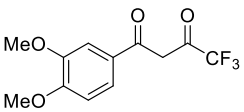
2,3,4,6-Tetrahydroxy-5*H*-benzo[7]annulen-5-one (111)



111

To a solution of pyrogallol (2.00 g, 15.85 mmol) in water (60 mL) was added sodium bicarbonate (3.30 g, 39.60 mmol). The solution was cooled in an ice-bath to approximately 5 °C and solid $K_3[Fe(CN)_6]$ was added. The solution was stirred at room temperature for 2h. The reaction mixture was acidified with aqueous solution of HCl 5% and extracted with EtOAc (3 × 20 mL). The combined organic layers were extracted with water, brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was suspended in EtOAc and filtrated over silica-gel and the filtrate was evaporated to dryness under reduced pressure. The residue was crystalized from MeOH to obtain the purpurogallin (0.10 g, 6%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 15.30 (s, 1H), 10.57 (bs, 1H), 9.42 (bs, 1H), 9.38 (s, 1H), 7.34 (d, *J* = 11.4 Hz, 1H), 7.07 (d, *J* = 9.5 Hz, 1H), 6.89 (s, 1H), 6.73 (dd, *J* = 11.4, 9.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 182.7, 155.1, 152.2, 152.0, 135.2, 134.8, 133.5, 124.0, 117.0, 115.3, 110.7. HRMS (ESI) *m/z* calcd for $C_{11}H_9O_5$ $[M+H]^+$ 221.0444, found 221.0443

1-(3,4-Dimethoxyphenyl)-4,4,4-trifluorobutane-1,3-dione (112)

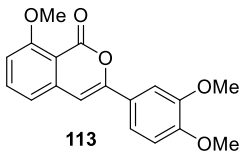


112

3,4-Dimethoxyacetophenone (1.34 g, 7.45 mmol) was dissolved in a freshly distilled THF (15 mL) at 0 °C under inert atmosphere of nitrogen. Sodium hydride 60% suspension in mineral oil (0.60 g, 14.90 mmol) was added and the reaction mixture was allowed to warm up to a room temperature and was stirred for 1h. Ethyl trifluoroacetate (1.58 g, 11.20 mmol) was added and the reaction mixture was stirred for 4h. After completion the reaction mixture was quenched by addition of 5% aqueous solution of HCl and extracted with EtOAc (3 × 15 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure to afford the intermediate **112** (2.00 g, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.48 (d, *J* = 2.1 Hz, 1H), 6.94 (d, *J* = 8.6 Hz, 1H), 6.52 (s, 2H), 3.97 (d, *J* = 5.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.8, 174.5 (q, *J* = 36.4 Hz), 154.4, 149.4, 125.9, 122.6,

118.9 (d, J = 282.3 Hz), 110.7, 109.7, 92.0 (d, J = 2.2 Hz), 56.2, 56.1. ^{19}F NMR (375 MHz, $CDCl_3$) δ -76.1. HRMS (EI) *m/z* calcd for $C_{10}H_{11}O_4F_3$ $[M]^{+\bullet}$ 276.0609, found 276.0608.

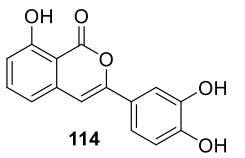
3-(3,4-Dimethoxyphenyl)-8-methoxy-1*H*-isochromen-1-one (113)



113

Compound **112** (0.50 g, 1.81 mmol) and cesium fluoride (1.37 g, 9.02 mmol) were dissolved in dry acetonitrile (50 mL) and 3-methoxy-2-(trimethylsilyl)phenyl-trifluoromethanesulfonate (0.77 g, 2.34 mmol) was added. The reaction mixture was refluxed for 4h. The reaction was quenched with water (70 mL) and extracted with EtOAc (3 × 40 mL). The combined organic layer were washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduce pressure. The residue was purified by column chromatography (cyclohexane/EtOAc 1:1) to obtain the intermediate **113** (0.20 g, 35%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 – 7.54 (m, 1H), 7.50 – 7.41 (m, 1H), 7.37 – 7.32 (m, 1H), 6.99 (d, J = 7.8 Hz, 1H), 6.96 – 6.85 (m, 2H), 6.73 (s, 1H), 4.01 (s, 3H), 3.97 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.8, 159.3, 154.1, 150.8, 149.3, 140.9, 135.9, 124.9, 118.7, 118.0, 111.2, 109.6, 109.0, 108.3, 100.7, 56.4, 56.3, 56.1. HRMS (ESI) *m/z* calcd for $C_{18}H_{16}O_5Na$ $[M+Na]^+$ 335.0889, found 335.0890.

Thunberginol A (114)



114

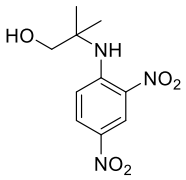
The intermediate **113** (0.050 g, 0.16 mmol) was suspended in 1M solution of BBr_3 in DCM (1.8 mL, 1.80 mmol). The reaction mixture was stirred for 2h at room temperature under inert atmosphere. The reaction mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC to afford the compound **114** (0.026 g, 60%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.85 (s, 1H), 9.58 (bs, 1H), 9.31 (bs, 1H), 7.68 (t, J = 8.0 Hz, 1H), 7.25 – 7.20 (m, 2H), 7.10 (dd, J = 7.9, 1.0 Hz, 1H), 6.92 (dd, J = 8.3, 1.0 Hz, 2H), 6.86 (d, J = 8.3 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.2, 160.5, 152.8, 147.8, 145.7, 138.6, 137.7, 122.3, 117.0, 116.6, 116.1, 114.1, 112.2, 105.3, 100.7. HRMS (ESI) *m/z* calcd for $C_{15}H_{10}O_5Na$ $[M+Na]^+$ 293.0420, found 293.0419.

General procedure for preparation of dinitrophenylamines

1-Chloro-2,4-dinitrobenzene (1.00 g, 4.93 mmol) and amine (1 eq.) were suspended in 1,4-dioxane (2.5 mL). Triethylamine (2 eq.) was added and the reaction mixture was stirred at 100 °C overnight in a sealed tube. The reaction mixture was quenched with water and extracted with

DCM. The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure.

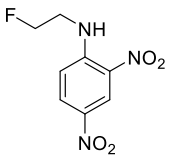
2-((2,4-Dinitrophenyl)amino)-2-methylpropan-1-ol (136)



136

2-Amino-2-methylpropan-1-ol was coupled using the condition described in the general procedure for preparation of dinitrophenylamines. The solid residue was purified by column chromatography (EtOAc) to afford the desired amine **136** (1.00 g, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.15 (s, 1H), 8.86 (d, J = 2.6 Hz, 1H), 8.19 (dd, J = 9.7, 2.7 Hz, 1H), 7.44 (d, J = 9.7 Hz, 1H), 5.56 (t, J = 5.2 Hz, 1H), 3.48 (d, J = 5.2 Hz, 2H), 1.42 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 147.7, 134.5, 130.3, 129.5, 124.0, 116.8, 69.2, 56.2, 22.9.

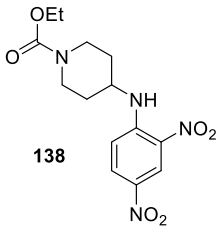
***N*-(2-Fluoroethyl)-2,4-dinitroaniline (137)**



137

2-Fluoroethan-1-amine hydrochloride was coupled using the condition described in the general procedure for preparation of dinitrophenylamines (3eq. triethylamine). The solid residue was purified by column chromatography (EtOAc/hexanes 1:2) to afford the desired amine **137** (0.93 g, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.14 (d, J = 2.7 Hz, 1H), 8.76 (s, 1H), 8.30 (ddd, J = 9.5, 2.7, 0.6 Hz, 1H), 6.99 (d, J = 9.5 Hz, 1H), 4.75 (dd, J = 47.0, 4.8 Hz, 2H), 3.78 (dq, J = 25.5, 5.6 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.4, 136.7, 131.0, 130.6, 124.4, 113.9 (d, $^{4}J_{C\text{-}F}$ = 1.8 Hz), 81.6 (d, $^{1}J_{C\text{-}F}$ = 172.2 Hz), 43.8 (d, $^{2}J_{C\text{-}F}$ = 20.4 Hz).

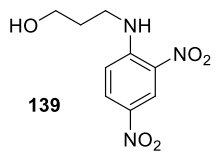
Ethyl 4-((2,4-dinitrophenyl)amino)piperidine-1-carboxylate (138)



138

Ethyl 4-aminopiperidine-1-carboxylate was coupled using the condition described in the general procedure for preparation of dinitrophenylamines. The solid residue was purified by column chromatography (DCM) to afford the desired amine **138** (1.57 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.15 (d, J = 2.7 Hz, 1H), 8.58 (d, J = 7.3 Hz, 1H), 8.28 (dd, J = 9.5, 2.6 Hz, 1H), 6.95 (d, J = 9.6 Hz, 1H), 4.31 – 3.99 (m, 4H), 3.92 – 3.62 (m, 1H), 3.11 (t, J = 11.4 Hz, 2H), 2.11 (dd, J = 13.0, 3.1 Hz, 2H), 1.75 – 1.55 (m, 2H), 1.28 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.5, 147.3, 136.3, 130.7, 130.6, 124.8, 114.0, 61.8, 50.2, 42.2, 31.6, 14.8.

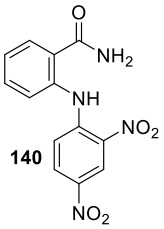
3-((2,4-Dinitrophenyl)amino)propan-1-ol (139)



139

3-Aminopropan-1-ol was coupled using the condition described in the general procedure for preparation of dinitrophenylamines. The solid residue was purified by column chromatography (MeOH/DCM 1:20) to afford the desired amine **139** (0.58 g, 48%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.12 (d, *J* = 2.7 Hz, 1H), 8.88 (s, 1H), 8.26 (ddd, *J* = 9.5, 2.7, 0.7 Hz, 1H), 6.97 (d, *J* = 9.6 Hz, 1H), 3.89 (t, *J* = 5.2 Hz, 2H), 3.59 (td, *J* = 6.6, 5.3 Hz, 2H), 2.03 (p, *J* = 5.6 Hz, 2H), 1.73 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.6, 136.0, 130.5(2x), 124.5, 114.0, 60.6, 41.5, 31.0.

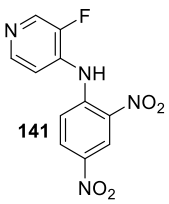
2-((2,4-Dinitrophenyl)amino)benzamide (140)



140

2-Aminobenzamide was coupled using the condition described in the general procedure for preparation of dinitrophenylamines. The solid residue was purified by column chromatography (DCM) to afford the desired amine **140** (1.15 g, 77%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 11.68 (s, 1H), 8.90 (d, *J* = 2.7 Hz, 1H), 8.29 (dd, *J* = 9.5, 2.7 Hz, 1H), 8.21 (s, 1H), 7.78 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.70 (s, 1H), 7.63 – 7.51 (m, 3H), 7.36 – 7.28 (m, 1H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 169.4, 144.5, 137.3, 137.2, 133.1, 131.6, 129.8, 129.3, 127.1, 125.0, 123.4, 122.6, 117.6.

***N*-(2,4-Dinitrophenyl)-3-fluoropyridin-4-amine (141)**



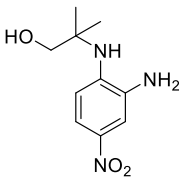
141

3-Fluoropyridin-4-amine was coupled using the condition described in the general procedure for preparation of dinitrophenylamines. The solid residue was purified by column chromatography (DCM) to afford the desired amine **141** (0.35 g, 25%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.88 (s, 1H), 9.19 (d, *J* = 2.4 Hz, 1H), 8.62 (d, *J* = 2.1 Hz, 1H), 8.48 (d, *J* = 5.3 Hz, 1H), 8.37 (dd, *J* = 9.4, 2.6 Hz, 1H), 7.47 – 7.37 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.5, 150.9, 147.1 (d, $^{4}J_{C\text{-}F}$ = 5.8 Hz), 143.3, 139.8, 139.8 (d, $^{2}J_{C\text{-}F}$ = 21.2 Hz), 134.1, 133.1 (d, $^{3}J_{C\text{-}F}$ = 9.2 Hz), 130.2, 123.8, 117.0 (d, $^{1}J_{C\text{-}F}$ = 47.5 Hz).

General procedure for reduction of dinitroanilines

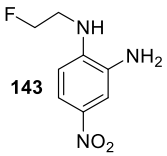
A mixture of sulphur (2 eq.), sodium sulfide nonahydrate (2 eq.) and water (0.5 M) was heated up to 60 °C under argon. Suspension of ethanol and the amine (1 eq.) was added. The mixture was stirred for 2h and then diluted with water and placed in an ice-bath. The dark red precipitate was collected, washed with water and dried under vacuum.

2-((2-Amino-4-nitrophenyl)amino)-2-methylpropan-1-ol (142)



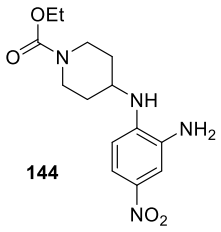
The amine **136** (1.00 g, 3.92 mmol) was reduced according to the general procedure for reduction of dinitroanilines to afford the amine **142** (0.48 g, 55%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 7.51 (dd, *J* = 9.0, 2.8 Hz, 1H), 7.45 (d, *J* = 2.8 Hz, 1H), 6.77 (d, *J* = 9.0 Hz, 1H), 5.09 (s, 2H), 5.07 (s, 1H), 5.02 (t, *J* = 5.7 Hz, 1H), 3.46 (d, *J* = 5.5 Hz, 2H), 1.31 (s, 6H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 141.9, 136.3, 135.5, 115.8, 109.7, 108.5, 68.4, 54.8, 23.6. HRMS (ESI) *m/z* calcd $C_{10}H_{15}N_3O_3Na$ $[M+Na]^+$ 248.1005, found 248.1005.

***N*^{1}-(2-Fluoroethyl)-4-nitrobenzene-1,2-diamine (143)**



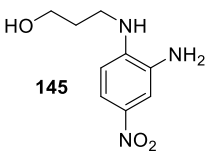
The amine **137** (0.93 g, 4.10 mmol) was reduced according to the general procedure for reduction of dinitroanilines to afford the amine **143** (0.32 g, 39%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 7.51 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.43 (d, *J* = 2.7 Hz, 1H), 6.55 (d, *J* = 8.9 Hz, 1H), 6.10 (t, *J* = 5.4 Hz, 1H), 5.20 (s, 2H), 4.62 (dt, *J* = 47.5, 4.9 Hz, 2H), 3.55 (dq, *J* = 27.3, 5.1 Hz, 2H). HRMS (ESI) *m/z* calcd $C_8H_{10}N_3O_2FNa$ $[M+Na]^+$ 222.0649, found 222.0649.

Ethyl 4-((2-amino-4-nitrophenyl)amino)piperidine-1-carboxylate (144)



The amine **138** (1.00 g, 2.95 mmol) was reduced according to the general procedure for reduction of dinitroanilines to afford the amine **144** (0.54 g, 60%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 7.50 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.41 (d, *J* = 2.6 Hz, 1H), 6.59 (d, *J* = 9.0 Hz, 1H), 5.64 (d, *J* = 7.5 Hz, 1H), 5.19 (s, 2H), 4.04 (q, *J* = 7.1 Hz, 2H), 3.97 (d, *J* = 12.2 Hz, 2H), 3.79 – 3.54 (m, 1H), 2.97 (s, 2H), 1.94 (d, *J* = 10.6 Hz, 2H), 1.34 (qd, *J* = 12.7, 4.1 Hz, 2H), 1.19 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 154.7, 141.2, 136.6, 134.6, 115.7, 107.6, 107.5, 60.7, 48.9, 42.4, 31.3, 14.6. HRMS (ESI) *m/z* calcd $C_{14}H_{20}N_4O_4Na$ $[M+Na]^+$ 331.1376, found 331.1377.

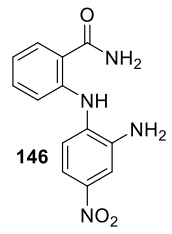
3-((2-Amino-4-nitrophenyl)amino)propan-1-ol (145)



The amine **139** (0.58 g, 2.40 mmol) was reduced according to the general procedure for reduction of dinitroanilines to afford the amine **145** (0.12 g, 24%). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 7.52 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.39 (d, *J* = 2.7 Hz, 1H), 6.47 (d, *J* = 8.9 Hz, 1H), 5.90 (t, *J* = 5.1 Hz, 1H), 5.14

(s, 2H), 4.54 (t, *J* = 5.1 Hz, 1H), 3.52 (q, *J* = 11.3, 6.1 Hz, 2H), 3.25 (q, *J* = 6.9 Hz, 2H), 1.76 (p, *J* = 6.5 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 142.7, 136.4, 134.4, 116.0, 107.2, 106.8, 58.4, 40.6, 31.5. HRMS (ESI) *m/z* calcd $C_9H_{13}N_3O_3Na$ $[M+Na]^+$ 234.0849, found 234.0848.

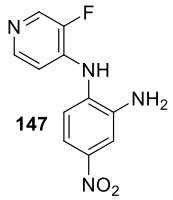
2-((2-Amino-4-nitrophenyl)amino)benzamide (146)



146

The amine **140** (1.10 g, 3.64 mmol) was reduced according to the general procedure for reduction of dinitroanilines to afford the amine **146** (0.55 g, 56%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.86 (s, 1H), 8.11 (s, 1H), 7.77 (d, *J* = 7.7 Hz, 1H), 7.65 (d, *J* = 2.6 Hz, 1H), 7.53 (s, 1H), 7.48 (dd, *J* = 8.8, 2.6 Hz, 1H), 7.40 (t, *J* = 7.3 Hz, 1H), 7.24 (dd, *J* = 16.0, 8.5 Hz, 2H), 6.95 (t, *J* = 7.4 Hz, 1H), 5.36 (s, 2H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 170.7, 143.2, 141.4, 140.3, 136.1, 132.0, 129.3, 119.8, 119.8, 117.8, 115.8, 113.6, 109.2. HRMS (ESI) *m/z* calcd $C_{13}H_{12}N_4O_3Na$ $[M+Na]^+$ 295.0801, found 295.0802.

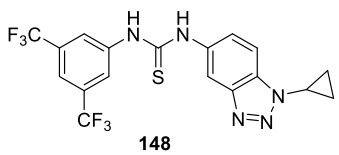
***N*^{1}-(3-Fluoropyridin-4-yl)-4-nitrobenzene-1,2-diamine (147)**



147

The amine **141** (0.35 g, 1.26 mmol) was reduced according to the general procedure for reduction of dinitroanilines to afford the amine **147** (0.12 g, 38%). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 8.32 (d, *J* = 3.7 Hz, 1H), 8.24 (s, 1H), 8.05 (d, *J* = 5.4 Hz, 1H), 7.64 (d, *J* = 2.7 Hz, 1H), 7.42 (dd, *J* = 8.7, 2.7 Hz, 1H), 7.18 (d, *J* = 8.6 Hz, 1H), 6.69 (dd, *J* = 7.7, 5.4 Hz, 1H), 5.65 (s, 2H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 150.79 (s), 148.3, 146.2 (d, $^4J_{C\text{-}F}$ = 4.6 Hz), 144.7 (s), 143.2, 138.4 (d, $^3J_{C\text{-}F}$ = 9.2 Hz), 136.7 (d, $^2J_{C\text{-}F}$ = 20.2 Hz), 130.9, 123.7, 110.7 (s), 110.4 (d, $^1J_{C\text{-}F}$ = 219.8 Hz). HRMS (ESI) *m/z* calcd $C_{11}H_{10}N_4O_2F$ $[M+H]^+$ 249.0782, found 249.0782.

1-(3,5-Bis(trifluoromethyl)phenyl)-3-(1-cyclopropyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)thiourea (148)

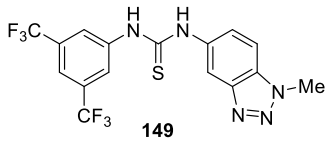


148

1-Cyclopropyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.10 g, 0.57 mmol) and 1-isothiocyanato-3,5-bis(trifluoromethyl)benzene (0.11 mL, 0.57 mmol) were dissolved in THF (3 mL) and the reaction mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure and the residue was crystalized from MeOH to afford the compound **148** (0.13, 51%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 10.56 – 10.36 (m, 1H), 10.24 (s, 1H), 8.27 (s, 2H), 8.09 (d, *J* = 1.4 Hz, 1H), 7.86 (dd, *J* = 8.8, 0.5 Hz, 1H), 7.80 (s, 1H), 7.61

(dd, J = 8.8, 1.8 Hz, 1H), 4.14 – 3.90 (m, 1H), 1.42 – 1.12 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.5, 145.4, 141.8, 135.1, 132.1, 130.0 (q, $^{2}J_{C\text{-}F}$ = 32.9 Hz), 126.2, 123.8, 123.3 (q, $^{1}J_{C\text{-}F}$ = 272.7 Hz), 117.0, 114.3, 110.6, 28.6, 6.0. ^{19}F NMR (375 MHz, DMSO-d_6) δ -61.53. HRMS (ESI) *m/z* calcd $C_{18}H_{13}N_5F_6NaS$ $[M+Na]^+$ 468.0688, found 468.0687.

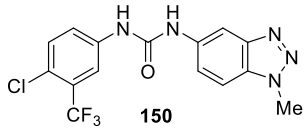
1-(3,5-Bis(trifluoromethyl)phenyl)-3-(1-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)thiourea (149)



149

1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.10 g, 0.67 mmol) and 1-isothiocyanato-3,5-bis(trifluoromethyl)benzene (0.13 mL, 0.67 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **149** (0.13 g, 48%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (s, 1H), 10.24 (s, 1H), 8.27 (s, 2H), 8.08 (d, J = 1.3 Hz, 1H), 7.85 (dd, J = 8.8, 0.3 Hz, 1H), 7.80 (s, 1H), 7.59 (dd, J = 8.8, 1.8 Hz, 1H), 4.31 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 145.3, 141.9, 134.8, 131.5, 130.0 (q, J = 32.9 Hz), 126.0, 123.8(2x), 123.3 (q, J = 272.8 Hz), 114.0, 110.7, 39.5, 34.3. HRMS (ESI) *m/z* calcd $C_{16}H_{11}ON_5F_6Na$ $[M+Na]^+$ 442.0531, found 442.0531.

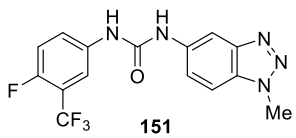
1-(4-Chloro-3-(trifluoromethyl)phenyl)-3-(1-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)urea (150)



150

1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.09 g, 0.61 mmol) and 1-chloro-4-isocyanato-2-(trifluoromethyl)benzene (0.13 g, 0.61 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **150** (0.16 g, 69%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.22 (bs, 1H), 9.05 (bs, 1H), 8.17 (d, J = 1.2 Hz, 1H), 8.11 (d, J = 2.3 Hz, 1H), 7.76 (d, J = 8.9 Hz, 1H), 7.67 (dd, J = 8.8, 2.3 Hz, 1H), 7.61 (d, J = 8.8 Hz, 1H), 7.52 (dd, J = 8.9, 1.7 Hz, 1H), 4.27 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 152.7, 145.7, 139.4, 135.6, 132.1, 129.9, 126.8 (q, $^{2}J_{C\text{-}F}$ = 30.4 Hz), 123.2, 122.9 (q, $^{1}J_{C\text{-}F}$ = 273.1 Hz), 122.4 (q, $^{4}J_{C\text{-}F}$ = 1.6 Hz), 121.3, 116.9 (q, $^{3}J_{C\text{-}F}$ = 5.7 Hz), 110.7, 106.5, 34.2. HRMS (ESI) *m/z* calcd $C_{15}H_{11}ON_5ClF_3Na$ $[M+Na]^+$ 392.0496, found 392.0497

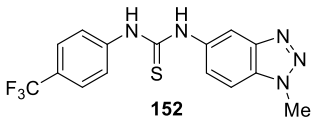
1-(4-Fluoro-3-(trifluoromethyl)phenyl)-3-(1-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)urea (151)



151

1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.15 g, 1.01 mmol) and 1-fluoro-4-isocyanato-2-(trifluoromethyl)benzene (0.21 g, 1.02 mmol) were dissolved in THF (3 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was triturated in MeOH to afford the compound **151** (0.25 g, 71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 1H), 9.02 (s, 1H), 8.17 (d, J = 1.3 Hz, 1H), 8.01 (dd, J = 6.4, 2.6 Hz, 1H), 7.76 (d, J = 8.9 Hz, 1H), 7.72 – 7.63 (m, 1H), 7.53 (dd, J = 8.9, 1.8 Hz, 1H), 7.44 (t, J = 9.8 Hz, 1H), 4.27 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 153.4 (d, $^{1}J_{C\text{-}F}$ = 245.8 Hz), 152.9, 145.6, 135.7, 129.8, 124.4 (d, $^{3}J_{C\text{-}F}$ = 7.9 Hz), 121.3 (q, $^{1}J_{C\text{-}F}$ = 272.9 Hz), 121.2, 117.6 (d, $^{2}J_{C\text{-}F}$ = 21.4 Hz), 116.1 (q, $^{4}J_{C\text{-}F}$ = 4.7 Hz), 110.7, 106.3, 39.5, 34.2. ^{19}F NMR (375 MHz, DMSO-d_6) δ -60.20 (d, $J_{F\text{-}C}$ = 12.6 Hz), -120.45 – -129.80 (m). HRMS (ESI) *m/z* calcd $C_{15}H_{11}ON_5F_4Na$ $[M+Na]^+$ 376.0791, found 376.0792.

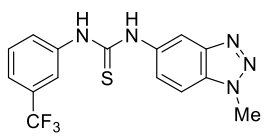
1-(1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)-3-(4-(trifluoromethyl)phenyl)thiourea (152)



152

1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.09 g, 0.61 mmol) and 1-isothiocyanato-4-(trifluoromethyl)benzene (0.12 g, 0.61 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was evaporated and the solid residue was triturated in MeOH to afford the compound **152** (0.13 g, 57%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.19 (d, J = 8.5 Hz, 2H), 8.09 (s, 1H), 7.81 (d, J = 8.8 Hz, 1H), 7.77 (d, J = 8.5 Hz, 2H), 7.69 (d, J = 8.7 Hz, 2H), 7.60 (dd, J = 8.9, 1.7 Hz, 1H), 4.30 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.4, 145.2, 143.4, 135.5, 131.3, 128.5, 126.0, δ 125.7 (q, J = 3.7 Hz), 124.1 (q, J = 31.8 Hz), 123.1, 113.4, 110.4, 34.3. ^{19}F NMR (375 MHz, DMSO-d_6) δ -60.39. HRMS (ESI) *m/z* calcd $C_{15}H_{12}N_5F_3NaS$ $[M+Na]^+$ 374.0657, found 374.0658.

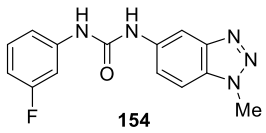
1-(1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)-3-(3-(trifluoromethyl)phenyl)thiourea (153)



1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.10 g, 0.67 mmol) and 1-isothiocyanato-3-(trifluoromethyl)benzene (0.10 ml, 0.67 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **153** (0.12 g, 47%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H),

10.06 (s, 1H), 8.08 (s, 1H), 7.98 (s, 1H), 7.88 – 7.73 (m, 2H), 7.60 (dd, J = 8.9, 1.8 Hz, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.47 (d, J = 7.8 Hz, 1H), 4.30 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.5, 145.4, 140.4, 135.5, 131.8, 129.5, 129.0 (q, $^{2}J_{C\text{-}F}$ = 31.8 Hz), 127.5, 126.2, 124.1 (q, $^{1}J_{C\text{-}F}$ = 272.4 Hz), 120.7 (q, $^{3}J_{C\text{-}F}$ = 3.6 Hz), 120.0 (q, $^{3}J_{C\text{-}F}$ = 3.9 Hz), 113.8, 110.3, 28.6. ^{19}F NMR (375 MHz, DMSO-d_6) δ -61.2. HRMS (ESI) *m/z* calcd $C_{15}H_{12}N_5F_3NaS$ $[M+Na]^+$ 374.0657, found 374.0659.

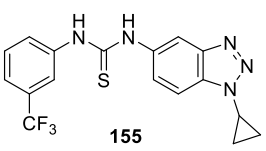
1-(3-Fluorophenyl)-3-(1-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)urea (154)



154

1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.09 g, 0.61 mmol) and 1-fluoro-3-isocyanatobenzene (70 μL, 0.61 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was triturated in MeOH to afford the compound **154** (0.12 g, 67%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (d, J = 6.4 Hz, 2H), 8.18 (s, 1H), 7.76 (d, J = 8.9 Hz, 1H), 7.63 – 7.44 (m, J = 7.0, 5.4 Hz, 2H), 7.31 (dd, J = 15.2, 8.0 Hz, 1H), 7.15 (d, J = 8.1 Hz, 1H), 6.79 (td, J = 8.5, 2.1 Hz, 1H), 4.27 (s, 3H). ^{19}F NMR (375 MHz, DMSO-d_6) δ -112.24. ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.5 (d, $^{1}J_{C\text{-}F}$ = 240.8 Hz), 152.7, 145.7, 141.6 (d, $^{3}J_{C\text{-}F}$ = 11.4 Hz), 135.9, 130.4 (d, $^{3}J_{C\text{-}F}$ = 9.8 Hz), 129.8, 121.1, 114.1 (d, $^{4}J_{C\text{-}F}$ = 2.4 Hz), 110.8, 108.3 (d, $^{2}J_{C\text{-}F}$ = 21.2 Hz), 105.0 (d, $^{2}J_{C\text{-}F}$ = 26.5 Hz), 106.1, 34.2. HRMS (ESI) *m/z* calcd $C_{14}H_{12}ON_5FNa$ $[M+Na]^+$ 308.0918, found 308.0918.

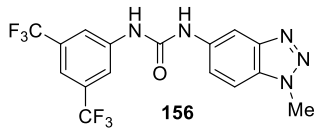
1-(1-Cyclopropyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)-3-(3-(trifluoromethyl)phenyl)thiourea (155)



155

1-Cyclopropyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.10 g, 0.57 mmol) and 1-isothiocyanato-3-(trifluoromethyl)benzene (87 μL, 0.57 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was evaporated and the solid residue was crystalizes from MeOH to afford the compound **155** (0.17 g, 77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.20 (s, 1H), 10.06 (s, 1H), 8.09 (s, 1H), 7.98 (s, 1H), 7.83 (d, J = 8.8 Hz, 1H), 7.78 (d, J = 7.9 Hz, 1H), 7.62 (d, J = 8.7 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 7.47 (d, J = 7.5 Hz, 1H), 4.17 – 3.83 (m, 1H), 1.49 – 0.95 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.5, 145.4, 140.4, 135.5, 131.8, 129.8, 129.0 (q, $^{2}J_{C\text{-}F}$ = 31.9 Hz), 127.5, 126.2, 124.1 (q, $^{1}J_{C\text{-}F}$ = 272.4 Hz), 120.69 (q, $^{3}J_{C\text{-}F}$ = 3.6 Hz), 120.0 (q, $^{3}J_{C\text{-}F}$ = 3.9 Hz), 113.8, 110.3, 28.6, 6.0. ^{19}F NMR (375 MHz, DMSO-d_6) δ -61.19. HRMS (ESI) *m/z* calcd $C_{17}H_{14}N_5F_3NaS$ $[M+Na]^+$ 400.0814, found 400.0815.

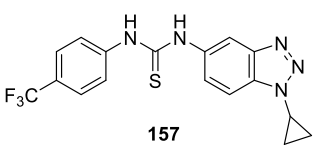
1-(3,5-Bis(trifluoromethyl)phenyl)-3-(1-methyl-1*H*-benzo[*d*][1,2,3]triaazol-5-yl)urea (156)



156

1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.08 g, 0.55 mmol) and 1-isocyanato-3,5-bis(trifluoromethyl)benzene (0.14 g, 0.55 mmol) were dissolved in THF (3 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **156** (0.15, 68%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.46 (s, 1H), 9.20 (s, 1H), 8.18 (d, *J* = 1.3 Hz, 1H), 8.16 (s, 2H), 7.78 (d, *J* = 8.9 Hz, 1H), 7.64 (s, 1H), 7.57 (dd, J = 8.9, 1.8 Hz, 1H), 4.28 (s, 3H). ^{19}F NMR (375 MHz, DMSO-*d*$_6$) δ -61.69. ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 194.1, 152.7, 145.6, 141.9, 135.4, 130.9, 130.6, 130.0, 121.4, 118.1, 110.1, 106.9, 34.2. HRMS (ESI) *m/z* calcd $C_{16}H_{11}ON_5F_6Na$ $[M+Na]^+$ 426.0760, found 426.0759.

