Charles University

Faculty of Science

Study program: Organic Chemistry

Branch of study: 4XORG

Carlos Berenguer Albiñana

Synthesis of Neuraminidase binders suitable for theranostics

Příprava inhibitorů Neuraminidasy vhodných pro teranostiku

Doctoral thesis

Supervisor: Dr. Aleš Machara

Prague, 2018

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 20.06.2018

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Acknowledgment

First, I would like to express my sincere gratitude to my mentor Dr. Aleš Machara for providing me the opportunity to carry out my PhD under his supervision. His guidance, patience, and determination has allowed me to develop into the chemist I am today.

In addition to my advisor, I would like to thank Dr. Milan Kožíšek for his immense help and guidance with the biochemical aspects of my work. Special thanks go to Dr. Jan Konvalinka, Dr. Pavel Šácha and Dr. Pavel Majer, it was extraordinary to have the opportunity to work for you.

I would like to thank my fellow lab mates and friends Martin and Václav for all the support and fun we had these last years, it has been a pleasure to work together. I want to thank also Adéla, Petr, Jan and all my colleagues at IOCB and Charles University for making this experience unforgettable.

I'm also deeply grateful to Prof. Tomáš Hudlický, Dr. John Hayward and the rest of co-workers at Brock University for their kindness and guidance during the months we worked together.

I'm especially thankful to Russell for the moral and emotional support during the toughest times. I am also grateful to all my friends both in Spain and the Czech Republic who have supported me along the way.

Finalment, vull donar les gràcies a les persones que més han influït en la meua carrera; els meus pares i el meu germà, no hauria pogut arribar fins ací sense el vostre suport, us ho dec tot a vosaltres. Moltes gràcies també als meus avis i a tota la meua família per donar-me el coratge necessari per a finalitzar el doctorat, Gràcies!

List of Abbreviations

Ac	Acetyl
AcOH	Acetic acid
Ac_2O	Acetic anhydride
AIBN	Azobisisobutyronitrile
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Bn	Benzyl
Boc	*tert*-Butyloxycarbonyl
Boc_2O	Di-*tert*-butyl dicarbonate
Cat.	Catalytic
CuAAC	Copper(I)-catalyzed alkyne-azide cycloaddition
Cys	Cysteine
DABCO	1,4-Diazabicyclo[2.2.2]octane
DANA	2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIAD	Diisopropyl azodicarboxylate
DIPEA	*N,N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethyl formamide
DMP	2,2-Dimethoxypropane
Dppf	1,1'-Bis(diphenylphosphino)ferrocene
EA	Ethyl acetate
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HA	Hemagglutinin
HBTU	*N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate
His	Histidine
HOTT	*S*-(1-Oxido-2-pyridyl)-*N,N,N′,N′*-tetramethyl-thiouronium hexafluorophosphate

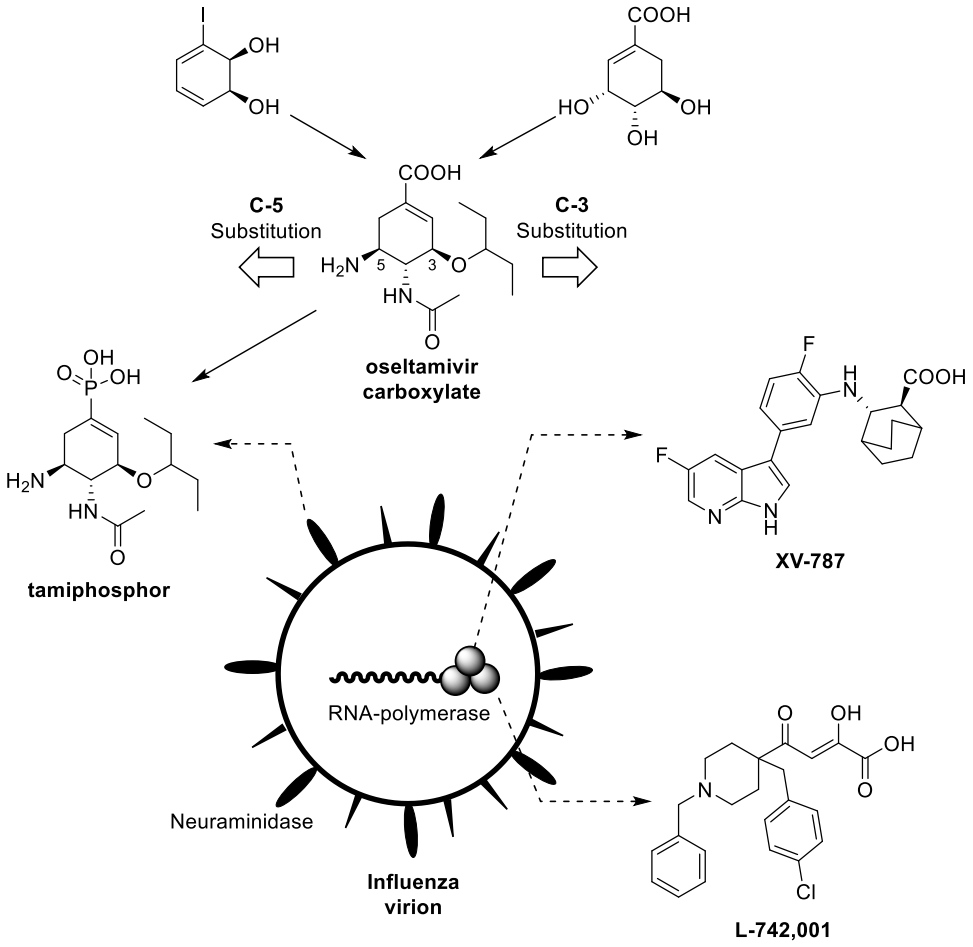
HR-ESI-MS	High resolution electro-spray ionisation mass spectroscopy
HTS	High-throughput screening
IBX	*o*-Iodoxybenzoic acid
Ile	Isoleucine
J	Coupling constant (Hz)
Leu	Leucine
Lys	Lysinie
Me	Methyl
Met	Methionine
MOM	Methoxymethyl
MOMCl	Methoxymethyl chloride
Ms	Mesylate
MUNANA	2'-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid
NA	Neuraminidase
NADP	Nicotinamide adenine dinucleotide phosphate
NaHMDS	sodium bis(trimethylsilyl)amide
NBS	*N*-bromosuccinimide
NMR	Nuclear magnetic resonance
NMP	*N*-Methyl-2-pyrrolidone
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
Phe	Phenylalanine
PPI	Protein-protein interactions
Pro	Proline
RNA	Ribonucleic acid
r.t.	Room temperature
SA	Sialic acid
Ser	Serine
S_N2	Bimolecular nucleophilic substitution
SPR	Surface plasmon resonance
TBAF	*tetra*-*n*-Butyl ammonium fluoride
TBTA	Tris(benzyltriazolylmethyl)amine
TBTU	2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
THF	Tetrahydrofuran

Thr	Threonine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TMSBr	Trimethylsilyl bromide
Tol	Toluene
Tr	Trityl
Trp	Tryptophan
TS	Trans sialidase
Ts	Tosyl
TsCl	*para*-Toluene sulfonylchloride
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine

Abstract

Influenza viruses cause respiratory illnesses which can vary in severity depending on the strain of the virus, as well as the age and health condition of the host. Influenza remains a major threat to public health due to its nature prone to suffer mutations. As a result, vaccines have to be reformulated annually and new strains may cause sporadic global pandemics. Furthermore, the recent emergence of resistant strains of the virus against the current standard of care (oseltamivir and zanamivir) underlines the need of novel anti-influenza therapeutics.

The aim of this dissertation work is to contribute to the discovery of new anti-influenza inhibitors either by rational drug-design and optimization of oseltamivir structure, or by developing screening assays suitable for the discovery of novel inhibitors of the enzymes neuraminidase or RNA-polymerase.



Scheme 1. Overview of the strategy used for the development of new anti-influenza therapeutics. The dashed arrows indicate the inhibitors that were converted into probes and their corresponding target enzymes

Two main modification points were explored for the improvement of oseltamivir properties (Scheme 1); modifications at carbon C-3 aimed to overcome oseltamivir resistance caused by common mutations like H274Y, meanwhile modifications at carbon C-5 have been used to explore the binding mode of the inhibitors to a cavity adjacent to the catalytic site known as the "150-cavity".

A second strategy was used in the pursuit of new anti-influenza inhibitors which involved the development of novel assays suitable for the screening of novel neuraminidase and RNA-polymerase inhibitors. Several detection probes based on the structure of known inhibitors (tamiphosphor, XV-787 and L-742,001) of the respective proteins were designed, prepared, optimized and successfully applied for the development of screening assays based on either DIANA or AlphaScreen technology. Two new inhibitors of the PA subunit of RNA-polymerase with IC_{50} in the low micromolar range were found during the first screening campaign using the developed AlphaScreen assay.

Keywords: *Influenza, oseltamivir, inhibitors, drug design, neuraminidase, RNA-polymerase, detection probes, DIANA assay, AlphaScreen, screening assays*

Abstrakt (Czech)

Četná onemocnění dýchacích cest jsou způsobena virem chřipky. Jejich závažnost se liší v závislosti na kmenu viru a na věku a zdraví hostitele. Chřipka je stále velkou zdravotní hrozbou zejména kvůli své přirozené náchylnosti k mutacím. Proto je nezbytné uvádět na trh každý rok nové vakcíny účinné proti mutovaným formám viru a snižovat tak riziko pandemií, které by tyto formy viru mohly vyvolat. Nedávný výskyt kmenů viru rezistentních vůči současné standardní léčbě chřipky (oseltamivir, zanamivir) dokazuje nutnost vývoje nových léčiv.

Cílem této disertační práce je přispět k vývoji nových látek účinných proti viru chřipky. Toho může být dosaženo racionálním designem a optimalizací stávajících inhibitorů nebo prostřednictvím vysokokapacitního testování, které může nalézt strukturně nové inhibitory neuraminidasy a RNA-polymerasy.

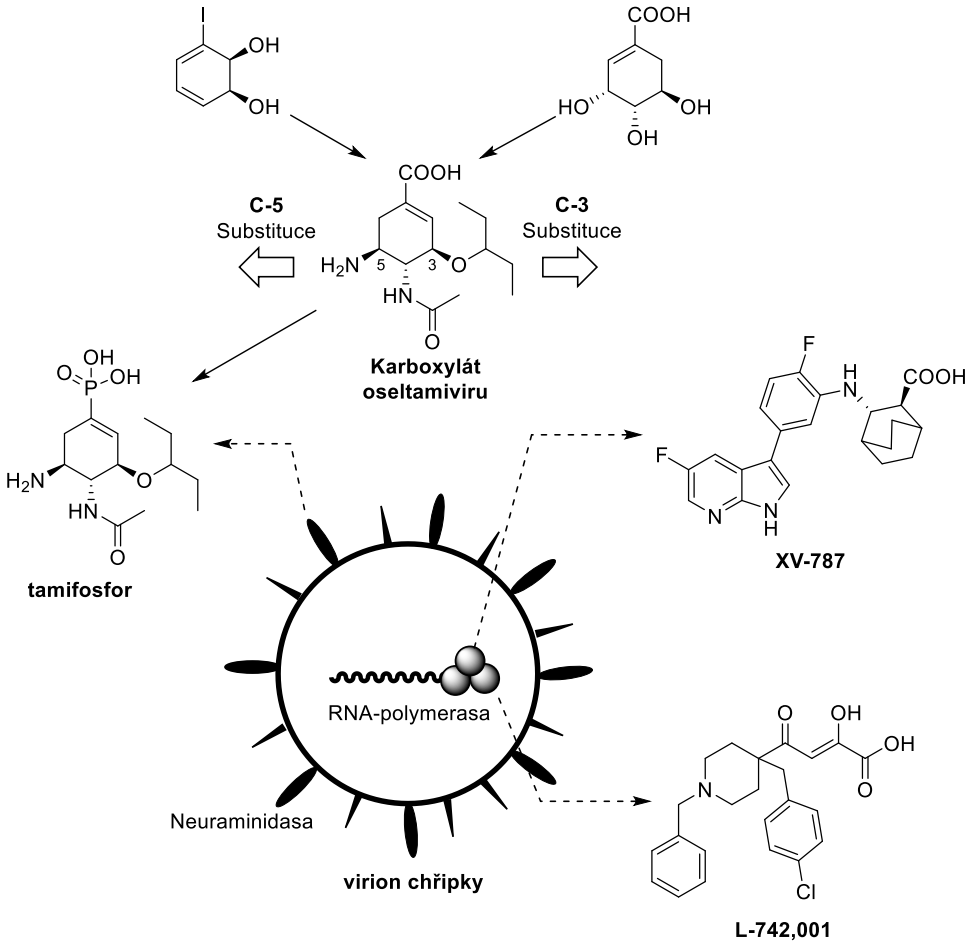


Schéma 1. Přehled strategie použité pro vývoj nových léčiv účinných proti chřipce. Přerušované šipky spojují inhibitory použité pro přípravu sond pro vysokokapacitní testování s enzymy, na které je sondami cíleno.

V rámci optimalizace struktury oseltamiviru byly studovány modifikace v pozicích C-3 a C-5. Zatímco změny v pozici C-3 měly za cíl zvýšení aktivity způsobené běžnou mutací H274Y, substituce v pozici C-5 byly použity pro studium vazby inhibitoru do dutiny úzce sousedící s aktivním místem enzymu.

Další strategií byl vývoj metody vysokokapacitního testování pro nalezení nových inhibitorů neuraminidasy a RNA-polymerasy. Návrh detekčních sond pro tuto strategii byl odvozen od struktur známých inhibitorů těchto enzymů (tamifosfor, XV-787 a L-742,001). Navržené sondy byly syntetizovány, optimalizovány a úspěšně použity pro vývoj testovacích metod technologiemi DIANA a AlphaScreen. Druhou zmíněnou metodou byly nalezeny dva nové mikromolární inhibitory PA-podjednotky virové RNA-polymerasy.

1. Introduction

1.1 Influenza virus

Influenza is an infection of the upper respiratory tract characterized by sudden onset of fever, dry couch, headache, muscle and joint pain, fatigue, sore throat and runny nose. Although most people recover from the fever and the other symptoms within a week, in high-risk patients it may lead to pneumonia, which can be fatal. According to a recent study (2017) from the World Health Organization (WHO)[1], 290.000 to 650.000 deaths annually are associated with respiratory diseases from seasonal influenza. In the EU/EEA, the number of fatal infections ranges from 15.000 to 70.000 according to the European Centre for Disease Prevention and Control (ECDC).[2] In addition, influenza has been the cause of 22 pandemics since the 1500s. The most recent ones were the Spanish flu (1918), which killed 20 – 50 million worldwide, the Asian flu (1957, 1 – 2 million casualties) and the Hong Kong flu (1968, 700.000 casualties).

Influenza viruses belong to the *Orthomyxoviridae* family which are negative-sense single-stranded RNA viruses. The viruses are classified into three types (A, B and C) depending on its nucleoproteins (NP) and the antigen determinants on its matrix protein (M). Further differentiation is found in the genomic RNA segments: influenza A viruses present eight segments that differ in size from the eight RNA segments found in B viruses. Influenza C viruses on the other hand, present only seven segments.[3] Viruses from type A and B are responsible for seasonal influenza, type C viruses also cause flu although the symptoms are much less severe. Influenza A viruses have attracted much more attention since they are accountable for most of the occasional pandemic outbreaks.

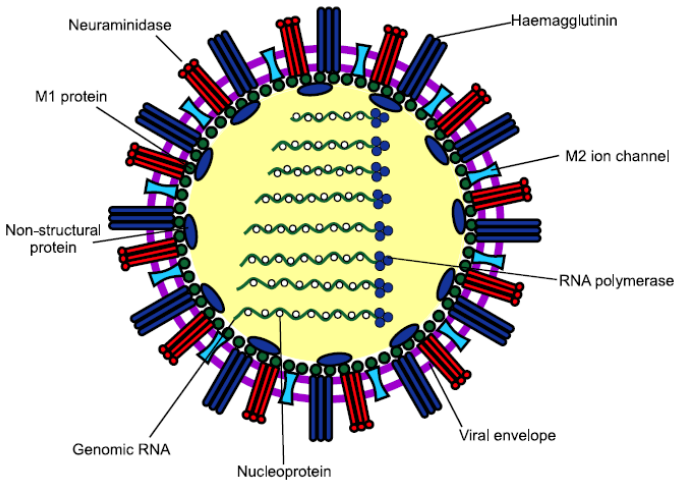


Figure 1. Influenza A virus particle according to Ludwig, S. et al 2003.[4]

Influenza A viruses (Figure 1) infect a wide range of avian and mammalian hosts, unlike influenza B viruses, which infect only humans and seals. The influenza A genome is composed of eight RNA segments, each one attached to an RNA polymerase

(formed by PA, PB1 and PB2 subunits) and coiled with nucleoproteins (NP). The viral envelope presents two glycoproteins (proteins linked to carbohydrates) on the surface: haemagglutinin (HA) and neuraminidase (NA). Also embedded in the lipid membrane is the M2 proton channel. Other important proteins are: matrix protein 1 (M1), non-structural protein 1 (NS1) and nuclear export protein (NEP). Additional proteins have been identified in different strains in recent years.[5, 6]

Influenza A viruses are classified by the antigenic HA and NA subtypes: eighteen HA (H1-H18) and eleven NA (N1-N11) have been identified so far. All subtypes of influenza viruses have been detected in avian species which are considered to be a reservoir of influenza A viruses. Additionally, new findings have proved that bats constitute an important reservoir of the virus as well.[7] On the other hand, influenza B makes up a more homogeneous group with only two different subgroups, the Victoria and Yamagata lineages.[8]

Antigenic shift and antigenic drift are responsible for the emergence of new strains[9] and new subtypes of the virus. These two phenomena are the key factors for understanding why influenza mutates in some extent every season. Moreover, this also explains why highly pathogenic strains could appear suddenly.

- **Antigenic Drift** (Figure 2, **A**)**:** occurs due to the accumulation of small mutations because RNA polymerase has no proof-reading mechanism. It's responsible for flu seasons and the reason why the flu vaccine must be reformulated every year. Influenza A and B viruses experience antigenic drift.
- **Antigenic Shift** (Figure 2, **B**)**:** occurs when a cell is infected with two or more different strains/subtypes of the virus, the viruses combine to form a new subtype with different surface antigens. Responsible for occasional pandemics and cross-species transmission of the virus, only influenza A viruses undergo an antigenic shift.

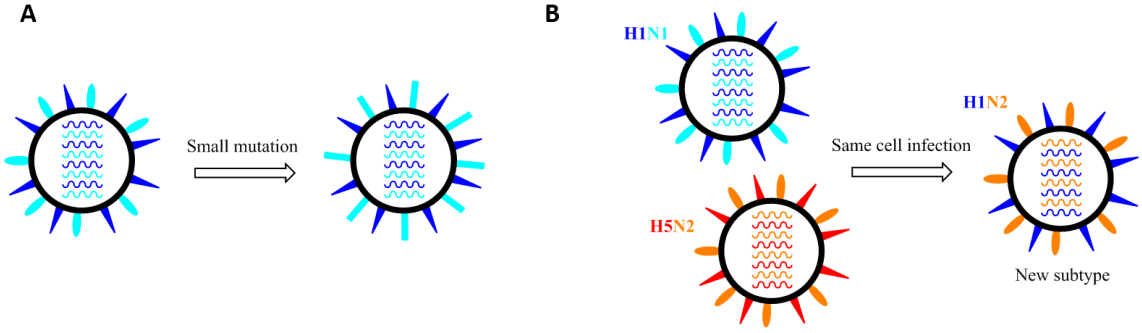


Figure 2. Representation of antigenic drift (left) and antigenic shift (right).

Although new vaccines must be reformulated annually, vaccination is still the primary method used for influenza prevention. In case of pandemic, long times (6-8 months) are required to manufacture enough vaccines to immunize a significant percentage of the population. Lamentably, during this extensive period of time, many human lives could have been taken by influenza. Therefore, an efficient pharmacotherapy needs to be accessible. Unfortunately, both antigenic drift and shift cause resistance of influenza towards recent drugs. The aforementioned disadvantages of immunization (vaccination) and drug therapy highlight the need to develop new small-molecular-weight inhibitors against crucial influenza enzymes.

1.1.1 Influenza viral life cycle

Influenza A virus presents lipid bilayer envelope derived from the previous host cell, within which are eight viral ribonucleoproteins, vRNPs (Figure 3). These vRNPs are formed by viral RNA (vRNA) genome segments in which the 5' and 3' termini are bound to an RNA polymerase complex, and the rest of the vRNA is associated to oligomeric nucleoproteins (NP).

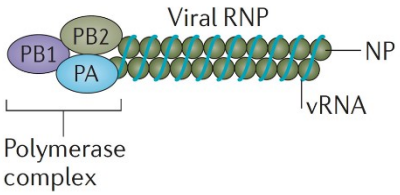


Figure 3. Structure of a vRNPs

Hemagglutinin (HA) is by far the most abundant protein in the viral envelope making up to eighty percent of all proteins. Neuraminidase (NA) is present in around seventeen percent and M2 ion channel is a very minor component with only 16-20 molecules per virion. M1 proteins keep the vRNPs attached to the inner layer of the envelope.

The first step during the infection of the host cell begins with the binding of surface glycoprotein hemagglutinin (HA) to sialic acid receptors from the host cell outer membrane[10] (usually cell membranes from the upper respiratory tract rich in sialic residues). After the binding to the receptor, the virus is transported into the cell in an endocytic vesicle. The lower pH in the endosome triggers two reactions: the fusion of the viral and endosomal membranes and initiates the flow of protons into the virus through the M2 ion channel, this results in the dissociation of the vRNPs from the M1 protein. Subsequently, nuclear localization sequences (NLSs) on nucleoproteins transport the free vRNPs from the cytoplasm to the nucleus.