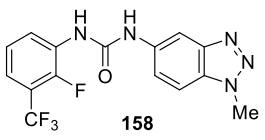
1-(1-Cyclopropyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)-3-(4-(trifluoromethyl)phenyl)thiourea (157)



157

1-Cyclopropyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.10 g, 0.57 mmol) and 1-isothiocyanato-4-(trifluoromethyl)benzene (0.21 g, 0.57 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **157** (0.16 g, 72%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 10.20 (d, *J* = 15.0 Hz, 2H), 8.11 (d, *J* = 1.5 Hz, 1H), 7.82 (d, *J* = 8.9 Hz, 1H), 7.78 (d, *J* = 8.5 Hz, 2H), 7.69 (d, *J* = 8.7 Hz, 2H), 7.64 (dd, *J* = 8.9, 1.8 Hz, 1H), 4.15 – 3.93 (m, 1H), 1.38 – 1.11 (m, 4H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 180.3, 145.3, 143.3, 135.6, 131.8, 126.2, 125.6 (q, $^{3}J_{C\text{-}F}$ = 3.7 Hz), 124.3 (q, $^{1}J_{C\text{-}F}$ = 271.3 Hz), 124.0 (q, $^{2}J_{C\text{-}F}$ = 31.9 Hz), 123.0, 113.6, 110.3, 28.6, 6.0. HRMS (ESI) *m/z* calcd $C_{17}H_{14}N_5F_3NaS$ $[M+Na]^+$ 400.0814, found 400.0815.

1-(2-Fluoro-3-(trifluoromethyl)phenyl)-3-(1-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)urea (158)

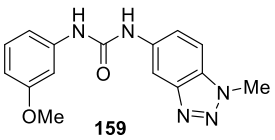


158

1-Methyl-1H-benzo[d][1,2,3]triazol-5-amine (0.10 g, 0.67 mmol) and 2-fluoro-1-isocyanato-3-(trifluoromethyl)benzene (0.10 mL, 0.67 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **158** (0.14 g, 56%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.34 (s, 1H), 8.88

(d, *J* = 2.1 Hz, 1H), 8.47 (td, *J* = 6.6, 3.2 Hz, 1H), 8.23 (d, *J* = 1.3 Hz, 1H), 7.78 (d, *J* = 8.9 Hz, 1H), 7.47 (dd, *J* = 8.9, 1.8 Hz, 1H), 7.42 – 7.31 (m, 2H), 4.28 (s, 3H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 152.3, 148.9 (d, *J* = 251.2 Hz), 145.6, 135.5, 128.9 (d, *J* = 9.3 Hz), 129.8, 125.2, 124.9 (d, *J* = 4.2 Hz), 122.7 (q, $^{1}J_{C\text{-}F}$ = 272.2 Hz), 120.8, 119.1 (d, *J* = 5.0 Hz), 116.6 (m) 110.9, 109.0, 34.2. ^{19}F NMR (375 MHz, DMSO-*d*$_6$) δ -59.86 (d, *J* = 12.8 Hz), -132.76 (m). HRMS (ESI) *m/z* calcd $C_{15}H_{11}ON_5F_4Na$ $[M+Na]^+$ 376.0791, found 376.0792.

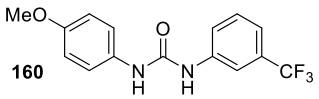
1-(3-Methoxyphenyl)-3-(1-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-yl)ure (159)



159

1-Methyl-1*H*-benzo[*d*][1,2,3]triazol-5-amine (0.10 g, 0.67 mmol) and 1-isocyanato-3-methoxybenzene (88 μL, 0.67 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **159** (0.14 g, 70%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 8.87 (s, 1H), 8.74 (s, 1H), 8.19 (d, *J* = 1.4 Hz, 1H), 7.75 (d, *J* = 8.9 Hz, 1H), 7.49 (dd, *J* = 8.9, 1.8 Hz, 1H), 7.21 (t, *J* = 2.1 Hz, 1H), 7.18 (d, *J* = 8.1 Hz, 1H), 6.97 (dd, *J* = 8.0, 1.2 Hz, 1H), 6.56 (dd, *J* = 8.0, 2.2 Hz, 1H), 4.27 (s, 3H), 3.74 (s, 3H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 159.7, 152.7, 145.7, 140.9, 136.1, 129.6, 129.6, 121.0, 110.6(2x), 107.3, 105.8, 104.1, 54.9, 34.2. HRMS (ESI) *m/z* calcd $C_{15}H_{16}O_2N_5$ $[M+H]^+$ 298.1298, found 298.1299.

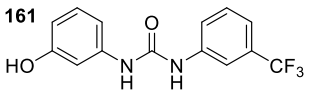
1-(3-Methoxyphenyl)-3-(3-(trifluoromethyl)phenyl)urea (160)



160

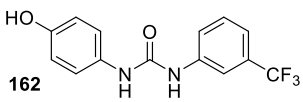
4-Methoxyaniline (0.15 g, 1.20 mmol) and 1-isocyanato-3-(trifluoromethyl)benzene (0.17 mL, 1.20 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was evaporated to dryness under reduced pressure and crystalized from EtOAc to afford the compound **160** (0.20 g, 53%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 8.94 (s, 1H), 8.57 (s, 1H), 8.01 (s, 1H), 7.56 (d, *J* = 10.3 Hz, 1H), 7.49 (t, *J* = 7.9 Hz, 1H), 7.37 (d, *J* = 9.0 Hz, 2H), 7.28 (d, *J* = 7.6 Hz, 1H), 6.88 (d, *J* = 9.0 Hz, 2H), 3.72 (s, 3H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 154.7, 152.7, 140.8, 132.3, 129.8, 129.5 (q, $^{2}J_{C\text{-}F}$ = 31.3 Hz), 124.4 (q, $^{1}J_{C\text{-}F}$ = 246.1 Hz), 121.7, 120.4, 117.8 (q, $^{3}J_{C\text{-}F}$ = 3.6 Hz), 114.0, 55.2. ^{19}F NMR (375 MHz, DMSO-*d*$_6$) δ -61.35. HRMS (ESI) *m/z* calcd $C_{15}H_{13}O_2N_2F_3Na$ $[M+Na]^+$ 333.0821, found 333.0821.

1-(3-Hydroxyphenyl)-3-(3-(trifluoromethyl)phenyl)urea (161)



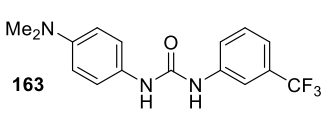
3-Aminophenol (0.15 g, 1.37 mmol) and 1-isocyanato-3-(trifluoromethyl)benzene (0.26 g, 1.37 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was evaporated under reduced pressure and crystalized from DCM to afford the compound **161** (0.09 g, 20 %). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.34 (s, 1H), 8.95 (s, 1H), 8.67 (s, 1H), 8.01 (s, 1H), 7.62 – 7.42 (m, 2H), 7.29 (d, *J* = 7.4 Hz, 1H), 7.13 – 6.94 (m, 2H), 6.80 (dd, *J* = 8.1, 1.0 Hz, 1H), 6.40 (dd, *J* = 8.0, 2.1 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 157.8, 152.4, 140.6, 140.4, 129.9, 129.6 (q, $^{2}J_{C-F}$ = 31.3 Hz), 129.5, 124.3 (q, $^{1}J_{C-F}$ = 272.3 Hz), 121.8, 118.0 (q, $^{3}J_{C-F}$ = 4.1 Hz), 114.1 (q, $^{3}J_{C-F}$ = 4.0 Hz), 109.4, 109.2, 105.6. ^{19}F NMR (375 MHz, DMSO-*d*$_6$) δ -61.35. HRMS (ESI) *m/z* calcd $C_{14}H_{12}O_2N_2F_3$ $[M+H]^+$ 297.0845, found 297.0846.

1-(4-Hydroxyphenyl)-3-(3-(trifluoromethyl)phenyl)urea (162)



4-Aminophenol (0.15 g, 1.37 mmol) and 1-isocyanato-3-(trifluoromethyl)benzene (0.19 mL, 1.37 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was evaporated to dryness under reduced pressure and crystalized from EtOAc to afford the compound **162** (0.12 g, 29 %). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.10 (s, 1H), 8.97 (s, 1H), 8.49 (s, 1H), 8.01 (s, 1H), 7.55 (d, *J* = 8.3 Hz, 1H), 7.47 (t, *J* = 7.9 Hz, 1H), 7.32 – 7.18 (m, 3H), 6.70 (d, *J* = 8.6 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 152.9, 152.8, 141.0, 130.7, 129.8, 129.5 (q, $^{2}J_{C-F}$ = 31.3 Hz), 124.3 (q, $^{1}J_{C-F}$ = 272.3 Hz), 121.6, 120.3, 117.7 (q, $^{3}J_{C-F}$ = 4.1 Hz), 115.2, 113.9 (q, $^{3}J_{C-F}$ = 4.0 Hz). ^{19}F NMR (375 MHz, DMSO-*d*$_6$) δ -61.4. HRMS (ESI) *m/z* calcd $C_{14}H_{12}O_2N_2F_3$ $[M+H]^+$ 297.0845, found 297.0845.

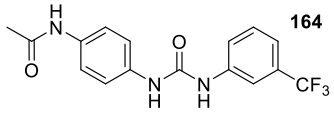
1-(4-(Dimethylamino)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (163)



N^{1},*N*^{1}-Dimethylbenzene-1,4-diamine (0.15 g, 1.10 mmol) and 1-isocyanato-3-(trifluoromethyl)benzene (0.15 mL, 1.10 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was evaporated to dryness under reduced pressure and crystalized from DCM to afford the compound **163** (0.11 g, 31%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 8.88 (s, 1H), 8.39 (s, 1H), 8.00 (s, 1H), 7.54 (d, *J* = 8.3 Hz, 1H), 7.48 (t, *J* = 7.9 Hz, 1H), 7.31 – 7.19 (m, 3H), 6.70 (d, *J* = 9.0 Hz, 2H), 2.84 (s, 6H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 152.7, 146.7, 141.0, 129.8, 129.5 (q, $^{2}J_{C-F}$ = 31.4 Hz), 129.0, 124.3 (q, $^{1}J_{C-F}$ = 272.3 Hz), 121.6,

120.5, 117.6 (q, $^{3}J_{C\text{-}F}$ = 4.0 Hz), 113.9 (q, $^{3}J_{C\text{-}F}$ = 4.0 Hz), 113.0, 40.7. ^{19}F NMR (375 MHz, DMSO-d_6) δ -61.30. HRMS (ESI) *m/z* calcd $C_{15}H_{16}ON_3F_3Na$ $[M+Na]^+$ 346.1137, found 346.1137.

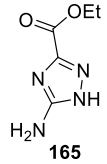
_N_-(4-(3-(3-(Trifluoromethyl)phenyl)ureido)phenyl)acetamide (164)



N-(4-Aminophenyl)acetamide (0.15 g, 1.00 mmol) and 1-isocyanato-3-(trifluoromethyl)benzene (0.14 mL, 1.00 mmol) were dissolved in THF (2 mL) and the reaction mixture was stirred overnight. The reaction mixture was filtered and the solid residue was crystalized from MeOH to afford the compound **164** (0.29 g, 86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.83 (s, 1H), 8.97 (s, 1H), 8.67 (s, 1H), 8.01 (s, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.52 – 7.46 (m, 3H), 7.43 – 7.34 (m, 2H), 7.29 (d, *J* = 7.5 Hz, 1H), 2.02 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.8, 167.9, 162.0, 152.5, 140.7, 134.5, 134.1, 129.9, 121.7, 119.6, 119.0, 117.9, 114.1, 23.9. ^{19}F NMR (375 MHz, DMSO-d_6) δ -61.33. HRMS (ESI) *m/z* calcd $C_{16}H_{14}O_2N_3F_3Na$ $[M+Na]^+$ 360.0930, found 360.0930.

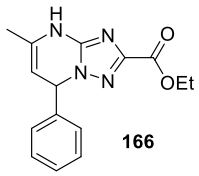
Published inhibitors

Ethyl 5-amino-1*H*-1,2,4-triazole-3-carboxylate (165)



5-Amino-1*H*-1,2,4-triazole-3-carboxylic acid was (0.50 g, 3.90 mmol) was suspended in EtOH (2.3 mL) and cooled down to 0 °C under inert atmosphere. $SOCl_2$ (0.28 mL, 3.90 mmol) was added and the reaction mixture was refluxed for 4h. More $SOCl_2$ (0.3 mL) was added at 0 °C and the reaction mixture was refluxed for further 2h. The reaction mixture was evaporated to dryness under reduced pressure and saturated solution of sodium acetate was added. White precipitate appeared and was collected and dried under vacuum to afford the ethyl ester derivative **165** (0.49 g, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.64 (bs, 1H), 6.23 (bs, 2H), 4.23 (q, *J* = 7.1 Hz, 2H), 1.26 (t, *J* = 7.1 Hz, 3H).

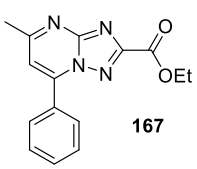
Ethyl 5-methyl-7-phenyl-4,7-dihydro-[1,2,4]triazolo[1,5-a]pyrimidine-2-carboxylate (166)



Compound **165** (0.20 g, 1.28 mmol) and benzylideneacetone (0.19 g, 1.28 mmol) were dissolved in dry DMF (3 mL) and refluxed in a pre-dried sealed tube for 5 h. The reaction was cooled down and quenched by addition of water (25 mL) and extracted with EtOAc (3 × 25 mL). The combined organic

fractions were evaporated to dryness and co-distilled with toluene. The solid residue was purified by column chromatography (EtOAc) to afford the compound **166** (0.20 g, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (s, 1H), 7.39 – 7.27 (m, 5H), 6.05 (dd, *J* = 3.4, 1.3 Hz, 1H), 4.72 – 4.61 (m, 1H), 4.45 – 4.29 (m, 2H), 2.06 (t, *J* = 1.2 Hz, 3H), 1.36 (t, *J* = 7.1 Hz, 3H).

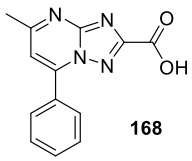
Ethyl 5-methyl-7-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-2-carboxylate (167)



167

Compound **166** (0.11 g, 0.44 mmol) and DDQ (0.096 g, 0.49 mmol) were suspended in dry 1,2-DCE (5 mL) and refluxed in a sealed tube for 1h. The reaction mixture was evaporated to dryness and the residue was purified by column chromatography (EtOAc) to furnish the desired compound **167** (0.086 g, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 – 8.00 (m, 2H), 7.65 – 7.53 (m, 3H), 7.17 (s, 1H), 4.53 (q, *J* = 7.1 Hz, 2H), 2.78 (s, 3H), 1.46 (t, *J* = 7.1 Hz, 3H).

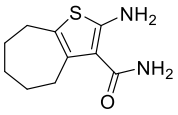
5-Methyl-7-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-2-carboxylic acid (168)



168

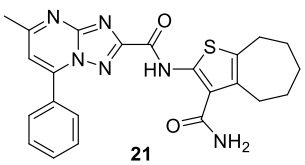
Compound **167** (0.13 g, 0.46 mmol) was suspended in MeOH (3.2 mL) and NaOH (0.025 g, 0.64 mmol) was added. The reaction mixture was refluxed for 2h. The reaction mixture was acidified with aqueous solution of HCl (1M, 15 mL) and a precipitate appeared. The precipitate was collected and dried on vacuum to afford the desired acid **168** (0.081 g, 70%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 8.27 – 7.99 (m, 2H), 7.84 – 7.43 (m, 4H), 2.71 (s, 3H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ 166.6, 161.4, 156.8, 155.7, 146.6, 131.7, 129.5, 129.4, 128.7, 111.9, 24.8.

2-Amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (169)



Cycloheptanone (0.50 g, 4.45 mmol), cyanoacetamide (0.37 g, 4.45 mmol) and sulphur (0.14 g, 4.45 mmol) were suspended in MeOH (5 mL). Diethylamine (0.50 mL, 4.90 mmol) was added and the reaction mixture was stirred overnight. The reaction was quenched with water (40 mL) and extracted with EtOAc/iPrOH 3:1 (3 × 15 mL). The combined organic layers were evaporated to dryness under reduced pressure and the solid residue was purified by column chromatography (DCM/MeOH 10:1) to obtain the desired amine **169** (0.16 g, 17%). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 6.80 (bs, 2H), 6.03 (bs, 2H), 2.88 – 2.60 (m, 2H), 2.50 (m, 2H overlap with solvent), 1.86 – 1.60 (m, *J* = 4.8 Hz, 2H), 1.64 – 1.42 (m, *J* = 5.1 Hz, 4H).

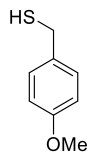
_N_-(3-Carbamoyl-5,6,7,8-tetrahydro-4_H_-cyclohepta[_b_]thiophen-2-yl)-5-methyl-7-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine-2-carboxamide (21)



21

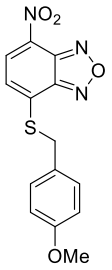
The acid **168** (0.074 g, 0.29 mmol) and TBTU (0.10 g, 0.32 mmol) were dissolved in dry DMF (3 mL) and trimethylamine (0.12 mL, 0.87 mmol) was added. The reaction mixture was stirred for 3 min. and the amine **169** (0.061 g, 0.29 mmol) was added. The reaction mixture was stirred for 4h. The reaction was quenched by addition of water (20 mL) and extracted with EtOAc. The combined organic layer was evaporated to dryness and the residue was purified by column chromatography (EtOAc) and crystalized from MeOH to yield the desired amide **21** (0.04 g, 31%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.09 (s, 1H), 8.28 – 8.08 (m, 2H), 7.94 – 7.45 (m, 4H), 2.89 – 2.80 (m, 2H), 2.80 – 2.71 (m, 5H), 1.94 – 1.74 (m, 2H), 1.73 – 1.49 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.3, 167.0, 157.1, 155.5, 155.1, 146.7, 136.3, 135.2, 131.8, 131.4, 129.6, 129.3, 128.8, 121.8, 111.9, 31.6, 28.3, 28.2, 27.5, 27.1, 24.9. Spectra are in agreement with the literature.[94]

(4-Methoxyphenyl)methanethiol (170)



1-(Chloromethyl)-4-methoxybenzene (0.60 g, 3.83 mmol) and thiourea (0.32 g, 4.21 mmol) were dissolved in EtOH (5 mL) and the reaction mixture was refluxed for 7h. An aqueous solution of ammonia (25%, 6 mL) was added and the reaction mixture was refluxed for 4h. The reaction mixture was concentrated under reduced pressure, acidified with 5% aqueous solution of citric acid (20 mL) and extracted with EtOAc (3 × 15 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, evaporated to dryness under reduced pressure, and purified by column chromatography (hexane/toluene 4:1) to obtain the thiol **170** (0.44 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 8.7 Hz, 2H), 3.80 (s, 3H), 3.71 (d, J = 7.3 Hz, 2H), 1.73 (t, J = 7.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.8, 133.4, 129.3, 114.2, 55.5, 28.5.

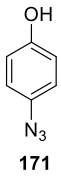
4-((4-Methoxybenzyl)thio)-7-nitrobenzo[*c*][1,2,5]oxadiazole (19)



19

(4-Methoxyphenyl)methanethiol (0.17 g, 1.10 mmol) and 4-Chloro-7-nitrobenzofurazan (0.20 g, 1.00 mmol) were added to a solution of EtOH (2 mL) and pyridine (0.1 mL) and the reaction mixture was refluxed for 1h. The reaction mixture was cooled down and a precipitate appeared. The precipitate was collected and dried under vacuum to give the product **19** (0.094 g, 29%).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (d, *J* = 7.9 Hz, 1H), 7.35 (d, *J* = 8.8 Hz, 2H), 7.19 (d, *J* = 7.9 Hz, 1H), 6.89 (d, *J* = 8.7 Hz, 2H), 4.48 (s, 2H), 3.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.8, 149.3, 142.6, 141.3, 133.0, 130.7, 130.2, 125.5, 121.4, 114.7, 55.5, 36.4. Spectra are in agreement with the literature.[95]

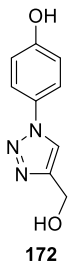
4-Azidophenol (171)



171

4-Aminophenol (2.00 g, 18.34 mmol) was dissolved in an aqueous solution of HCl (2M, 40 mL) and the solution was cooled down to 0 °C. A solution of $NaNO_2$ (1.51 g, 21.90 mmol) in water (6 mL) was cooled down to 0°C and was slowly added to the solution of phenol. The reaction mixture was stirred for 20 minutes at 0°C. NaN_3 (1.78 g, 27.40 mmol) solution in water (6 mL) was added and the reaction mixture was stirred for 1h at 0°C. The reaction mixture was extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with water, brine, dried with $MgSO_4$ and concentrated under reduced pressure to obtain the desired azide **171** (1.27 g, 51%).^{1}H NMR (300 MHz, $CDCl_3$) δ 6.99 – 6.87 (m, 2H), 6.86 – 6.78 (m, 2H).

4-(4-(Hydroxymethyl)-1*H*-1,2,3-triazol-1-yl)phenol (172)

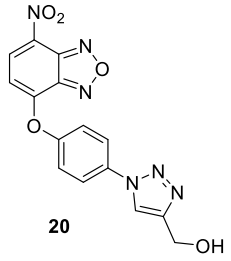


172

Azide **171** (0.70 g, 5.18 mmol) and propargyl alcohol (0.32 g, 5.70 mmol) were dissolved in a degassed mixture of H_2O/*t*BuOH 1:1 (15 mL) and $[Cu(CH_3CN)_4PF_6]$ (0.096 g, 0.26 mmol) was added. The reaction mixture was stirred overnight. The reaction was quenched with water (30mL) and extracted with EtOAc (3 × 15 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure to obtain the phenol **172** (0.81 g, 82%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.91 (s, 1H), 8.47 (s, 1H), 7.65 (d, *J* = 8.9 Hz, 2H), 6.92 (d, *J* = 8.9 Hz, 2H), 5.27 (t, *J* = 5.6 Hz, 1H), 4.58 (d, *J* = 5.5 Hz, 2H).

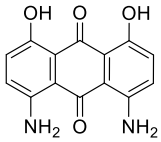
(1-(4-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)oxy)phenyl)-1*H*-1,2,3-triazol-4-yl)methanol (20)

4-Chloro-7-nitrobenzofurazan (0.36 g, 1.83 mmol) and phenol **172** (0.35 g, 1.83 mmol) were dissolved in EtOH (5 mL) and pyridine (0.3 mL, 3.72 mmol). Catalytic amount of KOAc was added and the reaction mixture was refluxed for 4h. The reaction mixture was evaporated to dryness under reduced pressure and purified by column chromatography (hexane/EtOAc 3/1) and crystalized from MeOH to yield to desired compound **20** (0.54 g, 83%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 (s, 1H), 8.66 (d, J = 8.4 Hz, 1H), 8.12 (d, J = 9.0 Hz, 2H), 7.66 (d, J = 9.0 Hz, 2H), 6.88 (d, J = 8.4 Hz, 1H), 5.37 (s, 1H), 4.63 (s, 2H). Spectra are in agreement with the literature.[96]



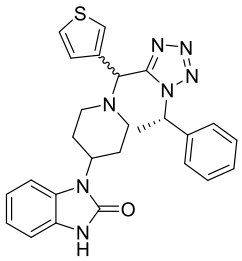
20

1,8-Diamino-4,5-dihydroxyanthracene-9,10-dione (21)



To a solution of 1,8-dihydroxy-4,5-dinitroanthracene-9,10-dione (1.00 g, 3.03 mmol) in EtOH (20 mL) was added tin(II) chloride dihydrate (6.80 g, 30.30 mmol) and the solution was stirred overnight at room temperature. The reaction mixture was evaporated and the residue was purified by column chromatography (DCM/MeOH 10:1) to obtain the pure product **21** (0.31 g, 38%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.75 (s, 1H), 7.91 (s, 2H), 7.26 (d, J = 9.3 Hz, 1H), 7.11 (d, J = 9.2 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 189.5, 184.0, 155.3, 146.3, 129.7, 125.7, 113.1, 109.1. Spectra are in agreement with the literature.[153]

(*S*)-1-(1-((1-(1-Phenylethyl)-1*H*-tetrazol-5-yl)(thiophen-3-yl)methyl)piperidin-4-yl)-1,3-dihydro-*2*H-benzo[*d*]imidazol-2-one (23a-b)



4-(2-Keto-1-benzimidazolinyl)piperidine (0.20 g, 0.92 mmol) and 3-thiophenecarboxaldehyde (0.10 g, 0.92 mmol) were stirred in MeOH (5 mL) for 15 minutes. (*S*)-(-)-α-methylbenzyl isocyanate (0.12 g, 0.92 mmol) and trimethylsilyl azide (0.11 g, 0.92 mmol) were added respectively. The reaction mixture was stirred overnight. The reaction mixture was evaporated and purified by preparative HPLC. Two diastereoisomers were isolated A (0.025 g, 6%); B (0.03 g, 7%). (a) ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.80 (s, 1H), 7.37 – 7.31 (m, 2H), 7.31 – 7.22 (m, 4H), 7.26 – 7.00 (m, 6H), 6.10 (q, J = 7.1 Hz, 1H), 5.07 (s, 1H), 4.19 (m, 1H), 3.13 – 2.98 (m, 1H), 2.76 (d, J = 9.0 Hz, 1H), 2.53 – 2.30 (m, 3H), 2.23 – 2.09

(m, 1H), 1.99 (d, *J* = 7.0 Hz, 3H), 1.85 – 1.67 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.1, 153.8, 139.4, 134.3, 129.3, 129.2, 128.8, 128.3, 128.1, 126.5, 126.1, 125.3, 121.4, 121.2, 109.9, 109.3, 60.0, 59.0, 51.6, 50.7, 49.3, 29.3, 22.7. (b) ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.82 (s, 1H), 7.46 – 7.33 (m, 4H), 7.28 – 7.18 (m, 4H), 7.12 – 6.99 (m, 4H), 5.69 (q, *J* = 7.0 Hz, 1H), 5.09 (s, 1H), 4.28 – 4.02 (m, 1H), 3.01 – 2.71 (m, 2H), 2.51 – 2.30 (m, 2H), 2.30 – 2.05 (m, 2H), 2.02 (d, *J* = 7.0 Hz, 3H), 1.86 – 1.57 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.1, 154.1, 139.6, 134.6, 129.3, 129.2, 128.8, 128.5, 128.0, 126.5, 126.3, 125.4, 121.3, 121.1, 109.8, 109.7, 59.6, 58.6, 51.0, 50.7, 49.0, 29.8, 29.5, 29.1, 22.8.

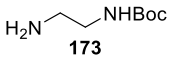
General procedure for *N,O*-deprotection

For 0.020 g of compound circa 2 mL of DCM and 1mL of TFA were added. The reaction progress was monitored by TLC. When there was no starting material detected by TLC the reaction mixture was evaporated to dryness under reduced pressure and the residue was purified by preparative HPLC.

General procedure for amide coupling

The acid (1 eq.) and TBTU (1.3 eq.) were dissolved in dry DMF or acetonitrile and TEA (4 eq.) was added under inter atmosphere of nitrogen. The reaction mixture was stirred for at least 5 minutes and then the amine (1.2 eq.) was added. The reaction progress was monitored by TLC. After completion the reaction was quenched by water and extracted with DCM. The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure.

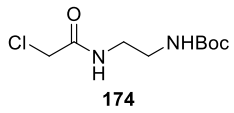
***tert*-Butyl (2-aminoethyl)carbamate (173)**



173

The solution of ethylenediamine (3.60 g, 60 mmol) in $CHCl_3$ (57 mL) was cooled down to 0 °C and the solution of Boc-anhydride (1.24 g, 5.70 mmol) in $CHCl_3$ (28 mL) was added dropwise over two hours. The reaction mixture was stirred for further 3 hours at room temperature. The reaction mixture was concentrated to obtain the amine **173** (0.86 g, 94 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.08 (bs, 1H), 3.25 – 3.05 (m, 2H), 2.81 (t, *J* = 5.9 Hz, 2H), 2.44 (bs, 2H), 1.43 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.4, 79.4, 43.0, 41.8, 28.5.

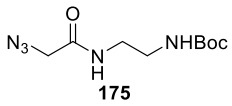
***tert*-Butyl (2-(2-chloroacetamido)ethyl)carbamate (174)**



174

Compound **173** (0.40 g, 1.69 mmol) and DIPEA (0.32 g, 2.71 mmol) were dissolved in THF (10 mL) and the solution was cooled down to -5°C under inert atmosphere of nitrogen. A solution of chloroacetyl chloride (0.28 g, 2.51 mmol) in THF (5 mL) was added dropwise. The reaction mixture was stirred for 30 min. at -5°C under inter atmosphere of nitrogen. The reaction mixture was concentrated under reduced pressure and subsequently quenched by addition of water (20 mL) the reaction mixture was washed with chloroform (3 × 20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness. The solid residue was crystalized from hexane/EtOAc 3:1 to afford the chloride **174** (0.38 g, 65%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20 (bs, 1H), 4.88 (bs, 1H), 4.03 (s, 2H), 3.45 – 3.36 (m, 2H), 3.36 – 3.23 (m, 2H), 1.44 (s, 9H).

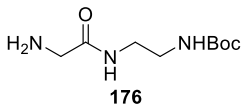
***tert*-Butyl (2-(2-azidoacetamido)ethyl)carbamate (175)**



175

Compound **174** (0.34 g, 1.46 mmol) was dissolved in a mixture EtOH/H_2O 5:1 (10 mL) and sodium azide (0.19 g, 2.93 mmol) was added. The reaction mixture was stirred overnight at 60 °C. The reaction mixture was carefully concentrated by evaporation of EtOH under reduced pressure and the water phase was extracted by DCM (3 × 13 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness to afford the azide **175** (0.34 g, 95 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.97 (bs, 1H), 4.90 (bs, 1H), 3.96 (s, 2H), 3.60 – 3.36 (m, 2H), 3.30 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 157.0, 80.1, 52.8, 40.9, 40.2, 28.5.

***tert*-Butyl (2-(2-aminoacetamido)ethyl)carbamate (176)**

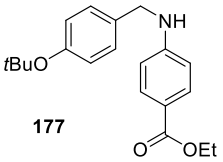


176

Compound **175** (0.33 g, 1.35 mmol) was dissolved in EtOH (14 mL) and 10 % Pd/C (0.033 g) was added. The reaction mixture was kept under positive pressure of hydrogen for 4h. After completion the reaction mixture was filtered and the filtrate was evaporated to obtain the amine **176** (0.26 g, 89 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (bs, 1H), 5.01 (bs, 1H), 3.59 – 3.33 (m, 4H), 3.33 – 3.11 (m, 2H), 1.90 (bs, 2H), 1.42 (s, 9H).

Ethyl 4-((4-(tert-butoxy)benzyl)amino)benzoate (177)

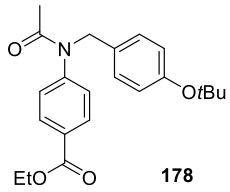
To a solution of benzocaine (0.37 g, 2.24 mmol) and 4-*tert*-butoxybenzaldehyde (0.40 g, 2.24 mmol) in MeOH (12 mL) was added 2-Pic-BH_3 complex (0.24 g, 2.24 mmol) and the reaction



177

mixture was stirred overnight. The reaction mixture was quenched by water (30 mL) and the mixture was extracted with DCM (3 × 30 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The solid residue was purified by column chromatography (hexane/EtOAc 4:1) to obtain 0.65 g of **177** (87 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.8 Hz, 2H), 7.23 (d, *J* = 8.5 Hz, 2H), 6.96 (d, *J* = 8.5 Hz, 2H), 6.62 (d, *J* = 8.7 Hz, 2H), 4.41 – 4.22 (m, 4H), 1.42 – 1.31 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.9, 155.0, 151.4, 132.9, 131.6, 128.3, 124.5, 119.5, 112.1, 78.7, 60.4, 47.7, 29.0, 14.6. HRMS (EI) *m/z* calcd $C_{22}H_{27}O_4N$ $[M]^{\bullet+}$ 327.1834 found 327.1839.

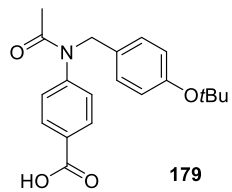
Ethyl 4-(*N*-(4-(tert-butoxy)benzyl)acetamido)benzoate (178)



178

Amine **177** (0.30 g, 0.91 mmol) and triethylamine (0.51 mL, 3.63 mmol) were dissolved in DCM (12 mL). Acetyl chloride (0.13 mL, 1.81 mmol) was added and the reaction mixture was stirred for 4h under inert atmosphere. The reaction was quenched by addition of water (30 mL). The mixture was extracted with DCM (3 × 20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and evaporated to dryness under reduced pressure. The solid residue was purified by column chromatography (hexanes/EtOAc 2:1) to obtain the amide **178** (0.29 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, *J* = 8.5 Hz, 2H), 7.02 (dd, *J* = 8.6, 2.9 Hz, 4H), 6.84 (d, *J* = 8.5 Hz, 2H), 4.84 (s, 2H), 4.35 (q, *J* = 7.1 Hz, 2H), 1.87 (s, 3H), 1.36 (t, *J* = 7.1 Hz, 3H), 1.29 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.9, 165.7, 154.9, 146.8, 132.0, 131.5, 130.9, 129.5, 128.3, 124.4, 124.1, 78.5, 61.3, 28.9, 22.9, 14.4. HRMS (EI) *m/z* calcd $C_{22}H_{27}O_4N$ $[M]^{\bullet+}$ 369.1940 found 369.1946.

4-(*N*-(4-(tert-butoxy)benzyl)acetamido)benzoic acid (179)

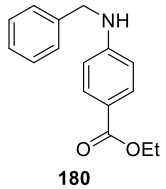


179

The ester **178** (0.24 g, 0.64 mmol) was dissolved in MeOH (15 mL) and 0.125 M aqueous solution of NaOH was added (10 mL). The reaction mixture was refluxed for 2h. The TLC analysis (DCM/MeOH/AcOH 46:2:1) showed consumption of starting material. The reaction mixture was acidified with 5% aqueous solution of citric acid and a precipitate appeared. The precipitate was collected, washed with water, and dried under vacuum to obtain the acid **179** (0.19 g, 84%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (d, *J* = 8.5 Hz, 2H), 7.21 (d, *J* = 8.5 Hz, 2H), 7.09 (d, *J* = 8.5 Hz, 2H), 6.89 (d, *J* = 8.5 Hz, 2H), 4.91 (s, 2H), 1.92 (s, 3H), 1.30 (s, 9H). ^{13}C NMR (100

MHz, CD_3OD) δ 171.0, 167.4, 154.7, 146.3, 131.9, 130.7, 130.3, 129.0, 128.1, 123.8, 78.3, 51.7, 27.8, 21.3. HRMS (ESI) *m/z* calcd $C_{20}H_{22}O_4N$ $[M–H]^-$ 340,1554 found 340,1551.

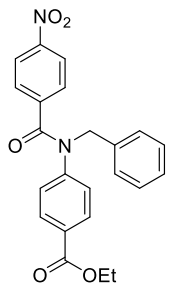
Ethyl 4-(benzylamino)benzoate (180)



180

To a solution of benzocaine (0.20 g, 1.21 mmol) and benzaldehyde (0.13 g, 1.22 mmol) in MeOH (7 mL) was added 2-Pic-BH_3 complex (0.14 g, 1.31) and the reaction mixture was stirred overnight. The reaction mixture was quenched by water (30 mL) and the mixture was extracted with DCM (3 × 30 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The solid residue was purified by column chromatography (hexane/EtOAc 4:1) to obtain **180** (0.24 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 8.8 Hz, 2H), 7.39 – 7.26 (m, 5H), 6.61 (d, *J* = 8.6 Hz, 2H), 4.79 (bs, 1H), 4.39 (s, 2H), 4.31 (q, *J* = 7.1 Hz, 2H), 1.36 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.9, 151.6, 138.4, 131.6, 128.9, 127.7, 127.6, 119.3, 111.9, 60.4, 47.9, 14.6. HRMS (EI) *m/z* calcd $C_{16}H_{17}N_1O_2Na$ $[M]^{+\bullet}$ 255.1259, found 255.1258.

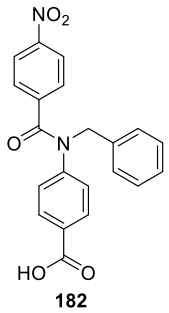
Ethyl 4-(*N*-benzyl-4-nitrobenzamido)benzoate (181)



181

To a solution of **180** (0.25 g, 0.98 mmol) and 4-nitrobenzoyl chloride (0.18 g, 1.00 mmol) in dry toluene (5 mL) was added DIPEA (0.31 mL, 1.85 mmol). The solution was stirred at 90 °C for 4h in a sealed tube. The reaction was quenched by addition of water (30 mL), and the mixture was extracted with DCM (3 × 15 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The solid residue was purified by column chromatography (hexane/EtOAc 4:1) to obtain the amide **181** (0.40 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 8.5 Hz, 2H), 7.48 (d, *J* = 8.8 Hz, 2H), 7.32 – 7.26 (m, 5H), 6.95 (d, *J* = 8.6 Hz, 2H), 5.16 (s, 2H), 4.32 (q, *J* = 7.1 Hz, 2H), 1.34 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.4, 165.5, 148.4, 146.4, 141.8, 136.5, 130.9, 129.8, 129.5, 128.9, 128.7, 128.0, 127.7, 123.4, 61.5, 53.9, 14.4. HRMS (ESI) *m/z* calcd $C_{23}H_{20}N_2O_5Na$ $[M+Na]^+$ 427.1264, found 427.1265.

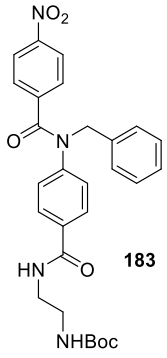
4-(*N*-Benzyl-4-nitrobenzamido)benzoic acid (182)



182

Compound **181** (0.16 g, 0.40 mmol) was dissolved in a mixture of THF/MeOH 1:1 (6.5 mL) and 0.125 M aqueous solution of NaOH (6.5 mL) was added. The reaction mixture was heated up to a 60 °C for 2.5 h. The reaction was quenched by addition of 5% aqueous solution of citric acid (15 mL). The mixture was extracted with DCM (3 × 25 mL) and the combined organic layers were evaporated to dryness under reduced pressure. The residue was purified by column chromatography (DCM/MeOH 20:1) to obtain the acid **182** (0.13 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, *J* = 8.9 Hz, 2H), 7.89 (d, *J* = 8.7 Hz, 2H), 7.50 (d, *J* = 8.9 Hz, 2H), 7.35 – 7.26 (m, 5H), 6.99 (d, *J* = 8.7 Hz, 2H), 5.17 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.8, 168.4, 148.5, 147.4, 141.6, 136.4, 131.6, 129.8, 128.9, 128.6, 128.1, 128.0, 127.7, 123.5, 53.9. HRMS (ESI) *m/z* calcd $C_{21}H_{15}N_2O_5$ $[M-H]^-$ 375.0986, found 375.0981.

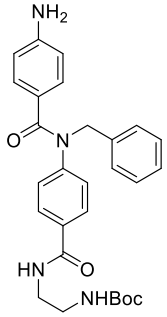
***tert*-Butyl (2-(4-(*N*-benzyl-4-nitrobenzamido)benzamido)ethyl)carbamate (183)**



183

Compounds **182** (0.13 g, 0.34 mmol) and **173** (0.054 g, 0.34 mmol) were coupled together using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **183** (0.15 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.9 Hz, 2H), 7.64 (d, *J* = 8.6 Hz, 2H), 7.46 (d, *J* = 8.9 Hz, 2H), 7.36 (bs, 1H), 7.31 – 7.20 (m, 5H), 6.92 (d, *J* = 8.5 Hz, 2H), 5.12 (bs, 1H), 3.45 (m, 2H), 3.33 (m, 2H), 1.34 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.3, 166.3, 162.6, 148.2, 145.0, 141.9, 136.5, 133.1, 129.7, 128.8, 128.6, 128.4, 127.9, 127.8, 123.3, 80.1, 53.9, 38.8, 38.7, 28.3. HRMS (ESI) *m/z* calcd $C_{28}H_{30}N_4O_6Na$ $[M+Na]^+$ 541.2057, found 541.2058.

***tert*-Butyl (2-(4-(4-amino-*N*-benzylbenzamido)benzamido)ethyl)carbamate (184)**

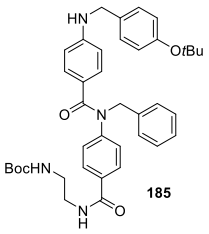


184

Compound **183** (0.15 g, 0.29 mmol) was dissolved in a mixture MeOH/EtOAc 2:1 (12 mL). NH_4Cl (0.076 g, 1.44 mmol) and tin(II) chloride dihydrate (0.26 g, 1.15 mmol) were added. The reaction mixture was stirred overnight at 60 °C. The reaction mixture was concentrated under reduced pressure and purified by column chromatography (DCM/MeOH 20:1) to afford the amine **184** (0.030 g, 21%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, *J* = 8.4 Hz, 2H), 7.35 – 7.20 (m, 5H), 7.16 (d, *J* = 8.6 Hz, 2H), 6.95 (d, *J* = 8.4 Hz, 2H), 6.43 (d, *J* = 8.1 Hz, 2H),

5.11 (bs, 2H), 5.08 (bs, 1H), 3.46 (m, 2H), 3.32 (m, 2H), 1.38 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 167.0, 157.7, 147.4, 137.6, 131.6, 131.3, 129.2, 128.6, 128.4, 128.3, 128.0, 127.5, 127.2, 125.4, 114.3, 80.1, 58.6, 53.9, 28.5. HRMS (ESI) *m/z* calcd $C_{28}H_{32}N_4O_4Na$ $[M+Na]^+$ 511.2315, found 511.2315.

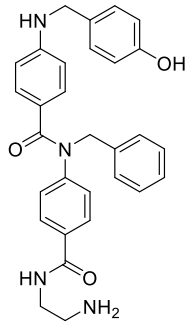
tert-Butyl (2-(4-(*N*-benzyl-4-((4-(*tert*-butoxy)benzyl)amino)benzamido)benzamido)ethyl) carbamate (185)



185

To a solution of **184** (0.03 g, 0.06 mmol) and 4-(*tert*-butoxy)benzaldehyde (0.01 g, 0.06 mmol) in MeOH (3 mL) was added 2-Pic-BH_3 complex (0.006 g, 0.06 mmol) and the reaction mixture was stirred overnight. The reaction mixture was evaporate to dryness under reduced pressure and the solid residue was purified by column chromatography (from cyclohexane/EtOAc 1:1 to EtOAc to EtOAc/MeOH 7:1) to afford the secondary amine **185** (0.031 g, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, *J* = 8.4 Hz, 2H), 7.30 – 7.22 (m, 5H), 7.20 (d, *J* = 8.8 Hz, 2H), 7.18 (d, *J* = 8.6 Hz, 2H), 6.98 (d, *J* = 8.7 Hz, 2H), 6.93 (d, *J* = 8.6 Hz, 2H), 6.35 (d, *J* = 8.8 Hz, 2H), 5.12 (s, 2H), 5.03 (s, 1H), 4.19 (s, 2H), 3.48 (m, 2H), 3.36 (m, 2H), 1.38 (s, 9H), 1.33 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3, 170.6, 167.0, 157.7, 154.9, 149.9, 147.8, 137.8, 133.3, 131.5, 131.3, 128.6, 128.3, 128.00, 127.4, 127.1, 124.5, 123.5, 111.5, 80.1, 78.7, 53.9, 47.4, 42.3, 40.0, 29.00, 28.4. HRMS (ESI) *m/z* calcd $C_{39}H_{46}N_4O_5Na$ $[M+Na]^+$ 673.3360, found 673.3360.

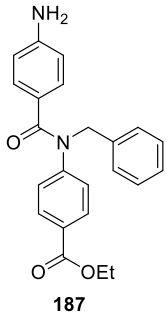
_N_-(4-((2-Aminoethyl)carbamoyl)phenyl)-_N_-benzyl-4-((4-hydroxybenzyl)amino) benzamide (186)



186

Compound **185** was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **186** (0.010 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, *J* = 8.6 Hz, 2H), 7.24 (m, 5H), 7.13 (m, 6H), 6.72 (d, *J* = 8.5 Hz, 2H), 6.39 (d, *J* = 8.8 Hz, 2H), 5.18 (s, 2H), 4.14 (s, 2H), 3.62 (m, 2H), 3.13 (t, *J* = 5.2 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 170.3, 157.5, 152.2, 148.8, 138.7, 132.3, 132.2, 131.2, 129.7, 129.5, 129.3, 129.2, 128.6, 128.4, 123.2, 116.2, 112.4, 54.5, 47.6, 41.0, 38.7. HRMS (ESI) *m/z* calcd $C_{30}H_{30}N_4O_3Na$ $[M+Na]^+$ 517.2210, found 517.2209.

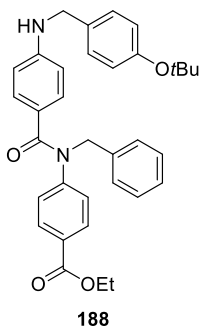
Ethyl 4-(4-amino-*N*-benzylbenzamido)benzoate (187)



187

Compound **181** (0.30 g, 0.74 mmol) was dissolved in a mixture EtOAc/MeOH 1:1 (10 mL) and NH_4Cl (0.59 g, 11.10 mmol) and activated zinc (0.72 g, 11.10 mmol) were added. The reaction mixture was stirred overnight. The reaction mixture was filtered over celite and the filtrate was evaporated under reduced pressure. The solid residue was suspended in 50 ml of water and by addition of saturated solution of sodium bicarbonate the pH was adjusted to 8-9. The mixture was extracted by DCM (3 × 50 mL) and the combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The solid residue was purified by column chromatography (cyclohexane/EtOAc 2:1) to afford the amine **187** (0.23 g, 74 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.8 Hz, 2H), 7.33 – 7.22 (m, 5H), 7.20 (d, *J* = 8.7 Hz, 2H), 6.99 (d, *J* = 8.8 Hz, 2H), 6.44 (d, *J* = 8.7 Hz, 2H), 5.15 (s, 2H), 4.32 (q, *J* = 7.1 Hz, 2H), 1.57 (bs, 2H), 1.35 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 166.0, 148.8, 137.7, 131.4, 130.5, 128.7, 128.2, 127.8, 127.5, 126.9, 125.1, 114.1, 113.00, 61.2, 53.9, 14.4. HRMS (ESI) *m/z* calcd $C_{23}H_{22}N_2O_3Na$ $[M+Na]^+$ 397.1522, found 397.1522.

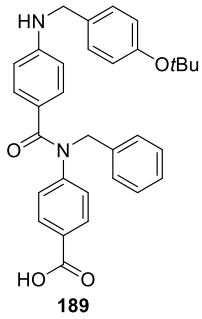
Ethyl 4-(*N*-benzyl-4-((4-(*tert*-butoxy)benzyl)amino)benzamido)benzoate (188)



188

To a solution of **187** (0.26 g, 0.69 mmol) and 4-(*tert*-butoxy)benzaldehyde (0.12 g, 0.67 mmol) in MeOH (16 mL) was added 2-Pic-BH_3 complex (0.072 g, 0.67 mmol) and the reaction mixture was stirred overnight. The reaction mixture was evaporated to dryness under reduced pressure and purified by column chromatography (cyclohexane/EtOAc 4:1 and toluene/EtOAc 10:1) to afford the secondary amine **188** (0.20 g, 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.8 Hz, 2H), 7.34 – 7.25 (m, 5H), 7.23 (d, *J* = 8.8 Hz, 2H), 7.18 (d, *J* = 8.6 Hz, 2H), 7.01 (d, *J* = 8.8 Hz, 2H), 6.94 (d, *J* = 8.6 Hz, 2H), 5.15 (s, 2H), 4.32 (q, *J* = 7.1 Hz, 2H), 4.20 (s, 2H), 1.36 (t, *J* = 7.1 Hz, 3H), 1.33 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.6, 166.1, 154.9, 149.7, 149.1, 137.8, 133.6, 131.5, 130.5, 128.6, 128.3, 128.1, 127.6, 127.4, 126.7, 124.4, 111.7, 78.7, 61.2, 53.9, 47.6, 29.00, 14.4. HRMS (ESI) *m/z* calcd $C_{34}H_{36}N_2O_4Na$ $[M+Na]^+$ 559.2567, found 559.2567.

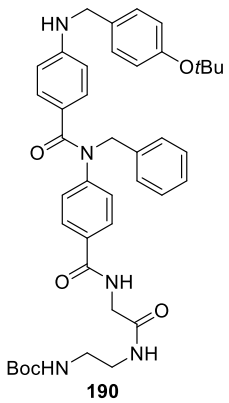
4-(*N*-benzyl-4-((4-(*tert*-butoxy)benzyl)amino)benzamido)benzoic acid 189



189

Compound **188** (0.10 g, 0.19 mmol) was dissolved in a mixture THF/MeOH 1:1 (8 mL) and a 0.125 M aqueous solution of NaOH (1.5 mL) was added. The reaction mixture was refluxed for 2.5 hours. The reaction mixture was acidified by addition of 5% aqueous solution of citric acid (10 mL) and extracted with DCM (3 × 30 mL). The combined organic layers were evaporated to dryness under reduced pressure and purified by column chromatography (DCM/MeOH 20:1) to afford the acid **189** (0.086 g, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, *J* = 8.7 Hz, 2H), 7.39 – 7.21 (m, 7H), 7.18 (d, *J* = 8.6 Hz, 2H), 7.04 (d, *J* = 8.7 Hz, 2H), 6.94 (d, *J* = 8.5 Hz, 2H), 6.38 (d, *J* = 8.8 Hz, 2H), 5.17 (s, 2H), 4.21 (s, 2H), 1.33 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8(2x), 154.9, 150.1, 150.00, 137.8, 133.3, 131.6, 131.2, 128.7, 128.3, 128.1, 127.5, 126.7, 124.5, 123.2, 111.6, 111.5, 78.7, 53.9, 47.4, 29.0. MS (ESI) *m/z* calcd $C_{32}H_{32}N_2O_4Na$ $[M+H]^+$ 509,2435, found 509.2721.

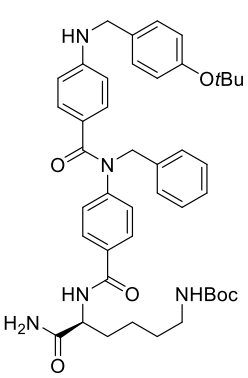
***tert*-Butyl (2-(2-(4-(*N*-benzyl-4-((4-(*tert*-butoxy)benzyl)amino)benzamido)benzamido) acetamido)ethyl)carbamate (190)**



190

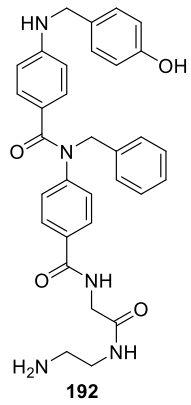
Acid **189** (0.043 g, 0.085 mmol) and amine **176** (0.019 g, 0.094 mmol) were coupled together using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide (0.039 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, *J* = 8.4 Hz, 2H), 7.35 – 7.21 (m, 8H), 7.20 (d, *J* = 8.5 Hz, 2H), 7.15 (s, 1H), 7.07 – 6.98 (m, *J* = 8.6 Hz, 3H), 6.95 (d, *J* = 8.5 Hz, 2H), 6.38 (d, *J* = 8.8 Hz, 2H), 5.15 (s, 2H), 5.12 (m, 1H), 4.21 (s, 2H), 4.04 (d, *J* = 4.9 Hz, 2H), 3.36 (m, 5.4 Hz, 2H), 3.25 (m, 2H), 1.40 (s, 9H), 1.34 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 169.4, 167.0, 157.0, 154.9, 149.9, 148.1, 137.8, 133.3, 131.5, 130.6, 128.7, 128.3, 128.2, 128.1, 127.4, 127.0, 124.4, 123.3, 111.6, 79.9, 78.7, 53.9, 47.4, 43.6, 41.0, 40.2, 29.0, 28.5. HRMS (ESI) *m/z* calcd $C_{41}H_{49}N_5O_6Na$ $[M+Na]^+$ 730.3575, found 730.3578.

***tert*-Butyl (*S*)-(6-amino-5-(4-(*N*-benzyl-4-((4-(*tert*-butoxy)benzyl)amino)benzamido) benzamido)-6-oxohexyl)carbamate (191)**



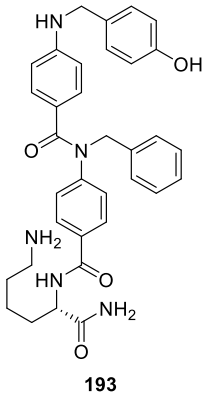
Acid **189** (0.043 g, 0.085 mmol) and (*S*)-1-amino-6-((*tert*-butoxycarbonyl)amino)-1-oxohexan-2-aminium chloride (0.026 g, 0.093 mmol) were coupled together using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **191** (0.048 g, 77%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.69 (d, *J* = 8.7 Hz, 2H), 7.32 – 7.16 (m, 7H), 7.14 (d, *J* = 8.8 Hz, 2H), 7.09 (d, *J* = 8.7 Hz, 2H), 6.92 (d, *J* = 8.5 Hz, 2H), 6.54 (s, 1H), 6.39 (d, *J* = 8.9 Hz, 2H), 5.16 (s, 2H), 4.19 (s, 2H), 3.03 (q, *J* = 6.5 Hz, 2H), 1.91 – 1.67 (m, 2H), 1.51 (m, 2H), 1.46 – 1.42 (m, 2H), 1.39 (s, 9H), 1.30 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD) δ 177.2, 173.3, 169.3, 158.6, 155.4, 152.3, 148.6, 138.8, 135.8, 132.8, 132.2, 129.5, 129.4, 129.2, 129.0, 128.5, 128.4, 125.3, 123.1, 112.2, 79.9, 79.6, 55.1, 54.6, 47.5, 38.9, 32.8, 30.6, 29.2, 28.8, 24.40. HRMS (ESI) *m/z* calcd $C_{43}H_{53}N_5O_6Na$ $[M+Na]^+$ 758.3888, found 758.3886.

***N*-(4-((2-((2-Aminoethyl)amino)-2-oxoethyl)carbamoyl)phenyl)-*N*-benzyl-4-((4-hydroxybenzyl)amino)benzamide (192)**



Compound **190** (0.038 g, 0.053 mmol) was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **192** (0.017 g, 58%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.72 (d, *J* = 8.8 Hz, 2H), 7.32 – 7.19 (m, 5H), 7.15 (d, *J* = 8.9 Hz, 2H), 7.13 – 7.07 (m, 4H), 6.71 (d, *J* = 8.7 Hz, 2H), 6.41 (d, *J* = 8.9 Hz, 2H), 5.17 (s, 2H), 4.14 (s, 2H), 3.97 (s, 2H), 3.50 – 3.43 (m, 2H), 3.05 (t, *J* = 5.7 Hz, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 173.3, 173.1, 169.9, 157.6, 151.9, 148.8, 138.7, 132.1, 130.9, 129.7, 129.5, 129.4, 129.3, 128.8, 128.6, 128.4, 123.5, 116.2, 112.7, 54.5, 49.0, 47.9, 44.3, 41.0, 38.1. HRMS (ESI) *m/z* calcd $C_{32}H_{34}N_5O_4$ $[M+H]^+$ 552.2605, found 552.2608.

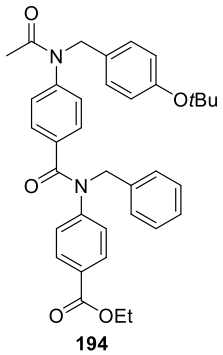
(*S*)-*N*-Benzyl-*N*-(4-((1,6-diamino-1-oxohexan-2-yl)carbamoyl)phenyl)-4-((4-hydroxybenzyl)amino)benzamide (193)



193

Compound **191** (0.048 g, 0.07 mmol) was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **193** (0.020 g, 54%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.69 (d, *J* = 8.7 Hz, 2H), 7.38 – 7.02 (m, 11H), 6.71 (d, *J* = 8.7 Hz, 2H), 6.52 – 6.37 (m, 2H), 5.17 (s, 2H), 4.51 (dd, *J* = 9.2, 5.3 Hz, 1H), 4.15 (s, 2H), 2.91 (td, *J* = 7.9, 2.9 Hz, 2H), 2.00 – 1.86 (m, 1H), 1.79 (dtd, *J* = 14.3, 9.4, 5.3 Hz, 1H), 1.73 – 1.59 (m, 2H), 1.58 – 1.36 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 176.8, 173.2, 169.4, 157.7, 151.3, 148.6, 138.7, 132.7, 132.1, 130.6, 129.9, 129.5, 129.4, 129.3, 128.6, 128.4, 121.7, 116.3, 113.2,113.1, 54.7, 54.5, 40.5, 32.5, 28.1, 24.0. HRMS (ESI) *m/z* calcd $C_{34}H_{37}N_5O_4Na$ $[M+Na]^+$ 602.2737, found 602.2739.

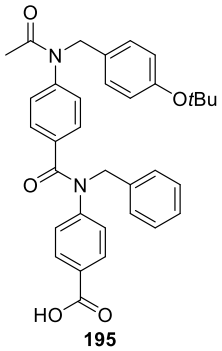
Ethyl 4-(*N*-benzyl-4-(*N*-(4-(*tert*-butoxy)benzyl)acetamido)benzamido)benzoate (194)



194

Acetyl chloride (10 µL, 0.14 mmol) was added to a solution of **188** (0.050 g, 0.09 mmol) and DIPEA (73 µL, 0.042 mmol) in dry DCM (2 mL). The reaction mixture was stirred for 4 h in a sealed tube. The reaction was quenched by addition of water (5 mL) and extracted with EtOAc (3 × 7 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness to afford the amide **194** (0.053 g, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.7 Hz, 2H), 7.30 – 7.24 (m, 7H), 6.92 (m, *J* = 8.7, 2.5 Hz, 4H), 6.82 (d, *J* = 8.4 Hz, 2H), 6.75 (d, *J* = 7.9 Hz, 2H), 5.15 (s, 2H), 4.73 (s, 2H), 4.33 (q, *J* = 7.1 Hz, 2H), 1.76 (s, 3H), 1.35 (t, *J* = 7.1 Hz, 3H), 1.31 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.9, 169.7, 165.7, 154.9, 147.4, 144.1, 137.0, 132.1, 130.5, 130.1, 129.7, 128.8, 128.4, 127.8, 127.4, 124.2, 78.6, 61.4, 53.7, 52.1, 29.0, 28.9, 22.8, 14.5, 14.4. HRMS (ESI) *m/z* calcd $C_{36}H_{38}N_2O_5Na$ $[M+Na]^+$ 601.2672, found 601.2675.

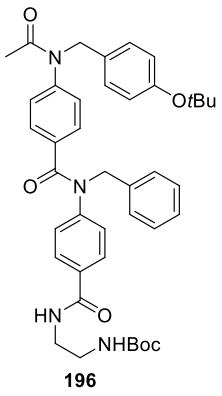
4-(*N*-Benzyl-4-(*N*-(4-(tert-butoxy)benzyl)acetamido)benzamido)benzoic acid (195)



195

Compound **194** (0.10 g, 0.30 mmol) was dissolved in a mixture of THF/MeOH 1:1 (8 mL) and a 0.125 M aqueous solution of NaOH (4.8 mL) was added. The reaction mixture was refluxed for 5 hours. The reaction mixture was acidified by addition of 5% aqueous solution of citric acid (20 mL) and extracted with DCM (4 × 50 mL). The combined organic layers were evaporated to dryness under reduced pressure and purified by column chromatography (DCM/MeOH 10:1) to afford the acid **195** (0.16 g, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 8.7 Hz, 2H), 7.35 – 7.18 (m, 7H), 6.98 – 6.87 (m, *J* = 8.6 Hz, 4H), 6.82 (d, *J* = 8.4 Hz, 2H), 6.75 (d, *J* = 7.9 Hz, 2H), 5.16 (s, 2H), 4.74 (s, 2H), 1.75 (s, 3H), 1.29 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4, 169.7, 169.5, 154.9, 147.9, 143.9 136.9, 135.4, 131.8, 131.0, 130.1, 129.7, 128.8, 128.3, 128.2, 127.9, 127.9, 127.5, 124.2, 78.7, 53.7, 52.2, 29.00, 22.6. HRMS (ESI) *m/z* calcd $C_{34}H_{33}N_2O_5$ $[M-H]^-$ 549.2395, found 549.2385.

***tert*-Butyl (2-(4-(*N*-benzyl-4-(*N*-(4-(*tert*-butoxy)benzyl)acetamido)benzamido) benzamido)ethyl)carbamate (196)**

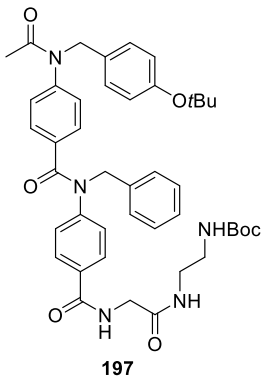


196

Compound **195** (0.05 g, 0.09 mmol) **173** (0.016 g, 0.01 mmol) were coupled together in acetonitrile using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **196** (0.037 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, *J* = 8.4 Hz, 2H), 7.32 – 7.21 (m, 7H), 6.92 (m, 4H), 6.82 (d, *J* = 8.4 Hz, 2H), 6.75 (d, *J* = 7.8 Hz, 2H), 5.12 (s, 2H), 5.06 (bs, 1H), 4.73 (s, 2H), 3.55 – 3.47 (m, 2H), 3.41 – 3.32 (m, 2H), 1.38 (s, 9H), 1.30 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.9, 169.5, 166.4, 154.8, 146,0 144.1, 137.0, 135.2, 132.5, 132.1, 130.2, 129.6, 128.7, 128.5, 128.0, 128.0, 127.8, 127.6, 124.2, 80.2, 78.7, 66.0, 53.9, 42.7, 39.9, 29.0, 28.4, 22.9, 15.4.

***tert*-Butyl (2-(2-(4-(*N*-benzyl-4-(*N*-(4-(*tert*-butoxy)benzyl)acetamido)benzamido) benzamido)acetamido)ethyl)carbamate (197)**

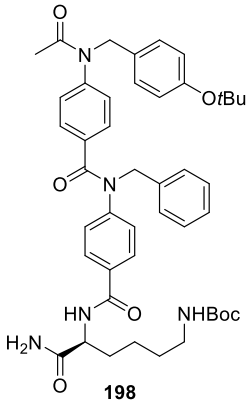
Acid **195** (0.050 g, 0.09 mmol) and amine **176** (0.018 g, 0.089 mmol) were coupled together using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **197** (0.029 g, 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, *J* = 8.5 Hz, 2H), 7.31 – 7.19 (m, 7H), 7.03 (bs, 1H), 6.98 – 6.91 (m, 4H), 6.88 (bs, 1H), 6.82 (d, *J* = 8.4 Hz, 2H), 6.77 (d, *J* = 7.7 Hz, 2H), 5.13 (s, 2H), 5.08 – 4.91 (m, 1H), 4.74 (s, 2H), 4.04 (d, *J* = 8.4 Hz, 2H), 3.44 – 3.30 (m, 2H), 3.25 (d, *J* = 4.9 Hz, 2H), 1.77 (s, 3H), 1.41 (d, *J* = 11.9 Hz, 9H), 1.32 – 1.28 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3, 169.9, 169.5, 169.1, 166.4, 157.2, 154.8, 146.4, 144.2, 136.9, 132.1, 131.9, 130.2, 129.6, 128.8, 128.4, 128.2, 128.0, 127.8, 127.6, 124.2, 80,1, 78.7, 60.5, 53.9, 43.5, 41.3, 40.2, 29.0, 28.5, 22.9. HRMS (ESI) *m/z* calcd $C_{43}H_{51}N_5O_7$ $[M+Na]^+$ 772.3680, found 772.3684.



197

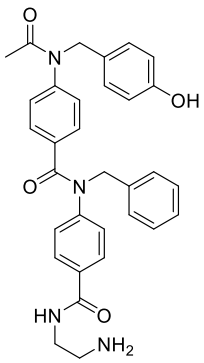
***tert*-Butyl (*S*)-(6-amino-5-(4-(*N*-benzyl-4-(*N*-(4-(*tert*-butoxy)benzyl)acetamido) benzamido)benzamido)-6-oxohexyl)carbamate (198)**

Acid **195** (0.050 g, 0.09 mmol) and (*S*)-1-amino-6-((*tert*-butoxycarbonyl)amino)-1-oxohexan-2-aminium chloride (0.028 g, 0.099 mmol) were coupled together in acetonitrile using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **198** (0.040 g, 57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 8.4 Hz, 2H), 7.31 – 7.22 (m, 7H), 7.02 (d, *J* = 7.5 Hz, 1H), 6.94 (dd, *J* = 8.5, 3.5 Hz, 4H), 6.83 (d, *J* = 8.4 Hz, 2H), 6.78 (d, *J* = 7.6 Hz, 2H), 6.46 (bs, 1H), 5.72 (bs, 1H), 5.12 (s, 2H), 4.73 (s, 2H), 4.70 (bs, 1H), 4.60 (m, 1H), 3.06 (m, 2H), 2.00 – 1.82 (m, 2H), 1.48 (m, 2H), 1.40 (m, 9H), 1.30 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0, 173.9, 170.0, 169.6, 166.2, 156.4, 154.8, 146.4, 144.2, 136.9, 135.2, 132.1, 132.0, 130.1, 129.5, 128.8, 128.3, 128.2, 128.0, 127.8, 127.6, 124.2, 79.3, 78.7, 53.9, 53.0, 40.0, 32.1, 29.7, 29.0, 28.5, 22.8, 22.5. HRMS (ESI) *m/z* calcd $C_{45}H_{55}N_5O_7Na$ $[M+Na]^+$ 800.3993, found 800.3997.