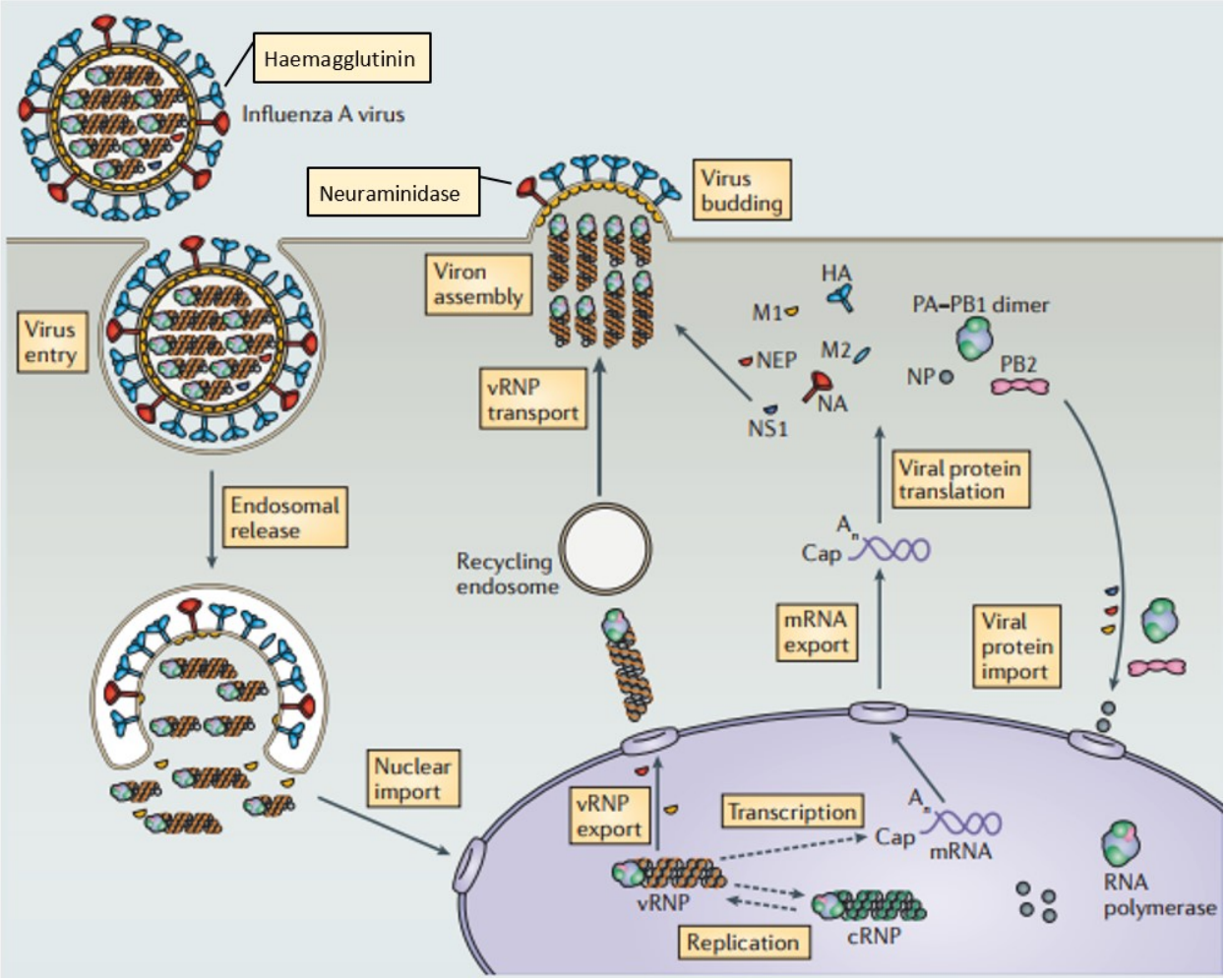


Figure 4. Influenza A life cycle according to Fodor et al., *Nature Rev Microb* **2016**[11]

Once in the nucleus, RNA-dependent RNA-polymerase initiates viral mRNA synthesis by using a 5'-capped RNA fragments from host pre-mRNA as primers, this process will be described thoroughly in following chapters. RNA-polymerase is not only responsible for mRNA synthesis; it also synthesizes unprimed replicas of vRNAs by making complementary copies of RNA (cRNA) that act as templates for replication of vRNAs. The viral mRNAs are transported to the cytoplasm for translation into proteins and the surface proteins (HA, NA and M2) are transported to the membrane of the host cell. Once formed, the NS1 protein suppresses the production of host mRNA. M1–NS2 complexes bound to the newly formed vRNPs transport them to the cytoplasm where they can reach the cell membrane to be incorporated in the budding new viruses. NA from the buddying viruses cleaves the sialic acid linkages between HA and the host cell, thereby releasing the new virions. This process will be described in more detail in upcoming chapters.

In the fight against influenza a better understanding of the role of the viral proteins and host cellular proteins is essential since they are potential targets for drug development.[12] At the time of writing, only a few drugs are currently FDA approved (NA inhibitors zanamivir, oseltamivir, peramivir and M2 ion channel inhibitors amantadine and rimantadine). The current standard of care for influenza cases in the United States is only the NA inhibitors oseltamivir and zanamivir.

Figure 5 depicts the structure of some of the known anti-influenza drugs. Although it may seem that there are enough drugs for the treatment of influenza, several related disadvantages reveal the need for new inhibitors. Amantadine and rimantadine were the first drugs to be approved but rapid drug resistance and neurotoxic side effects have limited their clinical use. Ribavirin also has serious side effects and Arbidol hydrochloride is only approved in Russia and China.

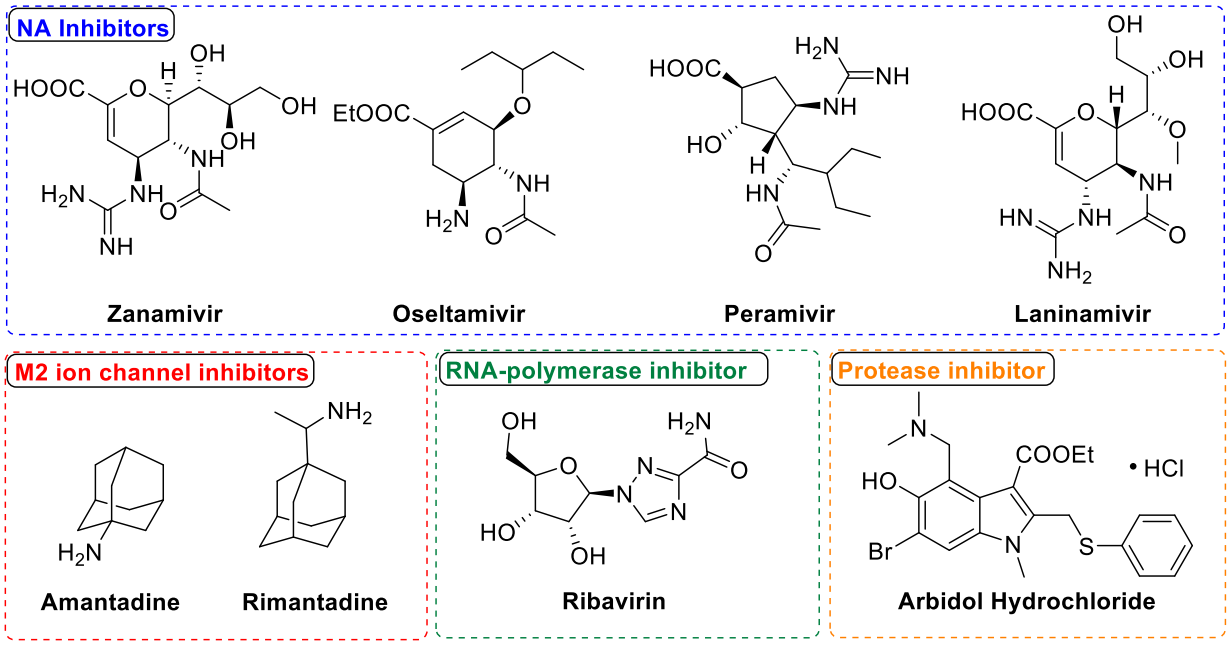


Figure 5. Structure of the available drugs for influenza's treatment.

Neuraminidase inhibitors are the most used antivirals for the treatment of influenza, in particular oseltamivir, since it's the only one that can be administrated orally. Unfortunately, the recent emergence of new drug-resistant strains generally caused due to prolonged treatment with NA inhibitors[13] (mostly oseltamivir), and the appearance of H5N1 viruses that have shown low sensitivity towards oseltamivir[14] has highlighted the need for new therapeutics.

1.1.2 Neuraminidase

Neuraminidase (NA) (also called sialidase) and Haemagglutinin (HA) are surface glycoproteins able to recognize carbohydrates, particularly sialic acid (*N*-acetylneuraminic acid or Neu5Ac) which is associated as the α-linked terminal carbohydrate unit of the upper respiratory tract and lung-associated glycoconjugates. The role of neuraminidase is to cleave the glycosidic linkage between the terminal sialic acid and the host glycoconjugate. By this process, neuraminidase facilitates the movement of the virus through the upper respiratory tract and more importantly, releases the budding virions from infected cells in the last step of the viral life cycle.

Influenzas NA is a mushroom-shaped enzyme attached to the viral membrane made up of four identical subunits. Each subunit is made from six β-sheets with a propeller disposition. Eleven subtypes of neuraminidase have been identified so far, these subtypes are further classified into two phylogenetically distinct groups. Group-1 contains N1, N4, N5 and N8 subtypes whereas group-2 contains N2, N3, N6, N7 and N9. These two groups are structurally distinct, the main difference being that group-1 has a cavity adjacent to the ligand binding site.[15]

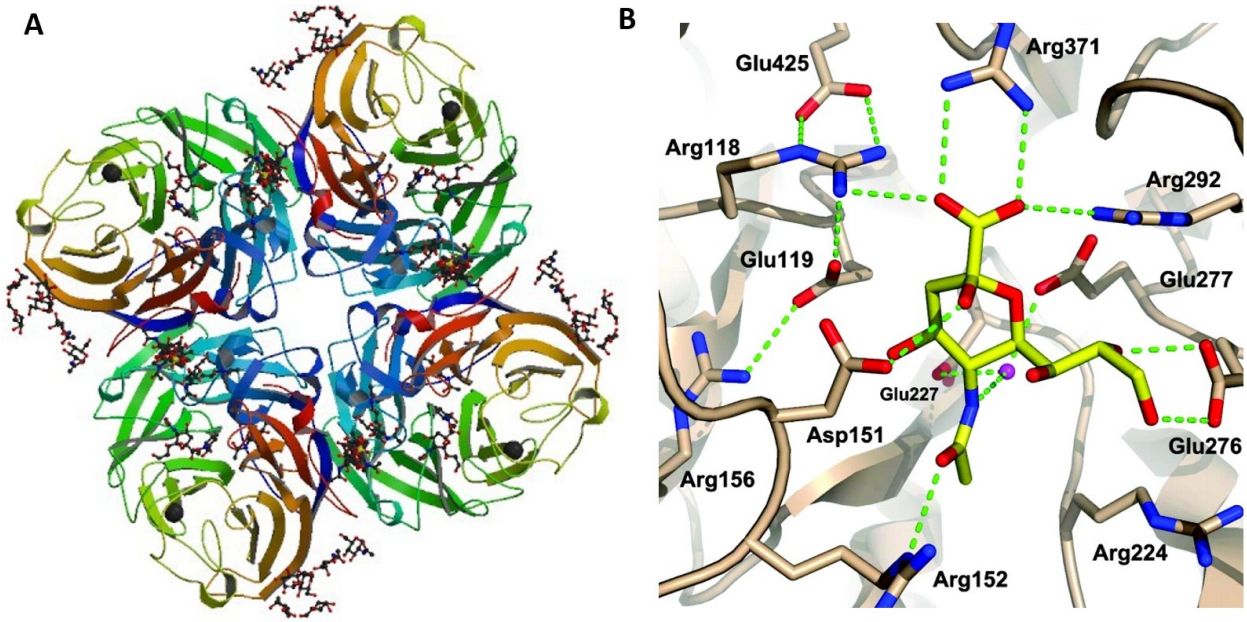
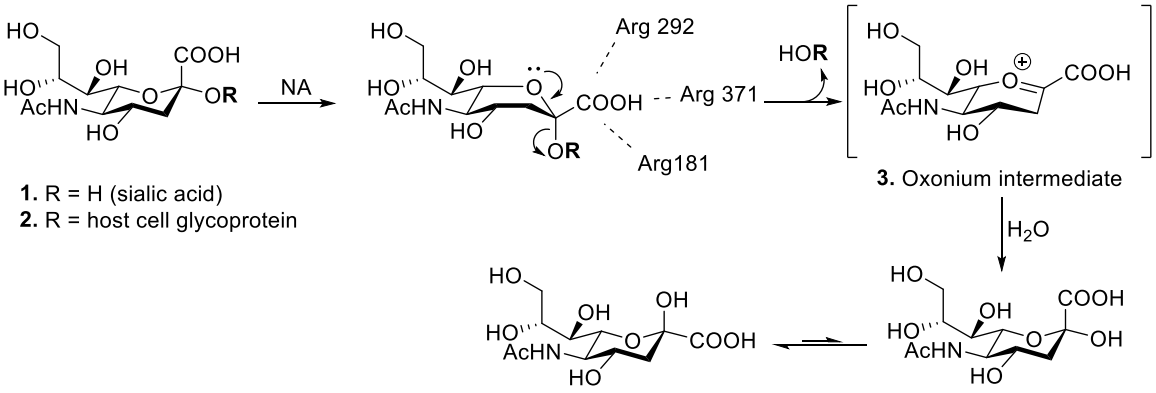


Figure 6 A. H2N2 influenza virus NA and sialic acid (PDB code: 2bat) **B.** The eight invariant amino-acid residues make direct contact with Neu5Ac, Hui-Ling Yen et al.[16]

The X-Ray structure of NA complex with sialic acid was resolved for the first time in 1983. Since then, the crystal structures of neuraminidase from group-1 (N8[15], N4[15] and N1[15]) and group-2 (N7[17], N6[17] and N3[18]) have been reported.

One remarkable feature of neuraminidase is that presents highly conserved amino-acids in the active site across all subtypes. These amino-acids can be grouped in three different pockets, pocket 1 is formed by E276, E277, R292, N294 and accommodates the glycerol chain of sialic acid. The pocket 2 forms a hydrophobic region made of the residues of A246, I222 and R224. The third and largest pocket is formed of polar negatively-charged amino-acids E119, E227 and D151.

When bound to the active site of neuraminidase, the sialic acid pyran ring flips from the most stable chair form to the boat conformation, this is due to the extra stabilization introduced by acid-base interactions between C-1 carboxylic acid of **1** and a highly conserved triarginyl cluster (Scheme 2). In the boat conformation, the lone pair of the pyran oxygen atom and the glycoside C-O bond are positioned in antiparallel which allows the overlap between the lone electron pair from the pyran oxygen and the σ* orbital of the C-OR bond, as result, the bond is broken and an oxonium intermediate **3** is formed, which is then intercepted by water generating a molecule of sialic acid.



Scheme 2. Mechanism of the hydrolysis of the sialic acid residue catalyzed by neuraminidase.

NA is a very attractive target for drug-development of small-molecular-weight inhibitors (less than 1000 Da). All NA inhibitors depicted in Figure 5 bind strongly the active site of NA due to their analogous structure to the oxonium intermediate **3**, competing directly with sialic acid residues and therefore called sialomimetics.

The recent progress in researching NA inhibitors has been mainly focused on modification or optimization of oseltamivir and zanamivir structures.

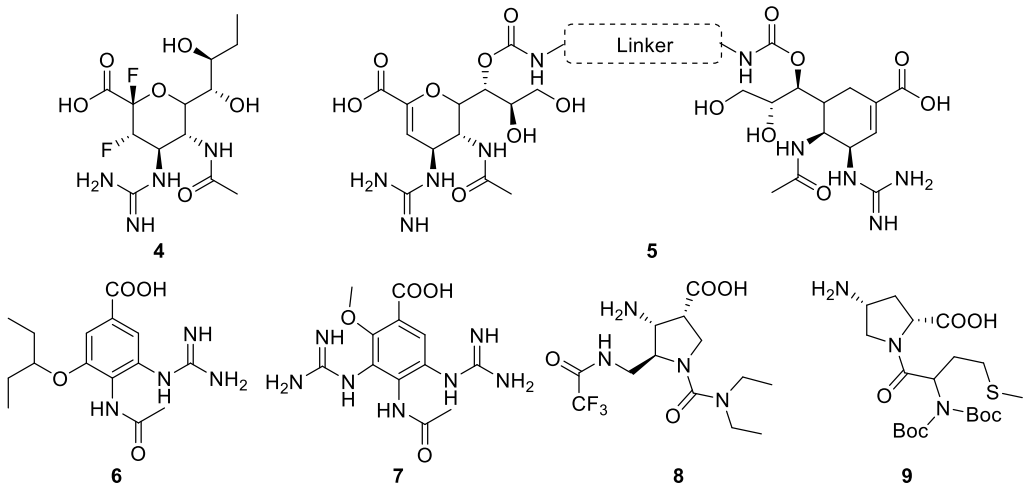


Figure 7. Structure of covalent inhibitor (**4**), dimeric zanamivir (**5**), benzoic acid derivatives (**6** and **7**) and pyrrolidine derivatives (**8** and **9**).

Also other types of compounds have been reported as NA inhibitors. Some of them are depicted in Figure 7. The irreversible inhibitor **4** is capable to form covalent intermediates with amino acids from NA-active site.[19] Dimeric zanamivir conjugates (**5**) were proven to be highly potent inhibitors but its deficient application in prophylaxis led to discontinuation of the whole project.[20] In recent studies was shown that some benzoic acid derivatives[21-24] (**6** and **7**) and pyrrolidine-based inhibitors[25] (**8** and **9**) were also effective NA inhibitors. Furthermore, several natural products have shown anti-influenza activities,[26, 27] but they are outside the scope of this work.

It is also worth to note that numerous compounds (flavonoids) that were described as NA inhibitors in the beginning were later on revealed as false-positive hits. Apparently, not only potent drugs for the treatment of influenza are required but also reliable and high-throughput screening assays are desperately needed. This Thesis described our recent effort to address both issues (*vide infra*).

1.1.3 150-cavity

As previously mentioned, NAs were classified into two phylogenetic groups based on sequence analysis. Russell et al[15] crystallized a series of NA from different subtypes and observed that group-1 (N1, N4, N5 and N8) NA feature a cavity near the active-site meanwhile group-2 (N3, N6, N7 and N9) did not. Group-1 enzymes presented an unusual conformation in active-site amino-

acids D151 and E119 which belong to the 150-loop (residues 147-152). As a result, a new cavity (150-cavity) of nearly 10 Å long, 5 Å wide and 5 Å deep opens up near the active-site. The proximity of the 150-cavity to the catalytic site could allow simultaneous binding of small molecules to both sites.

However, recent publications have shown that pandemic H1N1 neuraminidase N1 (group-1 NA) lacks the characteristic 150-cavity.[28] Subsequent MS simulations and crystallographic studies revealed that a salt bridge between D147-H/R150 controls the cavity formation in group-1 and group-2 enzymes, both of which are able to adopt flexible loop conformations.[29] Furthermore, a study reported the crystal structure a group-2 NA (N2) binding with oseltamivir presenting a partially open 150-loop,[30] which reinforces the assumption that 150-cavity is not a unique characteristic of group-1 NA. The fact that group-2 NA could also present the cavity makes it more attractive for drug development since new drugs targeting active-site and the 150-cavity could be applicable across all subtypes of NA.

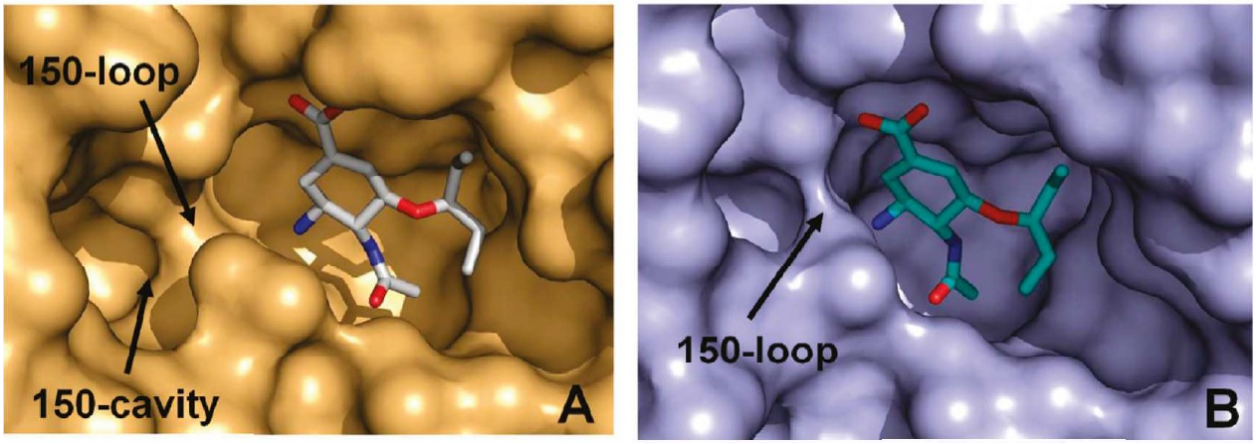


Figure 8 A. Oseltamivir carboxylate bound to the active-site of N1 neuraminidase **B**. Oseltamivir carboxylate bound to 5691the active site of N9 subtype. Figure from Mohan et al.[31]

Several research groups have reported neuraminidase inhibitors that were designed to take advantage of the existing cavity-150. The available crystal structures of NA binding to oseltamivir indicate that the amino group at the C-5 points towards the 150-cavity. Therefore, atom C-5 has been chosen as the modification site in most of the scientific reports.

A series of NA-inhibitors based on oseltamivir structure which bear a triazole moiety instead of the C-5 amino group showed that the new interactions gained by binding to the 150-cavity are weaker than the strong hydrogen bonding network that was disrupted by replacing the parent amine group by a less basic triazole (compare the K_i of **10**, **11** and **12** to oseltamivir in Figure

9, tested against virus-like particles that contain an influenza A virus N1 activity).[32] The mentioned work highlights the importance of having a basic group on C-5 for the inhibition activity. Compounds **13**[33] and **14**[34] are the most active from all the oseltamivir-based and zanamivir-based NA inhibitors designed to target the catalytic site and the 150-cavity so far.

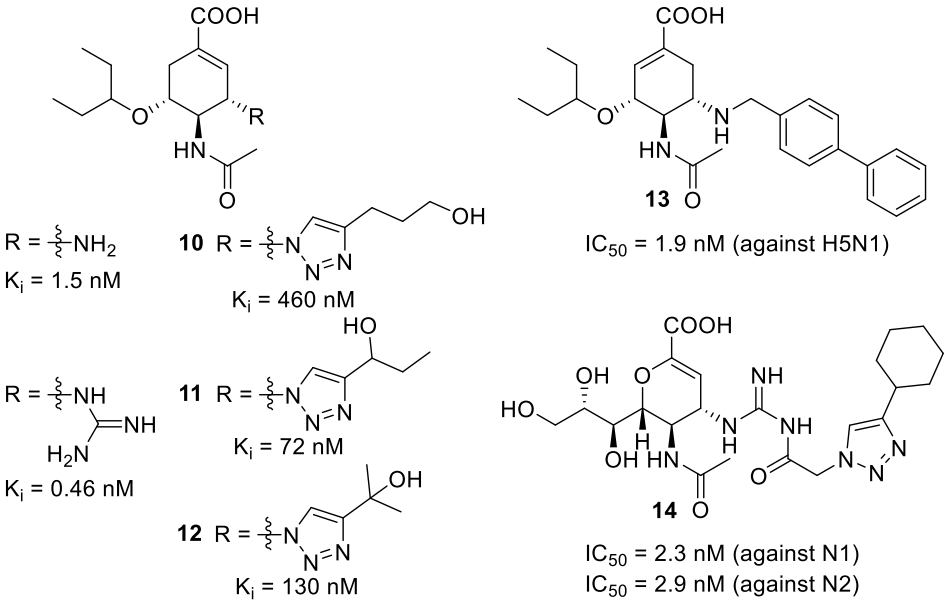


Figure 9. Structure of inhibitors designed to bind the catalytic site as well as the 150-cavity

Some of the reported inhibitors decorated with "150-cavity binding moieties" showed equal inhibition activity against enzymes from group-1 and group-2,[34] what implies that inhibitors targeting the cavity and the active-site may work well for all NA subtypes.

1.1.4 RNA-dependent RNA polymerase (RdRp)

The influenza RNA polymerase is a heterotrimeric complex formed by three subunits: PA (polymerase acidic protein), PB1 and PB2 (polymerase basic protein 1 and 2, respectively). The structure of RNA-polymerase complex appears to be highly complex, with all three subunits intricately involved in many of the important functional regions (Figure 10).[35] The crystal structures of the RNA polymerase complex (from bat FluA and human FluB) were firstly reported in 2014 by Cusack and co-workers.[35,36] The enzymatic complex presents a polymerase core made up of PB1, the C-terminal domain of PA and the N-terminal domain of PB2 as well as a flexible peripheral appendices formed by the endonuclease part of PA and the PB2 cap-binding, mid-linker, 627- and NLS domains. These appendices adopt two different conformations that either enable or disable cap-snatching, what implies that RdRp exists in several functional states.[11] RdRp is capable to perform the processes of transcription and replication using the same template of vRNA.

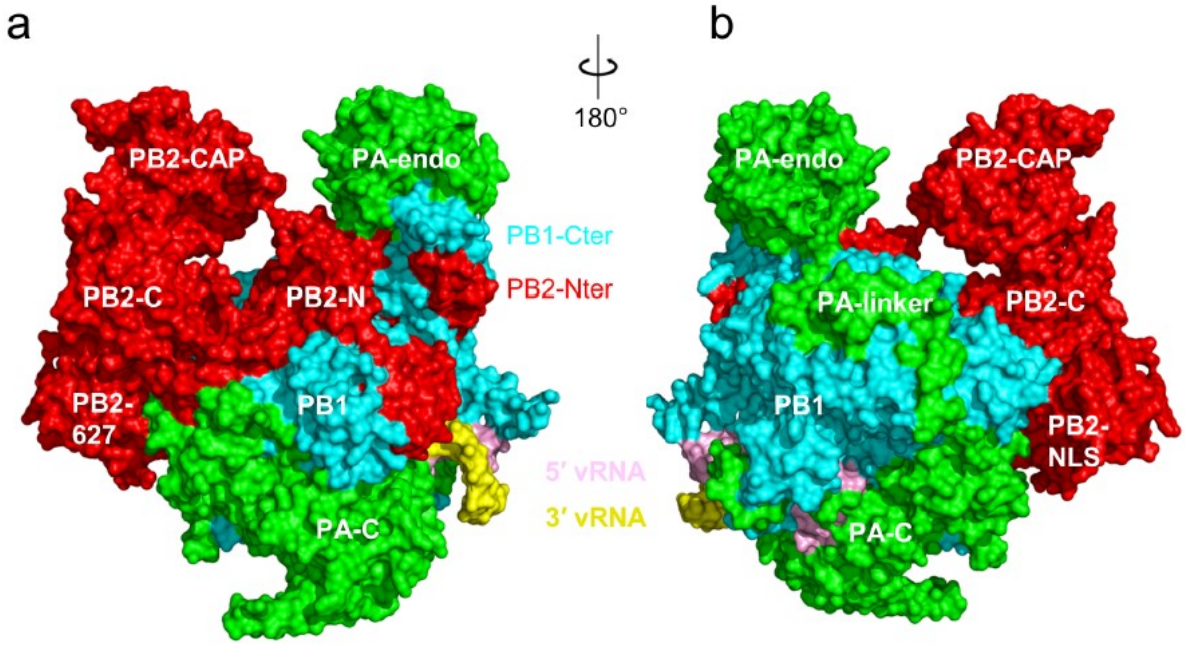


Figure 10. Surface views of the FluA heterotrimer with bound vRNA promoter **a)** at 0º **b)** 180 º **PB2**, **PA**, **PB1** and vRNA **5'** and **3'**. Figure from Pflug, A. et al[35] *Nature,* **2014**.

Transcription is a primer-dependent process; however, RNA-polymerase does not have capping activity and instead uses short capped oligomers from the host pre-mRNA as a primer for transcription, this process is called "cap-snatching" (Figure 11). PB2 has a cap binding domain and its function is to capture the 5'-cap from host pre-mRNAs (red).[37] Subsequently, an endonuclease in the amino-terminus of PA subunit (PA-Nter) cleaves the capped RNA 10-13 nucleotides

downstream.[38] This capped RNA-fragment is used as a primer and vRNA (green) as template by the PB1 subunit during the mRNA synthesis. Thus, the produced capped chimeric mRNA is ready to be translated into viral proteins in the cytoplasm.

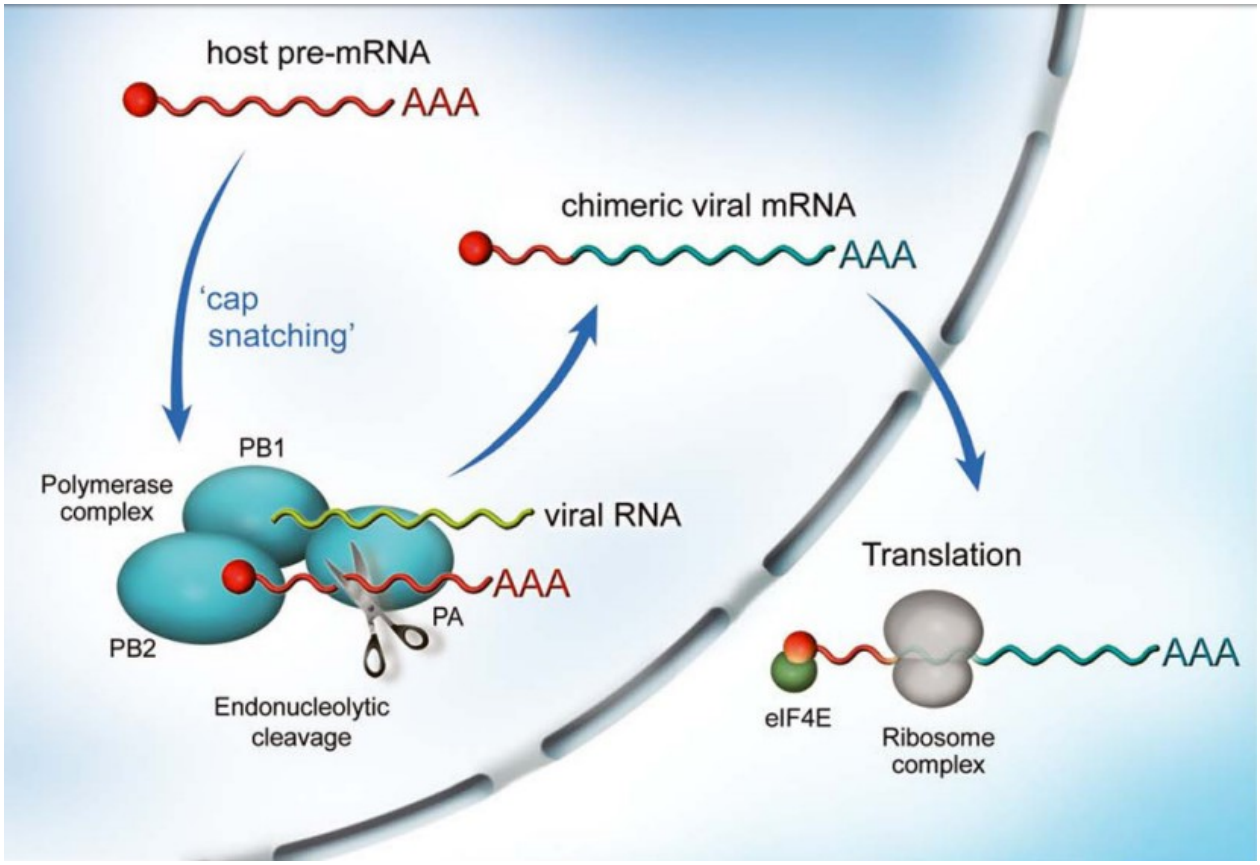


Figure 11. Cap-snatching transcription mechanism of influenza polymerase. Figure extracted from: Boivin et al.[39]

Contrary to the transcription process, the **replication** is primer independent. During this process a complementary RNA (cRNA) intermediate is synthesized as an exact, full-length copy of the vRNA, the cRNA is then used as a template to synthesize back vRNA. Nascent vRNA are immediately packed with nucleoproteins into new vRNPs, transported from the nucleus to the cell membrane and incorporated into the new virions.

Apart from being essential for the processes of transcription and replication, influenza's RNA-polymerase is also responsible for the evolution of the virus. This is due to the absence of a proof-reading mechanism in the enzyme which makes it prone to error during the RNA replication, the consequence is the production of mutant viral proteins which eventually lead to viruses that are better adapted to the host species.

RNA-dependent RNA polymerase is highly conserved across all strains and subtypes of influenza and it plays a critical role in the life cycle of the virus. Because of these reasons, RNA-dependent RNA polymerase is an exceptionally promising target for drug development.

1.1.4.1 Polymerase acidic protein (PA)

PA is a bridged dinuclear metalloenzyme in which the N-terminal (PA-Nter) and the C-terminal (PA-Cter) region are connected by a long flexible peptide chain. The PA-Nter region presents endonuclease activity meanwhile the PA-Cter part forms a "mouth" into which the small N-terminal domain of PB1 is inserted. The active-site of the PA-Nter is formed by His41, Lys134 and a group of the acidic residues Glu80, Asp108 and Glu119 that bind two divalent metal ions (Mn^{2+} or Mg^{2+}). Metal-chelating molecules able to bind these divalent metal ions and occupy the PA-Nter active site could work as inhibitors.

Three main reasons illustrate why the catalytic site of PA endonuclease is a very promising target for drug development:

- Endonuclease activity is essential for the life cycle of the virus.
- The PA-Nter is highly conserved in all strains and subtypes of the three families of viruses (A, B and C) which could lead to a universal inhibitor for influenza.
- No counterpart of PA-Nter exists in human cells which means that inhibitors have a good chance of being selective and non-toxic.

Although no FDA approved PA inhibitors have been reported till the date (2018), the PA inhibitor AL-794 (structures undisclosed) have entered clinical trials and the inhibitor S-033188 (recently named *baloxavir marbotil*) has been approved for sale in Japan (February 2018). These precedents validate PA-Nter as a target for drug development.

Many molecules with chelating groups have been reported as inhibitors of PA-Nter, a brief compilation of the most relevant is presented in the following lines. These inhibitors could be classified in the following groups:

I. ***Diketo Acid Derivatives****:* discovered through a random screening effort,[40] further research indicated/showed that derivatization of the carboxylic acid in the 2,4-dioxobutanoic side

chain is indispensable for the inhibition activity since it's responsible for the binding to the two metal ions (Figure 12). Piperidinyl-diketo acids were reported to be selective inhibitors of PA-endonuclease.[41] In particular **15** (L-742,001) is one of the most potent inhibitors. Compound **15** is able to chelate the two metal ions and at the same time; the two flexible benzyl rings (the "wings") occupy different hydrophobic pockets. It is worth to note that the piperidine nitrogen does not participate in hydrogen bonding. The structure of L-742,001 was used in this work as starting point for the design of a probe suitable for the AlphaScreen-based assay (*vide infra*).

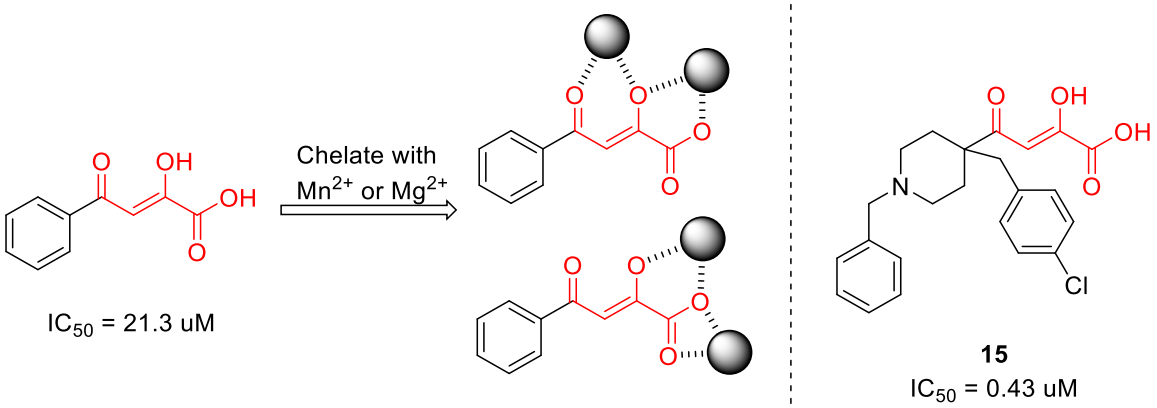


Figure 12. Two possible binding modes of a representative diketo acid and two metal ions, Structure of L-742,001 (**17**).

II. ***2,6-Diketopiperazine derivatives:*** Flutimide (Figure 13, **16**) was originally isolated from *Delitschia confertaspora*. This pyrazine derivative exhibits good inhibitory activity (IC_{50} = 5.1 μM) against endonuclease without affecting the activities of other polymerases.[42] Additional analogues with substitutions at positions 1, 3, and 5 of 3*H*-pyrazine-2,6-dione have been reported.[43] Although some flutimide-derivatives (**17** and **18**) showed enhanced potency *in vitro*, they also presented high cytotoxicity in cell culture. Other similar structures like **19** also showed inhibition activity although the selectivity is unacceptable.

III. ***Hydroxylated heterocycles:*** an extensive series of hydroxypyridines (**20** and **21**), hydroxypyrimidines and hydroxyquinolines have been reported to be not only efficient metal chelating agents but also PA inhibitors.[44] Compound **22** is a particularly promising lead compound suitable for further modification.[45]

IV. ***Catechol derivatives:*** Natural products containing catechol group have drawn attention from researchers in past years (**23**[46] and **24**[47]). Although these structures have not been

thoroughly investigated, they could be a promising scaffold for further structural optimization.

V. ***Others:*** An extensive list of other small molecules with diverse structures have shown binding activity to the PA endonuclease site. For example, 2,3-dihydroxybenzoic acid (**25**) and its bioisosteres (**26**)[48] represent some of them. Compound ANA-0 (**27**) features a unique scaffold and presumably utilizes different mechanism of the action. This compound belongs to a group of inhibitors without "textbook" chelating groups.[49]

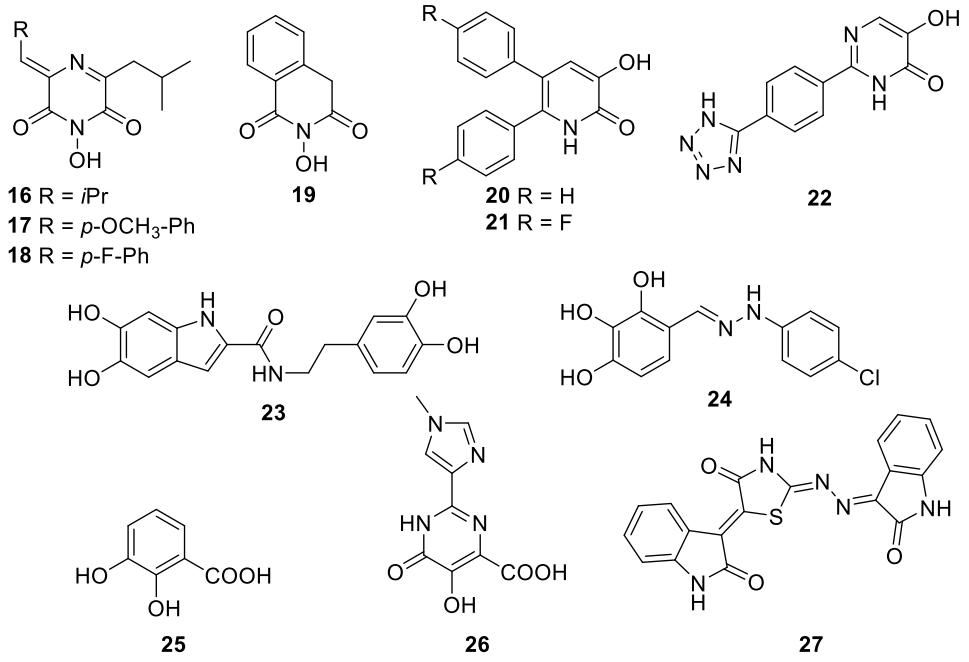


Figure 13. Structure of selected PA endonuclease inhibitors.

The list of possible PA endonuclease inhibitors is quite extensive but it's important to highlight that most of the reported structures were never further investigated after the initial report. Therefore, the pursuit of a powerful, selective, orally-available inhibitor of PA endonuclease is still a hot topic in medicinal chemistry.

1.1.4.2 Polymerase basic protein 2 (PB2)

The role of PB2 as the cap-binding part of the polymerase complex has been known for several decades. In 2008 the crystal structure of the PB2 cap-binding domain (PB2-CBD) was firstly reported.[50] The nature of the cap binding-site was thus unveiled and efficient structure-based design of drugs began to appear. The natural ligand of the PB2 domain is the 7-methyl GTP (m^7GTP) on the 5' end of the host pre-mRNA; the binding occurs in an independently folded

domain of PB2. Although the binding mode is similar to other cap-binding proteins it features important differences.[50] The binding of m^7GTP is shown in Figure 14, the methylated base is sandwiched between the residues of Phe404 and His357, and Phe323 stacks also with the ribose. The key acidic residue is Glu361 that forms hydrogen bonds with N1 and N2 and at the same time, the neighboring Lys376 interacts with O-6. The triphosphate group adopts a bent conformation and binds both Arg355 and Asn429.[50]

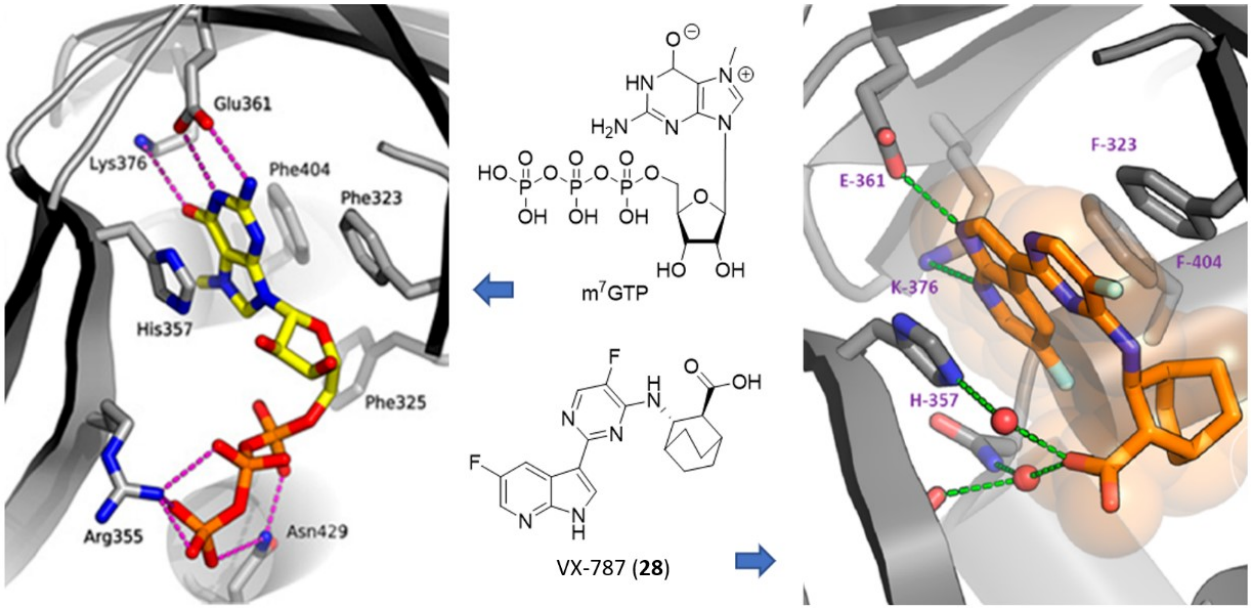


Figure 14. Structures of m^7GTP and VX-787 and the corresponding X-ray structures with PB2-CBD. Figure from Clark, P. M. et al.[51]

Clark et al. discovered a series of azaindole-based influenza PB2 inhibitors by phenotypic screening effort using cell protection assay. Rational optimization of the hits led to the discovery of VX-787 (**28**), which is an extremely potent, orally available and highly selective inhibitor of PB2 cap-binding domain.[51] VX-787 is in phase IIIb of clinical trial at the moment (March 2018).

The X-ray structure of **28** with PB2 (Figure 14) showed how does the azaindole core interact by π-stacking with His357, Phe323 and Phe404 residues. Simultaneously the two nitrogen atoms from the heterocycle bind with residues Lys376 and Glu361. The carboxylic acid from the [2.2.2]bicyclootane moiety is involved in two water-mediated interactions with the residues of His357, Gln406 and Arg355. The fluorine atom attached to the pyrimidine ring points outside the cap-binding site into the solvent.

Figure 15 shows other cap binding inhibitors. Compound **29** was the first cap-binding inhibitor ever reported and was based on the structure of m^7GTP.[52] Unfortunately due to the unfavorable pharmacokinetics the projects associated with compound **29** were discontinued.

Replacement of the carboxylic acid from **28** by charged and uncharged bioisosteres proved that a negatively charged moiety is important for the antiviral potency; unfortunately, none of the compounds reported showed superior inhibition activities to **28**, but compound **30** showed at least similar values.[53] Other reported inhibitors are Cap-3 and Cap-7, both exhibited EC_{50} in the micromolar range (Figure 15).

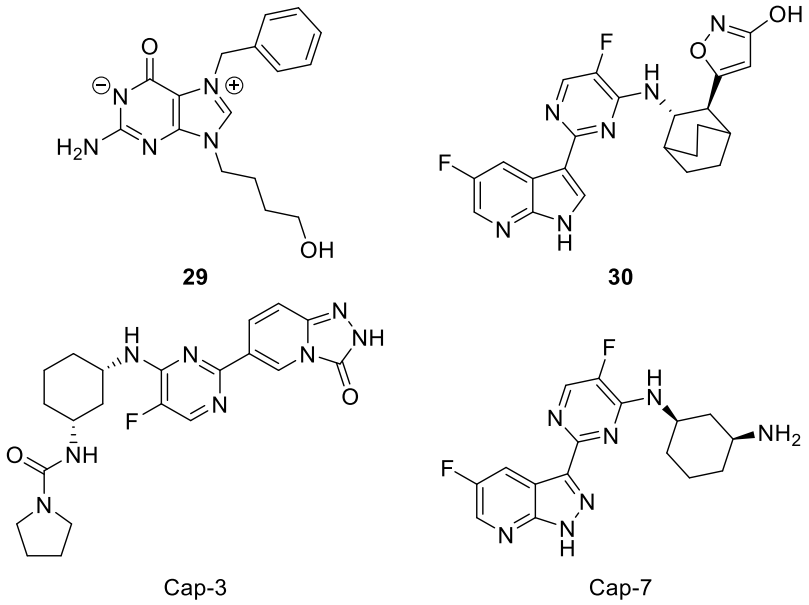


Figure 15. Structures of PB2 cap-binding inhibitors.

Due to the outstanding inhibition activity of VX-787 (**28**) and its well-known binding-mode to the active-site of PB2, we decided to use its scaffold as the binding part of a detection probe, which will be used in the development of a high throughput screening assay for possible PB2 cap-binding inhibitors.