198

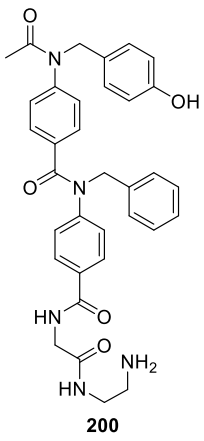
***N*-(4-((2-Aminoethyl)carbamoyl)phenyl)-*N*-benzyl-4-(*N*-(4-hydroxybenzyl)acetamido) benzamide (199)**



199

Compound **196** was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **199** (0.013 g, 46%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.69 (d, *J* = 8.6 Hz, 2H), 7.35 (d, *J* = 8.4 Hz, 2H), 7.31 – 7.21 (m, 5H), 7.10 (d, *J* = 8.6 Hz, 2H), 6.90 (d, *J* = 8.0 Hz, 2H), 6.82 (d, *J* = 8.2 Hz, 2H), 6.60 (d, *J* = 8.3 Hz, 2H), 5.19 (s, 2H), 4.71 (s, 2H), 3.62 (t, *J* = 5.9 Hz, 2H), 3.13 (t, *J* = 5.9 Hz, 2H), 1.75 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 169.2, 168.9, 166.9, 155.1, 144.2, 141.9, 135.1, 133.9, 130.2, 128.3, 128.0, 126.7, 126.5, 126.4, 126.4, 126.3, 125.9, 125.8, 113.2, 51.4, 49.9, 38.0, 35.9, 19.7. HRMS (ESI) *m/z* calcd $C_{32}H_{32}N_4O_4Na$ $[M+Na]^+$ 559.2315, found 559.2318.

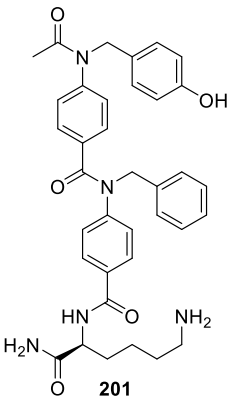
***N*-(4-((2-((2-Aminoethyl)amino)-2-oxoethyl)carbamoyl)phenyl)-*N*-benzyl-4-(*N*-(4-hydroxybenzyl)acetamido)benzamide**



200

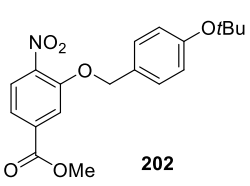
Compound **197** (0.029 g, 0.039 mmol) was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **200** (0.020 g, 87%). ^{1}H NMR (600 MHz, CD_3OD) δ 7.71 (d, *J* = 8.8 Hz, 2H), 7.34 (d, *J* = 8.6 Hz, 2H), 7.30 – 7.20 (m, 5H), 7.09 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 7.7 Hz, 2H), 6.81 (d, *J* = 8.1 Hz, 2H), 6.60 (d, *J* = 8.2 Hz, 2H), 5.19 (s, 2H), 4.71 (s, 2H), 3.99 (s, 2H), 3.52 – 3.43 (m, 2H), 3.05 (t, *J* = 5.8 Hz, 2H), 1.75 (s, 3H). ^{13}C NMR (150 MHz, CD_3OD) δ 173.0, 172.1, 171.9, 169.5, 158.0, 147.1, 144.7, 138.1, 136.8, 133.0, 131.3, 130.9, 129.6, 129.5, 129.4, 129.3, 129.2, 128.8, 128.7, 116.1, 54.3, 52.8, 44.2, 41.0, 38.2, 22.6. HRMS (ESI) *m/z* calcd $C_{34}H_{35}N_5O_5Na$ $[M+Na]^+$ 616.2530, found 616.2532.

(*S*)-*N*-Benzyl-*N*-(4-((1,6-diamino-1-oxohexan-2-yl)carbamoyl)phenyl)-4-(*N*-(4-hydroxybenzyl)acetamido)benzamide (201)



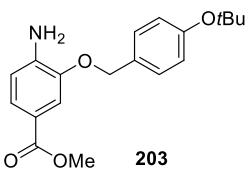
Compound **198** (0.037 g, 0.047 mmol) was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **201** (0.024 g, 82%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.70 (d, *J* = 8.8 Hz, 2H), 7.35 (d, *J* = 8.6 Hz, 2H), 7.30 – 7.19 (m, 5H), 7.09 (d, *J* = 8.6 Hz, 2H), 6.90 (d, *J* = 8.1 Hz, 2H), 6.80 (d, *J* = 8.1 Hz, 2H), 6.59 (d, *J* = 8.3 Hz, 2H), 5.19 (s, 2H), 4.70 (m, 2H), 4.53 (dd, *J* = 8.9, 5.4 Hz, 1H), 2.95 – 2.81 (m, 2H), 1.92 (m, 1H), 1.85 – 1.78 (m, 1H), 1.71 – 1.57 (m, 2H), 1.56 – 1.38 (m, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 176.7, 172.1, 171.9, 168.9, 158.0, 147.0, 144.7, 138.1, 136.9, 133.4, 131.3, 130.9, 129.6, 129.5, 129.4, 129.4, 129.2, 128.8, 128.7, 116.1, 54.7, 54.3, 52.8, 40.5, 32.5, 28.1, 24.0, 22.7. HRMS (ESI) *m/z* calcd $C_{36}H_{39}N_5O_5Na$ $[M+Na]^+$ 644.2843, found 644.2846.

Methyl 3-((4-(*tert*-butoxy)benzyl)oxy)-4-nitrobenzoate (202)



To an ice-cooled solution of (4-(*tert*-butoxy)phenyl)methanol (0.050 g, 0.27 mmol) and methyl 3-hydroxy-4-nitrobenzoate (0.12 g, 0.61 mmol) in THF (3 mL) under inert atmosphere was added triphenylphosphine (0.11, 0.42 mmol). 94% Diethylazadicarboxylate in toluene (85 µL, 0.42 mmol) was added dropwise to the solution and the reaction mixture was stirred overnight at room temperature under inert atmosphere. The reaction mixture was evaporated to dryness under reduced pressure and the residue was purified by column chromatography (cyclohexane/EtOAc 10:1) to afford the ether **202** (0.075 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, *J* = 5.8 Hz, 1H), 7.81 (d, *J* = 0.8 Hz, 1H), 7.68 (dd, *J* = 8.4, 1.5 Hz, 1H), 7.35 (d, *J* = 8.7 Hz, 2H), 7.00 (d, *J* = 8.6 Hz, 2H), 5.21 (s, 2H), 3.94 (s, 3H), 1.34 (s, 9H).

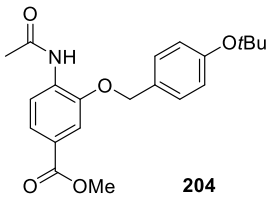
Methyl 4-amino-3-((4-(*tert*-butoxy)benzyl)oxy)benzoate (203)



Compound **202** (0.63 g, 1.75 mmol) was dissolved in a mixture of EtOAc/MeOH 1:1 (40 mL) and tin(II) chloride dihydrate (2.37 g, 10.53 mmol) was added. The reaction was stirred overnight. The reaction mixture was concentrated under reduced pressure and quenched by addition of saturated solution of sodium bicarbonate (50 mL). The reaction mixture was extracted with EtOAc (3 × 50 mL), the combined organic layers were washed with brine, dried

over $MgSO_4$ and evaporate to dryness under reduced pressure. The solid residue was purified by column chromatography (cyclohexane/EtOAc 4:1) to afford the amine **203** (0.46 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 – 7.51 (m, 2H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.01 (d, *J* = 8.5 Hz, 2H), 6.67 (d, *J* = 8.6 Hz, 1H), 5.05 (s, 2H), 4.13 (bs, 2H), 3.86 (s, 3H), 1.36 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.39, 155.5, 145.4, 141.6, 131.5, 128.8, 124.5, 124.2, 119.5, 113.4, 112.8, 78.8, 70.4, 51.8, 29.0. HRMS (ESI) *m/z* calcd $C_{19}H_{23}NO_4Na$ $[M+Na]^+$ 352.1519, found 352.1519.

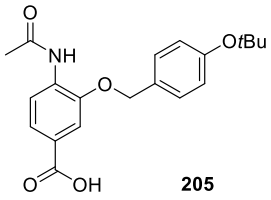
Methyl 4-acetamido-3-((4-(*tert*-butoxy)benzyl)oxy)benzoate (204)



204

To an ice-cooled solution of **203** (0.085 g, 0.258 mmol) and DIPEA (0.13 µL, 0.77 mmol) in DCM (2 mL) was added acetyl chloride (27 µL, 0.38 mmol). The reaction was stirred at room temperature for 40 min. The reaction mixture was quenched by addition of water (7 mL) and extracted with EtOAc (3 × 10 mL), the combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness to afford the acetamide **204** (0.094 g, 98%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 (d, *J* = 8.4 Hz, 1H), 7.91 (s, 1H), 7.68 (dd, *J* = 8.4, 1.7 Hz, 1H), 7.64 (d, *J* = 1.7 Hz, 1H), 7.31 (d, *J* = 8.6 Hz, 2H), 7.03 (d, *J* = 8.5 Hz, 2H), 5.09 (s, 2H), 3.88 (s, 3H), 2.16 (s, 3H), 1.36 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.5, 166.8, 155.9, 146.5, 132.4, 130.6, 128.9, 125.0, 124.3, 123.8, 118.8, 112.5, 78.9, 71.1, 52.2, 29.0, 25.2. HRMS (ESI) *m/z* calcd $C_{21}H_{25}NO_5Na$ $[M+Na]^+$ 394.1624, found 394.1625.

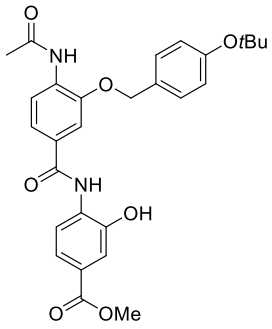
4-Acetamido-3-((4-(*tert*-butoxy)benzyl)oxy)benzoic acid (205)



205

Compound **204** (0.093 g, 0.25 mmol) was dissolved in a solution of THF/MeOH 1:1 (3 mL) and 0.125 M aqueous solution of NaOH (4 mL) was added. The reaction was refluxed for one hour. The reaction was quenched by addition of 5% aqueous solution of citric acid (20 mL) and extracted with DCM (3 × 20 mL). The combined organic layers were evaporated to dryness and the residue was purified by column chromatography (EtOAc) to afford the acid **205** (0.086 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (d, *J* = 8.5 Hz, 1H), 7.95 (s, 1H), 7.79 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.70 (d, *J* = 1.7 Hz, 1H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.05 (d, *J* = 8.5 Hz, 2H), 5.13 (s, 2H), 2.19 (s, 3H), 1.38 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.2, 168.7, 156.0, 146.5, 133.2, 130.5, 129.0, 124.8, 124.4, 124.1, 118.9, 112.9, 79.0, 71.3, 29.0, 25.2. HRMS (ESI) *m/z* calcd $C_{20}H_{22}NO_5^-$ $[M-H]^-$ 356.1503, found 356.1500.

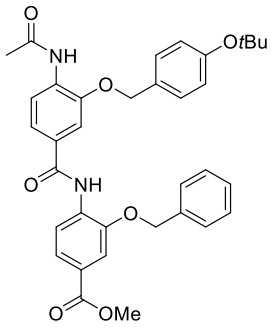
Methyl 4-(4-acetamido-3-((4-(*tert*-butoxy)benzyl)oxy)benzamido)-3-hydroxybenzoate (206)



206

Acid **205** (0.29 g, 0.81 mmol) and methyl 4-amino-3-hydroxybenzoate (0.15 g, 0.89 mmol) were coupled together using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (cyclohexane/EtOAc 1:1) to afford the amide **206** (0.22 g, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (d, *J* = 8.5 Hz, 1H), 7.98 (s, 1H), 7.88 (dd, *J* = 8.5, 1.8 Hz, 1H), 7.81 – 7.76 (m, 2H), 7.76 (d, *J* = 1.8 Hz, 1H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.04 (d, *J* = 8.5 Hz, 2H), 6.80 (d, *J* = 8.8 Hz, 1H), 5.13 (s, 2H), 4.14 (bs, 2H), 3.85 (s, 3H), 2.19 (s, 3H), 1.37 (s, 9H).

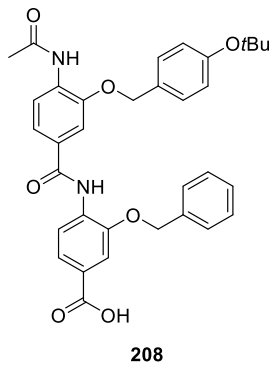
Methyl 4-(4-acetamido-3-((4-(*tert*-butoxy)benzyl)oxy)benzamido)-3-(benzyloxy)benzoate (207)



207

Compound **206** (0.21 g, 0.41 mmol) and potassium carbonate (0.28 g, 2.00 mmol) were suspended in DMF (7 mL) and benzyl bromide was added (68 µL, 0.57 mmol). The reaction mixture was heated up in an oil bath to 50 °C and stirred overnight. The reaction mixture was diluted with EtOAc (20 mL) and extracted with H_2O (5 × 15 mL). The organic phase was washed with brine, dried over $MgSO_4$, and evaporated to dryness under reduced pressure. The solid residue was purified by column chromatography (toluene/EtOAc 5:1) to afford the ester **207** (0.087 g, 34%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (bs, 1H), 8.64 (d, *J* = 8.5 Hz, 1H), 8.44 (d, *J* = 8.4 Hz, 1H), 7.88 (bs, 1H), 7.77 (dd, *J* = 8.5, 1.8 Hz, 1H), 7.71 (d, *J* = 1.8 Hz, 1H), 7.57 (d, *J* = 1.9 Hz, 1H), 7.49 – 7.31 (m, 6H), 7.29 (d, *J* = 8.5 Hz, 2H) 7.04 (d, *J* = 8.5 Hz, 2H), 5.21 (s, 2H), 5.03 (s, 2H), 3.92 (s, 3H), 2.17 (s, 3H), 1.38 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.6, 166.8, 164.5, 156.1, 147.2, 147.0, 136.0, 132.7, 131.7, 130.4, 129.45 (2x), 129.1, 128.9, 127.9, 125.2, 124.3, 124.1, 119.8, 119.1, 118.7, 112.4, 111.0, 79.0, 71.6, 71.1, 52.3, 29.0, 25.2. HRMS (ESI) *m/z* calcd $C_{35}H_{36}N_7O_2Na$ $[M+Na]^+$ 619.2414, found 619.2415.

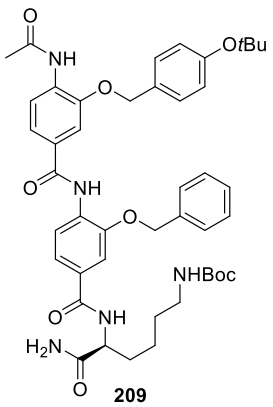
4-(4-Acetamido-3-((4-(*tert*-butoxy)benzyl)oxy)benzamido)-3-(benzyloxy)benzoic acid (208)



208

Compound **207** (0.082 g, 0.014 mmol) was dissolved in a mixture THF/MeOH 1:1 (4 mL) and) and 2 M aqueous solution of NaOH (1.5 mL) was added. The reaction mixture was refluxed for 3 hours. The reaction mixture was acidified by addition of 5% aqueous solution of citric acid (20 mL) and extracted with DCM (3 × 20 mL). The combined organic layer was evaporated to dryness and the residue was purified by column chromatography (DCM/MeOH 20:1) to afford the acid **208** (0.070g, 88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.11 (d, J = 8.6 Hz, 1H), 8.06 (d, J = 8.3 Hz, 1H), 7.70 – 7.59 (m, 3H), 7.55 (d, J = 7.3 Hz, 2H), 7.52 (dd, J = 8.5, 1.5 Hz, 1H), 7.41 (d, J = 8.4 Hz, 2H), 7.35 (m, 2H), 7.33 – 7.16 (m, 2H), 6.99 (d, J = 8.4 Hz, 2H), 5.29 (s, 2H), 5.19 (s, 2H), 2.11 (s, 3H), 1.29 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.0, 166.9, 164.3, 154.9, 149.2, 136.8, 131.7, 131.5, 131.1, 129.5, 128.6, 128.4, 128.4, 127.9, 127.4, 127.3, 123.4, 122.5, 122.3, 120.2, 113.1, 112.0, 78.0, 70.1, 70.0, 28.6, 24.1.

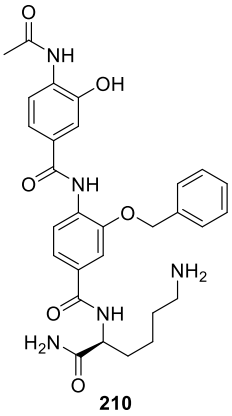
(*S*)-1-Amino-6-((*tert*-butoxycarbonyl)amino)-1-oxohexan-2-yl 4-(4-acetamido-3-((4-(*tert*-butoxy)benzyl)oxy)benzamido)-3-(benzyloxy)benzoate (209)



209

Acid **208** (0.069 g, 0.12 mmol) and (S)-1-amino-6-((tert-butoxycarbonyl)amino)-1-oxohexan-2-aminium chloride (0.036 g, 0.13 mmol) were coupled together using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **209** (0.069 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (s, 1H), 8.54 (d, J = 8.4 Hz, 1H), 8.37 (d, J = 8.4 Hz, 1H), 7.80 (s, 1H), 7.51 (dd, J = 15.8, 1.7 Hz, 2H), 7.42 – 7.36 (m, 3H), 7.35 – 7.30 (m, 2H), 7.29 – 7.20 (m, 4H), 7.03 – 6.92 (m, 3H), 6.35 (s, 1H), 5.50 (s, 1H), 5.12 (s, 2H), 4.94 (s, 2H), 4.67 – 4.51 (m, J = 14.4, 6.7 Hz, 2H), 3.24 – 2.99 (m, 2H), 2.10 (s, 3H), 2.01 – 1.64 (m, 2H), 1.54 – 1.38 (m, 2H), 1.35 (s, 9H), 1.31 (s, 9H).

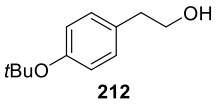
(*S*)-1,6-Diamino-1-oxohexan-2-yl 4-(4-acetamido-3-hydroxybenzamido)-3-(benzyloxy)benzoate (210)



210

Compound **209** (0.066 g, 0.08 mmol) was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **210** (0.025 g, 56%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.27 (d, *J* = 8.4 Hz, 1H), 7.95 (d, *J* = 8.4 Hz, 1H), 7.69 (d, *J* = 1.8 Hz, 1H), 7.57 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.53 – 7.47 (m, 2H), 7.45 – 7.30 (m, 4H), 7.26 (dd, *J* = 8.4, 2.1 Hz, 1H), 5.29 (s, 2H), 4.59 (dd, *J* = 9.1, 5.3 Hz, 1H), 2.94 (td, *J* = 8.0, 3.0 Hz, 2H), 2.21 (s, 3H), 2.08 – 1.81 (m, 2H), 1.81 – 1.63 (m, 2H), 1.63 – 1.41 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 176.96, 172.3, 169.4, 167.2, 150.1, 149.1, 137.9, 132.4, 131.8, 131.4, 131.2, 129.8, 129.1, 128.8, 122.5, 121.9, 121.7, 119.3, 115.7, 113.0, 72.4, 54.7, 40.5, 32.6, 28.1, 24.0, 23.8, 18.4. HRMS (ESI) *m/z* calcd $C_{29}H_{33}N_5O_6Na$ $[M+Na]^+$ 570.2323, found 570.2324.

2-(4-(*tert*-Butoxy)phenyl)ethan-1-ol (212)

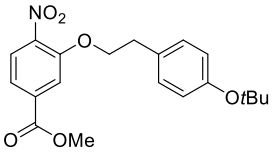


212

Dry (methoxymethyl)triphenylphosphonium chloride (3.84 g, 11.22 mmol) was suspended in a freshly distilled (Na/benzophenone) THF (50 mL) under inert atmosphere of nitrogen. The reaction mixture was cooled down to – 50 °C and 2.5 M solution of butyllithium in hexanes (4.5 mL) was added dropwise. The reaction mixture was stirred for 1 hour 20 min. at – 40 °C and 4-(*tert*-butoxy)benzaldehyde was added. The reaction was left to gradually heat up to a room temperature and after two hours the reaction mixture was concentrated under reduced pressure. The reaction mixture was quenched by water (50 mL) and extracted with EtOAc (50 mL). The organic phase was washed with water (20 mL), brine (20 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The solid residue was purified by column chromatography (cyclohexane/EtOAc/Et_3N 100/10/1) to afford the mixture of (*E*/*Z*)-1-(*tert*-butoxy)-4-(2-methoxyvinyl)benzene **211** (0.61 g, 52 %). (*E*/*Z*)-1-(*tert*-Butoxy)-4-(2-methoxyvinyl)benzene (0.61 g, 2.96 mmol) was dissolved in a mixture THF/H_2O 1:1 (18 mL) and mercury(II) acetate (0.94 g, 2.95 mmol) was added. The reaction mixture was stirred for 0.5 h and sodium borohydride (0.45 g, 11.85 mmol) and saturated solution of potassium carbonate (6 mL) were added. Mercury(0) appeared instantly. The reaction mixture was quenched by addition of water. The reaction mixture was extracted with EtOAc (3 × 30 mL). The liquid mercury was separated in a separatory funnel. The combined organic layers

were evaporated to dryness under reduced pressure and purified by column chromatography (cyclohexane/EtOAc 2:1) to afford the alcohol **212** (0.40 g, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, *J* = 8.6 Hz, 2H), 6.93 (d, *J* = 8.5 Hz, 2H), 3.84 (t, *J* = 6.5 Hz, 2H), 2.83 (t, *J* = 6.6 Hz, 2H), 1.33 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.0, 133.3, 129.5, 124.5, 78.5, 63.9, 38.7, 29.0. HRMS (EI) *m/z* calcd $C_{12}H_{18}O_2$ $[M]^{+\bullet}$ 194.1307, found 194.1308.

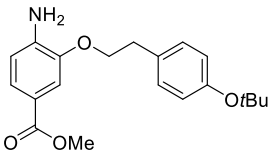
Methyl 3-(4-(*tert*-butoxy)phenethoxy)-4-nitrobenzoate (213)



213

To an ice-cooled solution of **212** (0.40 g, 2.05 mmol) and methyl 3-hydroxy-4-nitrobenzoate (0.40 g, 2.03 mmol) in dry THF (10 mL) was added triphenylphosphine (0.54, 2.06 mmol). 94% Diethylazadicarboxylate in toluene (0.430 µL, 2.03 mmol) was added dropwise to the solution and the reaction mixture was stirred overnight at room temperature under inert atmosphere. The reaction mixture was evaporated to dryness under reduced pressure and the residue was purified by column chromatography (cyclohexane/EtOAc 10:1) to afford the ether **213** (0.58 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.3 Hz, 1H), 7.70 (d, *J* = 1.5 Hz, 1H), 7.66 (dd, *J* = 8.3, 1.6 Hz, 1H), 7.17 (d, *J* = 8.5 Hz, 2H), 6.94 (d, *J* = 8.5 Hz, 2H), 4.32 (t, *J* = 6.9 Hz, 2H), 3.94 (s, 3H), 3.11 (t, *J* = 6.9 Hz, 2H), 1.33 (s, 9H). HRMS (ESI) *m/z* calcd $C_{20}H_{23}NO_6Na$ $[M+Na]^+$ 396.1417, found 396.1418.

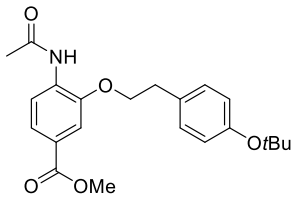
Methyl 4-amino-3-(4-(*tert*-butoxy)phenethoxy)benzoate (214)



214

Compound **213** (0.58 g, 1.54 mmol) was dissolved in EtOH (20 mL) and 10% Pd/C (0.058 g) was added. The reaction mixture was stirred under positive pressure of hydrogen for 2 hours. The reaction mixture was filtered over celite and concentrated under reduced pressure. The solid residue was purified by column chromatography (cyclohexane/EtOAc 4:1) to afford the amine **214** (0.45 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.46 (d, *J* = 1.8 Hz, 1H), 7.16 (d, *J* = 8.5 Hz, 2H), 6.94 (d, *J* = 8.5 Hz, 2H), 6.64 (d, *J* = 8.2 Hz, 1H), 4.25 (t, *J* = 6.9 Hz, 2H), 3.84 (s, 3H), 3.09 (t, *J* = 6.9 Hz, 2H), 1.34 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.4, 154.1, 145.4, 141.5, 133.1, 129.4, 124.4, 124.3, 119.6, 113.4, 112.7, 78.5, 69.3, 51.8, 35.3, 29.0. HRMS (ESI) *m/z* calcd $C_{20}H_{25}NO_4Na$ $[M+Na]^+$ 366.1675, found 366.1675.

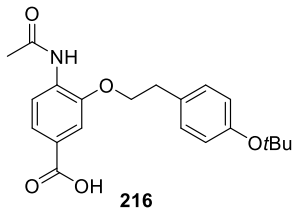
Methyl 4-acetamido-3-(4-(*tert*-butoxy)phenethoxy)benzoate (215)



215

To an ice-cooled solution of **214** (0.085 g, 0.258 mmol) and DIPEA (0.67 µL, 3.86 mmol) in DCM (8 mL) was added acetyl chloride (140 µL, 1.93 mmol). The reaction was stirred at room temperature under inert atmosphere for 40 min. The reaction mixture was quenched by addition of water (20 mL) and extracted with EtOAc (3 × 10 mL), the combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to dryness to afford the acetamide **215** (0.49 g, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (d, *J* = 8.5 Hz, 1H), 7.74 (s, 1H), 7.65 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.54 (d, *J* = 1.8 Hz, 1H), 7.15 (d, *J* = 8.6 Hz, 2H), 6.96 (d, *J* = 8.5 Hz, 2H), 4.31 (t, *J* = 6.6 Hz, 2H), 3.88 (s, 3H), 3.14 – 3.05 (m, 2H), 2.12 (s, 3H), 1.33 (s, 9H). HRMS (ESI) *m/z* calcd $C_{22}H_{27}NO_5Na$ $[M+Na]^+$ 408.1781, found 408.1780.

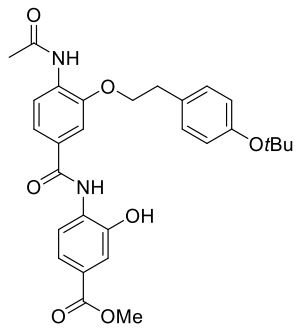
4-Acetamido-3-(4-(*tert*-butoxy)phenethoxy)benzoic acid (216)



216

Compound **215** (0.49 g, 1.28 mmol) was dissolved in a solution of THF/MeOH 1:1 (16 mL) and 0.2 M aqueous solution of NaOH (1.3 mL) was added. The reaction was refluxed for one hour. The reaction was quenched by addition of 5% aqueous solution of citric acid (20 mL) and extracted with DCM (3 × 20 mL). The combined organic layers were evaporated to dryness and the residue was purified by column chromatography (DCM/MeOH 20:1) to afford the acid **216** (0.45 g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, *J* = 8.5 Hz, 1H), 7.78 (s, 1H), 7.75 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.59 (d, *J* = 1.8 Hz, 1H), 7.17 (d, *J* = 8.5 Hz, 2H), 6.97 (d, *J* = 8.5 Hz, 2H), 4.33 (t, *J* = 6.6 Hz, 2H), 3.11 (t, *J* = 6.6 Hz, 2H), 2.14 (s, 3H), 1.34 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 168.6, 154.4, 146.3, 133.2, 132.6, 129.4, 124.6, 124.5, 124.0, 118.7, 112.5, 78.7, 69.5, 35.0, 29.0, 25.1. HRMS (ESI) *m/z* calcd $C_{21}H_{25}NO_5Na$ $[M+Na]^+$ 394.1624, found 394.1625.

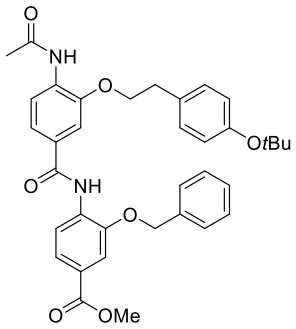
Methyl 4-(4-acetamido-3-(4-(*tert*-butoxy)phenethoxy)benzamido)-3-hydroxybenzoate (217)



217

Acid **216** (0.48 g, 1.30 mmol) and methyl 4-amino-3-hydroxybenzoate (0.26 g, 1.56 mmol) were coupled together in DMF (10 mL) using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (cyclohexane/EtOAc 2:1) to afford the amide **217** (0.17 g, 25%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J* = 8.5 Hz, 1H), 7.86 (dd, *J* = 8.5, 1.6 Hz, 1H), 7.82 – 7.75 (m, 3H), 7.68 (d, *J* = 1.7 Hz, 1H), 7.18 (d, *J* = 8.5 Hz, 2H), 6.97 (d, *J* = 8.5 Hz, 2H), 6.81 (d, *J* = 9.0 Hz, 1H), 4.35 (t, *J* = 6.5 Hz, 2H), 3.85 (s, 3H), 3.12 (t, *J* = 6.5 Hz, 2H), 2.16 (s, 3H), 1.34 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.6, 166.6, 164.2, 154.4, 146.5, 143.2, 137.1, 133.4, 132.6, 129.4, 129.0, 124.7, 124.6, 124.5, 123.4, 120.5, 118.9, 115.7, 112.7, 78.7, 69.6, 52.0, 35.0, 29.0, 25.2. HRMS (ESI) *m/z* calcd $C_{29}H_{32}N_2O_7Na$ $[M+Na]^+$ 543.2101, found 543.2101.

Methyl 4-(4-acetamido-3-(4-(*tert*-butoxy)phenethoxy)benzamido)-3-(benzyloxy)benzoate (218)

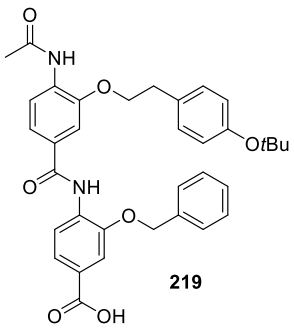


218

Compound **217** (0.17 g, 0.31 mmol) and potassium carbonate (0.22 g, 1.60 mmol) were suspended in DMF (5 mL) and benzyl bromide was added (0.081 g, 0.48 mmol). The reaction mixture was stirred for three days. The reaction mixture was diluted with EtOAc (60 mL) and extracted with H_2O (4 × 30 mL). The organic phase was washed with brine (20 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The soli residue was purified by column chromatography cyclohexane/EtOAc 1:1) and triturated (hexane/EtOAc 1:1) to afford the ether **218** (0.124 g, 34%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.78 (bs, 1H), 8.63 (d, *J* = 8.5 Hz, 1H), 8.38 (d, *J* = 8.4 Hz, 1H), 7.77 (dd, *J* = 8.5, 1.8 Hz, 1H), 7.73 – 7.67 (m, 2H), 7.51 – 7.35 (m, 6H), 7.23 (dd, *J* = 8.5, 1.9 Hz, 1H), 7.15 (d, *J* = 8.6 Hz, 2H), 6.96 (d, *J* = 8.6 Hz, 2H), 5.21 (s, 2H), 4.26 (t, *J* = 6.5 Hz, 2H), 3.92 (s, 3H), 3.08 (t, *J* = 6.5 Hz, 2H), 2.13 (s, 3H), 1.34 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.5, 166.8, 164.5, 154.4, 146.9, 146.9, 136.0, 132.7, 132.6, 131.7, 129.4, 129.3, 129.1, 128.9, 127.9, 125.2, 124.6, 124.1, 119.5, 118.9, 118.7, 112.41, 110.8, 78.6,

71.6, 69.4, 52.3, 35.0, 29.0, 25.1. HRMS (ESI) *m/z* calcd $C_{36}H_{38}N_2O_7Na$ $[M+Na]^+$ 633.2571, found 633.2574.

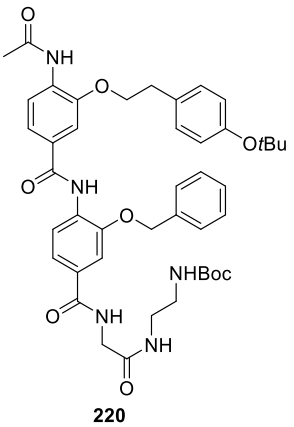
4-(4-Acetamido-3-(4-(*tert*-butoxy)phenethoxy)benzamido)-3-(benzyloxy)benzoic acid (219)



219

Compound **218** (0.13 g, 0.21 mmol) was dissolved in a mixture of THF/MeOH 1:1 (4 mL) and 0.2 M aqueous solution of NaOH (0.2 mL) was added. The reaction mixture was heated up to a 60 °C for 2 h. The reaction was quenched by addition of 10% aqueous solution of citric acid (15 mL). The mixture was extracted with DCM (3 × 25 mL) and the combined organic layers were evaporated to dryness under reduced pressure. The residue was purified by column chromatography (DCM/MeOH 20/1) and crystalized from MeOH to obtain the acid **219** (0.12 g, 98%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.93 (s, 1H), 9.49 (s, 1H), 9.03 (s, 1H), 8.13 (d, J = 8.3 Hz, 1H), 8.03 (d, J = 8.3 Hz, 1H), 7.67 (d, J = 1.7 Hz, 1H), 7.62 (dd, J = 8.3, 1.7 Hz, 1H), 7.60 – 7.44 (m, 4H), 7.37 – 7.30 (m, 3H), 7.27 (d, J = 8.4 Hz, 2H), 6.90 (d, J = 8.4 Hz, 2H), 5.26 (s, 2H), 4.26 (t, J = 6.9 Hz, 2H), 3.09 (t, J = 6.8 Hz, 2H), 2.13 (s, 3H), 1.25 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.7, 166.9, 164.3, 153.5, 149.4, 147.8, 136.7, 132.8, 131.7, 131.1, 129.6, 129.3, 128.4, 127.9, 127.4, 127.2, 123.7, 122.6, 122.4, 120.5, 120.2, 113.1, 110.9, 77.7, 70.1, 69.4, 34.1, 28.5, 24.1. HRMS (ESI) *m/z* calcd $C_{35}H_{36}N_2O_7Na$ $[M+Na]^+$ 619.2414, found 619.2416.