1.1.4.3 Polymerase basic protein 1 (PB1)

PB1 is located in the core of the heterotrimeric polymerase complex, bonded to PB2 and PA subunits. It's responsible for the polymerase activity including nucleotide polymerization and chain elongation. The polymerase active site is located at the edge of a large central cavity formed by PB1 and PB2-Nter.[35,36] This central cavity presents several channels that lead to the surface of

the protein, some of this channels are formed by amino acids from all three subunits meanwhile others, like the NTP entry channel, are made exclusively of residues from PB1 subunit.[54]

Very few compounds have been postulated as PB1 inhibitor, the inhibitors **31** and **32** (Figure 16) were found by high throughput screening assays and the mode-of-action was verified by mutation analysis.[55,56]

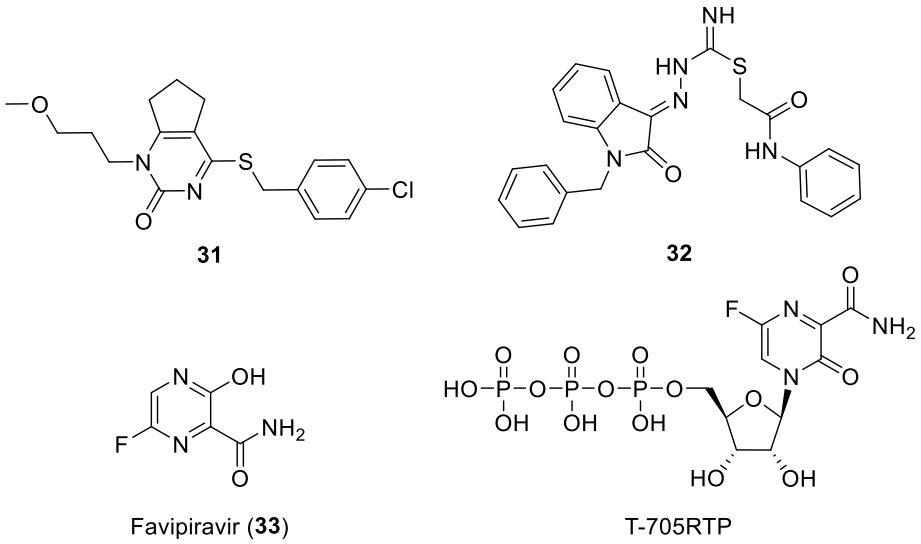


Figure 16. Structures of reported PB1 inhibitors.

Favipiravir (**33**) is likely the most promising anti-influenza drug in development, in the USA has advanced to phase II of clinical trials and in Japan is in phase III. Favipiravir, simple pyrazine derivative, was firstly reported in 2002 by Furuta et al.[57] Its mechanism of action begins with phosphorylation. At first, Favipiravir (also known as T-705) is converted to the monophosphate and this metabolite further undergoes phosphorylation by cellular kinases to the active triphosphate that are misidentified as a natural purine nucleotide by the virus RNA polymerase and is subsequently incorporated to the RNA. Once is introduced into the RNA it prevents further extension of the strand. Favipiravir inhibits the virus RNA polymerase but not cellular RNA and DNA synthesis since host cells enzymes can differentiate T-705 phosphates from the natural nucleotides.[58] Favipiravir has been tested against a wide range of influenza strains and it has shown high efficiency in nearly all, including strains resistant to neuraminidase and M2 ion channel inhibitors.

1.1.4.4 Protein-protein interactions

Disruption of the heterotrimeric polymerase complex is an alternative strategy for drug development against influenza since the protein-protein interactions (PPIs) between PB1, PB2 and PA are indispensable for the transcription and replication. Researchers have focused their work on the interaction between PA-C terminus and PB1-N terminus and the interaction of PB1-C terminus and PB2-N terminus.

- **PPI between PB1 and PB2**: The interaction was studied by protein crystallography. The contact between these two subunits depends on four salt bridges and eight H-bonds and the interface is quite extensive in sequence length what makes it an inappropriate target for drug development.[59] Despite not being the most promising target in influenza RNA polymerase there is some known compounds disrupting PB1-PB2 PPI.[60]
- **PPI between PA and PB1:** Crystallographic studies found that the C-terminal domain of PA forms a deep, highly hydrophobic groove formed by several α-helices, meanwhile, the N-terminal domain of PB1 forms a compact helix (residues 5-11) that is buried inside PAs

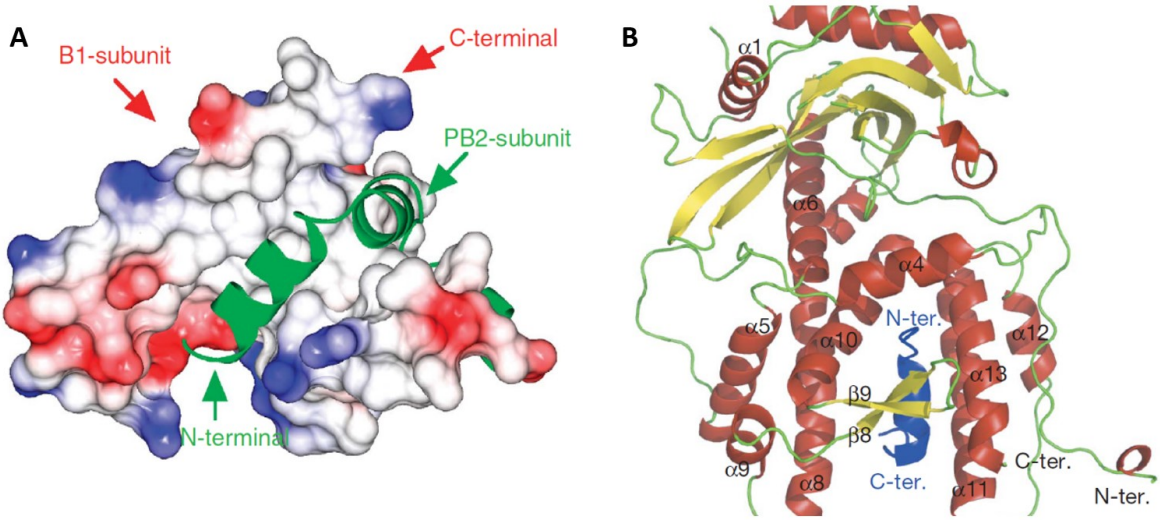


Figure 17. A. Schematic diagram showing PB1-PB2 PPI. The molecular surface of PB1 is colored by charge (blue = positive, red = negative). PB2 is shown as a green ribbon. Figure extracted from Sugiyama et al.[59] **B.** Crystal structure of PA-Cter (helices = red, strands = yellow, coil = green) bound to PB1-Nter peptide (blue). Figure extracted from Obayashi et al.[61]

groove.[61,62] The two domains interact largely by hydrophobic interactions, although hydrogen bonding and van der Waals forces were also detected. Short peptides (15 amino acids) derived from PB1-Nter showed inhibition activity not only against FluA polymerase but also FluB.[63]

Several small molecules and peptides have been reported as PA-PB1 disrupting compounds; benzofurazan derivatives were identified by HTS using ELISA-based screening method,[64] 4,6-diphenylpyridines, cycloheptathiophene-3-carboxamaides and triazolopyrimidines were discovered by a combination of virtual screening and ELISA assay.[65]

So far reported PB1 inhibitors are not meeting the requirement for being considerate as lead drug candidates due to a lack of potency or drug-like properties. Nevertheless, the recent elucidation of the structure of influenza's RNA polymerase[35] has been a game-changing success for the design and identification of PPI inhibitors.

1.2 Oseltamivir

In the previous chapters, we described some of influenza enzymes which are being targeted for therapeutic intervention. This chapter summarizes the chemistry of the pivotal compound of this Thesis, the neuraminidase inhibitor oseltamivir.

Oseltamivir (**34**, Tamiflu^{TM}, GS-4104-02, RO0640796) was firstly synthesized by Kim et al[66] (Gilead Science). The drug was approved by the FDA and commercially launched in 1999 after Gilead Science and F. Hoffmann-La Roche Ltd. signed a co-development contract. Oseltamivir is marketed as the phosphonate salt under the trade name of Tamiflu^{TM}. Tamiflu is an ethyl ester prodrug whose active form (oseltamivir carboxylate, **35**) is released after hepatic esterases hydrolyze the ethyl ester to the free acid.[67] Oseltamivir is the most used NA inhibitor at the moment, and it is prescribed for treatment and prophylaxis of both influenza A and B infections.

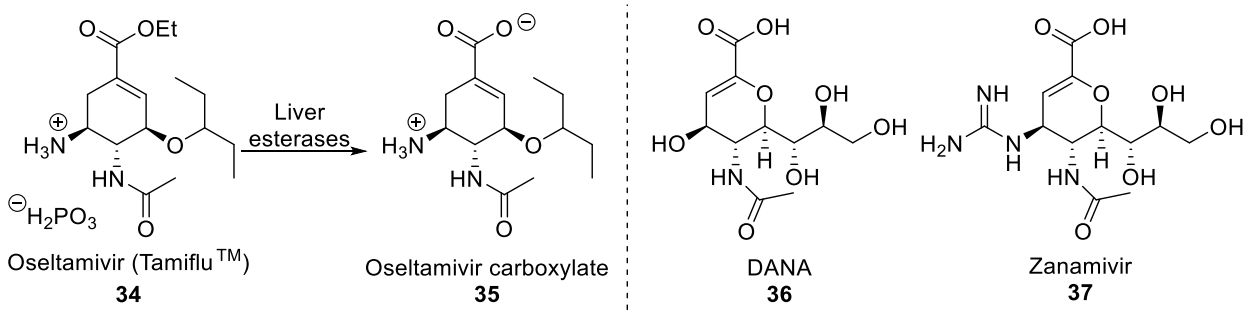


Figure 18. Structure of Oseltamivir, Oseltamivir carboxylate, Dana and Zanamivir.

The discovery of oseltamivir was the result of intense optimization of the structure of DANA (**36**) and zanamivir (**37**), which aimed for a less polar molecule with enhanced oral bioavailability. Replacing the glycerol moiety by a lipophilic group produced a change in the architecture of the active site. Glu276 (which usually interacts with the glycerol moiety in SA) gets reoriented and binds Arg224. That leads to the formation of a hydrophobic area where the pentyloxy group is accommodated.[68] Unlike zanamivir, oseltamivir (**34**) presents the double bound in a position that mimics more closely the oxocarbenium intermediate (Scheme 2), the position of the double bound is fundamental for the binding since the other regioisomer of oseltamivir presents no inhibition activity.[66]

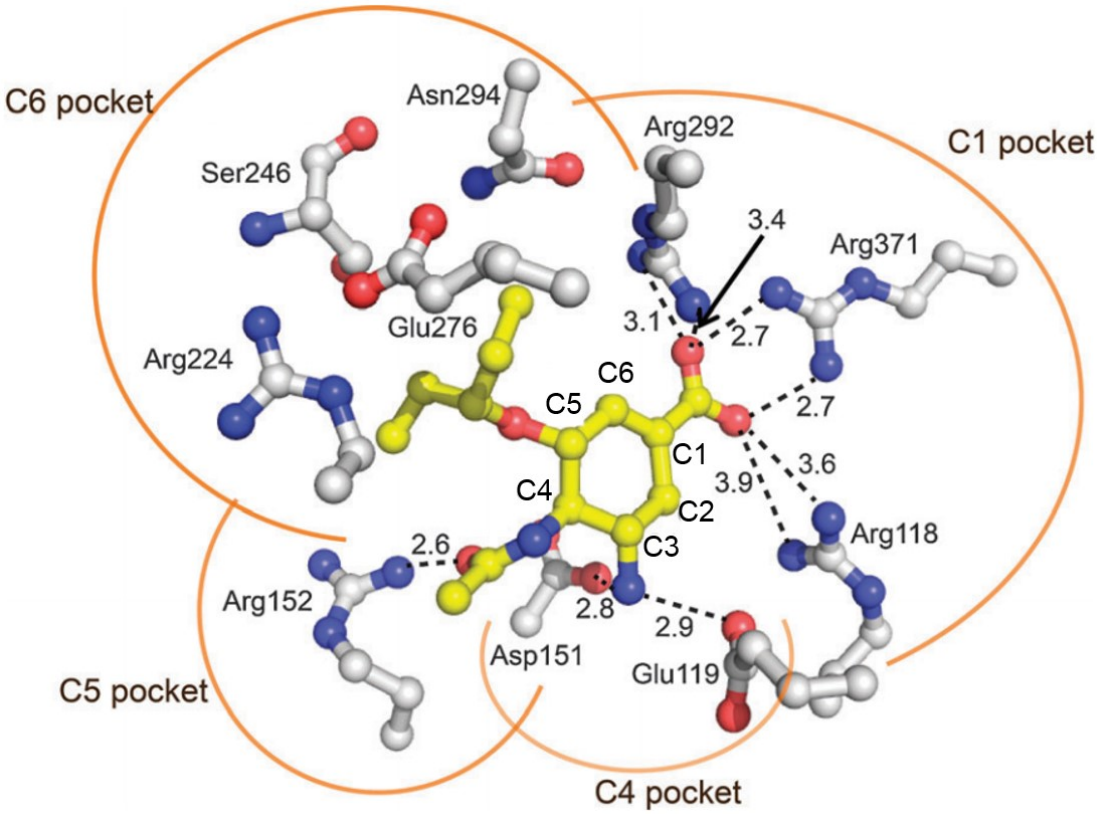


Figure 19. Intermolecular interactions of oseltamivir carboxylate **35** with 2009 pandemic H1N1 neuraminidase (PDB code: 2HUA). Figure from Naumov et al.[69]

The binding between oseltamivir carboxylate (**35**) and the active site of neuraminidase is depicted in Figure 19. The negatively charged carboxylate in C-1 binds by acid-base interaction with three basic arginine residues from C1 pocket (Arg292, Arg371 and Arg118). The other acid-base interaction takes place between the positively charged amine at C-5 and the acidic residues Glu119, Asp151 and Glu227. The acetamide moiety at C-4 forms a hydrogen bond with Arg152 meanwhile the pentoxy group binds by hydrophobic interactions with the hydrophobic pocket formed by Ile222, Arg224, Ala246 and Glu276.

Oseltamivir has shown *in vitro* activity not only against influenza A and B types but also across different influenza A subtypes. Furthermore, oseltamivir displays insignificant cross-reactivity with other sialic acid binding proteins from other viruses, bacteria or human microsomes.[70] Despite its success, the emergence of oseltamivir-resistant strains and some unwanted side effects are two concerning drawbacks that need to be addressed in further research.

1.2.1 Oseltamivir-Resistant strains

The original success of oseltamivir is partially based on the fact that the active site of neuraminidase is highly conserved among A and B viruses. Mutations leading to viral resistance against anti-influenza inhibitors often appears in humans undergoing treatment. Although resistance to NA inhibitors is less frequent than resistance to M2 ion channel inhibitors, some strains of the virus have shown low sensitivity towards oseltamivir.

During the 2007-2009 annual influenza epidemics spread a drug-resistant strain of the seasonal H1N1 virus containing the mutation H274Y worldwide. Typically, reduced susceptibility to neuraminidase inhibitors appears when a mutation alters the size and shape of the NA substrate binding site. In the mutation H274Y (Figure 20, **A**), His274 is replaced by a much bulkier Tyr274 which pushes Glu276 towards the active site. The new conformation of Glu276 prevents its rotation towards Arg224 and therefore the hydrophobic pocket that accommodates the pentyloxy group is not formed.[71] The H274Y appears in the N1 subtype including in the highly pathogenic H5N1. In contrast with oseltamivir, mutation H274Y has little effect in the inhibition of zanamivir, this is due to the H-bonding between the glycerol and Glu276 is not disrupted.[72]

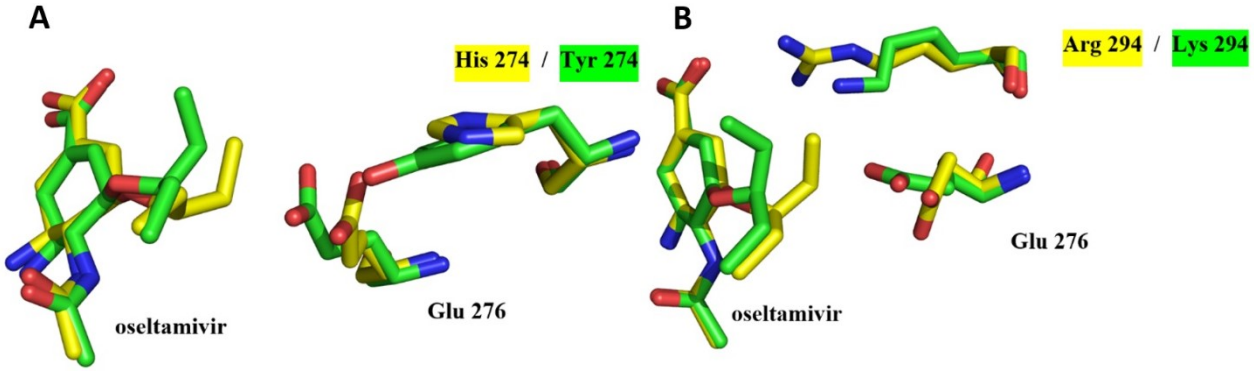
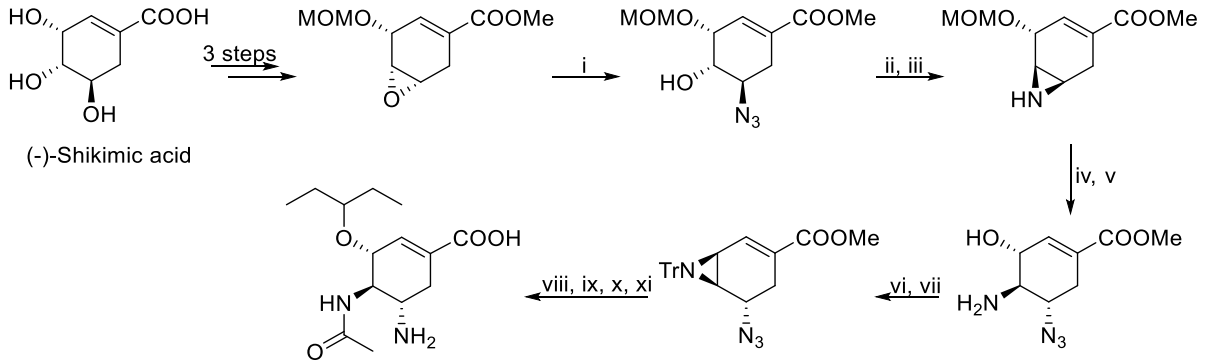


Figure 20. **A**. Effect of mutation H274Y on E276 and oseltamivir in the wild-type N1 NA (yellow) and mutant N1 NA (green). **B.** Effect of mutation R294K on E276 and oseltamivir in the wild-type N9 NA and mutant N9 NA.

Although less common than mutation H274Y, the widespread mutation R294K appears in the N2 subtype and markedly reduces the sensitivity towards oseltamivir. X-Ray structural analysis showed that a salt bridge formed between the new Lys294 and Glu276 pushes the formed towards the pentyloxy group moving the molecule of oseltamivir away from the active site.[73] R294K confers less resistance towards zanamivir than to oseltamivir.

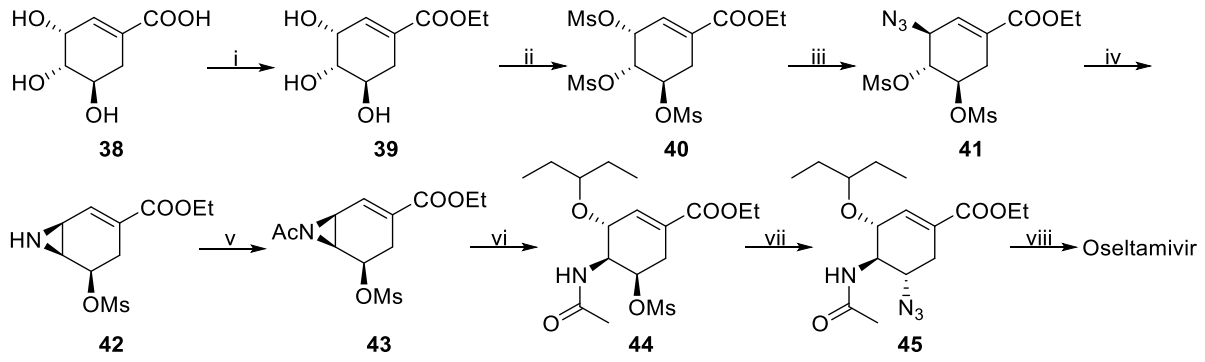
1.2.2 Oseltamivir syntheses

The first synthesis reported by Kim[66] (Scheme 3) used (-)-shikimic acid as starting material for the oseltamivir carboxylate synthesis. Shikimic acid as starting material was a reasonable choice since it already presents the cyclohexene scaffold and the key carboxylic acid moiety. In addition, all carbons have the same oxidation states as in oseltamivir carboxylate.



Scheme 3. Gilead Science's first synthetic route.[66] *Reagents and conditions.* (i) NaN_3, NH_4Cl; (ii) MsCl, Et_3N; (iii) Ph_3P, THF/H_2O, Et_3N; (iv) NaN_3, NH_4Cl; (v) HCl-MeOH 5%; (vi) TrCl, Et_3N; (vii) MsCl, Et_3N; (viii) 3-pentanol, $BF_3{\cdot}OEt_2$; (ix) Ac_2O, py, DMAP; (x) PhP_3, THF, H_2O; (xi) KOH, THF, H_2O.

The 3-pentoxy moiety is introduced *via* aziridine-opening reaction at a late stage of the synthesis because it could be easily diversified, which is preferable in the drug discovery stage. The synthetic route reported by Shi et al[74] (Scheme 4) exhibits similar features. With only eight steps and 47% overall yield, Shi's approach is an excellent route for the synthesis of oseltamivir-derivatives with different moieties in C-3.



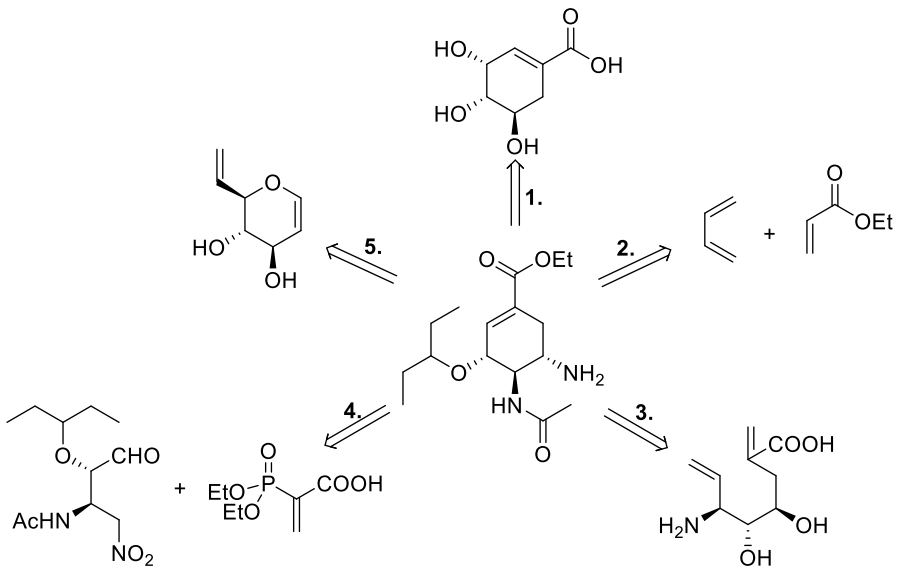
Scheme 4. Shi et al synthetic route.[74] *Reagents and conditions.* (i) $SOCl_2$, EtOH; (ii) MsCl, Et_3N, DMAP; (iii) NaN_3, acetone/water, 0 °C; (iv) Ph_3P, THF/water; (v) Ac_2O, Et_3N; (vi) 3-pentanol, $BF_3{\cdot}OEt_2$; (vii) NaN_3, EtOH/water, reflux; (viii) Lindlar cat., H_2.