***tert*-Butyl (2-(2-(4-(4-acetamido-3-(4-(*tert*-butoxy)phenethoxy)benzamido)-3-(benzyloxy)benzamido)acetamido)ethyl)carbamate (220)**

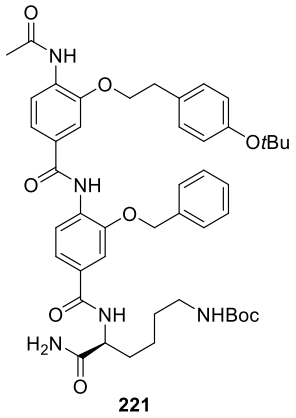


220

Acid **219** (0.023 g, 0.04 mmol) and amine **176** (0.010 g, 0.05 mmol) were coupled together in DMF with HATU using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **220** (0.024 g, 77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.48 (s, 1H), 9.02 (s, 1H), 8.72 (t, J = 5.8 Hz, 1H), 8.12 (d, J = 8.3 Hz, 1H), 8.02 – 7.89 (m, 2H), 7.70 (d, J = 1.7 Hz, 1H), 7.62 – 7.46 (m, 5H), 7.37 – 7.22 (m, 5H), 6.94 – 6.87 (m, 2H), 6.81 (t, J = 5.4 Hz, 1H), 5.24 (s, 2H), 4.26 (t, J = 6.9 Hz, 2H), 3.85 (d, J = 5.7 Hz, 2H), 3.16 – 3.04 (m, 4H), 3.04 – 2.94 (m, 2H), 2.13 (s, 3H), 1.37 (s, 9H), 1.25 (s, 9H). ^{13}C NMR

(125 MHz, DMSO-d_6) δ 169.2, 168.8, 165.8, 164.3, 155.7, 153.5, 149.8, 147.7, 136.8, 132.9, 130.9, 130.9, 130.2, 129.7, 128.4, 127.9, 127.5, 127.4, 123.8, 123.1, 120.5, 120.3, 120.1, 113.9, 111.9, 110.8, 77.7, 70.1, 69.4, 56.1, 42.8, 34.1, 28.6, 28.3, 24.2, 18.7. HRMS (ESI) *m/z* calcd $C_{44}H_{53}N_5O_9Na$ $[M+Na]^+$ 818.3735, found 818.3721.

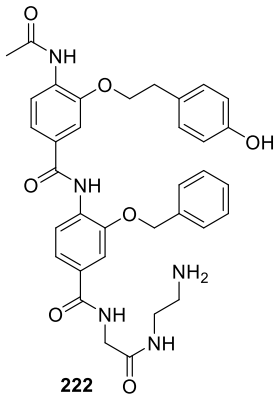
tert-Butyl (_S_)-(5-(4-(4-acetamido-3-(4-(_tert_-butoxy)phenethoxy)benzamido)-3-(benzyloxy)benzamido)-6-amino-6-oxohexyl)carbamate (221)



221

Acid **219** (0.048 g, 0.08 mmol) and (*S*)-1-amino-6-((*tert*-butoxycarbonyl)amino)-1-oxohexan-2-aminium chloride (0.027 g, 0.10 mmol) were coupled together in DMF using the conditions described in the general procedure for amide coupling. The solid residue was purified by column chromatography (DCM/MeOH 20:1) to afford the amide **221** (0.050 g, 75%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.51 (s, 1H), 9.06 (s, 1H), 8.35 (d, J = 7.7 Hz, 1H), 8.13 (d, J = 7.8 Hz, 1H), 7.92 (d, J = 8.3 Hz, 1H), 7.70 (s, 1H), 7.63 – 7.49 (m, 5H), 7.43 (s, 1H), 7.37 – 7.24 (m, 5H), 7.04 (s, 1H), 7.00 – 6.86 (m, 2H), 6.81 (t, J = 5.0 Hz, 1H), 5.26 (s, 2H), 4.48 – 4.33 (m, 1H), 4.26 (t, J = 6.4 Hz, 2H), 3.51 – 3.42 (m, 2H), 3.17 – 3.04 (m, 2H), 2.90 (s, 2H), 2.14 (s, 3H), 1.85 – 1.64 (m, 2H), 1.38 (s, 2H), 1.36 (s, 9H), 1.26 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 174.0, 168.8, 165.7, 164.3, 155.6, 153.5, 149.8, 147.7, 136.8, 132.9, 131.0, 130.9, 130.1, 129.7, 128.4, 127.9, 127.5, 123.8, 123.0, 120.5, 120.3, 112.0, 110.8, 77.7, 77.4, 70.1, 69.4, 66.4, 56.1, 55.0, 53.3, 34.1, 29.3, 28.6, 28.3, 24.4, 23.3, 18.6. HRMS (ESI) *m/z* calcd $C_{46}H_{57}N_5O_9Na$ $[M+Na]^+$ 846.4048, found 846.4060.

4-Acetamido-_N_-(4-((2-((2-aminoethyl)amino)-2-oxoethyl)carbamoyl)-2-(benzyloxy)phenyl)-3-(4-hydroxyphenethoxy)benzamide (222)

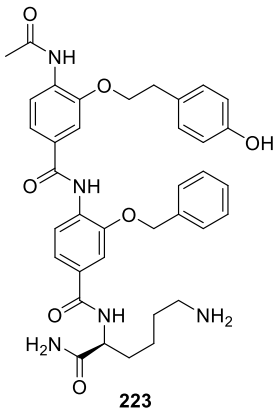


222

Compound **220** (0.024 g, 0.03 mmol) was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **222** (0.016 g, 84%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.54 (s, 1H), 9.27 (s, 1H), 9.01 (s, 1H), 8.81 (t, J = 5.7 Hz, 1H), 8.24 – 8.05 (m, 2H), 7.94 (d, J = 8.3 Hz, 1H), 7.77 (s, 3H), 7.70 (s, 1H), 7.66 – 7.42 (m, 5H), 7.33 (d, J = 7.6 Hz, 3H), 7.16 (d, J = 8.4 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 5.24 (s, 2H), 4.19 (t, J = 6.9 Hz, 2H), 3.89 (d, J = 5.6 Hz, 2H), 3.35 – 3.23 (m, 3H), 3.02 (t, J = 6.8 Hz, 2H), 2.87 (s, 2H),

2.14 (s, 3H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 170.0, 168.8, 166.0, 164.4, 156.0, 149.9, 147.7, 136.8, 131.0, 130.7, 130.3, 130.1, 129.4, 128.6, 128.4, 128.2, 128.0, 127.4, 123.2, 120.4, 120.3, 120.1, 115.2, 111.9, 110.8, 70.1, 69.8, 42.9, 36.4, 34.0, 24.2. HRMS (ESI) *m/z* calcd $C_{35}H_{38}N_5O_7$ $[M+H]^+$ 640.2765, found 640.2759.

(*S*)-4-Acetamido-*N*-(2-(benzyloxy)-4-((1,6-diamino-1-oxohexan-2-yl)carbamoyl)phenyl)-3-(4-hydroxyphenethoxy)benzamide (223)



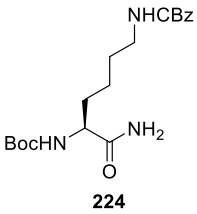
223

Compound **221** (0.046 g, 0.05 mmol) was deprotected using the conditions described in the general procedure for *N,O*-deprotection to afford the amine **223** (0.034 g, 90%). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 9.52 (s, 1H), 9.28 (s, 1H), 9.01 (s, 1H), 8.40 (d, *J* = 8.1 Hz, 1H), 8.13 (d, *J* = 8.2 Hz, 1H), 7.92 (d, *J* = 9.4 Hz, 1H), 7.79 – 7.63 (m, 4H), 7.59 (dd, *J* = 8.3, 1.4 Hz, 1H), 7.58 – 7.47 (m, 4H), 7.43 (s, 1H), 7.41 – 7.24 (m, 3H), 7.16 (d, *J* = 8.4 Hz, 2H), 7.07 (d, *J* = 16.7 Hz, 1H), 6.70 (d, *J* = 8.4 Hz, 2H), 5.25 (s, 2H), 4.39 (td, *J* = 9.1, 5.1 Hz, 1H), 4.19 (td, *J* = 6.9 Hz, 2H), 3.02 (t, *J* = 6.8 Hz, 2H), 2.78 (s, 2H), 2.14 (s, 3H), 1.85 – 1.64 (m, 2H), 1.63 – 1.49 (m, 2H), 1.49 – 1.28 (m, 2H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 174.0, 168.9, 165.8, 164.5, 156.0, 149.9, 147.8, 136.9, 131.0, 131.0, 130.2, 130.1, 129.4, 128.5, 128.3, 128.0, 127.5, 123.2, 120.5, 120.4, 120.3, 115.2, 112.1, 110.9, 70.2, 69.8, 53.1, 38.8, 34.0, 31.2, 26.8, 24.2, 22.9. HRMS (ESI) *m/z* calcd $C_{37}H_{42}N_5O$ $[M+H]^+$ 668.3078, found 668.3077.

General procedure for peptide synthesis in a liquid phase

Boc-protected peptide (1 eq.) is deprotected using the solution TFA/DCM 1:1 (1 g of peptide/10 mL) for 1h. The solution is evaporated to dryness and the residue is dissolved in DMF (1M) with DIPEA to ensure basic pH. Boc-protected amino acid (1.1 eq.), TBTU (1.1) and DIPEA (3 eq.) are stirred in DMF (0.5M) for 15 min and the solution of deprotected peptide is added. The solution is stirred for 2.5h. 2-Aminoethylmorpholine (0.3 eq.) is added and the reaction mixture is stirred for further 30 min. The reaction mixture is diluted with EtOAc (70 mL) and extracted twice with 5% aqueous solution of HCl, twice with saturated aqueous solution of $NaHCO_3$ and with brine. The organic phase is concentrated to a minimum volume and the peptide is precipitated by TBME.

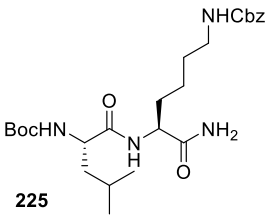
N^{ε}-Benzylcarbonyl-N^{α}-*tert*-butoxycarbonyl-*L*-Lysine-NH_2 = Boc-K(Z)NH_2 (224)



224

N^{ε}-Benzylcarbonyl-N^{α}-*tert*-butoxycarbonyl-*L*-Lysine-OH (3.00 g, 7.89 mmol), TBTU (2.78 g, 8.67 mmol), and DIPEA (4.12 mL, 23.60 mmol) were stirred in DMF (15 mL) for 30 min. Ammonium chloride (0.63 g, 11.80 mmol) was added and the reaction mixture was stirred for 3h. The reaction mixture was diluted with EtOAc (70 mL) and extracted twice with 5% aqueous solution of HCl, twice with saturated aqueous solution of $NaHCO_3$ and with brine. The organic phase was concentrated to a minimum volume and the product was precipitated by TBME to obtain the amide **224** (2.35 g, 78 %).^{1}H NMR (300 MHz, DMSO-d_6) δ 7.42 – 7.27 (m, 5H), 7.27 – 7.17 (m, 2H), 6.92 (s, 1H), 6.70 (d, J = 8.2 Hz, 1H), 5.00 (s, 2H), 3.80 (td, J = 8.8, 5.1 Hz, 1H), 2.96 (q, J = 6.6 Hz, 2H), 1.65 – 1.42 (m, 2H), 1.43 – 1.30 (m, 11H), 1.31 – 1.19 (m, 2H). The spectrum is in agreement with the literature.[154]

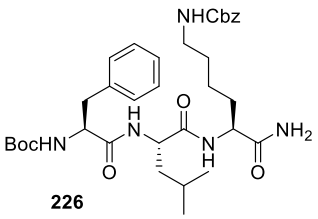
Boc-LK(Z)-NH_2 (225)



225

Compound **224** (2.35 g, 6.00 mmol) and Boc-Leu-OH (1.57 g, 6.82 mmol) were coupled together using the condition described in General procedure for peptide synthesis in a liquid phase to obtain the dipeptide **225** (1.98 g, 65%). MS ESI $[M+Na]^+$ 515.12.

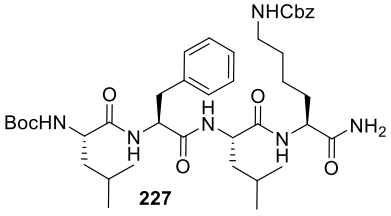
Boc-FLK(Z)-NH_2 (226)



226

Compound **225** (1.98 g, 4.03 mmol) and Boc-Phe-OH (1.17 g, 4.44 mmol) were coupled together using the condition described in General procedure for peptide synthesis in a liquid phase to obtain the tripeptide **226** (2.15 g, 83%). MS ESI $[M+Na]^+$ 662.35.

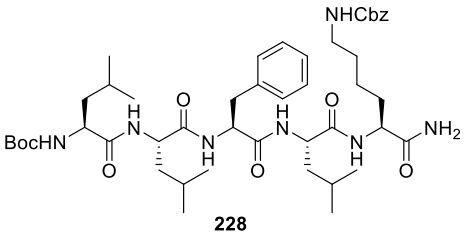
Boc-LFLK(Z)-NH_2 (227)



227

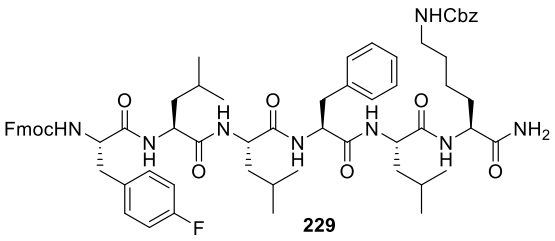
Compound **226** (2.15 g, 3.37 mmol) and Boc-Leu-OH (0.86 g, 3.70 mmol) were coupled together using the condition described in General procedure for peptide synthesis in a liquid phase to obtain the tetrapeptide **227** (1.95 g, 77%). MS ESI $[M+Na]^+$ 775.55.

Boc-LLFLK(Z)-NH_2 (228)



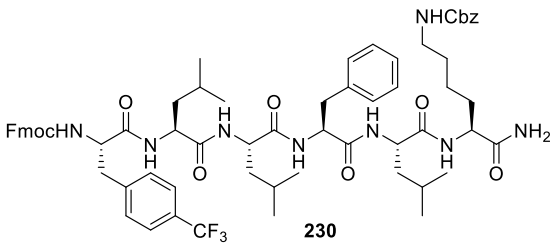
Compound **226** (1.95 g, 2.59 mmol) and Boc-Leu-OH (0.66 g, 2.86 mmol) were coupled together using the condition described in General procedure for peptide synthesis in a liquid phase to obtain the pentapeptide **228** (1.93 g, 86%). MS ESI $[M+Na]^+$ 888.57.

Fmoc-ØLLFLK(Z)-NH_2, Ø: 4-Fluorophenylalanine (229)



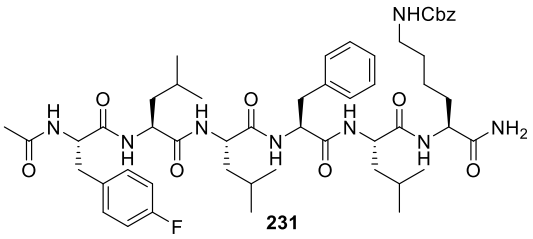
Pentapeptide **228** (0.10 g, 0.12 mmol) and Fmoc-4-fluorophenylalanine (0.05 g, 0.13 mmol) were coupled together using the condition described in General procedure for peptide synthesis in a liquid phase to obtain the protected hexapeptide X (0.10 g, 78%). MS ESI $[M+Na]^+$ 1175.57.

Fmoc-ØLLFLK(Z)-NH_2, Ø: 4-trifluoromethyphenylalanine (230)



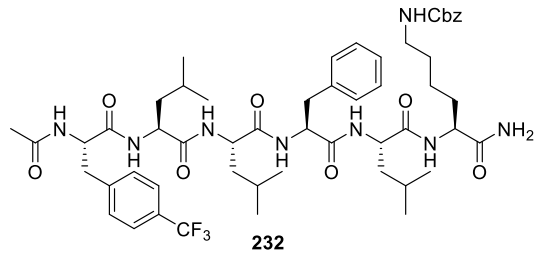
Pentapeptide **228** (0.15 g, 0.17 mmol) and *L*-Fmoc-4-trifluoromethylphenylalanine (0.9 g, 0.20 mmol) were coupled together using the condition described in General procedure for peptide synthesis in a liquid phase to obtain the protected hexapeptide **230** (0.12 g, 60%). MS ESI $[M+H]^+$ 1203.70.

Ac-ØLLFLK(Z)-NH_2, Ø: 4-Fluorophenylalanine (231)



To a solution of **229** (0.10 g, 0.09 mmol) in DMF (2 mL) was added DMAP (0.055 g, 0.45 mmol) and acetanhydride (17 µL, 0.18 mmol). The reaction mixture was stirred for 2 days. The reaction mixture was co-distilled with toluene to get rid of DMF. The residue was suspended in 5% aqueous solution of HCl and the precipitate was collected and dried under vacuum to give the hexapeptide **231** (0.050 g, 56%). MS ESI $[M+Na]^+$ 995.50.

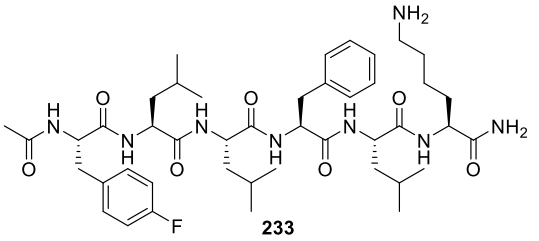
Ac-ØLLFLK(Z)-NH_2, Ø: 4-trifluoromethyphenylalanine (232)



232

To a solution of **230** (0.12 g, 0.10 mmol) in DMF (2 mL) was added DMAP (0.063 g, 0.51 mmol) and acetanhydride (19 µL, 0.21 mmol). The reaction mixture was stirred for 6 days. The reaction mixture was co-distilled with toluene to get rid of DMF. The residue was suspended in 5% aqueous solution of HCl and the precipitate was collected and dried under vacuum to give the hexapeptide **232** (0.054 g, 51%). MS ESI $[M+H]^+$ 1045.64.

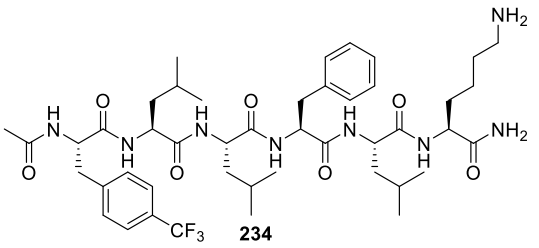
Ac-ØLLFLK-NH_2, Ø: 4-Fluorophenylalanine (233)



233

20% Pd/C (6 mg) was added to a solution of **231** (0.05 g, 0.05 mmol) in EtOH/CH_3COOH 10/1 (6 mL) and the reaction mixture was stirred under positive pressure of hydrogen, the reaction was monitored by UPLC-MS. After 5h the reaction mixture was filtered and the residue was evaporated to dryness under reduced pressure and purified by preparative HPLC (0.015 g, 35%). HRMS (ESI) *m/z* calcd for $C_{44}H_{68}O_7N_8F$ $[M+H]^+$ 839.5189, found 839.5192.

Ac-ØLLFLK-NH_2, Ø: 4-trifluoromethyphenylalanine (234)

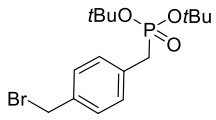


234

20% Pd/C (6 mg) was added to a solution of **232** (0.05 g, 0.05 mmol) in EtOH/CH_3COOH 10/1 (4 mL) and the reaction mixture was stirred under positive pressure of hydrogen, the reaction was monitored by UPLC-MS. After 8h the reaction mixture was filtered and the residue was evaporated to dryness under reduced pressure and purified by preparative HPLC (0.014 g, 30%). HRMS (ESI) *m/z* calcd for $C_{45}H_{68}O_7N_8F_3$ $[M+H]^+$ 889.5157, found 889.5155.

Di-*tert*-butyl (4-(bromomethyl)benzyl)phosphonate (235)

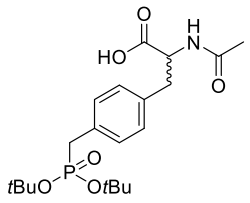
To a solution of di-t*ert*-butyl phosphite (1.00 g, 5.10 mmol) in THF (15 mL) was added 60% oil suspension of NaH (0.20 g, 5.00 mmol) and the solution was stirred 1h under inert



235

atmosphere of nitrogen. 1,4-bis(bromomethyl)benzene (1.49 g, 5.66 mmol) was added and the solution was stirred overnight at 80 °C in a sealed tube. The reaction was quenched by addition of saturated aqueous solution of $NaHCO_3$ (50 mL) and the mixture was extracted with EtOAc (3 × 50 mL), the combined organic layer was washed with brine, dried over $MgSO_4$ and evaporated to dryness. The solid residue was purified by column chromatography (EtOAc/cyclohexane 2:1) and crystallization from petroleum ether to give the bromide (0.21 g, 23%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (d, *J* = 8.1 Hz, 2H), 7.24 (dd, *J* = 8.3, 2.4 Hz, 2H), 4.48 (s, 2H), 3.03 (d, *J* = 21.5 Hz, 2H), 1.41 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.0 (d, *J* = 3.8 Hz), 134.1 (d, *J* = 9.8 Hz), 130.4 (d, *J* = 6.5 Hz), 129.0 (d, *J* = 2.9 Hz), 82.4 (d, *J* = 8.9 Hz), 37.8 (d, *J* = 142.4 Hz), 33.7 (s), 30.4 (s). ^{31}P NMR (160 MHz, $CDCl_3$) δ 20.2.

2-Acetamido-3-(4-((di-*tert*-butoxyphosphoryl)methyl)phenyl)propanoic acid (237)

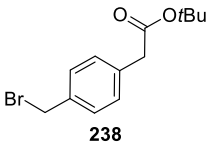


237

A 40% solution of benzyltrimethylammonium hydroxide in H_2O (0.28 mL, 0.70 mmol) was added to a solution of **235** (0.26 g, 0.70 mmol), potassium iodide (0.011 g, 0.07 mmol) and ethyl 2-((diphenylmethylene)amino)acetate (0.19 g, 0.70 mmol) in dry 1,4-dioxane (3 mL). The reaction mixture was stirred overnight. A 10% aqueous solution of citric acid (20 mL) and THF (10 mL) were added and the reaction mixture was stirred for further 2h. The reaction mixture was washed with diethyl ether (3 × 20 mL) and the organic fractions were discarded. The reaction mixture was basified with saturated aqueous solution of sodium bicarbonate, extracted with DCM (3 × 20 mL) and dried over $MgSO_4$. The residue was purified by column chromatography to obtain the desired amine **236** (0.13 g, 45%). The product was used without further purification. 1M aqueous solution of NaOH (1.8 mL) was added to a solution of **236** (0.15 g, 0.36 mmol) in 1,4-dioxane (3 mL). The reaction progress was monitored by UPLC-MS. After 20 min. the reaction mixture was made neutral by addition of 1M HCl solution in diethyl ether (1.8 mL). DIPEA (0.13 mL, 0.70 mmol) and *N*-Acetoxysuccinimide (0.068 g, 0.40 mmol) were added to the reaction mixture. The reaction mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure and 10% aqueous solution of citric acid was added (40 mL). The mixture was extracted with EtOAc (3 × 10 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, evaporated to dryness under reduced pressure and the residue was purified by column chromatography (DCM/MeOH 10:1) to obtain the acid **237** (0.094 g, 32%, over two steps). ^{1}H

NMR (400 MHz, $CDCl_3$) δ 7.19 (dd, *J* = 8.1, 2.6 Hz, 2H), 7.09 (d, *J* = 7.8 Hz, 2H), 6.40 (d, *J* = 7.7 Hz, 1H), 4.85 (ddd, *J* = 7.6, 6.2, 3.8 Hz, 1H), 3.36 – 2.89 (m, 4H), 1.92 (s, 3H), 1.40 (s, 9H), 1.38 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1 (s), 170.1 (s), 134.9 (d, *J* = 4.0 Hz), 131.2 (d, *J* = 9.7 Hz), 130.1 (d, *J* = 6.6 Hz), 129.6 (d, *J* = 3.3 Hz), 83.5 (dd, *J* = 9.5, 5.0 Hz), 53.0 (s), 37.9 (s), 36.8 (s), 36.5 (s), 30.4 (t, *J* = 3.8 Hz), 23.1 (s).

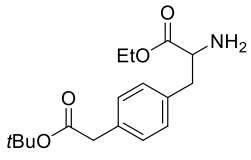
tert-Butyl 2-(4-(bromomethyl)phenyl)acetate (238)



238

Trifluoroboron etherate (0.19 mL, 1.74 mmol) was added to a solution of 2-(4-(bromomethyl)phenyl)acetic acid (0.20 g, 0.87 mmol) and *tert*-butyl 2,2,2-trichloroacetimidate (0.40 mL, 1.74 mmol) in dry THF (2.9 mL). The reaction mixture was stirred overnight under inert atmosphere of argon. Saturated solution of sodium bicarbonate was added (30 mL) and the mixture was extracted with EtOAc (3 × 12 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (cyclohexane/EtOAc 10:1) to obtain the desired bromide **238** (0.21 g, 85%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, *J* = 8.2 Hz, 2H), 7.25 (d, *J* = 8.3 Hz, 2H), 4.49 (s, 2H), 3.52 (s, 2H), 1.44 (s, 9H).

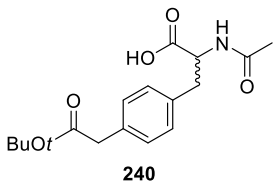
Ethyl 2-amino-3-(4-(2-(*tert*-butoxy)-2-oxoethyl)phenyl)propanoate (239)



239

A 40% solution of benzyltrimethylammonium hydroxide in H_2O (0.29 mL, 0.74 mmol) was added to a solution of **238** (0.21 g, 0.74 mmol), potassium iodide (0.012 g, 0.07 mmol) and ethyl 2-((diphenylmethylene)amino)acetate (0.20 g, 0.74 mmol) in dry 1,4-dioxane (3 mL). The reaction mixture was stirred overnight. A 10% aqueous solution of citric acid (20 mL) and THF (10 mL) were added and the reaction mixture was stirred for further 2h. The reaction mixture was washed with diethyl ether (3 × 20 mL) and the organic fractions were discarded. The reaction mixture was basified with saturated aqueous solution of sodium bicarbonate, extracted with DCM (3 × 20 mL) and dried over $MgSO_4$. The residue was purified by column chromatography to obtain the desired amine **239** (0.11 g, 48%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, *J* = 8.2 Hz, 2H), 7.15 (d, *J* = 8.2 Hz, 2H), 4.16 (q, *J* = 7.1 Hz, 2H), 3.70 (dd, *J* = 7.9, 5.3 Hz, 1H), 3.49 (s, 2H), 2.95 (ddd, *J* = 21.5, 13.6, 6.6 Hz, 2H), 1.43 (s, 9H), 1.24 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.1, 171.1, 136.0, 133.4, 129.6, 129.5, 81.0, 61.1, 56.0, 42.3, 40.9, 28.2, 14.3.

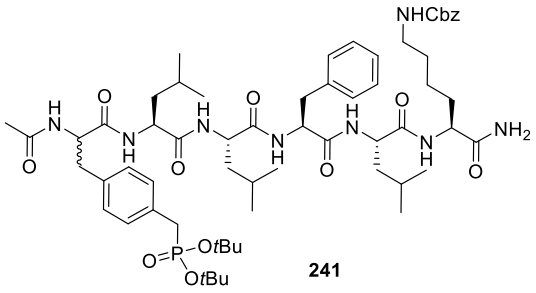
2-Acetamido-3-(4-(2-(*tert*-butoxy)-2-oxoethyl)phenyl)propanoic acid (240)



240

1M aqueous solution of NaOH (1.7 mL) was added to a solution of **239** (0.10 g, 0.34 mmol) in 1,4-dioxane (2 mL). The reaction progress was monitored by UPLC-MS. After 20 min. the reaction mixture was made neural by addition of 1M HCl solution in diethyl ether (1.7 mL). DIPEA (0.12 mL, 0.68 mmol) and N-Acetoxysuccinimide (0.064 g, 0.40 mmol) were added to the reaction mixture. The reaction mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure and 10% aqueous solution of citric acid was added (30 mL). The mixture was extracted with DCM (3 × 17 mL). The combined organic layer was evaporated to dryness and the residue was purified by column chromatography (DCM/MeOH 10:1) to obtain the acid **240** (0.077 g, 72%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.18 (s, 4H), 4.64 (dd, *J* = 9.0, 5.1 Hz, 1H), 3.49 (s, 2H), 3.06 (ddd, *J* = 23.0, 14.0, 7.1 Hz, 2H), 1.90 (s, 3H), 1.42 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD) δ 174.8, 173.1, 173.0, 137.1, 134.6, 130.3, 130.3, 82.0, 55.1, 43.0, 38.0, 28.3, 22.3. Spectra are in agreement with the literature.[155]

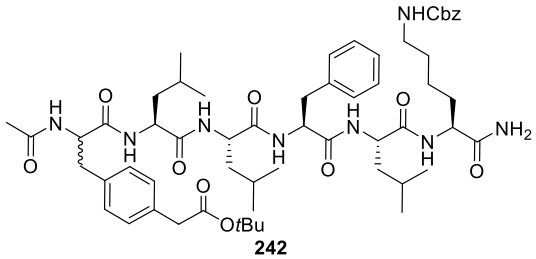
Ac-ØLLFLK(Z)-NH$_2$, Ø: 4-((di-*tert*-butoxyphosphoryl)methyl)phenylalanine (241)



241

Pentapeptide **228** (0.15 g, 0.17 mmol) and **237** (0.09 g, 0.20 mmol) were coupled together using the modified condition described in General procedure for peptide synthesis in a liquid phase. The reaction mixture was concentrated by co-distillation with toluene. The residue was suspended in 5% aqueous solution of HCl and ultrasound was applied for 1 min and precipitate appeared. The precipitate was collected, washed with 5% HCl, diethylether and dried under vacuum to obtain the peptide **241** (0.22 g, 84%). MS ESI $[M+Na]^+$ 1183.71.

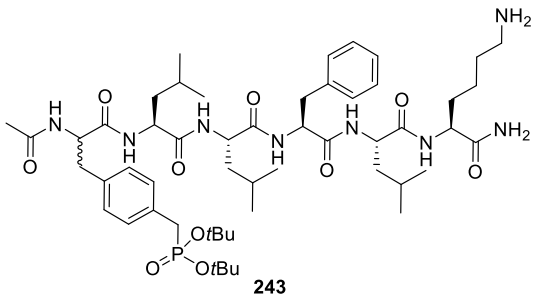
Ac-ØLLFLK(Z), Ø: 4-((*tert*-butylcarboxylate)methyl)phenylalanine (242)



242

Pentapeptide **228** (0.22 g, 0.26 mmol) and **240** (0.075 g, 0.28 mmol) were coupled together using the modified condition described in General procedure for peptide synthesis in a liquid phase to obtain the hexapeptide **242** (0.26 g, 94%). MS ESI $[M+Na]^+$ 1091.56.

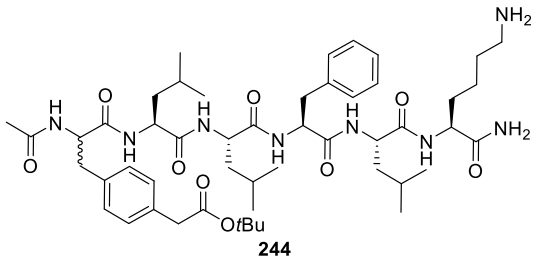
Ac-ØLLFLK, Ø: 4-((di-*tert*-butoxyphosphoryl)methyl)phenylalanine (243)



243

Powder 20% Pd/C (22 mg) was added to a solution of **241** (0.22 g, 0.19 mmol) in EtOH/CH_3COOH 10/1 (11 mL) and the reaction mixture was stirred under positive pressure of hydrogen, the reaction was monitored by UPLC-MS. After 7h the reaction mixture was filtered and the residue was evaporated to dryness under reduced pressure to obtain the hexapeptide **243** (0.21 g, 97%). MS ESI $[M+H]^+$ 1027.68.