Roche's current production process relies also on shikimic acid and is suitable for mass production.[75] More than sixty oseltamivir syntheses have been reported since Kim's publication in

1997, some have been focused on improving the original synthesis by shortening the number of steps or increasing the overall yield.[76-82] Several reviews have summarized the progress made in past twenty years.[83-87]

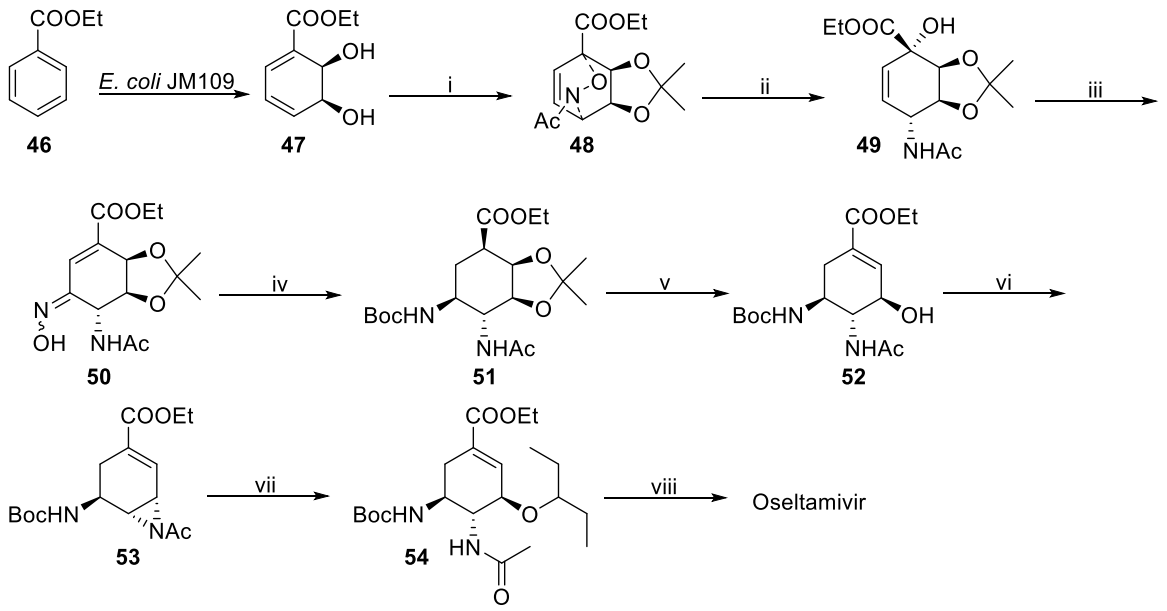
The numerous retrosynthetic approaches towards oseltamivir could be divided into five different strategies (Scheme 5):[87]

1. Syntheses which use 6-membered rings as a starting material (shikimic acid, quinic acid, cyclohexane-1,2-diol, etc.).
2. Construction of the ring by Diels-Alder reaction using acrylates as dienophile.
3. Formation of the cyclohexene by ring-closing metathesis reaction.
4. Assembly of the cyclohexene core *via* Michael addition to a nitroalkene and subsequent Horner−Wadsworth−Emmons.
5. Approach starting with *D*-glucal and utilizing Claisen rearrangement.



Scheme 5. Five different retrosynthetic approaches towards oseltamivir synthesis.

Hudlicky's approach will be described in more detail here since part of our research was based on his work. Hudlicky and coworkers reported a 13-steps synthesis which uses inexpensive ethyl benzoate as the starting material with an overall yield of 7%.[88] Further optimization lead to the much simpler and azide-free synthetic pathway outlined in the Scheme 6.[89]



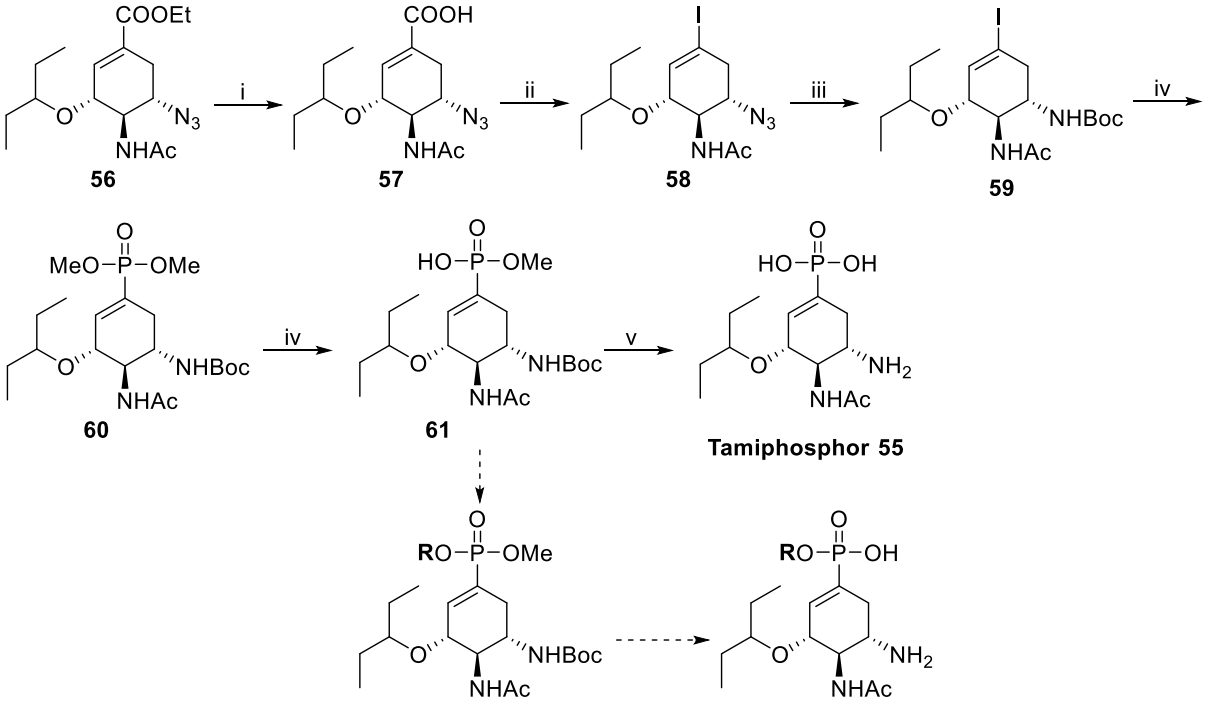
Scheme 6. Oseltamivir synthesis reported by Hudlicky et al.[89] *Reagents and conditions.* (i) 1. DMP, TsOH; 2. CH_3CONOH, $NaIO_4$, MeOH; (ii) $Mo(CO)_6$, MeCN, H_2O; (iii) 1. CrO_3, Ac_2O; 2. $NH_2OH{\cdot}HCl$, EtOH, py; (iv) 5% Rh/Al_2O_3 60 psi H_2, Boc_2O; (v) 0.05M EtONa; (vi) Me_2PPh, DIAD; (vii) 3-pentanol, $Cu(OTf)_2$; (viii) TFA.

The synthesis starts with the fermentation of ethyl benzoate **46** with a recombinant strain of *Escherichia coli* JM109 (pDTG601A) to produce the diol **47**. The diol was firstly protected as the acetonide which underwent an inverse electron-demand Diels-Alder cycloaddition with an acyl nitroso species formed *in situ*. The resulting oxazine **48** was generated as a single isomer. The N-O bond was then reduced to yield the allylic alcohol **49** which underwent Dauben-Michno oxidative transposition under treatment with chromium trioxide. The formed enone was directly converted to the oxime **50**. Hydrogenation of the oxime under the presence of Boc-anhydride afforded the Boc-protected saturated intermediate **51**, which after treatment with sodium ethoxide underwent elimination of the ether in C-2. The allylic alcohol **52** can't be directly alkylated and it's necessary to introduce the pentoxy group *via* aziridine opening; the requested aziridine **53** was formed using Mitsunobu conditions. Treatment of aziridine **53** with 3-pentanol in the presence of a Lewis acid yielded Boc-protected oseltamivir **54**.

1.2.3 Tamiphosphor and other bioisosteres

Design of bioisosteres is a convenient strategy for structural modification in drug design.[90] In particular, phosphonate group is a well-known biosisostere of the carboxylate moiety in medicinal chemistry. The phosphonate moiety is able to bind guanidinium cations with stronger electrostatic interactions than carboxylate anion, which could be advantageous for the binding to NA active side since an arginine triad (Arg292, Arg371 and Arg118) interact with the acidic functional group at carbon C-1 of oseltamivir.

Fang et al reported a synthesis of a phosphonate derivative of oseltamivir (Tamiphosphor, **55**) which showed enhanced potency in the NA inhibition and antiflu assays, high selectivity index and no toxicity to the host cell.[91] Streicher and coworkers reported a short and efficient approach to convert the available azide **56**, the precursor of Tamiflu, into tamiphosphor **55** (Scheme 7).[92]



Scheme 7. Tamiphosphor synthesis reported by Streicher,[92] possible pathway to monoalkylated tamiphosphor derivatives represented in dashed arrows. *Reagents and conditions.* (i) NaOH 0.5M; (ii) Vilsmeier reagent, *N*-hydroxypyridinethione, CF_3CH_2I, irradiation; (iii) 1. PMe_3, THF; 2. Boc_2O, THF; (iv) $(CH_3O)_2P(O)H$, $[Pd(PPh_3)_4]$, Et_3N; (v) NaOH 0.5M; (vi) 1. TMSBr; 2. TFA.

Moreover, Streicher and co-workers were the first who reported that monoalkylated phosphonate derivatives are able to retain a negative charge on C-1 under physiological conditions which confers them essentially the same properties than oseltamivir.[92] Later, Fang's group also

demonstrated that monoalkylated tamiphosphor and guanidine-tamiphosphor derivatives maintain their activity by testing two sets of derivatives in a cell-based assay, the results showed potent anti-influenza activities for all the monoalkylated derivatives with EC_{50} in the low nanomolar range.[93] The guanidine-tamiphosphor analogs tested showed strong potency also against the oseltamivir-resistant H274Y mutant virus.

Later on, Streicher reported a solution for the tethering of tamiphosphor without impairing its binding potency. In the same publication, he probed that tamiphosphor could be used as a valid alternative to oseltamivir in the construction of neuraminidase-binding diagnostic tools (Figure 21).[94]

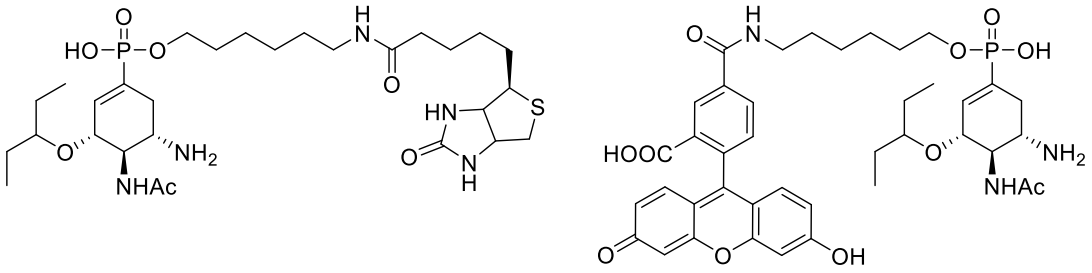


Figure 21. Probes prepared by Streicher based on tamiphosphor bearing the reporter groups biotin (left) and fluorescein (right)[94]

It is worth to mention that other bioisosteres of carboxylic acid, like hydroxamates and acyl sulfonamides, have been prepared but none of the reported derivatives showed higher activities than oseltamivir.[95]

The aforementioned synthetic route (Scheme 7) inspired our approach for the production of tamiphosphor-based probes (*vide infra*). The key intermediate **61** could be easily alkylated and regioselectively *O*-demethylated to afford monoalkylated tamiphosphor derivatives. Thus the furnished compound is suitable for the preparation of detection probes in a similar fashion to Streicher's approach.

1.3 The Inhibitor Screening Assays.

An important part of the research presented in this Thesis was dedicated to the synthesis of detection probes suitable for the development of different screening assays against several enzymes from influenza (*vide supra*). In the following pages a brief description of the two main assays for which the probes are intended will be provided. Both assays have been developed by our collaborators from the Department of Proteases of human Pathogens.

1.3.1 DNA-linked Inhibitor Antibody Assay

A multiwell plate-based DNA-linked Inhibitor Antibody Assay (DIANA) has been reported by Konvalinka's group recently as an ultrasensitive assay suitable for enzyme quantification as well as for quantitative screening of enzyme inhibitors with either purified or unpurified enzymes.[96] In DIANA, the wells of the titration plate are coated with immobilized antibodies able to capture the target enzyme. Subsequently, the enzyme binds the detection probe, which consists of a known inhibitor covalently bound to a reporter DNA oligonucleotide. The DNA is then detected by quantitative PCR.[96]

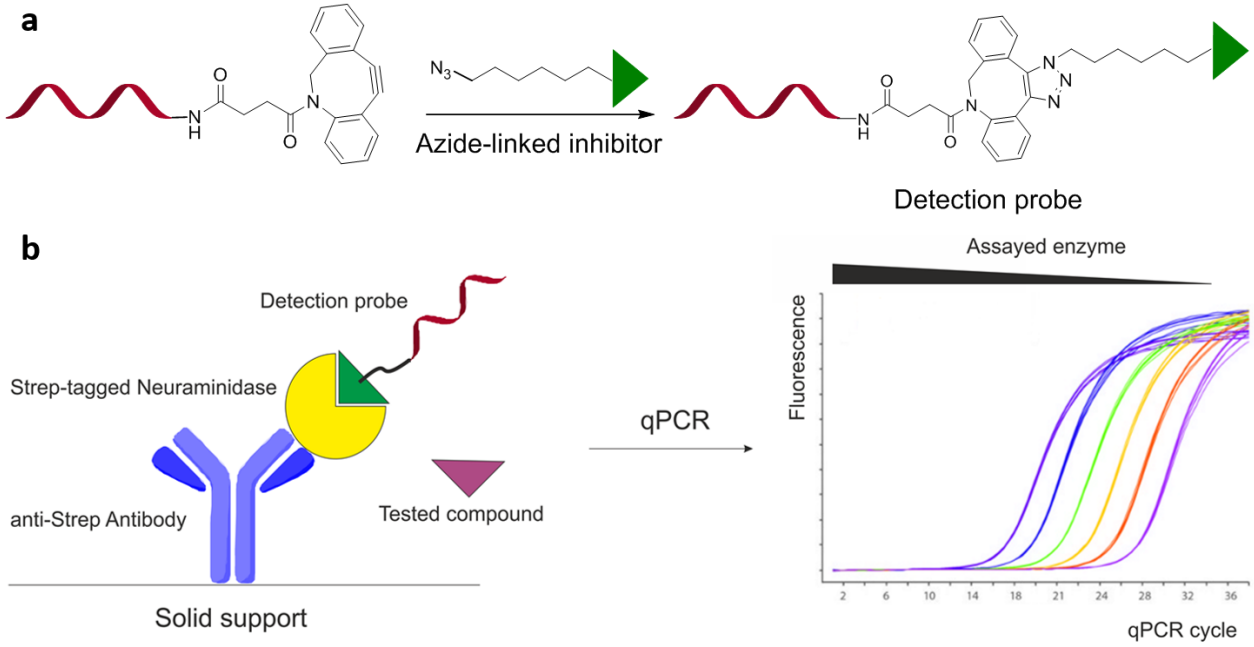


Figure 22. A. Outline of the assembly of the detection probe. **B.** Schematic representation of DIANA assay. The detection probe binds to the active site of enzyme captured on the solid support of the well after binding to the immobilized anti-Strep antibody. The detection probe competes with the tested compound, the amount of detection probe binding to the enzyme is determined by quantitative PCR.

The selectivity emerges from the dual recognition of the enzyme by the antibody and the detection probe meanwhile the high sensitivity is a consequence of the broad linear range of qPCR.

DIANA has a simple and reliable protocol, doesn't require the use of purified enzymes, is compatible with multiwell plates and has the exceptional ability to calculate the K_i of a compound from a single tested concentration. We demonstrate in this work that DIANA assay could be successfully used for screening of small-molecule inhibitors against neuraminidase and the PB2-cap-binding domain of influenza's RNA-polymerase.

1.3.2 Amplified Luminescent Proximity Homogenous Assay Screen

Amplified Luminescent Proximity Homogenous Assay Screen (Known as AlphaScreen) is an example of bead-based proximity assay and it was developed from a Luminescent Oxygen Channeling Immunoassay reported in 1994.[97] AlphaScreen relies on the use of a photoexitable latex bead (donor) and a second bead able to produce a chemiluminescent response (acceptor) when is placed in close proximity to the donor bead.

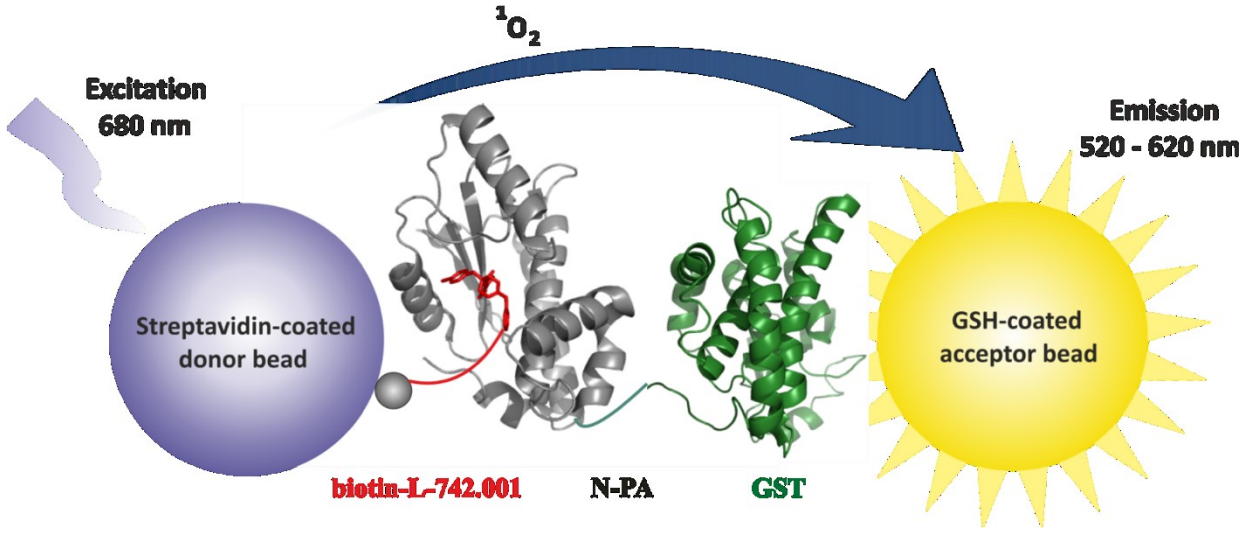


Figure 23. Outline of AlphaScreen Assay. Streptavidin-coated donor bead is attached to a biotin-tethered inhibitor of PA endonuclease. The acceptor bead is coated with GSH ligated to GST fused to the N-terminal part of PA subunit. When there is binding between the inhibitor and the enzyme the two beads are into close proximity and singlet oxygen can travel from the donor to the acceptor bead and produce the emission of light.

Upon laser excitation, the donor bead produces molecular singlet oxygen which diffuses across to react with a chemiluminescer in the acceptor bead which then emits light at 520–620

nm.[98] Singlet oxygen has a very short half-life (~ 4 μs) and it only reaches the acceptor bead if there is co-recognition of the analytes coated on the surface of both beats (in Figure 23 these are the biotinylated inhibitor L-742.001 at donor beat and the N-terminal part of subunit PA form RNA-polymerase at acceptor bead).

This technology is used for screening of low-molecular-weight inhibitors that compete with the biotin-tethered inhibitor (probe) for the binding to the enzyme. Better binders would displace the probe and as a result, the beads would be too distant which produces a detectable change in the intensity of the emission of light by the acceptor bead.

2. Objectives of the Thesis

- Find a convenient synthetic pathway for the introduction of modifications at position C-3 in oseltamivir's structure. Adapt and optimize the procedure for a rapid and direct production of oseltamivir derivatives designed to overcome oseltamivir-resistant mutations.
- Design a short and efficient modular synthesis for the production of C-5 derivatives of oseltamivir.
- Synthesize a set of C-5 oseltamivir derivatives designed to explore the binding mode to the 150-cavity.
- Prepare tamiphosphor and tamiphosphor-based inhibitors. Explore conditions for the decarboxylative halogenation step and for the introduction of phosphonate moiety into the cyclohexene ring.
- Design and synthesize neuraminidase-binding probes containing a sialylmimetic group suitable for DIANA assay.
- Design and prepare from commercially available building blocks probes based on the structure of the PB2 inhibitor VX-787. The probes prepared must be suitable for SPR and DIANA assay.
- Design and prepare probes based on the structure of the PA-endonuclease inhibitor L-742,001. The probes prepared must be suitable for the AlphaScreen assay.

3. Results and Discussion

The herein reported work is subdivided in two main sections; the first part is centered on the synthesis of new inhibitor candidates by modification of the structure of oseltamivir. For that, we used rational drug design approach to pursuit an improvement in the inhibition activity of the molecule. The second part involves the synthesis of molecular tools (also known as probes) suitable for the development of assays which final objective is to screen libraries of small molecules for new lead inhibitors against influenza's neuraminidase, PB2 cap-binding domain, and PA endonuclease.

3.1 Synthesis of Oseltamivir derivatives

As mentioned above, oseltamivir is the most extensively used drug for the treatment and prophylaxis of influenza infections. Unfortunately, recent appearance of oseltamivir-resistant strains of the virus could compromise its efficiency in the event of future epidemics. In this work, we intended to synthesize NA inhibitors capable to overcome oseltamivir-resistance by the introduction of polar moieties at position C-3 in oseltamivir (Figure 24).

The structure of oseltamivir was also used as a template in the synthesis of C-5 derivatives. Unlike the C-3 derivatives, C-5 derivatives have the objective to target the 150-cavity adjacent to the active site and explore the binding in this region.

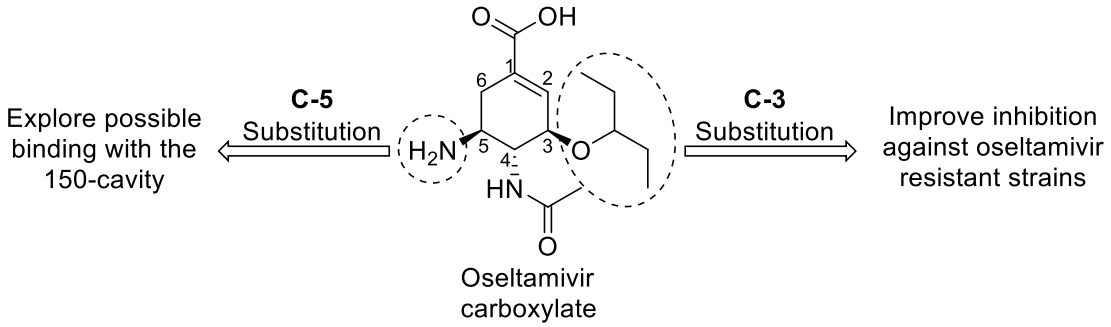


Figure 24. Two sets of oseltamivir-derivatives were synthesized by modification of either position C-3 or C-5.

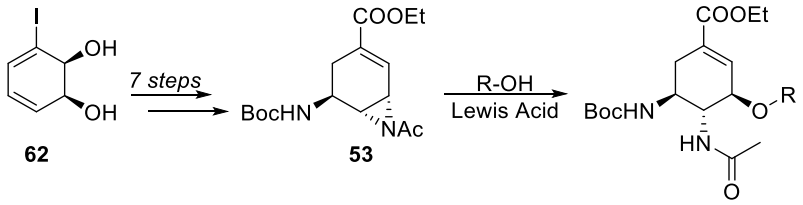
3.1.1 C-3 Modification of oseltamivir

Mutations H274Y and R294K which cause oseltamivir resistance were described previously. Both mutations cause the blocking the rotation of E276 and therefore the hydrophobic pocket cannot be formed. As a result, the binding of oseltamivir to the active site is destabilized.

On the other hand, zanamivir, which presents a glycerol group at C-3 instead of a pentoxy group, is less sensitive towards these mutations. We hypothesize that replacement of the hydrophobic pentoxy moiety by a polar sidechain capable of binding E276 would improve the inhibition activity. To demonstrate our hypothesis a set of new oseltamivir derivatives had to be synthesized.

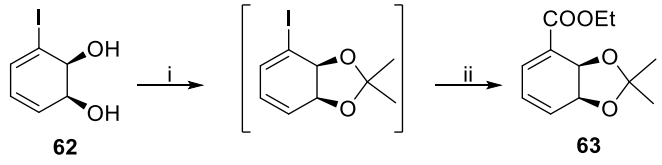
3.1.1.1 Hudlicky's approach

The Hudlicky's approach to oseltamivir was firstly selected as the synthetic pathway to follow for the production of C-3 derivatives of oseltamivir. The advantage of this approach is the late stage introduction of the pentoxy moiety *via* aziridine ring opening with pentan-3-ol (showed previously in Scheme 6). In a similar way, the opening of the aziridine **53** with different alcohols could potentially be a simple and direct approach towards derivatives with a polar sidechain (Scheme 8).



Scheme 8. Outline of the strategy for the synthesis of C-3 derivatives following Hudlicky's approach

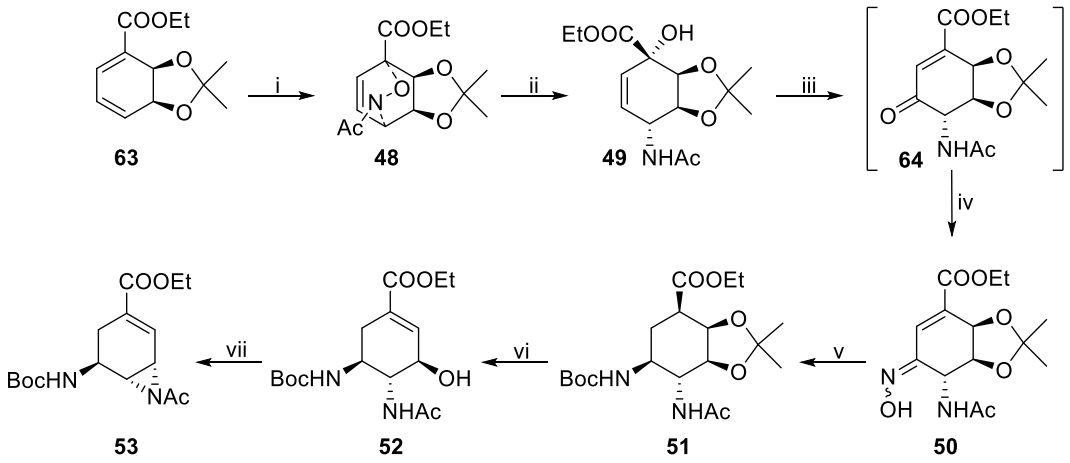
A second advantage is our direct collaboration with Hudlicky research group which provided access to large quantities of the starting material 3-iodocyclohexa-3,5-diene-1,2-diol **62**. The unstable 3-iododiol was extracted from a buffered solution and protected as the acetonide under acid catalyzed conditions (Scheme 9). The resulting product was then directly submitted for palladium-catalyzed carbonylation using standard conditions to afford the ethyl ester **63** in 85% yield over the two chemical tranformations.[99]



Scheme 9. *Reagents and conditions:* (i) TsOH ·HCl, 2,2-dimethoxypropane; (ii) CO (g), $Pd(OAc)_2$, PPh_3, Et_3N, EtOH, 40 °C, 2h, 85% yield.

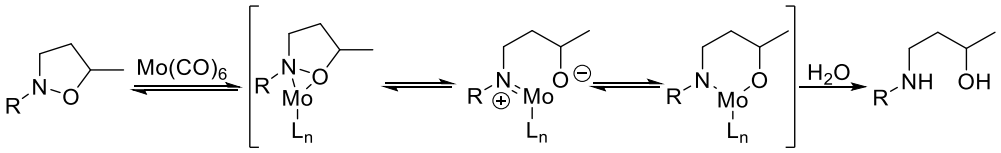
The subsequent steps leading to the synthesis of the aziridine **53** follow closely the synthetic pathway described in Scheme 6 (page 37). The diene **63** undergoes inverse-electron demand Diels-

Alder cycloaddition with an acylnitroso dienophile component generated *in situ* by oxidation of hydroxamic acid with sodium periodate, the resulting bicyclic oxazine **48** was obtained as a single isomer in 88% yield (Scheme 10).



Scheme 10. *Reagents and conditions:* (i) $NaIO_4$, acetohydroxamic acid, MeOH, 16h, 88% yield; (ii) $Mo(CO)_6$, $AcCN/H_2O$, 85 °C, 4h, 77% yield; (iii) CrO_3, Ac_2O, 0 °C, 5 min; (iv) $NH_2OH{\cdot}HCl$, py, $NaHCO_3$, EtOH, 16 h, 71% yield; (v) Rh/Al_2O_3 5%, 60 psi H_2, Boc_2O, EtOH, 16h, 69% yield; (vi) NaOEt 0.05M, EtOH, 1h, 87% yield; (vii) DIAD, $PPhMe_2$, Et_3N, -40 °C, 66% yield.

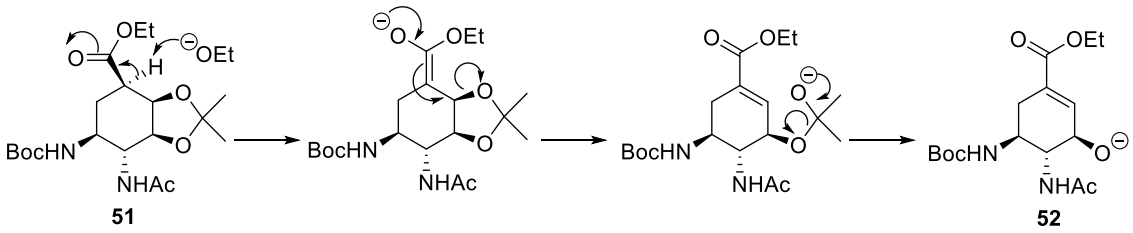
The N-O bond was then reduced using molybdenum hexacarbonyl under reflux in wet acetonitrile. The mechanism of the reduction is represented in Scheme 11; water acts as the source of protons and pushes the equilibrium to the right.[100]



Scheme 11. Mechanism for the reduction of a generic isoxazole using molybdenum hexacarbonyl according to Cicchi et al[100]

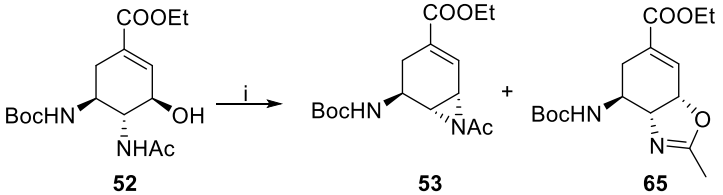
The resulting allylic alcohol **49** was obtained in 77% yield. Compound **49** underwent [3,3] oxidative transposition when a solution of chromium trioxide in acetic anhydride was added, the formed enone **64** was not isolated and transformed into the stable oxime **50** directly. The overall yield from the allylic alcohol **49** to the oxime **50** was around 70%. Special caution should be taken when the chromium oxide is being dissolved in acetic anhydride since the solution needs to be heated up to 80 °C and it tends to bubble vigorously upon addition of the chromium oxide. The unsaturated oxime **53** is then hydrogenated using a Parr shaker apparatus with a hydrogen pressure of 60 psi overnight period. The reduction is done in the presence of Boc-anhydride to yield the

protected amine **51** in a 69% yield. The acetonide **51** collapses under treatment with sodium ethoxide to produce the allylic alcohol **52** *via* E1cB elimination in 87% yield. The suggested mechanism is depicted in Scheme 12.



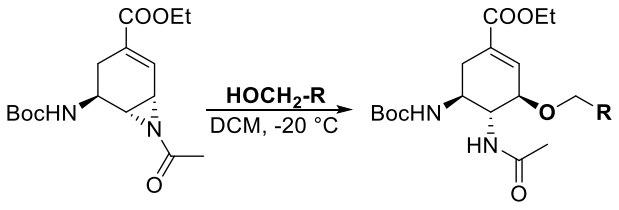
Scheme 12. Proposed mechanism for the elimination of the acetonide.

Direct alkylation of the allyl alcohol **52** has been reported to be inefficient with yields lower than 10 %.[89] Therefore, the introduction of the ether functional group at C-3 had to be done indirectly *via* opening of aziridine. The aziridine **53** was formed by intramolecular Mitsunobu reaction following the conditions reported by Shibasaki.[101] Despite the fact that the conditions applied were optimized to minimize the formation of the oxazoline **65** I always observed the formation of a non-negligible amount of by-product **65** which lowered the yield of the reaction to 60-65% in the best cases (Scheme 13).



Scheme 13. *Reagents and conditions:* (i) DIAD, Me_2PPh, Et_3N, -40 °C, 10 min.

Once the aziridine **53** was obtained, I attempted its opening with a set of relatively complex alcohols (Table 1). Two Lewis acids were used; $Cu(OTf)_2$ in catalytic amounts inspired by Corey's conditions[102] and stoichiometric excess of $BF_3{\cdot}OEt_2$ following Shibasaki's method.[101]



Scheme 14. Opening of the aziridine, structure of the alcohols used depicted in Table 1.

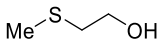
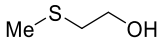
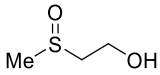
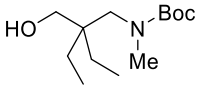
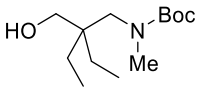
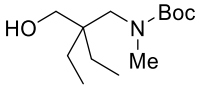
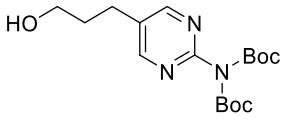
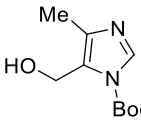
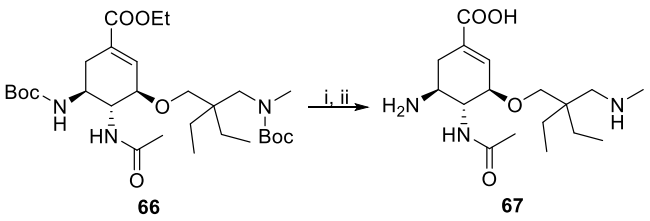
Alcohol	N° Run	Eq. Alc.	Lewis A.	Eq. LA	T (°C)	t (h)	Yield
	**1**	60	$BF_3 \cdot OEt_2$	1.5	-20	0.25	-
	**2**	10	$Cu(OTf)_2$	0.16	-20	16	-
	**3**	1.5	$BF_3 \cdot OEt_2$	0.16	-20	16	-
	**4**	2	$BF_3 \cdot OEt_2$	2	-20	0.5	8%
	**5**	5	$Cu(OTf)_2$	0.16	-20	16	34%
	**6**	5	$BF_3 \cdot OEt_2$	2	-10	1	34%
	**7**	4.5	$Cu(OTf)_2$	0.16	-20	16	-
	**8**	3.4	$Cu(OTf)_2$	0.16	-20	16	-

Table 1. The columns represent (from left to right); structure of the alcohols, number of the run, equivalents of the alcohol, Lewis acid used, equivalents of the Lewis acid, temperature, time and yield. All reactions were carried out in DCM under inert atmosphere.

2-(Methylthio)ethanol (run **1** and **2**) and its derivative 2-hydroxyethyl methyl sulfoxide (**3**) were the first alcohols used for the opening of the aziridine. Condition **1** used the alcohol as a co-solvent meanwhile conditions **2** and **3** used it in large and small excess respectively. Unfortunately, no formation of product was observed for either of these conditions. In the runs **4**, **5** and **6** I used the same alcohol but with different amount of equivalents and reaction times. In these runs, I obtained similar yields of the product by using either $Cu(OTf)_2$ or $BF_3 \cdot OEt_2$ as Lewis acid. Higher yields were actually obtained when the alcohol was used in larger excess. Disappointingly, when I used similar conditions to run **5** but for different alcohols (run **7** and **8**) I did not obtain the desired products.



Scheme 15. *Reagents and conditions:* (i) NaOH 0.5 M, 1,4-dioxane; (ii) neat TFA, 1h

Despite the low yields, enough of the ether **66** was obtained to finish up the synthesis. The following step involved the hydrolysis of the ethyl ester and Boc-deprotection of the two amines by treatment with neat TFA. Compound **67** has a similar structure to oseltamivir but featured a secondary amine in the sidechain introduced at C-3 position. The reason to have an amine group in this position is to attempt binding of the basic moiety with the acidic Glu276 in a similar way as zanamivir does.

Compound **67** should be tested for inhibition of the neuraminidase activity of an H1N1 virus in collaboration with Konvalinka's group at the Institute of Organic Chemistry and Biochemistry of the CAS.

3.1.1.2 Conclusions from Hudlicky's approach

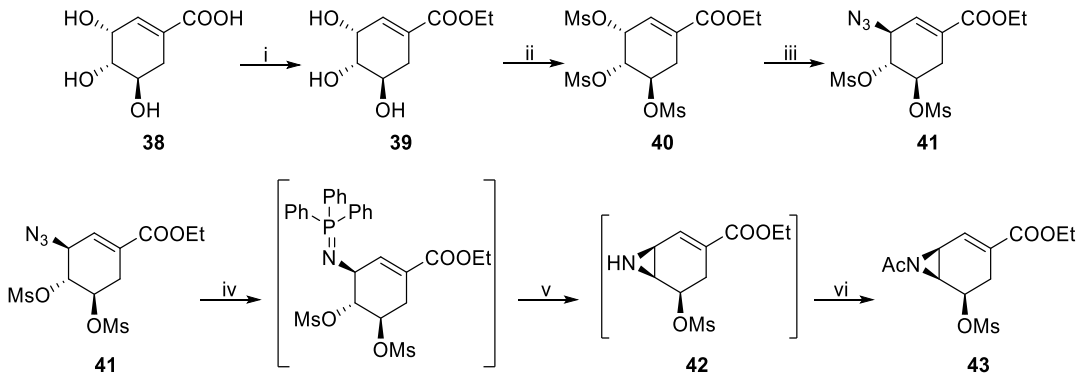
1. I was able to follow and repeat the synthetic approach reported by Hudlicky.
2. I investigated the key aziridine-opening step with different alcohols and under catalytic and stoichiometric conditions.
3. A new oseltamivir derivative was synthesized and purified, the compound is undergoing testing.

Although I was able to complete the synthesis and prepare a promising new oseltamivir derivative we decided to abandon this approach due to the complexity of the reactions involved. Other important reasons that led to the discontinuation of this pursue were the low overall yield (19% from the 3-iododiol **62** to the aziridine **53**), the limited scope of alcohols that were successfully introduced and the large excess of alcohol needed for the aziridine-opening reaction.

3.1.1.3 Shi's approach

The second synthetic strategy used for the preparation of C-3 modified oseltamivir derivatives followed closely the approach reported by Shi et al. depicted in Scheme 3.[74] The advantages of this approach stem from the simple chemical transformations that are utilized and the use of commercially available (-)-shikimic acid as a starting material. Another positive aspect is the rapid formation of key aziridine (only four steps from the starting material) and the introduction of the ether moiety at C-3 in the late stage of the synthesis.

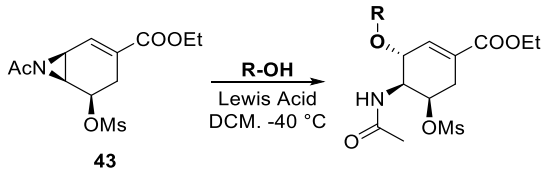
The first three steps of the synthesis are identical to the ones described previously in Scheme 4 (page 35). Esterification of (-)-shikimic **38** acid under acidic conditions with ethanol yielded the ethyl shikimate **39** in 86% yield.[103] The three hydroxyl groups were mesylated simultaneously using an excess of methanesulfonyl chloride and trimethylamine, the mesylated product **40** was obtained in quantitative yields (97%). Highly regioselective nucleophilic substitution at the allylic position by azido moiety can be achieved by carefully adding an aqueous solution of sodium azide to the mesylate meanwhile keeping the temperature always below 5 °C, the reaction gives the corresponding azide **41** with the (*S*)-configuration at C-3 in an 89% yield.



Scheme 16. Compound **44** was transformed into **46** without isolation of the intermediates. *Reagents and conditions.* (i) $SOCl_2$, EtOH; (ii) MsCl, Et_3N, DMAP; (iii) NaN_3, acetone/water, 0 °C; (iv) Ph_3P, THF; (v) H_2O, Et_3N; (vi) Ac_2O, Et_3N, 51% overall yield.

Staudinger reduction of the azide **41** gave the free amine which in the presence of triethylamine undergoes nucleophilic attack at C-4 and closes the aziridine ring *in situ*. Aziridine **42** was difficult to separate from the triphenylphosphine oxide by column chromatography. Shi and co-workers solved this problem by diluting the aziridine **42** in an acidic aqueous solution and extracting the triphenylphosphine oxide into the organic layer, the pH of the aqueous solution was

then set up to basic and the neutral aziridine was extracted to the organic layer. Low yields were always obtained by using this purification method hence I decided to submit the crude aziridine mixture for the acetylation step and purify it afterwards. By this method I was able to transform the azide **41** into the *N*-acetyl aziridine **43** in a 51% yield over the three chemical steps which is considerably higher than the yield obtained when the aziridine **42** was isolated.



Scheme 17. The opening of the aziridine, alcohols used are depicted in table 2

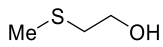
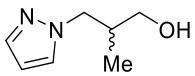
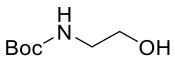
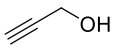
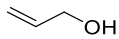
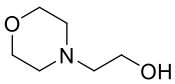
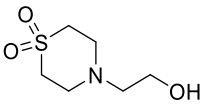
Alcohol	N° Run	Eq. Alc	Lewis A.	Eq. LA	T (°C)	t (h)	Yield
	**1**	10	$BF_3 \cdot OEt_2$	2	-40	16*	-
	**2**	8	$Cu(OTf)_2$	0.1	0	16*	>10%
	**3**	10	$BF_3 \cdot OEt_2$	1.5	-40	1	60%
	**4**	10	$BF_3 \cdot OEt_2$	1.5	-40	1	58%
	**5**	10	$BF_3 \cdot OEt_2$	1.5	-40	1	64%
	**6**	10	$BF_3 \cdot OEt_2$	1.5	-40	16*	-
	**7**	7	$BF_3 \cdot OEt_2$	2.5	-40	16*	-

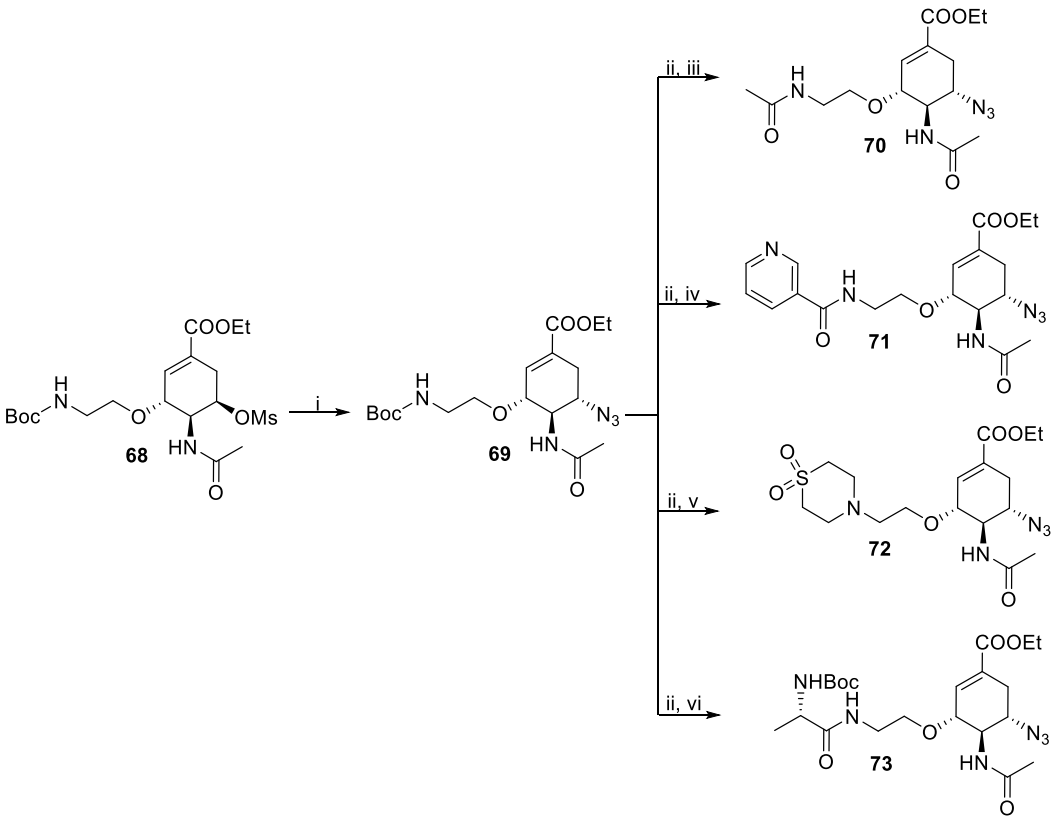
Table 2. The columns represent (from left to right); structure of the alcohols, number of the run, equivalents of the alcohol, Lewis acid used, equivalents of the Lewis acid, temperature, time and yield. All reactions were carried out in DCM under inert atmosphere, $BF_3 \cdot OEt_2$ was used as 1.65 M in DCM. *The reaction was monitored for 1 h but no product was observed, therefore reaction was left overnight at r.t.