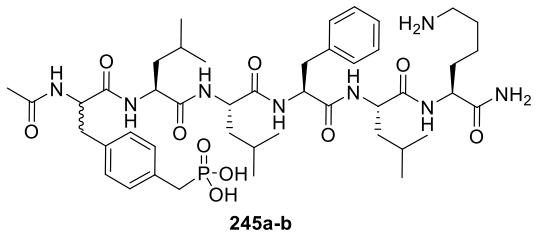
Ac-ØLLFLK, Ø: 4-((*tert*-butylcarboxylate)methyl)phenylalanine (244)



244

20% Pd/C (26 mg) was added to a solution of **242** (0.26 g, 0.19 mmol) in EtOH/CH_3COOH 10/1 (18 mL) and the reaction mixture was stirred under positive pressure of hydrogen, the reaction was stirred overnight. The reaction mixture was filtered and the residue was evaporated to dryness under reduced pressure to obtain the hexapeptide **244** (0.25 g, 96%). MS ESI $[M+H]^+$ 935.65.

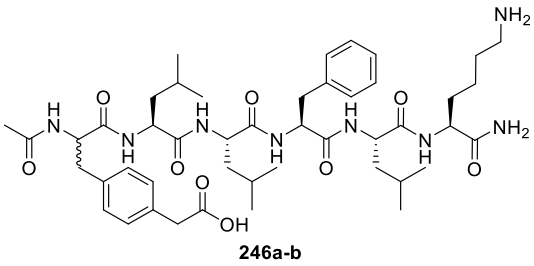
Ac-ØLLFLK, Ø: 4-((phosphoryl)methyl)phenylalanine (245a-b)



245a-b

Hexapeptide **243** (0.21 g, 0.20 mmol) was dissolved in a mixture TFA/DCM 2:1 (4 mL) and a few drops of water. The reaction was monitored by UPLC-MS. After 0.5h the reaction mixture was evaporated under reduced pressure and the residue was purified by preparative HPLC. Both diastereomers were isolated and separated: isomer a. (0.020 g, 9%) isomer b (0.020 g, 9%). HRMS (ESI) *m/z* calcd for $C_{45}H_{72}O_{10}N_8P$ $[M+H]^+$ 915.5103, found 915.5104.

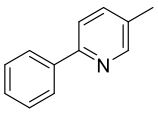
Ac-ØLLFLK, Ø: 4-((carboxyl)methyl)phenylalanine (246a-b)



246a-b

Hexapeptide **244** (0.25 g, 0.20 mmol) was dissolved in a mixture TFA/DCM 2:1 (5 mL) and a few drops of water. The reaction was monitored by UPLC-MS. After 0.5h the reaction mixture was evaporated under reduced pressure and the residue was purified by preparative HPLC. Both diastereomers were isolated and separated: isomer a. (0.022 g, 9%) isomer b (0.021 g, 9%). MS ESI $[M+H]^+$ 879.67.

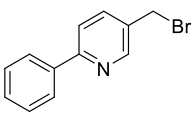
5-Methyl-2-phenylpyridine (247)



247

Tetrakis(triphenylphosphine)palladium(0) (0.14 g, 0.12 mmol) was added to a solution of 2-brom-5-methylpyridine (0.68 g, 4.00 mmol), phenylboronic acid (0.64 g, 5.20 mmol) and sodium carbonate (3.20 g, 30.21 mmol) in toluene/H_2O/EtOH 14:14:5 (33 mL). The reaction mixture was refluxed overnight under inert atmosphere of argon. The reaction was quenched by addition of saturated aqueous solution of NH_4Cl (60 mL). The mixture was extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (DCM) to obtain the desired methylpyridine derivative **247** (0.61 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (dd, J = 1.5, 0.7 Hz, 1H), 8.04 – 7.91 (m, 2H), 7.67 – 7.60 (m, 1H), 7.60 – 7.52 (m, 1H), 7.46 (m, 2H), 7.40 (dt, J = 9.5, 4.3 Hz, 1H), 2.38 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.8, 150.0, 139.3, 137.4, 131.6, 128.7, 128.6, 126.7, 120.1, 18.2.

5-(Bromomethyl)-2-phenylpyridine (248)

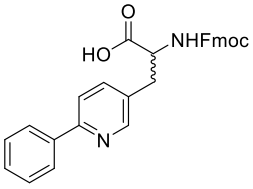


248

Dibenzoyl peroxide (0.015 g, 0.062 mmol) was added to a solution of **247** (0.21 g, 1.25 mmol) and *N*-bromosuccinimide (0.24 g, 1.37 mmol) in dry chloroform (10 mL). The reaction mixture was refluxed for 3h under flow of nitrogen. The reaction mixture was absorbed on silicagel (2.50 g) and purified by column chromatography (DCM/toluene 10:1) to obtain the desired bromide **248** (0.19 g, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 – 8.62 (m, 1H), 8.08 – 7.93 (m, 2H), 7.80 (dd, J = 8.2, 2.4 Hz, 1H), 7.73 (dd, J = 8.2, 0.7 Hz, 1H), 7.55 – 7.39 (m, 3H), 4.53 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$)

δ 157.5, 149.8, 138.8, 137.6, 132.1, 129.4, 129.0, 127.1, 120.6, 29.9. Spectra are in agreement with the literature.[156]

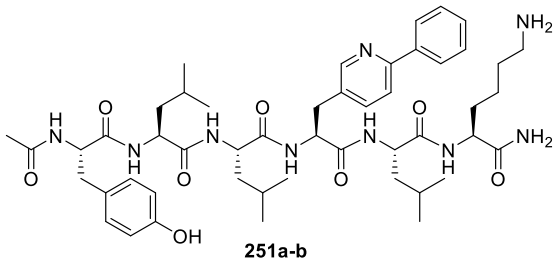
2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(6-phenylpyridin-3-yl)propanoic acid (250)



250

A 40% solution of benzyltrimethylammonium hydroxide in H_2O (1.01 mL, 2.29 mmol) was added to a solution of **248** (0.57 g, 2.29 mmol), potassium iodide (0.038 g, 0.23 mmol) and ethyl 2-((diphenylmethylene)amino)acetate (0.61 g, 2.29 mmol) in dry 1,4-dioxane (10 mL). The reaction mixture was stirred overnight. A 10% aqueous solution of citric acid (15 mL) and THF (15 mL) were added and the reaction mixture was stirred for further 4h. The reaction mixture was washed with diethyl ether (3 × 20 mL) and the organic fractions were discarded. The reaction mixture was basified with saturated aqueous solution of sodium bicarbonate, extracted with DCM (3 × 20 mL) and dried over $MgSO_4$. The residue was purified by column chromatography (DCM/MeOH 20:1) to obtain the desired amine **249** (0.52 g, 84%). The product was used without further purification. 1M aqueous solution of NaOH (9.6 mL) was added to a solution of **249** (0.52 g, 1.91 mmol) in 1,4-dioxane (10 mL). The reaction progress was monitored by UPLC-MS. After 20 min. the reaction mixture was made neutral by addition of 1M HCl solution in diethyl ether (9.6 mL). DIPEA (0.67 mL, 3.82 mmol) and *N*-Acetoxysuccinimide (0.78 g, 2.29 mmol) were added to the reaction mixture. The reaction mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure and 10% aqueous solution of citric acid was added (40 mL). The mixture was extracted with DCM/iPrOH 5:1 (3 × 10 mL). The combined organic layer was evaporated to dryness under reduced pressure and the residue was purified by column chromatography (DCM/MeOH/AcOH 46:2:1) to obtain the acid **250** (0.59 g, 66%, over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.65 (s, 1H), 8.57 (s, 1H), 7.92 – 7.32 (m, 16H), 5.59 (d, J = 6.2 Hz, 1H), 4.78 – 4.68 (m, 1H), 4.64 (dd, J = 10.6, 6.8 Hz, 1H), 4.43 (dd, J = 10.7, 6.7 Hz, 1H), 4.27 (t, J = 6.7 Hz, 1H), 3.47 – 3.27 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1, 155.5, 155.2, 147.1, 143.9, 143.8, 141.8, 141.5, 141.3, 136.6, 132.3, 129.9, 129.1, 127.8, 127.7, 127.1, 127.1, 125.2, 125.0, 122.6, 120.1, 120.0, 66.5, 54.7, 47.3, 34.5.47.4, 34.6.

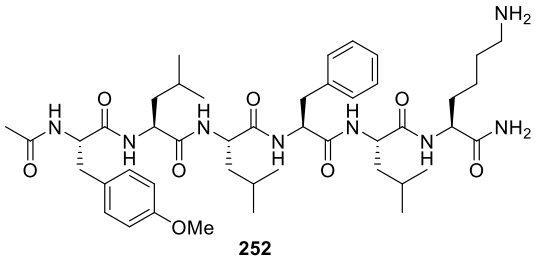
Ac-YLLØLK-NH_2, Ø: 3-(6-phenylpyridin-3-yl)-*L*-alanine (251a-b)



251a-b

The first two amino acids were introduced using PS3 using rink amide resin (0.10 mmol) Compound **250** was introduced using the general procedure for SPPS in a syringe. Last three amino acids were introduced using PS3, acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the isomer a (0.011 g, 12%) isomer b (0.013 g, 14%). MS ESI $[M+H]^+$ 914.56.

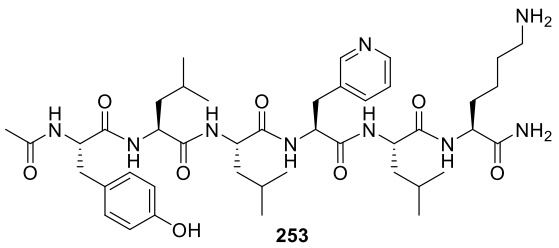
Ac-ØLLFLK-NH_2, Ø: 4-Methoxyphenyl-*L*-alanine (252)



252

The peptide was synthesized using the general procedure for SPPS in a syringe using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **252** (0.023 g, 27%). HRMS (ESI) *m/z* calcd for $C_{43}H_{67}O_9N_8Na$ $[M+Na]^+$ 851.5389, found 860.5392.

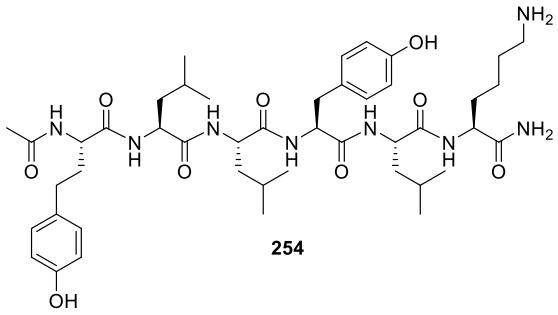
Ac-YLLØLK-NH_2, Ø: 3-(3-Pyridyl)-*L*-alanine (253)



253

The peptide was synthesized using the general procedure for SPPS in a syringe using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **253** (0.014 g, 16%). HRMS (ESI) *m/z* calcd for $C_{43}H_{67}O_9N_8Na$ $[M+Na]^+$ 860.5004, found 860.5007.

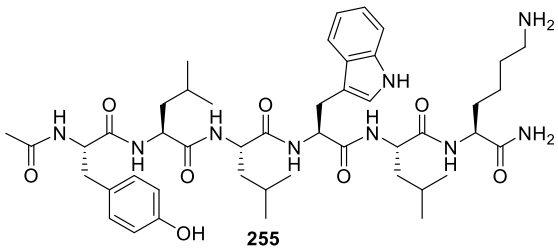
Ac-ØLLYLK-NH$_2$, Ø: homotyrosine (254)



254

The NH_2-LLYLK-(resin) peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), fmoc-HomoTyr-OH (0.13 g, 0.30 mmol) was coupled using HATU (0.11 g, 0.30 mmol), NMM (100 µL, 1.00 mmol) in DMF (2 mL), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **254** (0.015 g, 17%). HRMS (ESI) *m/z* calcd for $C_{45}H_{71}O_9N_8$ $[M+H]^+$ 867.5338, found 867.5341.

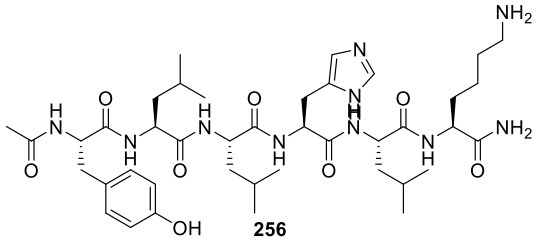
Ac-YLLWLK (255)



255

The peptide was synthesized on a peptide synthesizer PS3 using rink amide resin (0.125 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **255** (0.022 g, 20%). HRMS (ESI) *m/z* calcd for $C_{46}H_{70}O_8N_9$ $[M+H]^+$ 876.5341, found 876.5339.

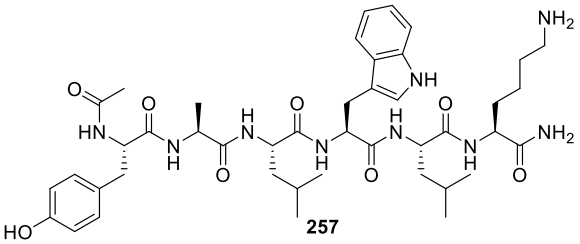
Ac-YLLHLK-NH$_2$ (256)



256

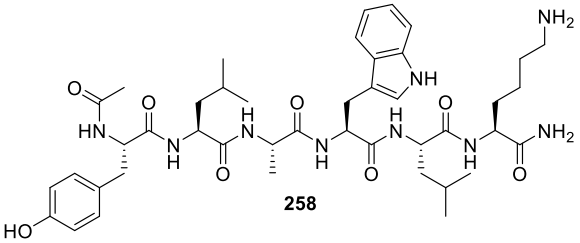
The peptide was synthesized on a peptide synthesizer PS3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **256** (0.018 g, 21%). HRMS (ESI) *m/z* calcd for $C_{41}H_{67}O_8N_{10}$ $[M+H]^+$ 827.5137, found 827.5139.

Ac-YALWLK-NH$_2$ (257)



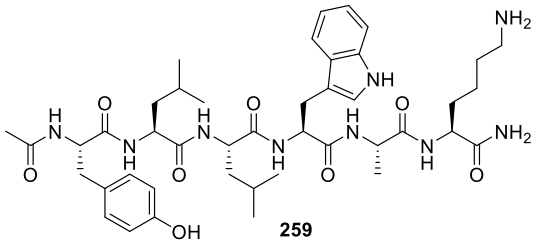
The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **257** (0.011 g, 13%). MS ESI $[M+H]^+$ 834.71.

Ac-YLAWLK-NH$_2$ (258)



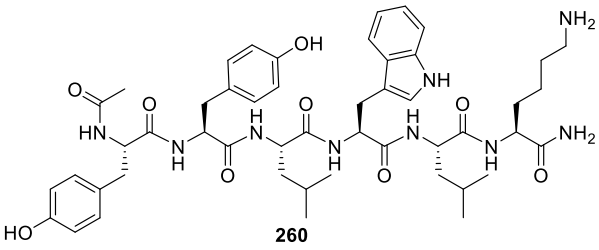
The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **258** (0.019 g, 23%). MS ESI $[M+H]^+$ 834.60.

Ac-YLLWAK-NH$_2$ (259)



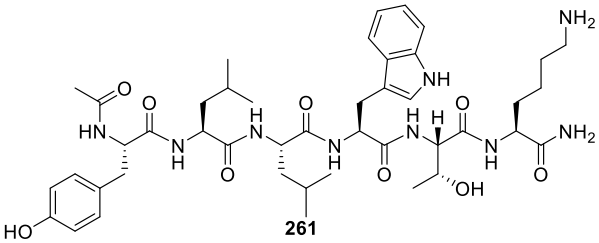
The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **259** (0.012 g, 14%). MS ESI $[M+H]^+$ 834.50.

Ac-YYLWLK-NH$_2$ (260)



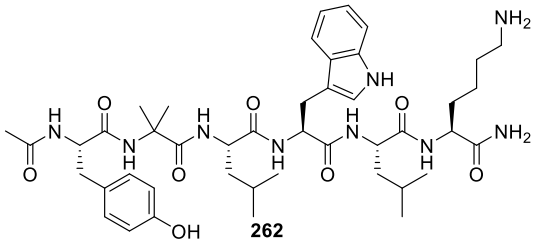
The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide (0.021 g, 23%). HRMS (ESI) *m/z* calcd for $C_{49}H_{68}O_9N_9$ $[M+H]^+$ 926.5134, found 926.5129.

Ac-YLLWTK-NH$_2$ (261)



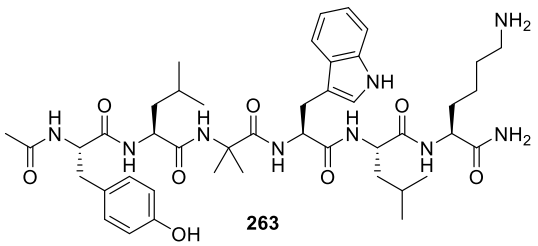
The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **261** (0.014 g, 16%). HRMS (ESI) *m/z* calcd for $C_{44}H_{66}O_9N_9$ $[M+H]^+$ 864.4978, found 864.4971.

Ac-Y<u>Aib</u>LWLK-NH$_2$ (262)



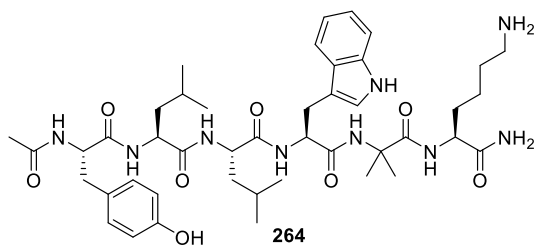
The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **262** (0.023 g, 27%). HRMS (MALDI) *m/z* calcd for $C_{44}H_{66}O_8N_9$ $[M+H]^+$ 848.5028, found 848.5029.

Ac-YL<u>Aib</u>WLK-NH$_2$ (263)



The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **263** (0.019 g, 22%). HRMS (MALDI) *m/z* calcd for $C_{44}H_{66}O_8N_9$ $[M+H]^+$ 848.5028, found 848.5029.

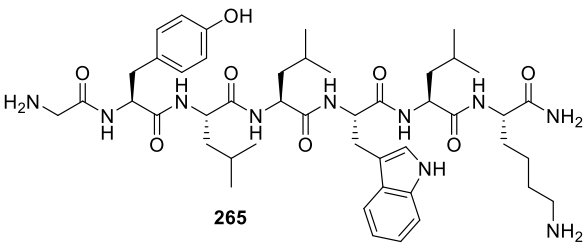
Ac-YLLW<u>Aib</u>K-NH$_2$ (264)



The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), acetylated using the general procedure Y, cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the

peptide **264** (0.025 g, 29%). HRMS (MALDI) *m/z* calcd for $C_{44}H_{66}O_8N_9$ $[M+H]^+$ 848.5028, found 848.5029.

GYLLWLK-NH₂ (265)

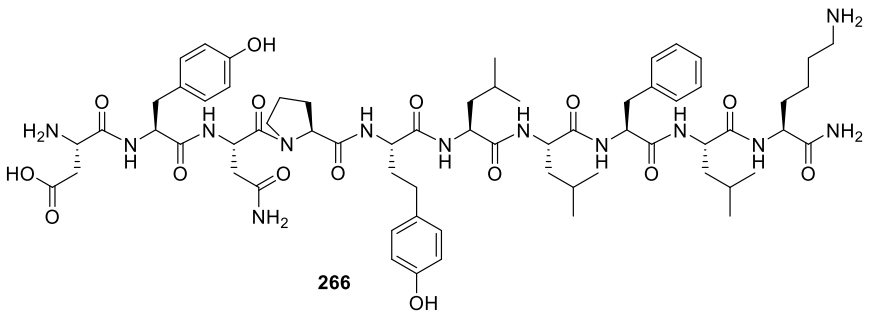


265

The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.10 mmol), cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **265** (0.013 g, 15%). HRMS (MALDI) *m/z* calcd for $C_{46}H_{71}O_8N_{10}$ $[M+H]^+$ 891.5451, found 891.5464.

Dekapeptides

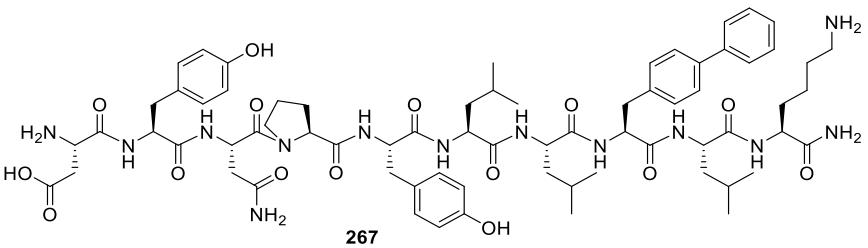
DYNPØLLFLK-NH₂, Ø: homotyrosine (266)



266

Peptide NH_2-LLFLK-(resin) was prepared on automatic peptide synthesizer PS-3. Fmoc-homo-*l*-tyrosine-OH was coupled using the general procedure for SPPS in a syringe. The rest of the peptide was synthesized on an automatic peptide synthesizer PS-3, cleaved from the resin using general procedure X and purified by preparative HPLC to obtain the peptide **266** (0.014 g, 11%). HRMS (ESI) *m/z* calcd for $C_{65}H_{96}O_{15}N_{13}$ $[M+H]^+$ 1298.7143, found 1298.7148.

DYNPYLLØLK-NH₂ Ø: 3-(4-Biphenylyl)-*l*-alanine (267)

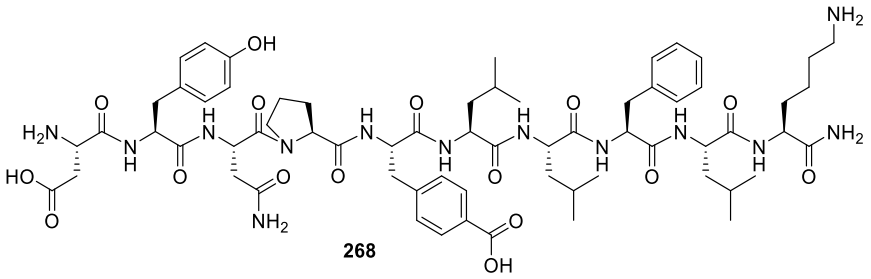


267

Dipeptide NH_2-LK-(resin) was prepared on automatic peptide synthesizer PS3. (S)-3-biphenyl-4-yl-2-(9H-fluoren-9-ylmethoxycarbonylamino)propionic acid (0.23 g, 0.50 mmol) was

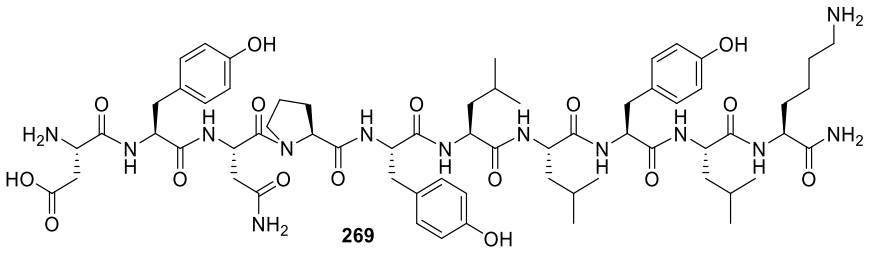
coupled using the general procedure for SPPS in a syringe. The rest of the peptide was synthesized on an automatic peptide synthesizer PS-3, cleaved from the resin using general procedure X and purified by preparative HPLC to obtain the peptide **267** (0.068 g, 50%). HRMS (ESI) *m/z* calcd for $C_{70}H_{98}O_{15}N_{13}$ $[M+H]^+$ 1360.7299, found 1360.7287.

DYNPØLLYLK-NH₂ Ø: phenyl-(4-carboxylic acid)-*l*-alanine (268)



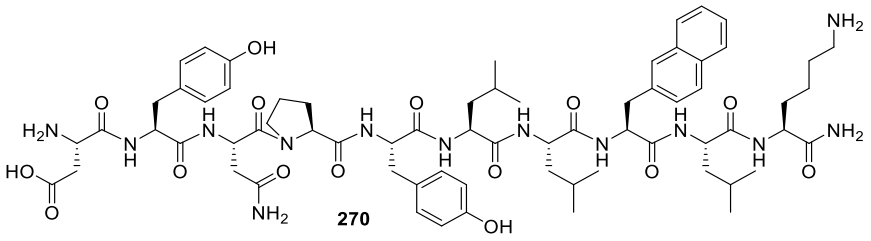
Dipeptide NH_2-LLFLK-(resin) was prepared on automatic peptide synthesizer PS3. (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(*tert*-butoxycarbonyl)phenyl)propanoic acid was coupled using the general procedure for SPPS in a syringe. The rest of the peptide was synthesized on an automatic peptide synthesizer PS-3, cleaved from the resin using general procedure X and purified by preparative HPLC to obtain the peptide **268** (0.018 g, 14%). HRMS (ESI) *m/z* calcd for $C_{65}H_{94}O_{16}N_{13}$ $[M+H]^+$ 1312.6936, found 1312.6928.

DYNPYLLYLK-NH₂ (269)



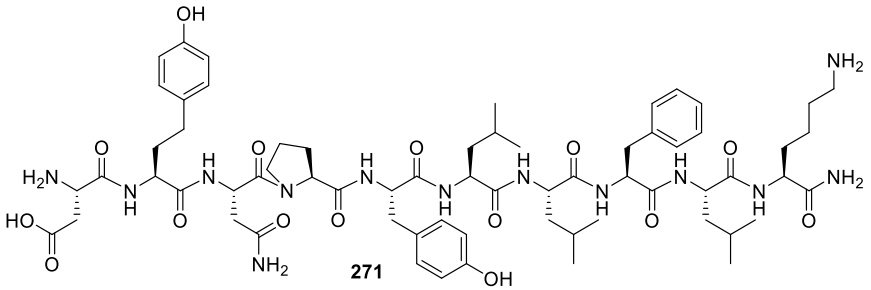
Peptide was prepared on automatic peptide synthesizer PS3 using RINK amide resin (0.15 g, 0.10 mmol), cleaved from the resin using general procedure X and purified by preparative HPLC to obtain the peptide **269** (0.014 g, 11%). MS ESI $[M+2H]^{2+}$ 650.5 (m/2z).

DYNPYLLØLK-NH_2 Ø: 3-(2-naphthyl)-*l*-alanine (270)



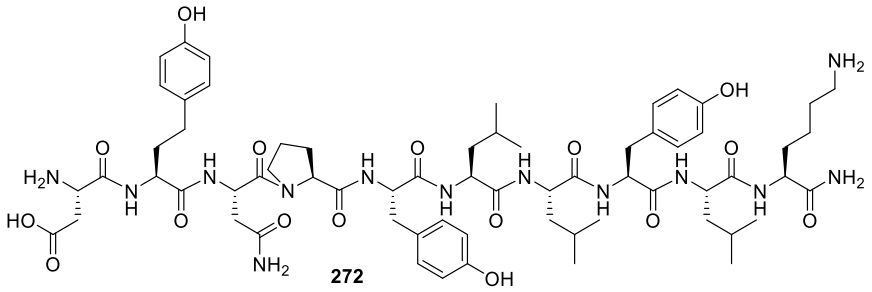
Dipeptide NH_2-LK-(resin) was prepared on automatic peptide synthesizer PS-3 using RINK amide resin (0.15 g, 0.1 mmol). Fmoc-3-(2-naphthyl)-*l*-alanine-OH (0.22 g, 0.50 mmol) was coupled using the general procedure for SPPS in a syringe. The rest of the peptide was synthesized on an automatic peptide synthesizer PS-3, cleaved from the resin using general procedure X and purified by preparative HPLC to obtain the peptide **270** (0.058 g, 42%). HRMS (ESI) *m/z* calcd for $C_{70}H_{98}O_{15}N_{13}$ $[M+H]^+$ 1360.7299, found 1360.7287.

DØNPYLLWLK-NH_2, Ø: homo-*l*-tyrosine (271)



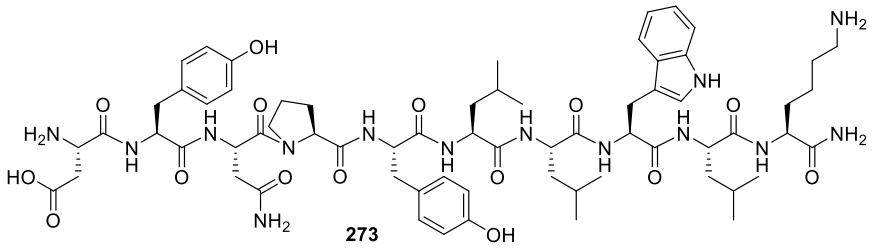
The peptide NH_2-NPYLLFLK-(resin) was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.071 mmol), fmoc-homo-*l*-tyrosine-OH and fmoc-aspartic(O*t*Bu)-OH were coupled individually using the general procedure for SPPS in a syringe. The peptide was cleaved from the resin using general procedure X and purified by preparative HPLC to obtain the peptide **271** (0.035 g, 26%). HRMS (ESI) *m/z* calcd for $C_{65}H_{96}O_{15}N_{13}$ $[M+H]^+$ 1298.7143, found 1298.7146.

DØNPYLLWLK-NH$_2$, Ø: homo-*l*-tyrosine (272)



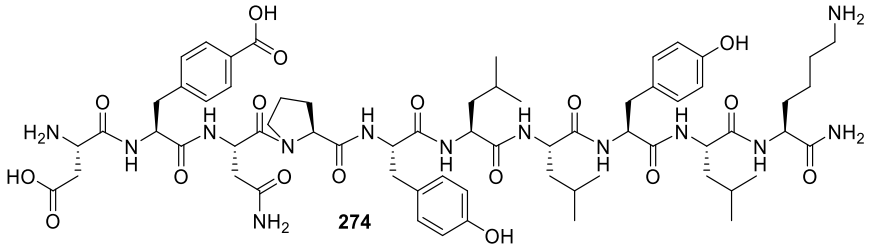
The peptide NH$_2$-NPYLLYLK-(resin) was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.071 mmol), fmoc-homo-*l*-tyrosine-OH and fmoc-aspartic(O*t*Bu)-OH were coupled individually using the general procedure for SPPS in a syringe. The peptide was cleaved from the resin using general procedure X and purified by preparative HPLC to obtain the peptide (0.035 g, 26%). HRMS (ESI) *m/z* calcd for $C_{65}H_{96}O_{16}N_{13}$ $[M+H]^+$ 1314.7092, found 1314.7095.

DYNPYLLWLK-NH$_2$ (273)



The peptide was synthesized on a peptide synthesizer PS-3 using rink amide resin (0.125 mmol), cleaved from the resin using the general procedure X and purified by preparative HPLC to obtain the peptide **273** (0.021 g, 13%). HRMS (ESI) *m/z* calcd for $C_{66}H_{95}O_{15}N_{14}$ $[M+H]^+$ 1323.7095, found 1323.7098.

DØNPYLLYLK-NH$_2$, Ø: phenyl-(4-carboxylic acid)-*l*-alanine (274)



FmocNH-NPYLLYLK-Resin was prepared on PS3 automatic synthesizer using RINK amide resin (0.10 g, 0.071 mmol). ((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(*tert*-butoxycarbonyl)phenyl)propanoic acid (0.18 g, 0.37 mmol) and fmoc-Asp(OtBu)-OH (0.15 g, 0.35 mmol) was coupled using the general procedure for coupling in a syringe. The peptide was cleaved from the resin using the general procedure X. The residue was purified by preparative HPLC to obtain the peptide **274** (0.012 g, 13%). HRMS (ESI) *m/z* calcd for $C_{65}H_{94}O_{17}N_{13}$ $[M+H]^+$ 1328.6885, found 1328.6889.

Solid phase

General procedure X

The resin was stirred in a mixture of TFA/TIS/H_2O; 95/2.5/2.5 (10 mL/ 0.1 mmol of resin) for 1h. The solution was filtered, washed with DCM and the filtrate was concentrated using the flow of N_2. The peptide was precipitated by *tert*-butyl methyl ether. The precipitate was collected by filtration or centrifugation.

General procedure Y

HOBT (0.15 g, 1.00 mmol), DIC (0.15 µL, 1.00 mmol) and CH_3COOH (60 µL, 1.00 mmol) were dissolved in DMF (6 ml/0.1 mmol resin) and the resin was incubated in the solution for 1h. The resin was subsequently washed with DMF (10 ×).

General procedure Z

The resin was stirred in a mixture of TFA/thioanisole/anisole/1,2-ethanedithiol 9/0.5/0.2/0.3 (10 mL/ 0.1 mmol of resin) for 1h. The solution was filtered, washed with DCM (3×) and the filtrate was concentrated using the flow of N_2. The peptide was precipitated by *tert*-butyl methyl ether. The precipitate was collected by filtration or centrifugation.

General procedure for peptide synthesis on synthesizer PS3

Table 12. Details of the programs used on PS3 synthesizer

Program	1 [min]	2 [min]	3 [min]
Solvation	3 × 1	3 × 1	3 × 3
Deprotection	2 × 10	2 × 10	2 × 10
Solvation	6 × 1	6 × 1	6 × 1
Activation	1 × 2	1 × 2	
Amide coupling	1 × 30	1 × 30	

Activation		1 × 2
Amide coupling		1 × 30
Solvation	3 × 1	3 × 1

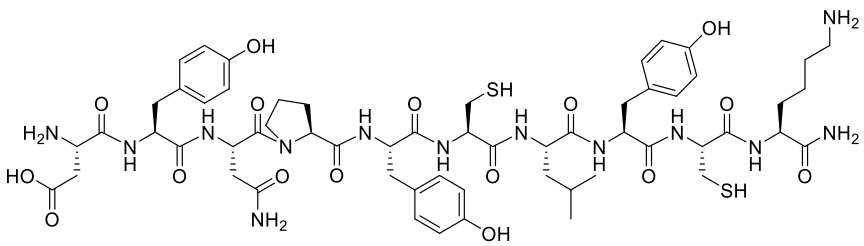
PS3 synthesizer was used for solid phase peptide synthesis. Three programs were used. Program 1 was used for simple amide coupling. Program 2 was used for double amide coupling. Double amide coupling was necessary for certain amino acids in the peptide sequence that are known to be problematic, or for the last amino acids in the sequence. Program three was used for deprotection of Fmoc-group from the alpha-aminogroup. For solvation NMP was used. Deprotection was achieved with 20% piperidine in DMF. Amino acid and HBTU were mixed in a ration 1:1 (10 eq./resin) and resin was placed in a reactor. 20% NMM in NMP was used for activation.