Once I had the key aziridine **43** in hands, I attempted the opening with 2-(methylthio)ethanol (run **1**). Unfortunately, as it happened for the previous aziridine **53**, the reaction was unsuccessful. The reaction using a more complex alcohol (run **2**) resulted in the formation of product but only in negligible quantities. On the other hand, when much simpler

alcohols were used (run **3**, **4** and **5**) I obtained the corresponding ether in fair yields. Increasing the complexity of the alcohols used led always to unsuccessful results as runs **6** and **7** demonstrate.

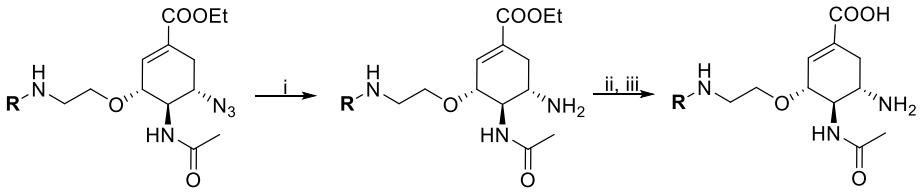
Due to the narrow range of alcohols that were suitable for the aziridine opening, we decided to change slightly our strategy and open the aziridine only with Boc-protected ethanolamine, propargyl alcohol, and allyl alcohol (runs **3**, **4** and **5**). The corresponding products (**68**, **79** and **81** respectively) were supposed to be used as branching points for introduction of more complex moieties and will be described in the following paragraphs.

The ether **68** resulting from opening the aziridine with Boc-protected ethanolamine, was transformed into the corresponding azide **69** by nucleophilic substitution of the mesyl group in C-5 with sodium azide, this second substitution was performed by refluxing in aqueous ethanol overnight in contrast with the C-3 substitution that needed low temperature and short reaction times to enhance the regioselectivity.



Scheme 18. *Reagents and conditions.* (i) NaN_3, EtOH/H_2O 5:1, reflux 24h, 81% yield; (ii) TFA/DCM 1:5, 1h; (iii) Ac_2O, Et_3N, 3h, r.t., 88% yield; (iv) nicotinoyl chloride hydrochloride, Et_3N, 4h, 71% yield; (v) divinyl sulfone, Et_3N, 3h, 60 °C, 89% yield; (vi) Boc-*L*-alanine, TBTU, Et_3N, 16h, 58% yield.

The amine of **69** was then de-protected by treatment with TFA, the free amine was used as a vector for introduction of several groups for the coverage of a broader chemical space. Namely, the free amine was treated with acid anhydrides, acyl chlorides, Michael acceptors and activated amino acids (Scheme 18). These reactions afforded the products **70**-**73**. This "proof of concept" set of compounds showed that introduction of ethanolamine could be a viable and flexible vector for further derivatization at the C-3 position.



Scheme 19. *Reagents and conditions.* (i) Lindlar catalyst (60 w%), 1 atm H_2, EtOH, 3h r.t.; (ii) NaOH 0.5M, 1,4-dioxane; (iii) neat TFA, 1h.

The furnished azides **70**-**73** were reduced by hydrogenation using Lindlar catalyst to prevent the hydrogenation of the C_1=C_2 double bond. The last step was the hydrolysis of the ethyl ester moiety by treatment with sodium hydroxide solution and further Boc-deprotection with TFA if needed (Scheme 19). Purification using preparative HPLC furnished five oseltamivir derivatives bearing polar sidechains at C-3 (Figure 25).

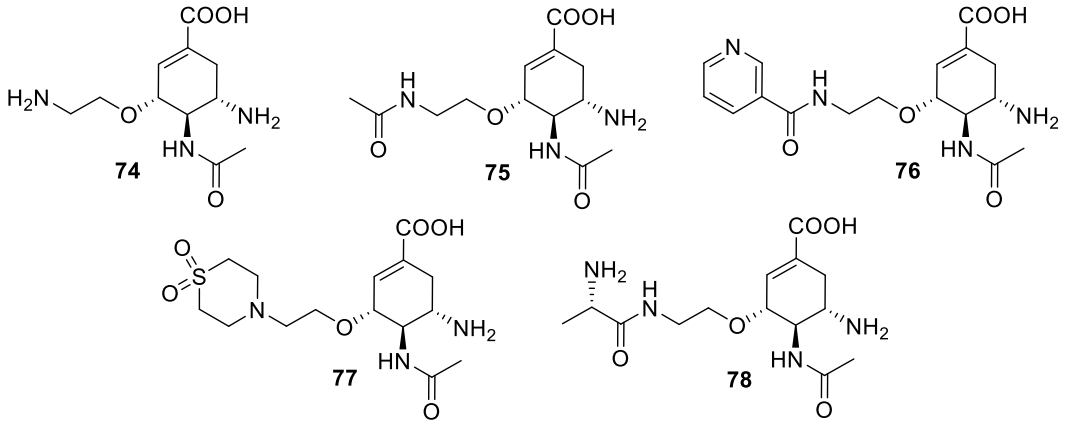
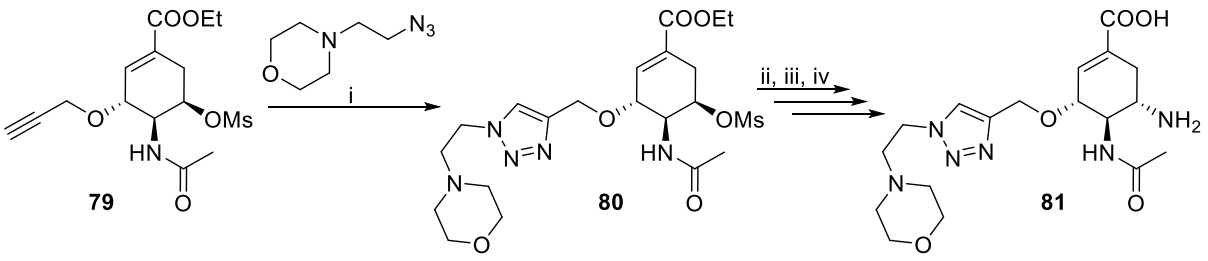


Figure 25. Structure of five new oseltamivir derivatives**.**

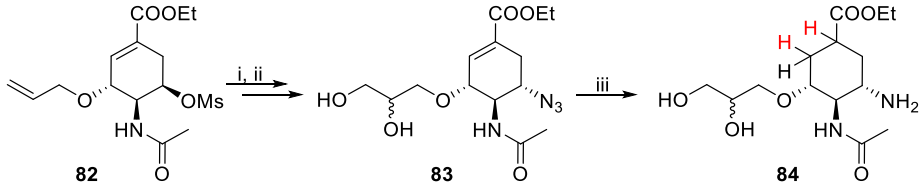
On the other hand, the ether intermediate **79** obtained by opening the aziridine with propargyl alcohol was designed to be used for introduction of different sidechains *via* CuAAC (Scheme 20). In this case, the click reaction had to be done prior to the azide introduction at C-5. The reduction of the azide and hydrolysis of the ester steps were identical to the conditions described in Scheme 19.



Scheme 20. *Reagents and conditions.* (i) $[Cu(CH_3CN)_4]PF_6$, TBTA, THF, 24 h, r.t., 77% yield; (ii) NaN_3, EtOH/H_2O 5:1, reflux 24h, 37% yield; (iii) Lindlar catalyst (60 w%), 1 atm H_2, EtOH, 3h r.t., 85% yield; (iv) NaOH 0.5M, 1,4-dioxane, 57% yield.

Only one compound was synthesized following the CuAAC approach due to changes in the priority of the research. This method became the central work of one of my co-workers and further research is being done solely by him.

Lastly, the introduction of the allyl alcohol in C-3 position allowed us to perform asymmetric dihydroxylation of the double bound (Scheme 21). AD-mix-α was used for the dihydroxylation of olefin **82**; the oxidized intermediate **83** was obtained in 61% yield. Unfortunately, when I attempted to reduce the azido moiety I have observed rapid hydrogenation of the double bond despite the fact that Lindlar catalyst was used. This could happen due to the presence of two vicinal hydroxyl groups which could bind tightly the catalyst and direct it towards C_1=C_2 double bond.



Scheme 21. *Reagents and conditions.* (i) NaN_3, EtOH/H_2O 5:1, reflux 24h, 37% yield; (ii) AD-mix-α, *t*-BuOH/H_2O 1:1, 0 °C, 24h, 61% yield; (iii) Lindlar catalyst; (60 w%), 1 atm H_2, EtOH, 3h r.t., 95% yield.

Due to time restriction and the project moving on, I had no time to improve the aforementioned approach utilizing allyl alcohol. The problems encountered with the reduction of the azide could apparently be solved by using Staudinger reduction instead of catalytic hydrogenation.

3.1.1.4 Conclusions from Shi's approach.

1. I was able to follow and replicate the synthetic approach reported by Shi et al.

2. The yield of the aziridine formation step has been improved.
3. Several alcohols were screened for the aziridine-opening reaction.
4. Boc-protected ethanolamine, propargyl alcohol and allyl alcohol were proven to be suitable for the opening of the aziridine, further derivatization could be done allowing us to explore a broad chemical space.
5. Six new C-3 modified oseltamivir derivatives were prepared, the compounds are undergoing testing at IOCB.

The aforementioned approach represents a simple and efficient way for the synthesis of broad number of oseltamivir derivatives. Unfortunately, due to time restriction and the project moving on, a large library of oseltamivir derivatives has not been prepared.

3.1.2 C-5 Modifications of oseltamivir

As described in the chapter discussing the 150-cavity, recent studies showed that the 150-cavity is not only present in group A but also in group B neuraminidases. The so-called 150-loop is flexible and allows the cavity to adopts "open" and "closed" conformations.[30] Molecules capable of binding the active site of NA and simultaneously interact with the aminoacids forming the 150-cavity could feature better affinity towards particular subtypes of neuraminidases.

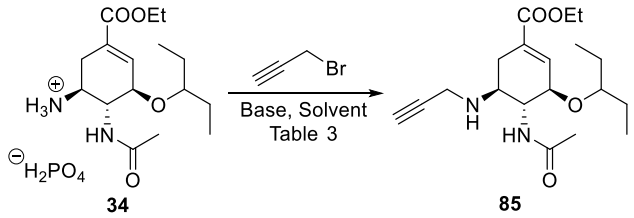
Modifications at position C-5 of oseltamivir have the objective of exploring the binding mode of new inhibitors to the 150-cavity without compromising the interactions with the NA catalytic site. The amine group located at C-5 in the oseltamivir structure is directed towards the cavity and therefore has been used as the modification site (Figure 8).

3.1.2.1 1,2,3-Triazole derivatives

With the objective of preparing a set of C-5 modified oseltamivir derivatives in a short period of time, I designed a fast and efficient modular approach which relies on the introduction of several moieties *via* copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC). Previous research highlighted the importance of having a basic group at carbon C-5. Hence I prepared the first set of C-5 oseltamivir derivatives that conserve the basic amine in this position. We postulated that direct

alkylation of the amine group with propargyl alcohol followed CuAAC reaction with a collection of azides could be a rapid way to achieve a broad number of derivatives.

Direct alkylation of amines usually leads to a mixture of products because the generated secondary amine retains its nucleophilicity and usually react rapidly with a second equivalent of alkylating agent. After a short screening of conditions for the alkylation of oseltamivir phosphate (Table 3), I was able to obtain the mono-propargylated oseltamivir **85** in reasonable yields minimizing the formation of side-products.

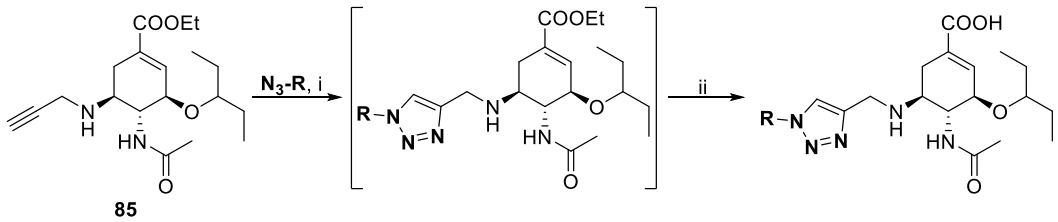


Scheme 22. Alkylation of oseltamivir phosphate

Base	Base Eq.	Reagent Eq.	Solvent	Temp. (°C)	Time (h)	Yield
K_2CO_3	3	1	CH_3CN	r.t.	24	40 %
K_3PO_4	3	1	CH_3CN	r.t.	24	24 %
K_2CO_3	5	1	CH_3CN	r.t.	24	63 %
LiOH	3	1.2	DMF*	r.t.	24	33 %
K_2CO_3	5	1.2 + 0.1**	CH_3CN	r.t. then 50 °C	24 + 4	65 %

Table 3. *DMF was mixed with powdered molecular sieves 4 Å. **1.2 equivalents of propargyl bromide were used, the reaction was stirred 24h at r.t. followed by addition of 0.1 eq. of propargyl bromide and heating to 50 °C for 4 h.

Propargylated oseltamivir **85** was combined with a series of azide-containing fragments by CuAAC. For this click reaction, I used an excess of copper(II) sulfate pentahydrate which was reduced *in situ* to Cu(I) by sodium L-ascorbate. The resulting crude triazole intermediates were directly hydrolyzed and after purification by preparative HPLC, the desired set of C-5 oseltamivir derivatives was obtained.



Scheme 23. *Reagents and conditions.* (i) $CuSO_4 \cdot 5H_2O$, (+)-sodium L-ascorbate, THF/H_2O 2:1, 45 °C, 2h; (ii) NaOH 0.5 M, 1,4-dioxane, 2h, r.t.

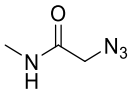
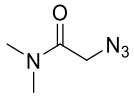
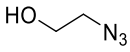
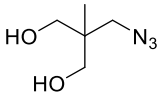
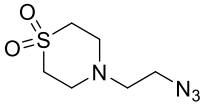
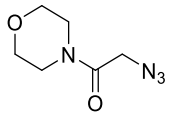
Azide	N° Run	Eq. Azide	Eq. $CuSO_4 \cdot 5H_2O$	Eq. L-ascorbate	Yield*
	**1**	1.2	2	5	55%
	**2**	1.2	2	5	21%
	**3**	1.2	2	5	48%
	**4**	1.2	2	5	45%
	**5**	1.2	2	5	30%
	**6**	1.2	2	5	12%**

Table 4. Azides and conditions used for the CuAAC reaction. *Yields after hydrolysis of the ester moiety and preparative HPLC purification. **The yield corresponds to the free acid formed by hydrolysis of the amide.

The employed azide-containing synthons possess either hydrogen bonding donor or hydrogen bonding acceptor moieties. All the CuAAC reactions generated the desired products (showed in Figure 26). Unfortunately, during the hydrolysis of the ester in run **6** I observed the hydrolysis of the amide from the morpholine ring. The unexpected free carboxylic acid **91** was identified, isolated and submitted for testing with the other derivatives since it could also be a valid inhibitor candidate.

Compounds **86** and **87** (Figure 26) differ in their ability to participate in hydrogen bonding with the residues from the cavity. Compounds **88** and **91** present the same short chain but differ in the oxidation state of the terminal functional group. Finally, compounds **89** and **90** bear much bulkier substituents which could fit tighter into the cavity helping to delimitate the size of it. We intended to use these derivatives to shed light on the mode of binding to the 150-cavity. These compounds are undergoing testing at Konvalinka's group in IOCB, the results will be soon published in the form of a research paper.

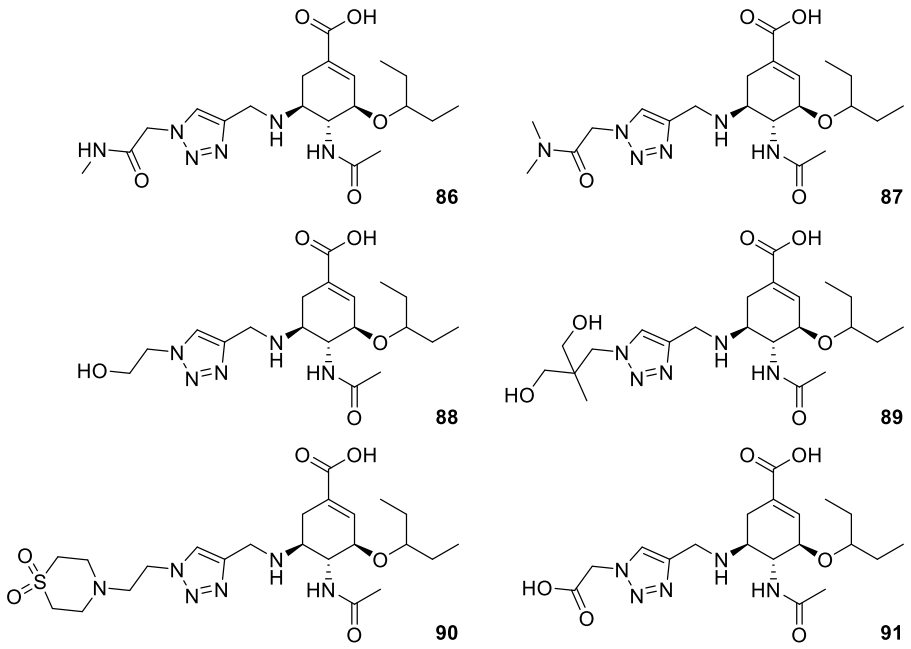
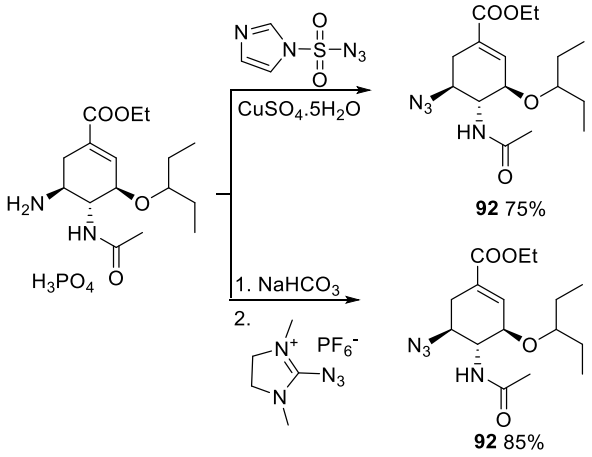
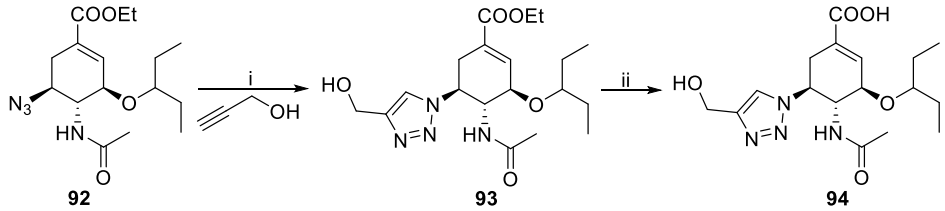


Figure 26. Structures of the C-5 oseltamivir derivatives synthesized using the CuAAC approach.

The other possible approach, in which the oseltamivir core bears the azido moiety and the fragment introduced contains the triple bond was also attempted. Although the binding activity could be impaired by replacing the basic amine group with a triazole moiety, we decided to synthesize some of these derivatives and observe how this modification affects the binding. The first step was to transform oseltamivir phosphate into the azide **92** by treatment with diazo-transfer reagents. Imidazole-1-sulfonyl azide hydrochloride and 2-azido-1,3-dimethylimidazolinium hexafluorophosphate were prepared according to the literature and successfully used for the synthesis of **92** (Scheme 24).[104,105]



Scheme 24. Two diazo-transfer reagents were used for the synthesis of azide **92**.



Scheme 25. *Reagents and conditions.* (i) CuI, DIPEA, toluene, 2h, 91% yield; (ii) NaOH 0.5M, 1,4-dioxane, 24 h, r.t., 85% yield.

After CuAAC with propargyl alcohol I obtained the triazole **93** in quantitative yields, further hydrolysis of the ethyl ester group to the acid furnished the new neuraminidase inhibitor **94** (Scheme 25). Although the inhibition potency of **94** was nearly 20 times worse than oseltamivir, the crystal structure of the inhibitor binding to the catalytic site of neuraminidase was obtained (Figure 27). This crystal structure as well as the binding activities of the inhibitors designed to bind the 150-cavity will be published in the form of a research publication.