General procedure for SPPS in a syringe

Fmoc protected resin was placed in a syringe with filter. Fmoc-protected resin was incubated with 20% piperidine in DMF (2 × 10 min.). The resin was washed with DMF (10 ×) to get rid of piperidine. Amino acid (10 eq.) and HBTU (9.5 eq.) were dissolved in DMF and DIPEA was added. The activation took 1 minute. The resin was incubated for 45 min. with the solution of activated amino acid. The resin was washed with DMF (ten times) and it was ready for another coupling cycle. Special amino acids were used only in 5 equivalents excess.

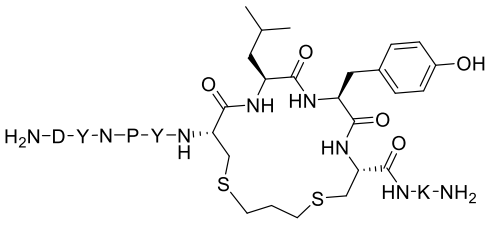
Peptide stapling

DYNPYCLYCK-NH_2 (275)



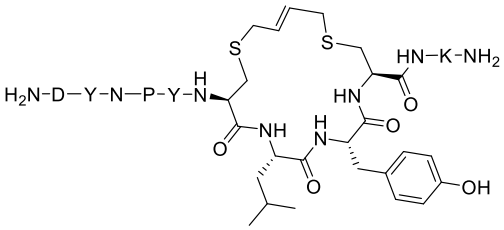
The peptide was synthesized on an automatic peptide synthesizer PS2, cleaved from the resin using general procedure Z and purified by preparative HPLC to obtain the peptide **275** (0.050 g, 26%). HRMS (MALDI) *m/z* calcd for $C_{58}H_{82}N_{13}O_{16}S_2$ $[M+H]^+$ 1280.5438, found 1280.5442.

Stapled peptide (276)



A 0.3 M solution of 1,3-dibrompropane in THF (75 µL) was added to a solution of peptide **275** (0.010 g, 7.81 µm), TCEP (0.0045 g, 18.00 µm) and ammonium bicarbonate (0.19 g, 2.40 mmol) in degassed CH_3CN/H_2O 1:1 (100 mL). The solution was stirred for 2 days under inert atmosphere on nitrogen. The reaction mixture was lyophilized and the residue was purified by semi-preparative HPLC to obtain the peptide **276** (0.002 g, 20%). HRMS (ESI) *m/z* calcd for $C_{61}H_{86}N_{13}O_{16}S_2$ $[M+H]^+$ 1320.5751, found 1320.5755.

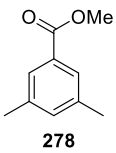
Stapled peptide (277)



A 0.3 M solution of 1,4-dibrombut-2-en in THF (60 µL) was added to a solution of peptide **275** (0.008 g, 6.25 µm), TCEP·HCl (0.0036 g, 12.60 µm) and ammonium bicarbonate (0.15 g, 1.94 mmol) in degassed CH_3CN/H_2O 1:1 (80 mL). The solution was stirred for 2h under inert atmosphere on nitrogen. The reaction mixture was lyophilized and the residue was purified by semi-preparative HPLC to obtain the peptide **277** (0.005 g, 63%). HRMS (ESI) *m/z* calcd for $C_{62}H_{86}N_{13}O_{16}S_2$ $[M+H]^+$ 1332.5751, found 1320.5736.

Bicyclization

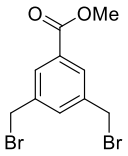
Methyl 3,5-dimethylbenzoate (278)



278

Sulfuric acid (2.7 mL) was added to a solution of 3,5-dimethylbenzoic acid (8.00 g, 53.33 mmol) in methanol (30 mL). The reaction mixture was refluxed overnight. The reaction mixture was evaporated to dryness and the residue was partitioned between water (70 mL) and toluene (70 mL). Water phase was washed with toluene (2 × 30 mL). The combined organic phase was washed with brine, dried over $MgSO_4$ and evaporated to dryness to obtain the desired ester **278** (8.05 g, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, J = 0.6 Hz, 2H), 7.19 (s, 1H), 3.90 (s, 3H), 2.36 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.6, 138.2, 134.7, 130.1, 127.4, 52.1, 21.3.

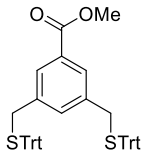
Methyl 3,5-bis(bromomethyl)benzoate (279)



279

Compound **278** (4.00 g, 24.40 mmol) and *N*-bromosuccinimide (8.67 g, 49.00 mmol) were added to chloroform (30 mL). Dibenzoyl peroxide (0.29 g, 1.20 mmol) was added and the reaction mixture was refluxed for 3h under inert atmosphere of nitrogen. The reaction mixture was concentrated under reduced pressure and the residue was purified by column chromatography (cyclohexane/EtOAc 10:1 to 4:1) and crystallized from n-hexane to obtain the desired dibromide **279** (3.46 g, 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (d, *J* = 1.8 Hz, 2H), 7.61 (t, *J* = 1.8 Hz, 1H), 4.50 (s, 4H), 3.93 (s, 3H).

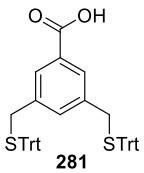
Methyl 3,5-bis((tritylthio)methyl)benzoate (280)



280

Compound **279** (0.25 g, 0.77 mmol) was dissolved in DMF (6 mL) and DIPEA (1.10 mL, 6.21 mmol) was added. Triphenylmethanethiol (0.45 g, 1.63 mmol) was added and the reaction mixture was stirred for 4h under inert atmosphere. The reaction was quenched by addition of water (30 mL) and the mixture was extracted with EtOAc (3 × 15 mL). The combined organic fractions were washed with brine, dried over $MgSO_4$ and concentrated to dryness under reduced pressure to obtain the desired compound **280** (0.53 g, 97%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.61 (d, *J* = 1.7 Hz, 2H), 7.49 – 7.39 (m, 12H), 7.35 – 7.26 (m, 12H), 7.26 – 7.17 (m, 6H), 6.95 (t, *J* = 1.6 Hz, 1H), 3.88 (s, 3H), 3.29 (s, 4H).

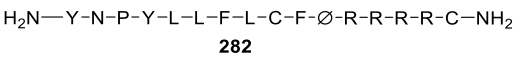
3,5-Bis((tritylthio)methyl)benzoic acid (281)



281

1M aqueous solution of NaOH was added to a solution of **280** (0.52 g, 0.73 mmol) in 1,4-dioxane (4 mL). The reaction mixture was stirred for 2h at 60 °C. The reaction was quenched by addition of 10% aqueous solution of citric acid (20 mL). The mixture was extracted with DCM (4 × 30 mL). The combine organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (DCM/MeOH 100:1 to 20:1) to obtain the desired acid **281** (0.33 g, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (s, 2H), 7.51 – 7.40 (m, *J* = 8.2, 0.9 Hz, 12H), 7.35 – 7.27 (m, *J* = 10.2, 5.0 Hz, 13H), 7.25 – 7.17 (m, 6H), 7.00 (s, 1H), 3.31 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.6, 138.2, 135.2, 129.8, 129.7, 129.6, 128.2, 127.9, 127.0, 67.8, 36.5.

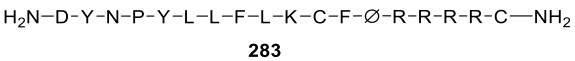
Peptide (282)



282

The peptide was synthesized on an automatic peptide synthesizer PS2, cleaved from the resin using general procedure Z and purified by preparative HPLC to obtain the peptide **282** (0.028 g, 5%). HRMS (MALDI) *m/z* calcd for $C_{106}H_{155}O_{19}N_{30}S_2$ $[M+H]^+$ 2216.1521, found 2216.1515.

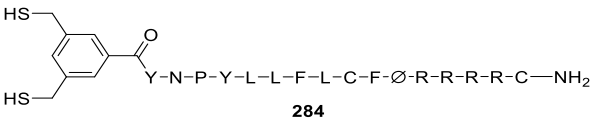
Peptide (283)



283

The peptide was synthesized on an automatic peptide synthesizer PS2, cleaved from the resin using general procedure Z and purified by preparative HPLC to obtain the peptide **283** (0.020 g, 3%). HRMS (MALDI) *m/z* calcd for $C_{116}H_{172}O_{23}N_{33}S_2$ $[M+H]^+$ 2459.2740, found 2459.2715.

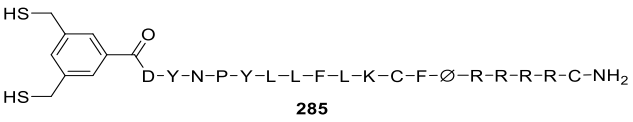
Peptide (284)



284

Resin with peptide (0.50 µm) was swelled with DMF in a syringe. The resin was treated with a solution of HATU (0.07 g, 0.18 mmol), 3,5-bis((tritylthio)methyl)benzoic acid **281** (0.16 g, 0.22 mmol) and DIPEA (0.17 mL, 1.00 mmol) in DMF (5 mL) for 2h. The resin was cleaved according to the procedure X to obtain the peptide **284** (0.0034 g, 3%). HRMS (MALDI) *m/z* calcd $C_{115}H_{163}N_{30}O_{20}S_4$ $[M+H]^+$ 2412.1543, found 2412.0362.

Peptide (285)



285

The peptide was synthesized on an automatic peptide synthesizer PS2. The N-terminus was fmoc-deprotected and the resin was treated with a solution of HATU (0.07 g, 0.18 mmol), 3,5-bis((tritylthio)methyl)benzoic acid **281** (0.16 g, 0.22 mmol) and DIPEA (0.17 mL, 1.00 mmol) in DMF (5 mL) for 2h. The resin was cleaved according to the procedure X to obtain the peptide

285 (0.0047 g, 3%). HRMS (MALDI) *m/z* calcd $C_{125}H_{179}N_{33}O_{24}S_4$ $[M+H]^+$ 2655.2762, found 2655.3199.

Peptide (286)

H_2N—R-R-R-R-Ø-F-C-Y-N-P-Y-L-L-F-L-C-NH_2

286

The peptide was synthesized on an automatic peptide synthesizer PS2, cleaved from the resin using general procedure Z and purified by preparative HPLC to obtain the peptide **286** (0.024 g, 4%). HRMS (MALDI) *m/z* calcd for $C_{106}H_{155}O_{19}N_{30}S_2$ $[M+H]^+$ 2216.1521, found 2216.1585.

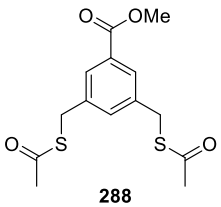
Peptide (287)

H_2N-R-R-R-R-Ø-F-C-D-Y-N-P-Y-L-L-F-L-K-C-NH_2

287

The peptide was synthesized on an automatic peptide synthesizer PS2, cleaved from the resin using general procedure Z and purified by preparative HPLC to obtain the peptide **287** (0.036 g, 6%). HRMS (MALDI) *m/z* calcd for $C_{116}H_{172}O_{23}N_{33}S_2$ $[M+H]^+$ 2459.2740, found 2459.2714.

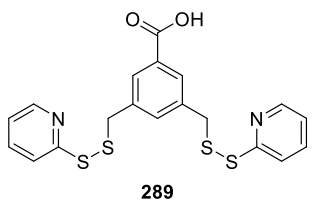
Methyl 3,5-bis((acetylthio)methyl)benzoate (288)



288

Potassium thioacetate (0.47 g, 4.10 mmol) was added to a solution of **279** (0.32 g, 1.71 mmol) in acetone (10 mL). The reaction mixture was refluxed for 3h. The reaction mixture was diluted with water (40 mL) and extracted with EtOAc (3 × 15 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was purified by column chromatography (cyclohexane/EtOAc 10:1 to 5:1) to obtain the desired thioacetate **288** (0.50 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 1.7 Hz, 2H), 7.41 (t, *J* = 1.7 Hz, 1H), 4.11 (s, 4H), 3.90 (s, 3H), 2.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.9, 166.6, 138.7, 133.9, 131.1, 129.0, 52.4, 33.0, 30.5.

3,5-Bis((pyridin-2-yldisulfanyl)methyl)benzoic acid (289)



289

Methyl 3,5-bis((acetylthio)methyl)benzoate (0.50 g, 1.60 mmol) was dissolved in MeOH (15 mL, degassed by a flow of argon) and 7M solution of NaOH was added (3 mL, degassed by a flow of argon). The reaction mixture was stirred overnight under inert atmosphere of argon. Consumption of the starting material was controlled by TLC

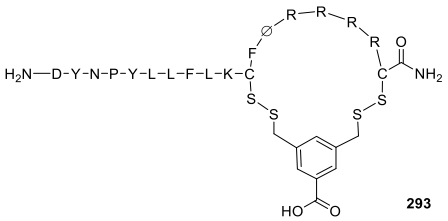
(cyclohexane/EtOAc 4:1). The reaction mixture was acidified by acetic acid (2.4 mL) and 2,2'-dipyridyl disulfide was added. The reaction mixture was stirred for 3h. The reaction mixture was evaporated to dryness under reduced pressure and purified by a column chromatography (cyclohexane/EtOAc/acetic acid 50/50/1) to obtain the desired acid **289** (0.10 g, 15%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 – 8.40 (m, 2H), 7.85 (d, *J* = 1.6 Hz, 2H), 7.58 – 7.50 (m, 2H), 7.50 – 7.40 (m, 3H), 7.05 (ddd, *J* = 7.2, 4.9, 1.1 Hz, 2H), 3.97 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 159.4, 149.2, 137.6, 137.5, 135.2, 130.4, 130.4, 121.1, 120.3, 43.0. Spectra are in agreement with the literature.[115]

General procedure for bicyclization

1. The corresponding protected peptide was prepared on a PS2. The Acm groups were deprotected with saturated solution of mercury acetate in DMF overnight under argon. The resin was washed with DMF (10×) to get rid of mercury acetate and subsequently washed with 2-mercaptoethanol in DMF (20%) for 2h (twice). The resin was washed with DMF (12×) to get rid of the reducing agent. The resin was treated with 1 eq. 3,5-bis((pyridin-2-yldisulfanyl)methyl)benzoic acid in DMF with 1% CH_3COOH overnight. The Fmoc group was deprotected with 20% solution of piperidine in DMF (2 × 10 min.). The resin was washed with DMF (12×) to get rid of piperidine.

2. The resin was treated with solution of HOBt in DMF (1M) twice for 1h to get rid of any remaining piperidine. Amide coupling was done in a presence of PyBOP (10 eq.) and DIPEA (20 eq.) in DMF for 2h. The resin was washed with DMF and MeOH and dried on vacuum. The peptide was cleaved from the resin with the mixture TFA/TIS/H_2O 95/2.5/2.5 (10 ml/0.1 mmol) for 2.5 h. The mixture was filtered and the solution was concentrated using a flow of nitrogen. The peptide was precipitated with *tert*-butyl methyl ether and the precipitate was collected. The solid residue was purified by preparative HPLC.

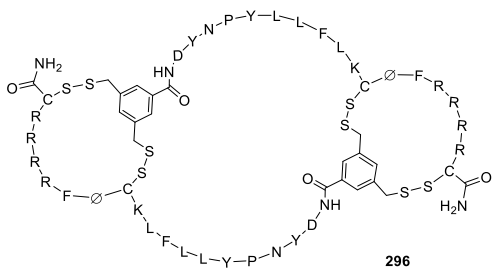
Peptide (293)



293

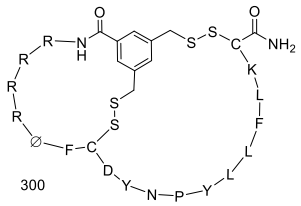
The peptide **293** was prepared using the conditions described in the general procedure for bicyclization 1 (Rink Amide; 0.2 mmol). The peptide was cleaved from the resin using the modified (cleavage for 2.5 h) general procedure X. The peptide was purified by preparative HPLC (0.050 g, 10%).

Peptide (296)



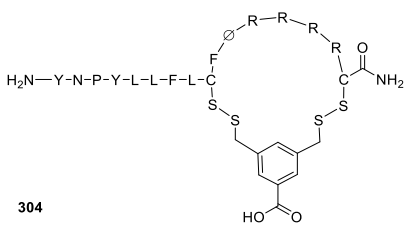
The peptide **296** was prepared using the conditions described in the general procedure for bicyclization (TentaGel R RAM resin; 0.15 mmol). The peptide was cleaved from the resin using the modified (cleavage for 2.5 h) general procedure X. The peptide was purified by preparative HPLC (0.0008 g, 0.2%).

Peptide (300)



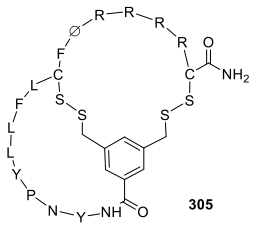
The peptide **300** was prepared using the conditions described in the general procedure for bicyclization (TentaGel R RAM; 0.14 mmol, ChemMatrix resin; 0.16 mmol). The peptide was cleaved from the resin using the modified (cleavage for 2.5 h) general procedure X. The peptide was purified by preparative HPLC. TentaGel R RAM (0.003 g, 0.7%) ChemMatrix (0.004 g, 0.9%)

Peptide (304)



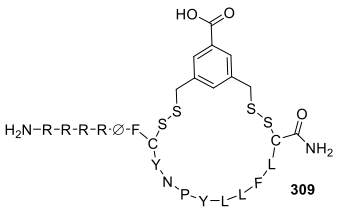
The peptide **304** was prepared using the conditions described in the general procedure for bicyclization 1 (Rink Amide, 0.25 mmol). The peptide was cleaved from the resin using the modified (cleavage for 2.5 h) general procedure X. The peptide was purified by preparative HPLC (0.054 g, 9%).

Peptide (305)



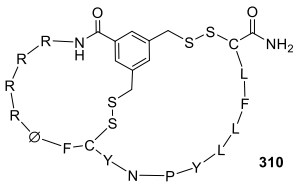
Peptide **304** (0.025 g, 0.010 mmol) in DMF (3 mL) was added via syringe pump (3 mL/8h) to the solution of PyBOP (0.050 g, 0.096 mmol) and DIPEA (50 µL, 0.28 mmol) in DMF (30 mL) under argon. The reaction was stirred for further 4h. The reaction mixture was lyophilized and purified by preparative HPLC to furnish the peptide **305** (0.004 g, 16%).

Peptide (309)



The peptide **309** was prepared using the conditions described in the general procedure for bicyclization 1 (Rink Amide, 0.25 mmol). The peptide was cleaved from the resin using the modified (cleavage for 2.5 h) general procedure X. The peptide was purified by preparative HPLC (0.060 g, 10%).

Peptide (310)



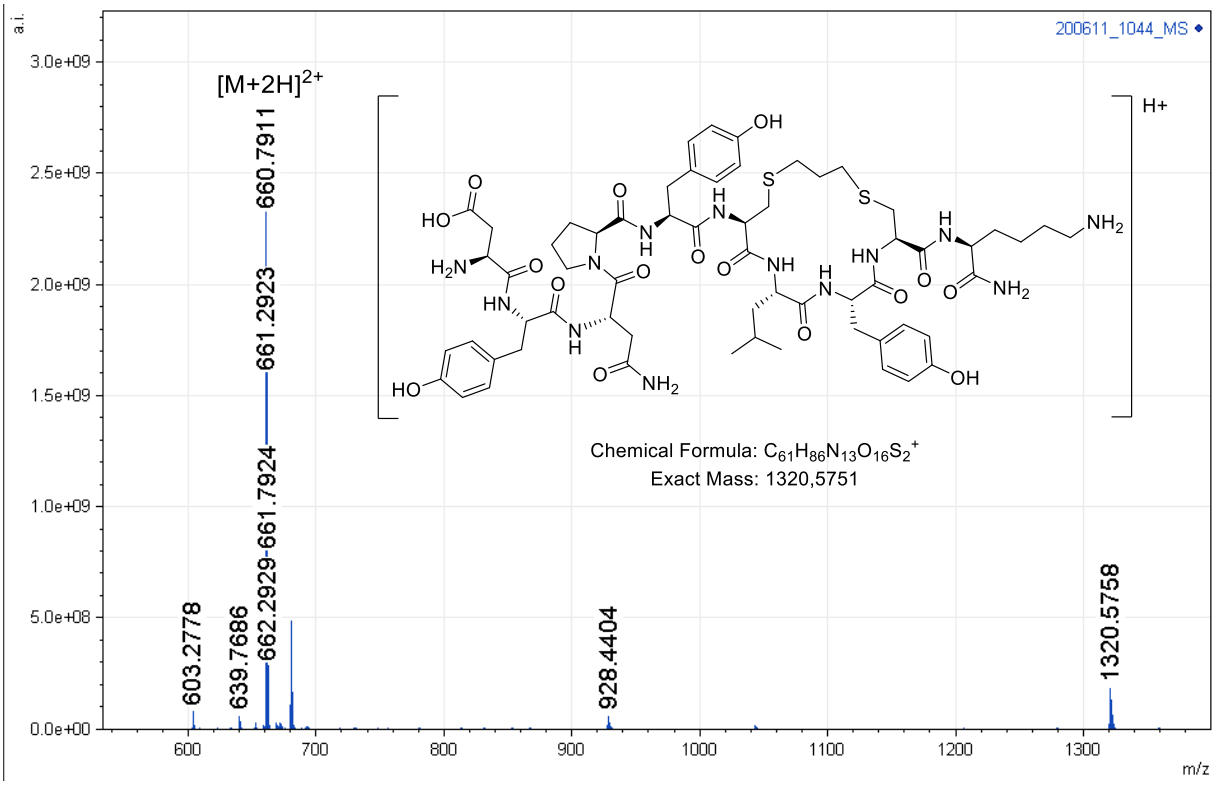
The peptide **310** was prepared using the conditions described in the general procedure for bicyclization (TentaGel R RAM; 0.14 mmol). The peptide was cleaved from the resin using the modified (cleavage for 2.5 h) general procedure X. The peptide was purified by preparative HPLC (0.003 g, 0.9 %).

Peptide **309** (0.026 g, 0.011 mmol) in DMF (3 mL) was added via syringe pump (3 mL/8h) to the solution of PyBOP (0.050 g, 0.09 mmol) and DIPEA (50 µL, 0.28 mmol) in DMF (30 mL) under nitrogen. The reaction was stirred for further 4h. The reaction mixture was lyophilized and purified by preparative HPLC to furnish the peptide **310** (0.003 g, 11%).

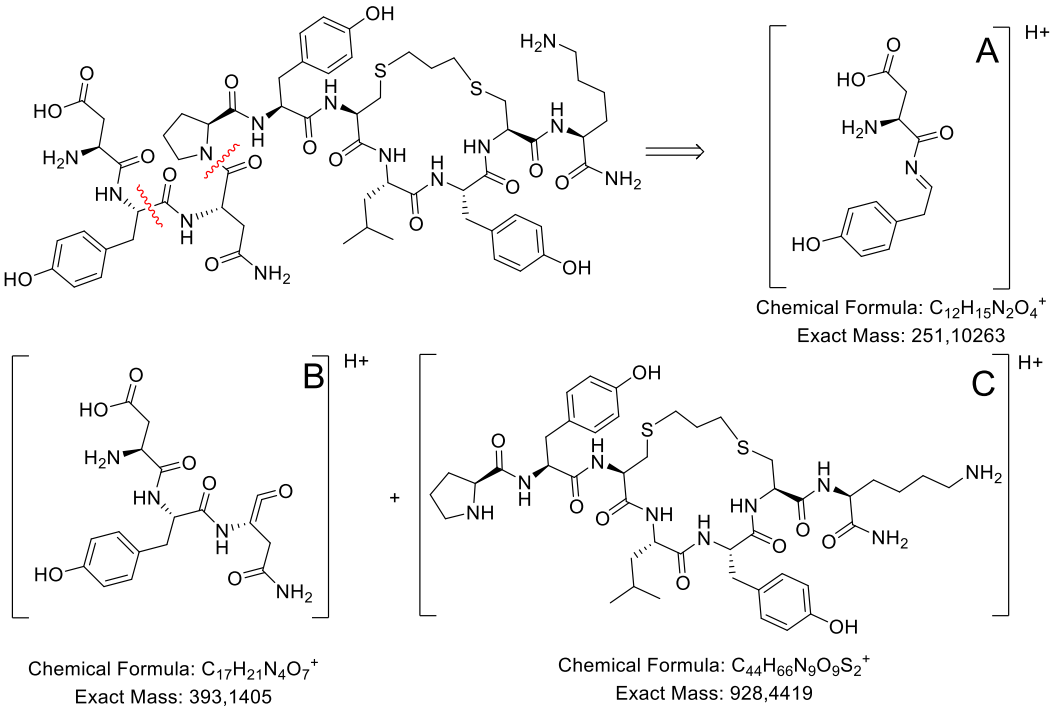
6. Attachment

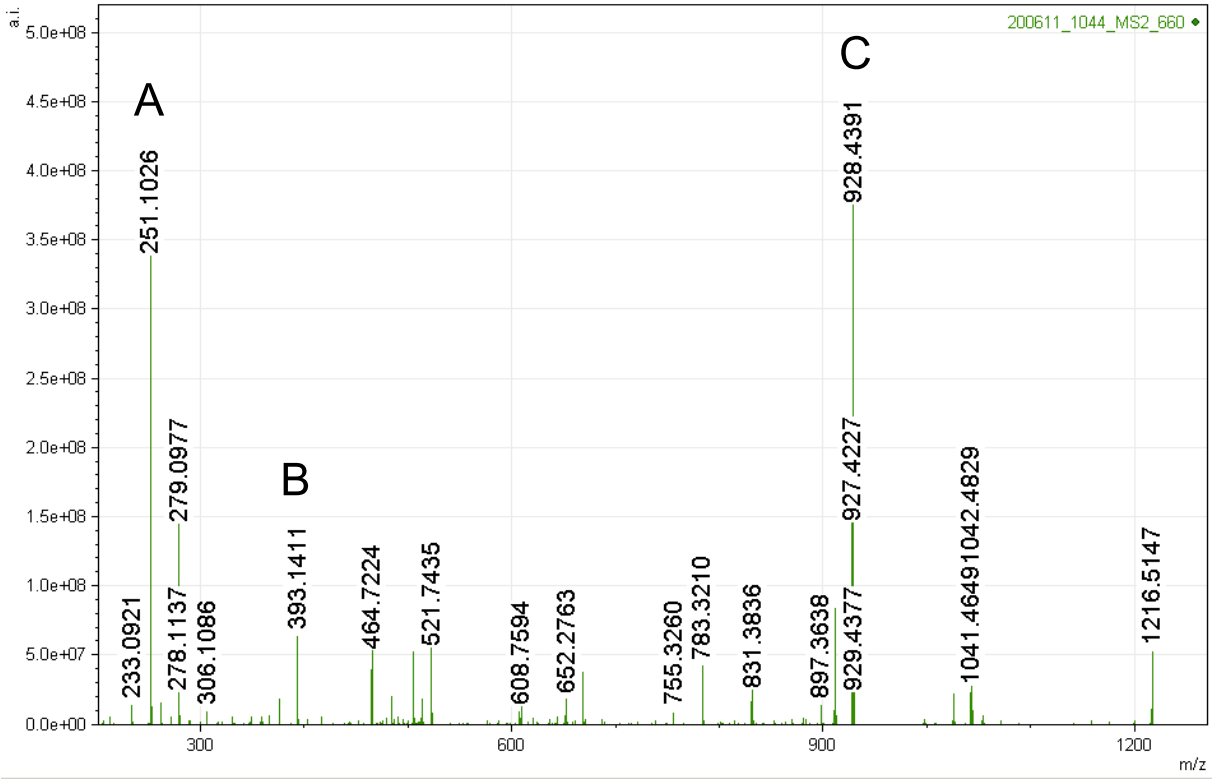
CID assignment

Full HRMS spectrum MS1

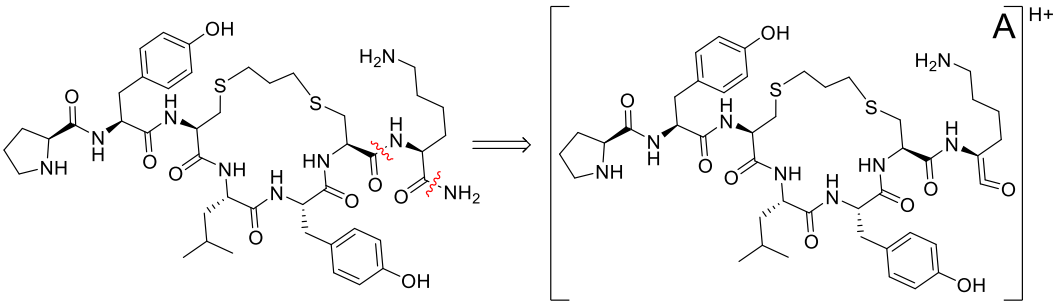


Collision induced dissociation (CID) MS1 → MS2 [660]

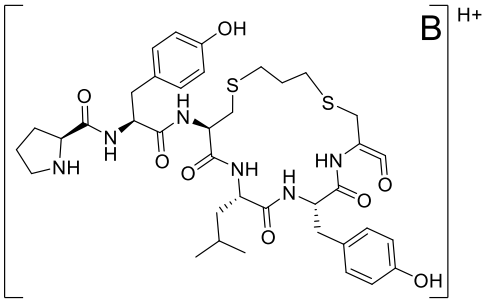




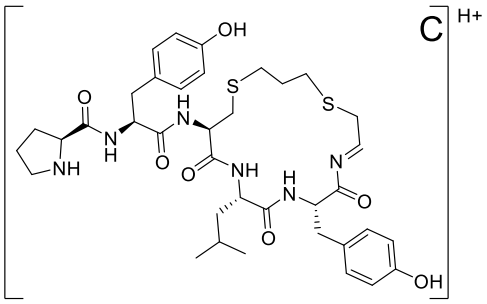
CID: MS1 → MS2 [660] → MS3 [928]



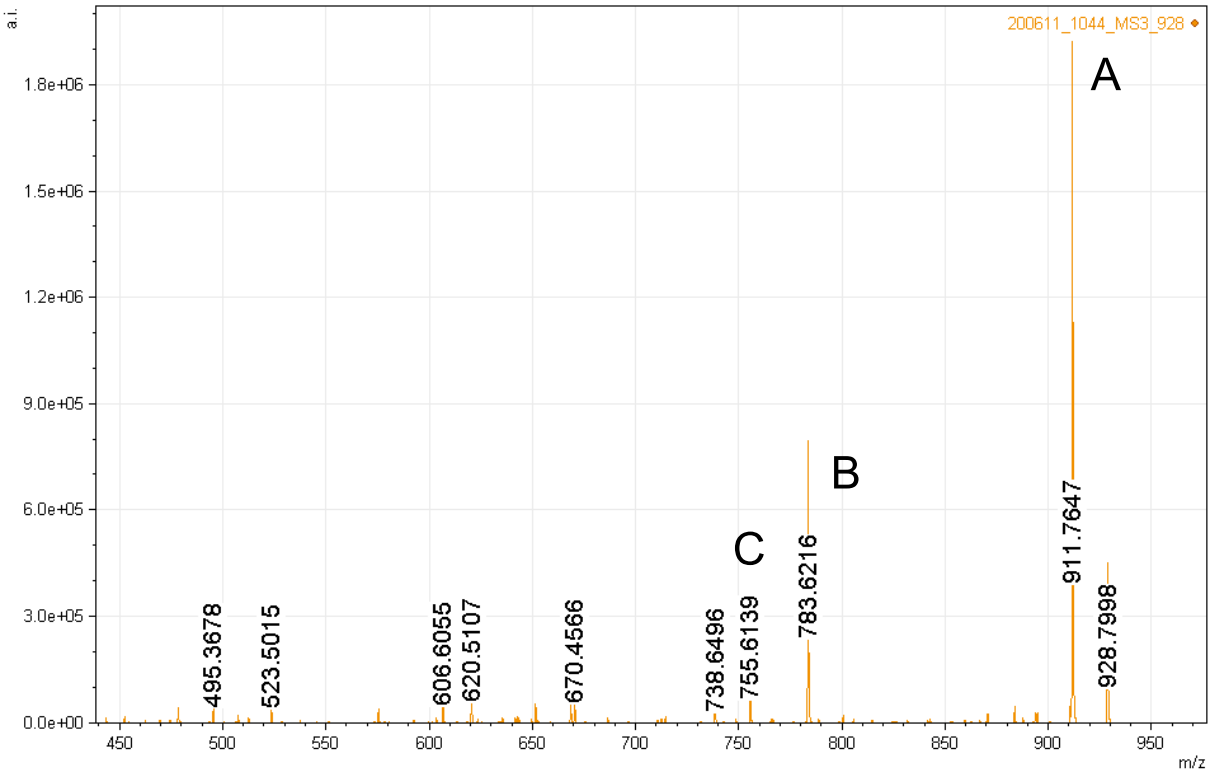
Chemical Formula: $C_{44}H_{63}N_8O_9S_2^+$
Exact Mass: 911,4154



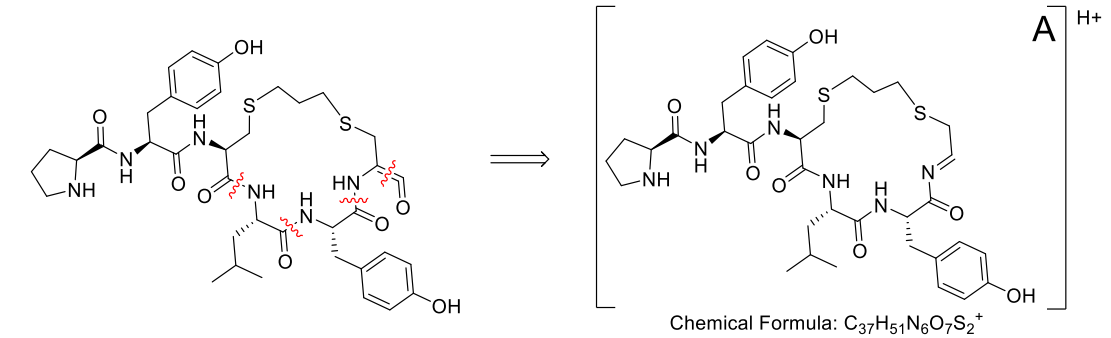
Chemical Formula: $C_{38}H_{51}N_6O_8S_2^+$
Exact Mass: 783,3204



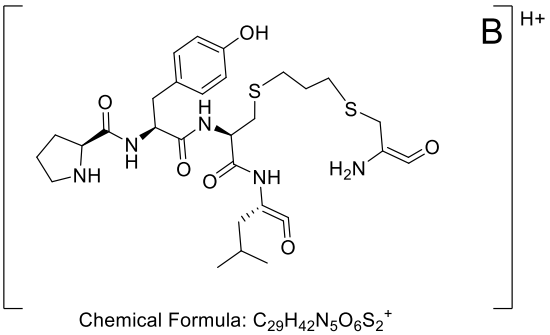
Chemical Formula: $C_{37}H_{51}N_6O_7S_2^+$
Exact Mass: 755,3255



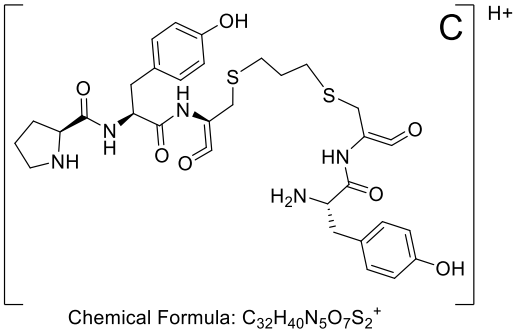
CID: MS1 → MS2 [660] → MS3 [928] → MS4 [783]



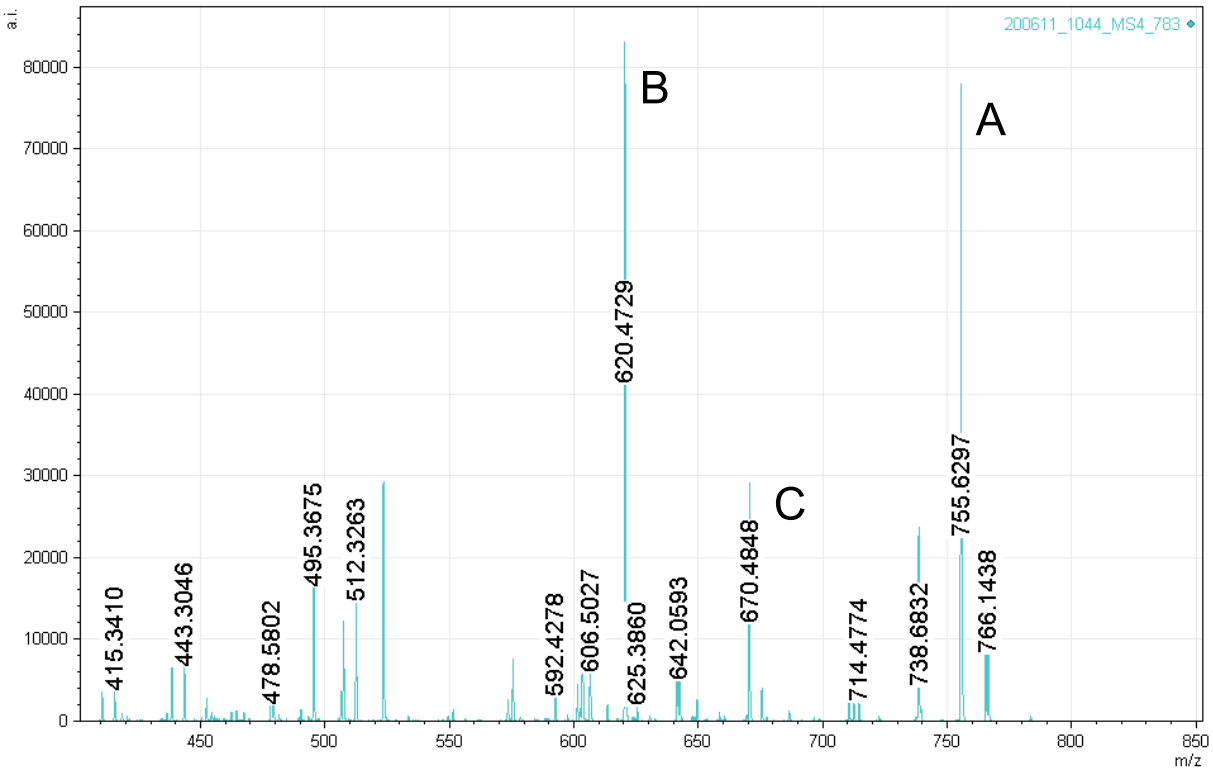
Chemical Formula: $C_{37}H_{51}N_6O_7S_2^+$
Exact Mass: 755,3255



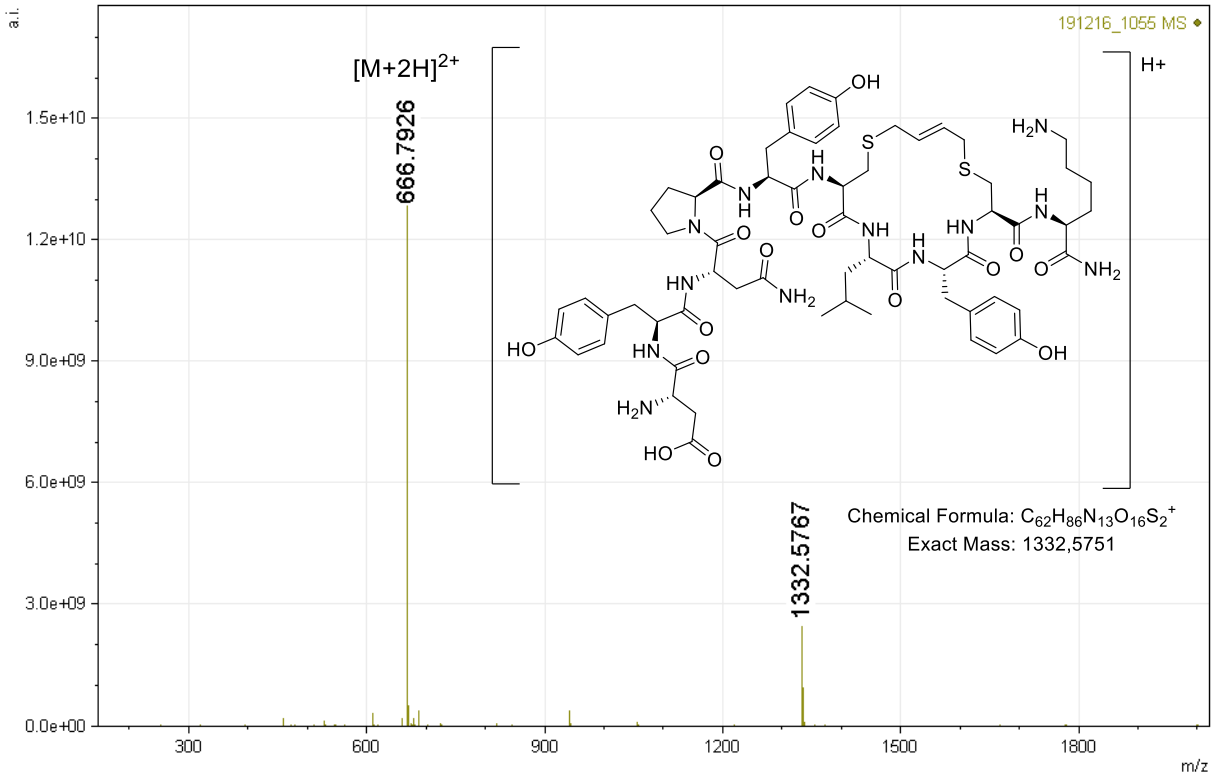
Chemical Formula: $C_{29}H_{42}N_5O_6S_2^+$
Exact Mass: 620,2571



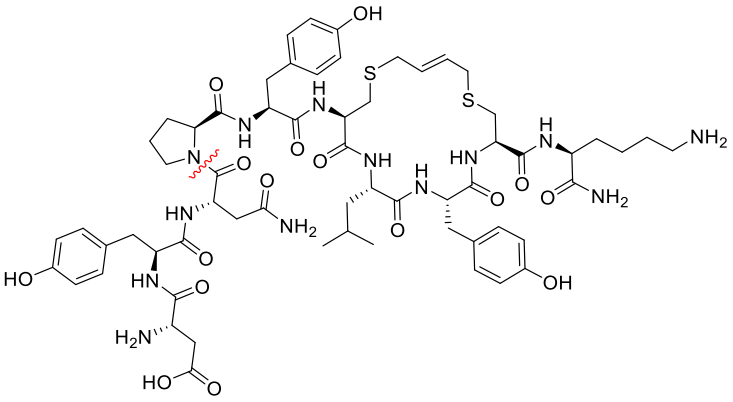
Chemical Formula: $C_{32}H_{40}N_5O_7S_2^+$
Exact Mass: 670,2364

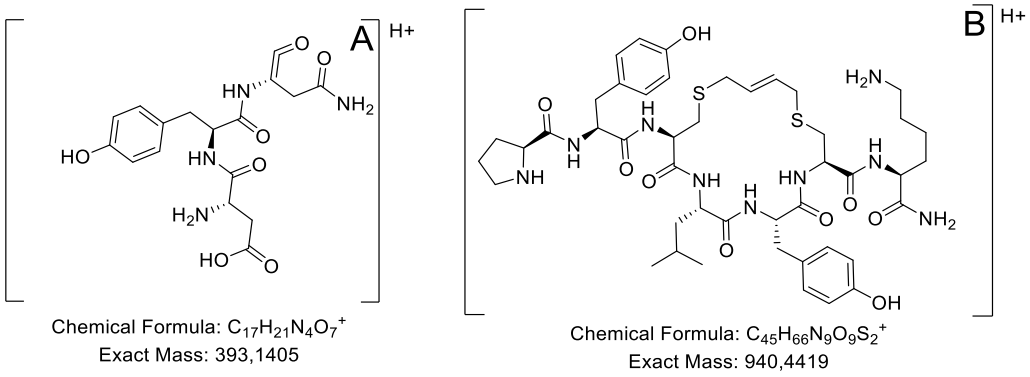


Full HRMS spectrum MS1



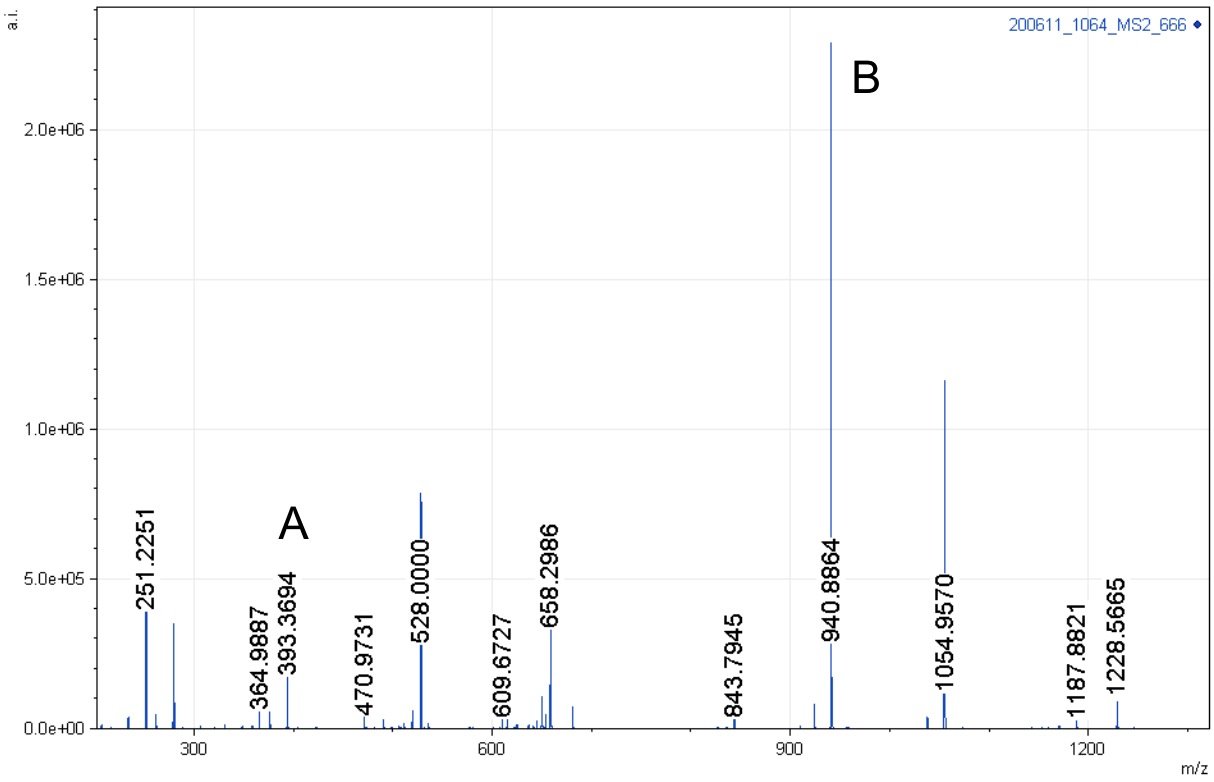
Collision induced dissociation (CID) MS1 → MS2 [666]



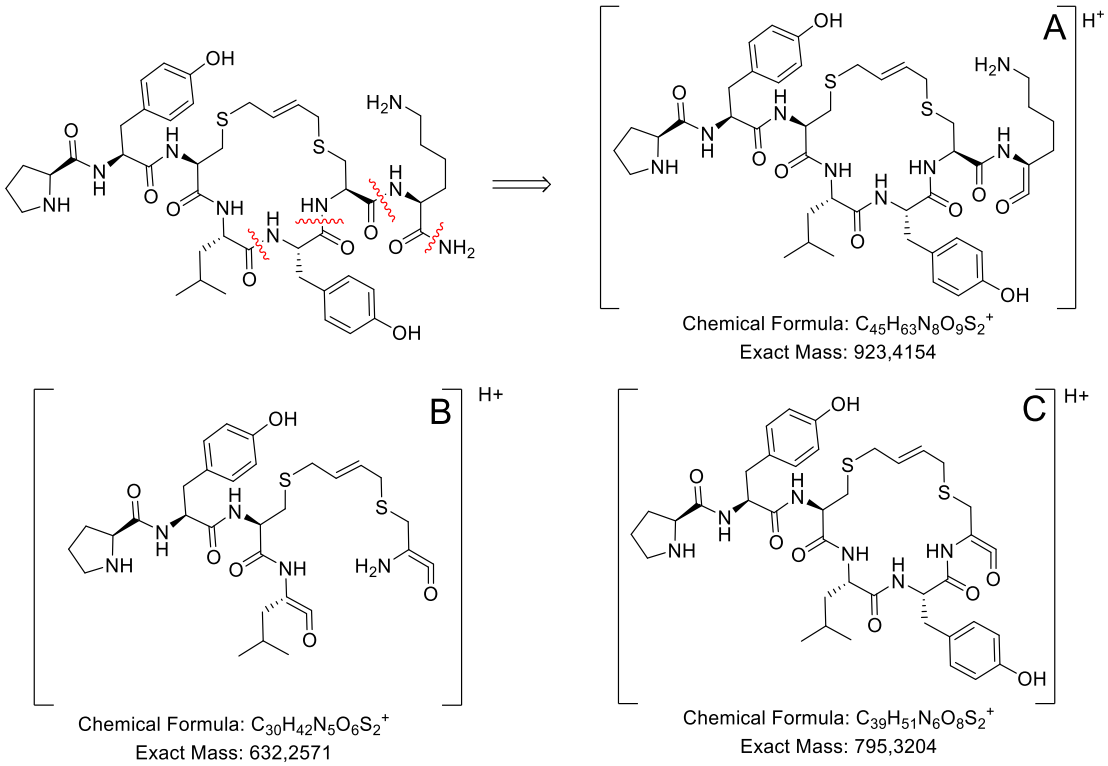


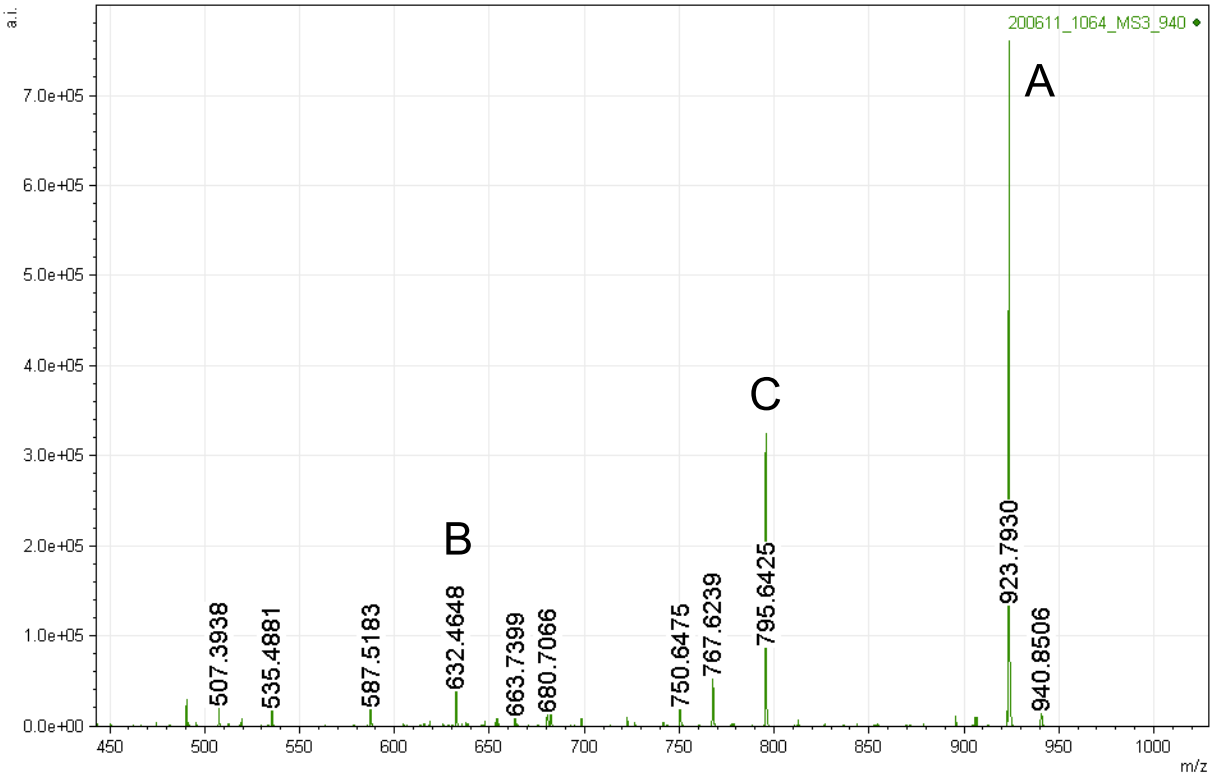
Chemical Formula: $C_{17}H_{21}N_4O_7^+$
Exact Mass: 393,1405

Chemical Formula: $C_{45}H_{66}N_9O_9S_2^+$
Exact Mass: 940,4419

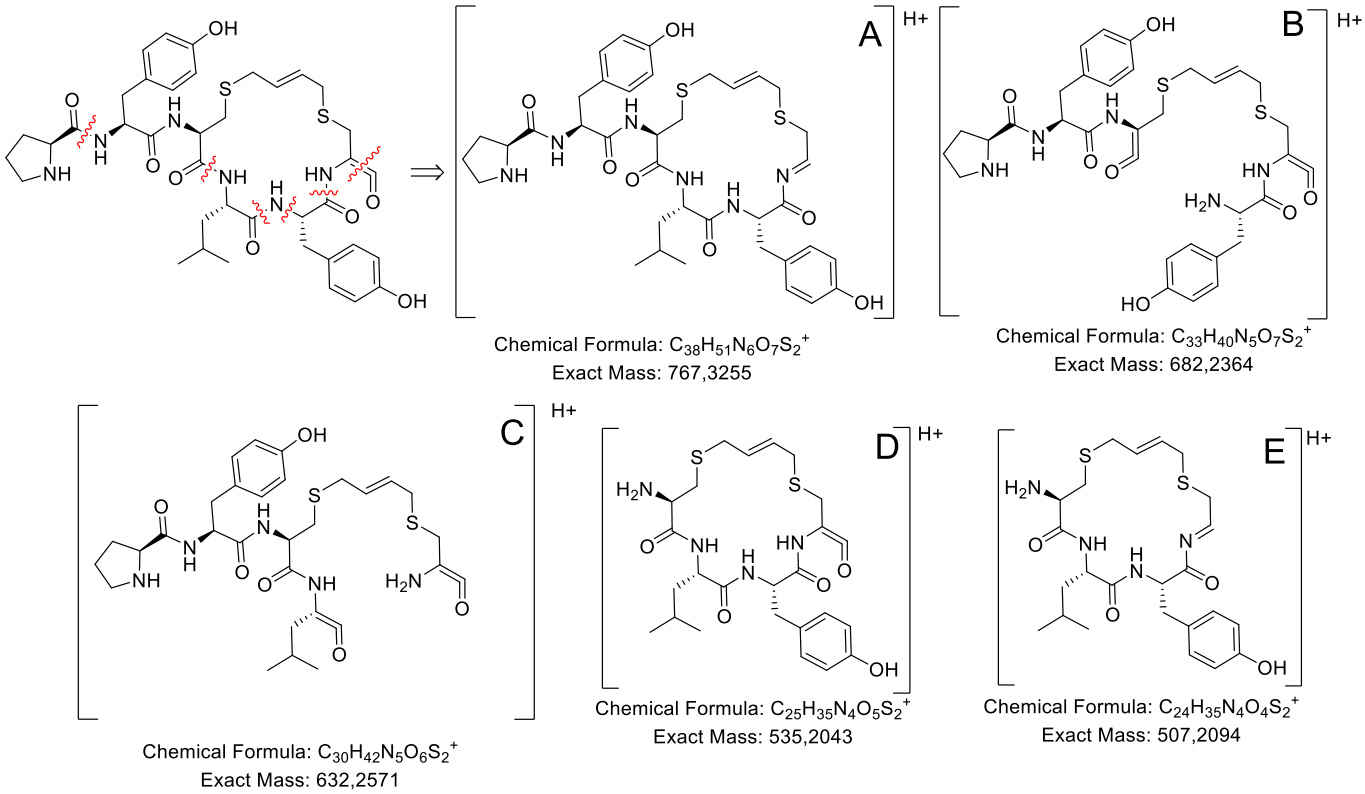


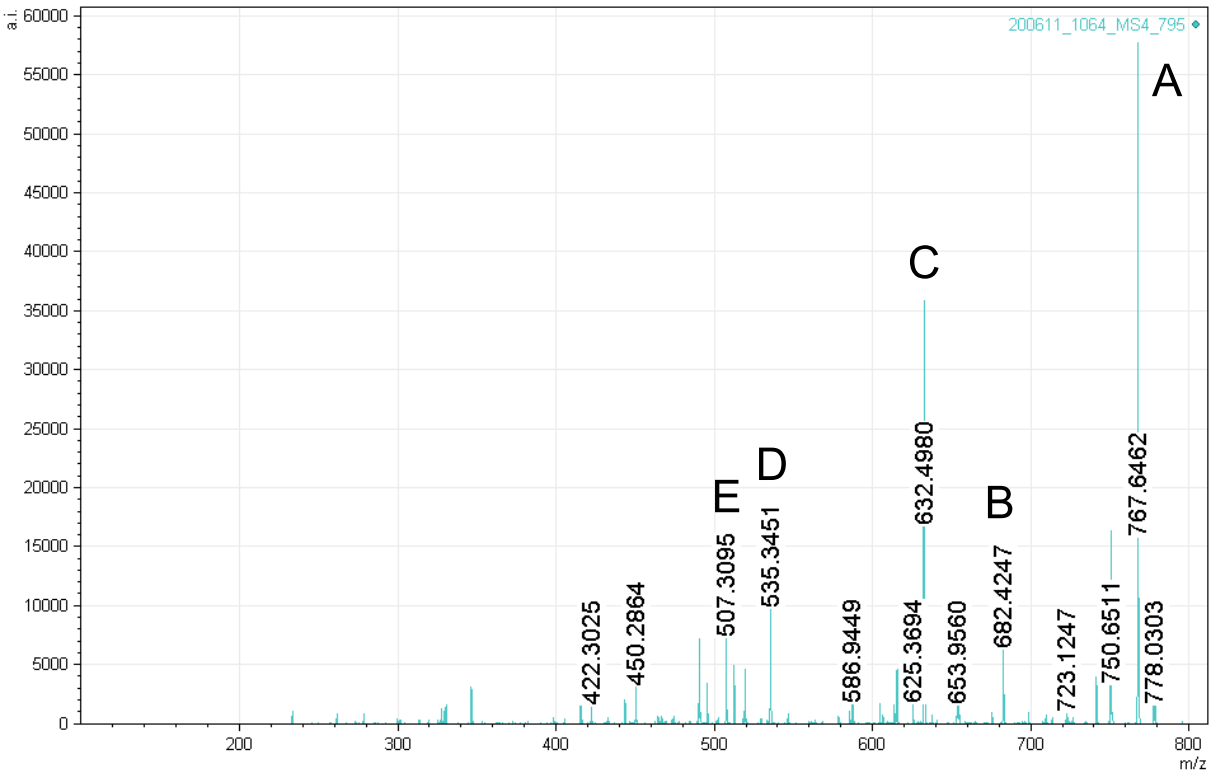
CID: MS1 → MS2 [666] → MS3 [940]



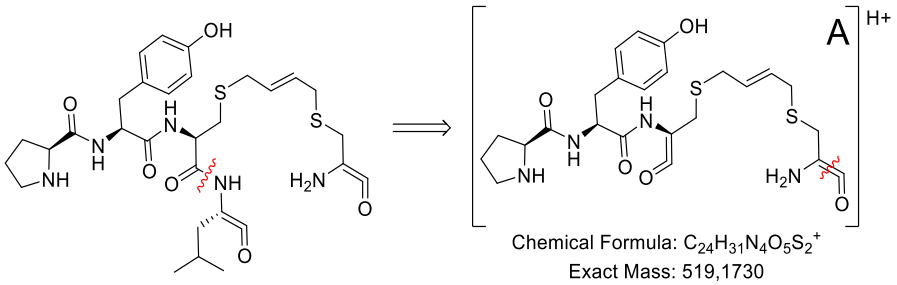


CID: MS1 → MS2 [666] → MS3 [940] → MS4 [795]

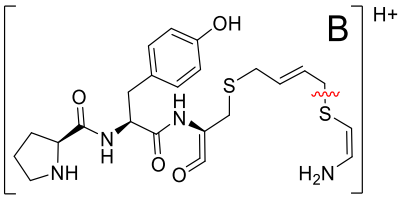




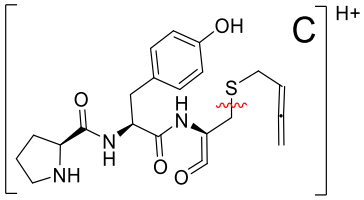
CID: MS1 → MS2 [666] → MS3 [940] → MS4 [632]



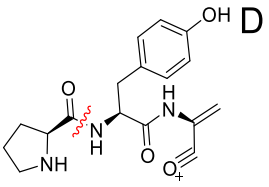
Chemical Formula: $C_{24}H_{31}N_4O_5S_2^+$
Exact Mass: 519,1730



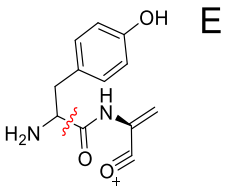
Chemical Formula: $C_{23}H_{31}N_4O_4S_2^+$
Exact Mass: 491,1781



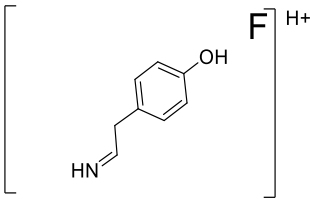
Chemical Formula: $C_{21}H_{26}N_3O_4S^+$
Exact Mass: 416,1639



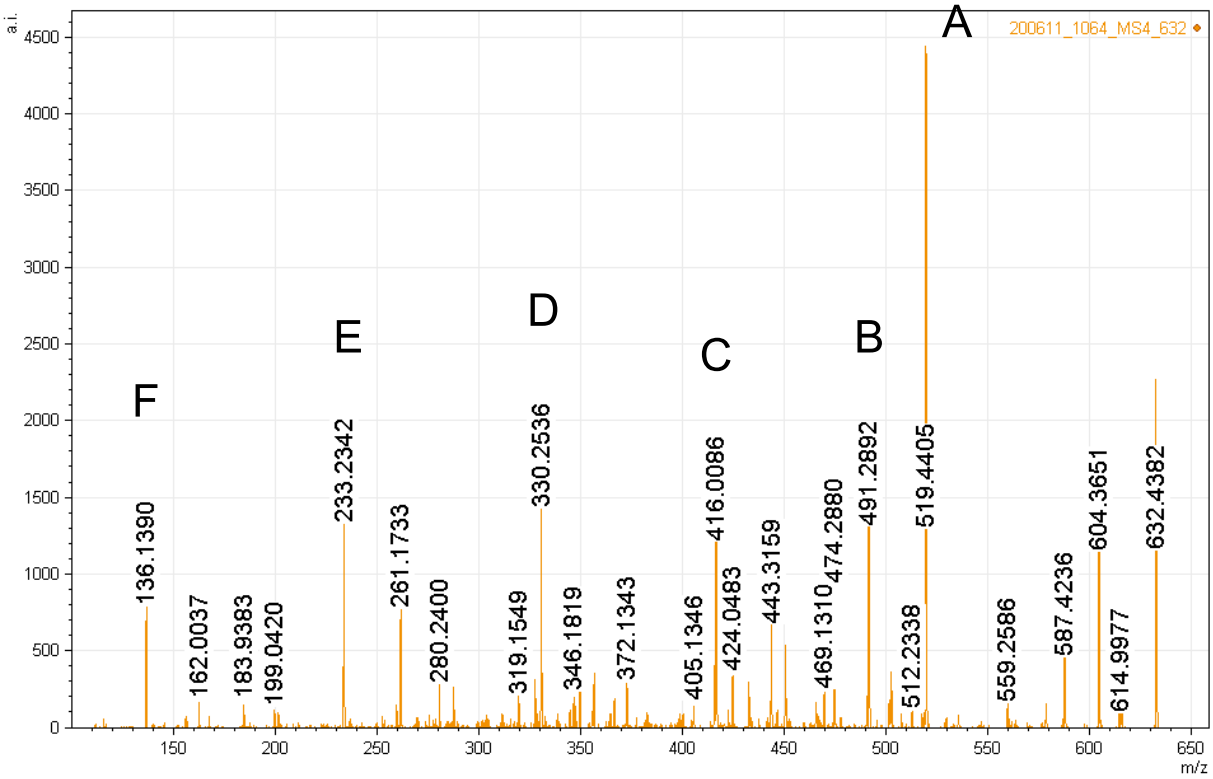
Chemical Formula: $C_{17}H_{20}N_3O_4^+$
Exact Mass: 330,1448



Chemical Formula: $C_{12}H_{13}N_2O_3^+$
Exact Mass: 233,0921



Chemical Formula: $C_8H_{10}NO^+$
Exact Mass: 136,0757



7. Literature

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