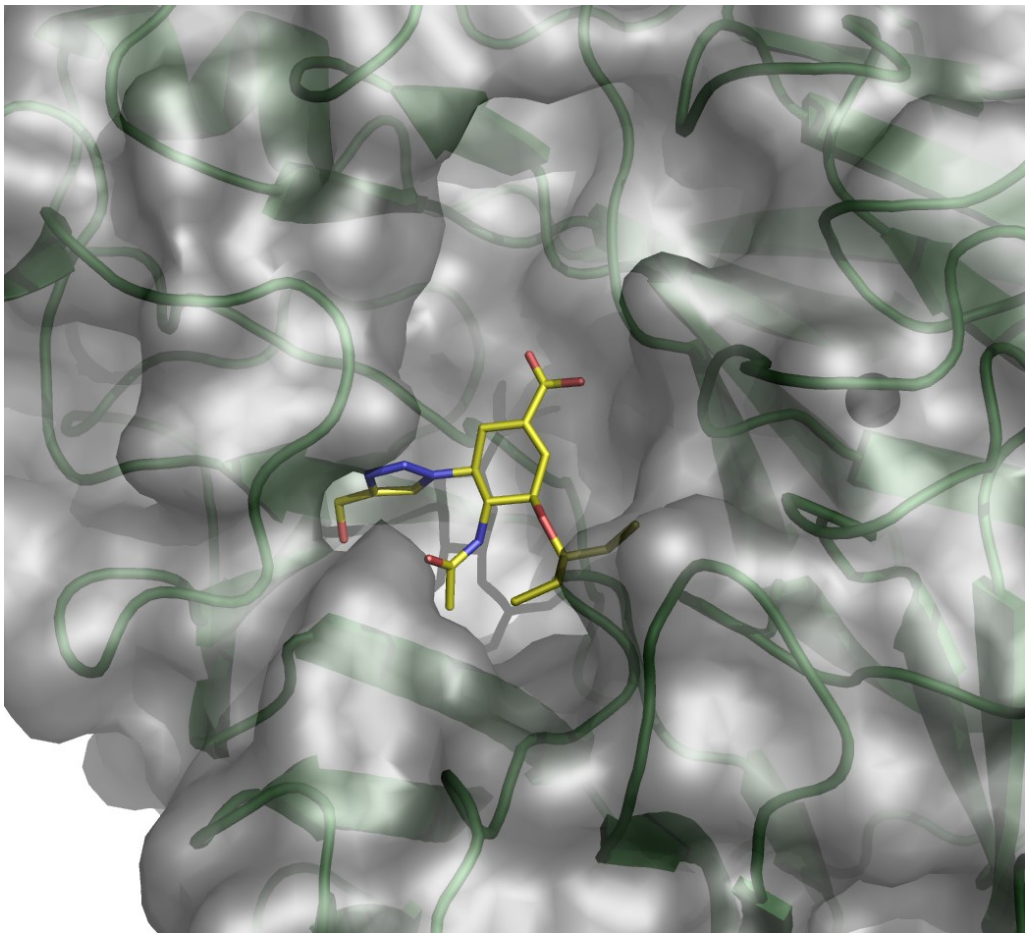
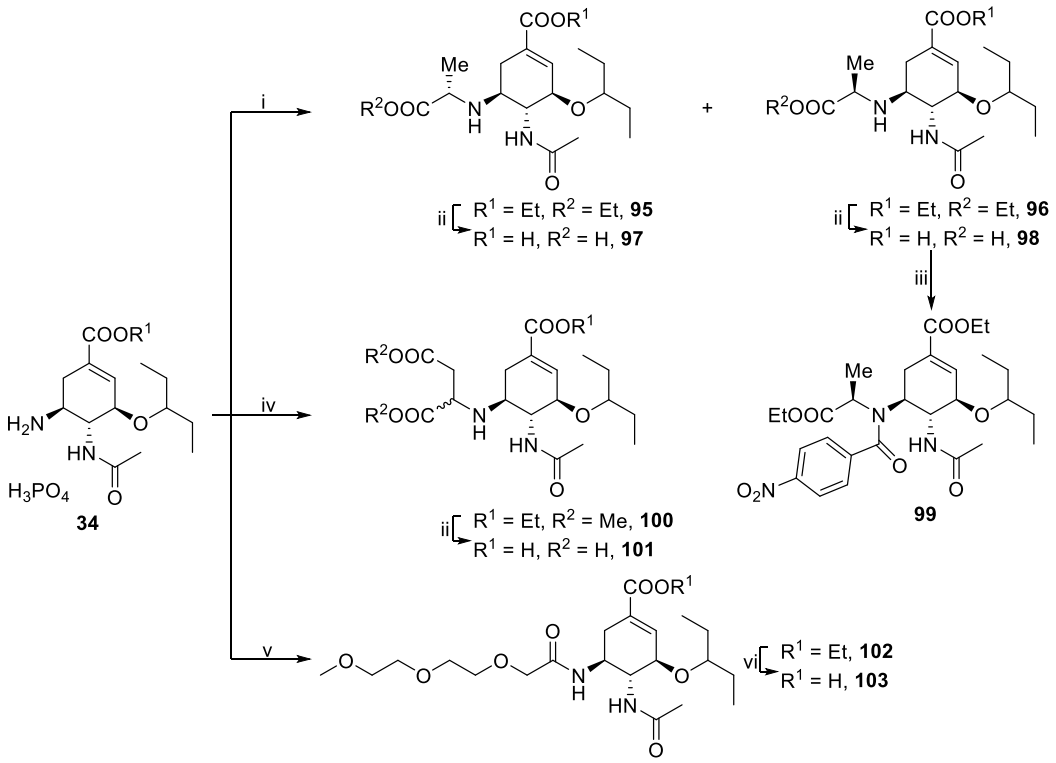


Figure 27. Crystal structure of the **94** (shown as a stick model) binding to the active site of neuraminidase

3.1.2.2 Alkylation at the C-5 amine

The amine at carbon C-5 is the most nucleophilic moiety present in the oseltamivir structure. Therefore, it could be selectively used to produce a broad number of oseltamivir derivatives in a direct and straightforward way. Unsurprisingly this approach has been extensively studied in the past and many C-5 derivatives have been reported,[106-110] nevertheless in this section we describe the synthesis of some new C-5 derivatives that were prepared. The prepared inhibitors were intended to be used to demonstrate the validity of DIANA assay as a screening method. Moreover, a set of well-known inhibitors of neuraminidase had to be prepared to compare the inhibition values obtained from our assay with the previously published data based on fluorometric enzyme (MUNANA) assay.



Scheme 26. *Reagents and conditions.* (i) Ethyl 2-bromopropionate, $NaHCO_3$, 73% yield; (ii) 0.5 M NaOH, 1,4-dioxane; (iii) 4-nitrobenzoyl chloride, Et_3N, 36% yield; (iv) $NaHCO_3$, dimethyl maleate, 75% yield; (v) 2-[2-(2-methoxyethoxy)-ethoxy]acetic acid, TBTU, Et_3N, DMF, 93% yield; (vi) 0.5 M NaOH, 1,4-dioxane, 70% yield.

Treatment of oseltamivir phosphate with ethyl 2-bromopropionate under basic conditions produced a mixture of the two diastereomers **95** and **96.** After their separation from each other followed by saponification of the ester, the diastereomers **97** and **98** were obtained in 38% and 34%

yields respectively. In order to determine the absolute configuration of the diastereomers I attempted several crystallization experiments. Unfortunately, no suitable monocrystal of compound **98** for X-ray analysis has been obtained. We decided to solve this issue by introducing a group that facilitates the crystallization. Hence compound **99** was prepared, which eventually yielded a suitable monocrystal whose X-ray structure (depicted in Figure 28) revealed the absolute configuration.

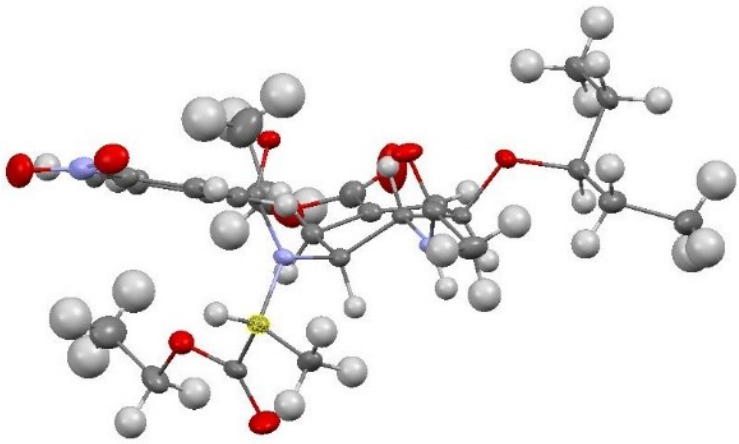
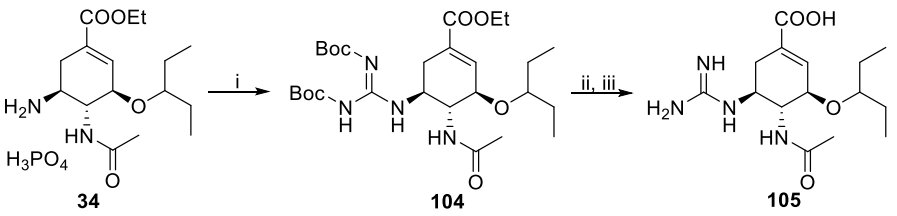


Figure 28. Crystal structure of compound **99**. The stereogenic center is depicted in yellow color (*R*).

When oseltamivir phosphate was alkylated with dimethyl maleate I obtained **100** as an inseparable mixture of diastereomers, after hydrolysis of the ester the free acid **101** was obtained and used as a diastereomeric mixture.

In the case of the next inhibitor, we intentionally decreased the basicity at C-5 by forming an amide at carbon C-5. This poor inhibitor was prepared to demonstrate the viability of DIANA assay on a broader range of K_i values. In this case, oseltamivir phosphate was submitted to standard amide coupling with 2-[2-(2-methoxyethoxy)ethoxy]acetic acid using TBTU as the coupling agent, subsequent hydrolysis of the ester gave the compound **103** in 65% overall yield.

The last inhibitor that was prepared by C-5 modification was the oseltamivir analogue **105** which is a well-known and potent neuraminidase inhibitor firstly prepared by Gilead Science in 2000 (Scheme 27).[111] Compound **105** was prepared to provide a comparison between the inhibition constants obtained by novel DIANA assay with the results from well-established MUNANA assays.



Scheme 27. *Reagents and conditions.* (i) *N,N*'-di-(*tert*-butoxycarbonyl)thiourea, HgCl, Et_3N, DMF, 24 h, 89% yield; (ii) NaOH 0.5 M, 1,4-dioxane, 24 h, 48% yield; (iii) TFA, 1h, r.t., 43% yield.

The binding constants of the inhibitors reported in this section will be discussed in the upcoming chapter covering the synthesis of detection probe suitable for DIANA assay.

3.1.3 C-1 Modifications (Tamiphosphor)

Tamiphosphor is a well-known bioisostere of oseltamivir which presents enhanced potency and high selectivity. Oseltamivir derivatives bearing other bioisosteres of carboxylic acid at C-1 have been reported but none showed better binding activities than tamiphophor (Figure 29).[95]

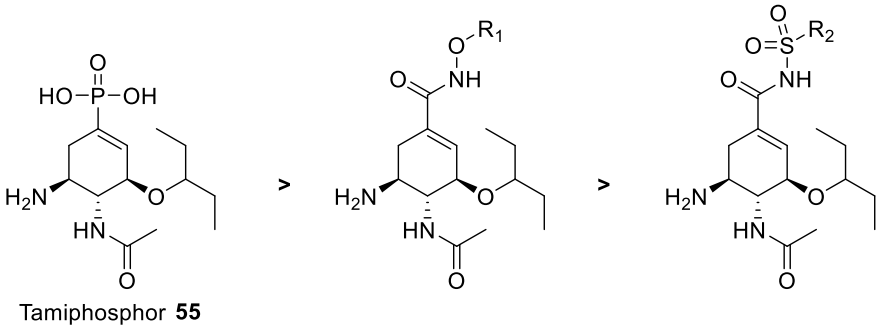


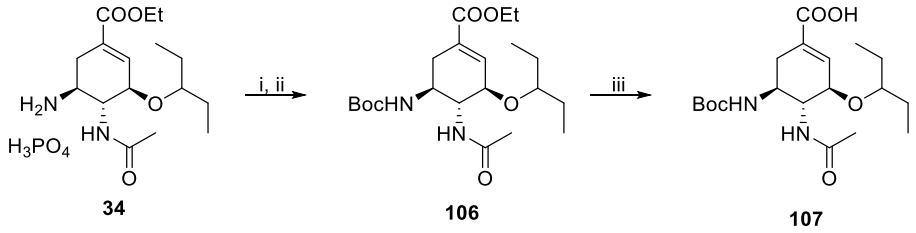
Figure 29. Tamiphosphor **55** exhibits higher inhibitory potency than the hydroxamate derivatives (center) and the sulfonamine derivatives (right).[95]

Tamiphosphor plays a pivotal role in this Thesis since it was used as a cornerstone compound to prepare particular neuraminidase inhibitors as well as to prepare the detection probes for the development of DIANA assay. Moreover, a tamiphosphor derivative was also used for the construction of a polymeric material that could be used in the treatment of patient in life-treating condition. This side-project is not cover by the Thesis since we do not want to disclose our findings at this time.

The first synthetic approach that I empoyed for preparation of tamiphosphor **55** was inspired by the work reported by Streicher (see Scheme **7**, page 38). Even though Streicher's procedure (which features a decarboxylative halogenation of the azide intermediate **57**) was quite

optimal, a more convenient synthesis could be done by using commercially available oseltamivir phosphate **34** as the starting material instead of the azide intermediate **56** used by Streicher.

Tamiphosphor **55** was prepared in my case from commercially available oseltamivir phosphate **34**. The synthesis starts with free basing of oseltamivir followed by protection of the amine at carbon C-5 with Boc-anhydride. The ethyl ester **106** was then hydrolyzed to form the corresponding free acid **107** in quantitative yields (Scheme 28).



34 **106** **107**

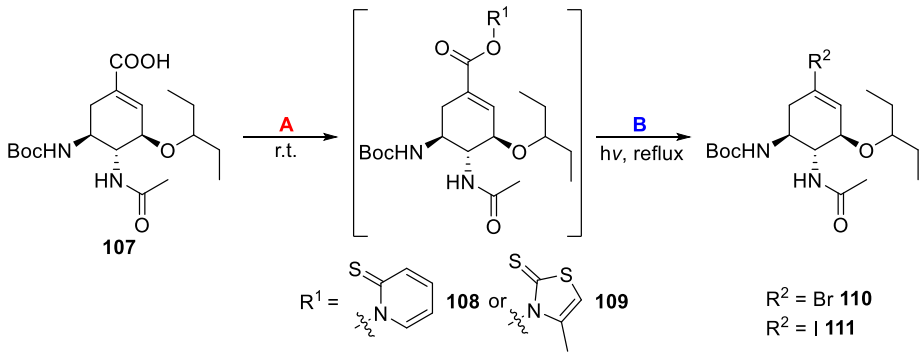
Scheme 28. *Reagents and conditions.* (i) $NaHCO_3/H_2O$; (ii) Boc_2O, Et_3N, 98% yield; (iii) 0.5 M NaOH, 1,4-dioxane, 95% yield.

The following step is the decarboxylation of **107**; the conditions used by Streicher[92] (Vilsmeier reagent and *N*-hydroxypyridinethione) were initially attempted but gave low yields. An alternative approach described by Gunasekera was subsequently explored.[112] In this case, the decarboxylative halogenation was done *via* formation of a standard Barton ester intermediate (Scheme 29). Although the reaction was successful I decided to optimize and simplify the procedure.

The conditions used for the Barton decarboxylation are summarized in Table 5, in all cases the Barton thioester was formed by activating the acid with coupling agents. Once formed, the Barton thioester undergoes radical decarboxylation upon irradiation with a flood lamp which produces a vinyl radical. This free radical could be trapped with a halide from the solvent to yield the corresponding vinyl bromide or vinyl iodide (**110** and **111**).

Mercaptopyridine *N*-oxide was firstly used for the Barton ester formation with different coupling reagent. The coupling with propylphosphonic anhydride and HBTU were unsuccessful (run **1** and **3**) meanwhile TBTU gave low yields (run **2**). When 2-mercapto-4-methylthiazole 3-oxide was used instead of 2-mercaptopyridine (run **4**) a significant improvement in the yield was observed. *S*-(1-Oxido-2-pyridyl)-*N,N,N′,N′*-tetramethyl-thiuronium hexafluorophosphate (also known as HOTT) is a coupling reagent which contains the 2-mercaptopyridine *N*-oxide part and

could be used to form the Barton ester in a much straightforward way. After a short optimization (runs **5**, **6**, and **7**) I was able to obtain the desired vinyl bromide **110** and vinyl iodide **111** by using $CBrCl_3$ or 2,2,2-trifluoroiodoethane as radical trapping agents, respectively. Higher yields were obtained when the decarboxylation step was performed without the presence of the radical initiator AIBN and using $CBrCl_3$ as radical trapping agent (run **7**).



Scheme 29. A. Formation of the Barton ester of oseltamivir **B.** Radical decarboxylation step.

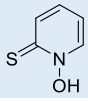
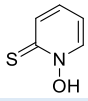
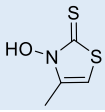
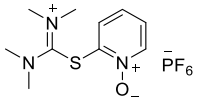
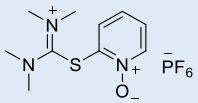
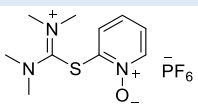
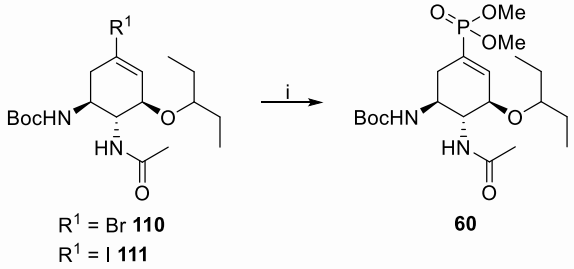
	A				B		
N° Run	**N-oxide heterocycle**	**Coupling reagent**	**Base**	**Solvent**	**Eq. AIBN**	**Halogenation agent**	**Yield (product)**
1		T3P	DABCO	DCM	0.05 eq.	$CBrCl_3$	-
2		TBTU	Et_3N	DMF	0.05 eq.	$CBrCl_3$	27 % (**100**)
3		HBTU	Et_3N	CH_3CN	-	-	-
4		TBTU	DIPEA	DMF	0.05 eq.	$CBrCl_3$	64 % (**100**)
5	-		Et_3N	THF	0.25 eq.	$CBrCl_3$	38 % (**100**)
6	-		Et_3N	THF	-	CF_3CH_2I	39 % (**101**)
7	-		Et_3N	THF	-	$CBrCl_3$	78 % (**100**)

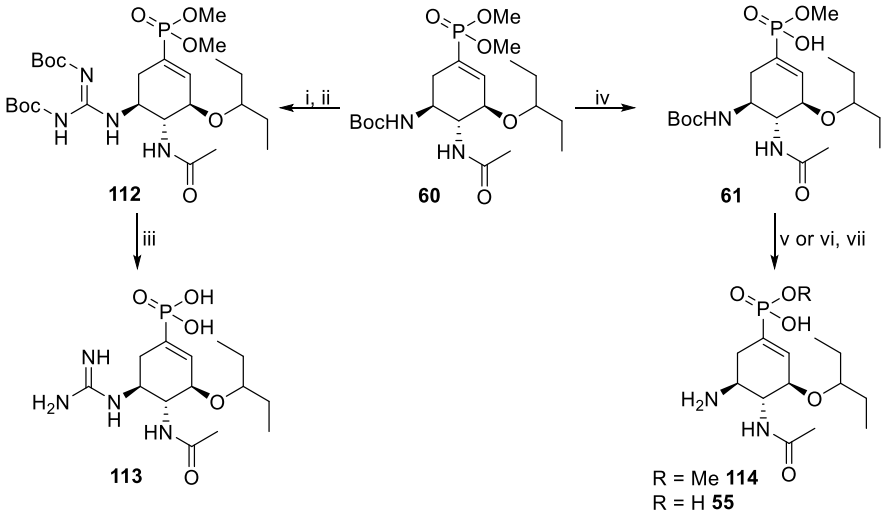
Table 5. Conditions for the Barton ester formation **A** and for the radical decarboxylation **B**. Yields over two steps

The carbon-phosphorous bond was introduced *via* palladium-catalyzed Hirao coupling of the bromide **110** with dimethyl phosphite, which afforded the dimethyl phosphonate **60** in very good yield (Scheme 30). Vinyl iodide **111** was also successfully used for Hirao coupling with comparable efficiency to the bromide. When 0.15 equivalents of palladium catalyst were used erratic yields were observed, this problem was solved by increasing the catalyst loading to 0.3 equivalents that gave consistently yields around 80%.



Scheme 30. *Reagents and conditions.* (i) Dimethyl phosphite, $Pd(PPh_3)_4$, Et_3N, 80 °C, 90 min, 86% yield.

The dimethyl ester **60** was selectively mono-*O*-demethylated by treatment with sodium hydroxide solution in 1,4-dioxane. This mild reaction afforded compound **61** in quantitative yields. Intermediate **61** could be either further demethylated and deprotected to yield tamiphosphor **55** or just Boc-deprotected to yield O-methoxytamiphosphor **114** (Scheme 31).



Scheme 31. *Reagents and conditions.* (i) TFA; (ii) *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine, Et_3N, 47% yield; (iii) TMSBr, DCM, 21% yield; (iv) NaOH 0.5M, 1,4-dioxane, 18h r.t., 91% yield; (v) TFA/H_2O, 43% yield of compound **114**; (vi) TMSBr, 2,6-lutidine; (vii) TFA/H_2O.

The parental compound **60** was also used to produce the known inhibitor **113**. One pot deprotection of the amine followed by installation of the guanidium moiety furnished **112** which

was treated with trimethylsilyl bromide to afford fully deprotected compound with the guanidinium moiety. Naturally, all prepared compounds were purified by preparative HPLC and submitted for biochemical tests.

Kinetic analysis of the binding between the compounds depicted in Figure 30 and pandemic A/California/07/2009 (H1N1) influenza virus (NA2009wt) was done by Dr. Milan Kožíšek from Konvalinka's group. The results show that oseltamivir carboxylate **34** and tamiphosphor **55** had equal affinity for NA2009wt. It also proved that a negative charge at carbon C-1 is fundamental as the low K_i value of ethyl ester **34** indicates. From the inhibitory potency of **114**, we could extract that introduction of methoxy group as replacement of one hydroxyl from the phosphonic acid has a negligible effect on the binding activity which is in full agreement with Streicher and Fang findings. Replacement of the amine at C-5 by guanidine moiety did not produce an improvement in the inhibition.

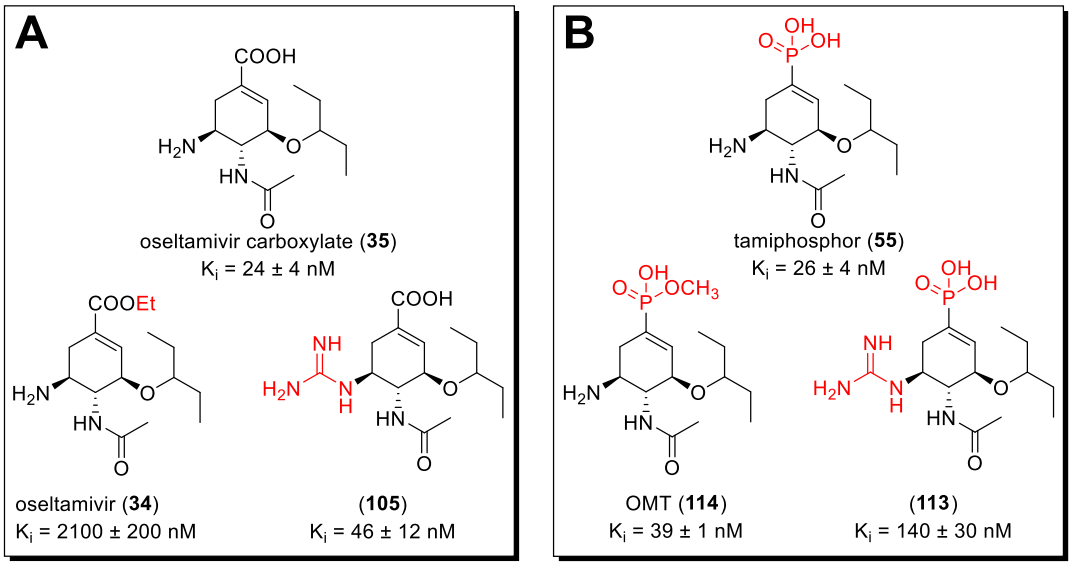


Figure 30. Oseltamivir derivatives (A) and tamiphosphos derivatives (B). The inhibition constants were determined by fluorimetric assay

The first crystal structure of tamiphosphor with the catalytic domain of neuraminidase (NA2009wt) was resolved by our collaborators Dr. Milan Kožíšek and Dr. Pavlína Maloy Řezáčová at 1.8 Å resolution (Figure 31). They used for his pioneering work tamiphosphor prepared by me.

The synthesis of the compounds showed in Figure 30 as well as their binding activities were published by us in 2016. The publication also included the crystal structure of tamiphosphor and

neuraminidase complex (Figure 31) as well as the first thermodynamic characterization (ITC) of the binding mode of oseltamivir carboxylate and tamiphosphor to neuraminidase.

Albiñana, C. B.; Machara, A.; Řezáčová, P.; Pachl, P.; Konvalinka, J.; Kožišek, M. Kinetic, thermodynamic and structural analysis of tamiphosphor binding to neuraminidase of H1N1 (2009) pandemic influenza. *European Journal of Medicinal Chemistry*. **2016**, 121, 100-109.[113]
https://doi.org/10.1016/j.ejmech.2016.05.016

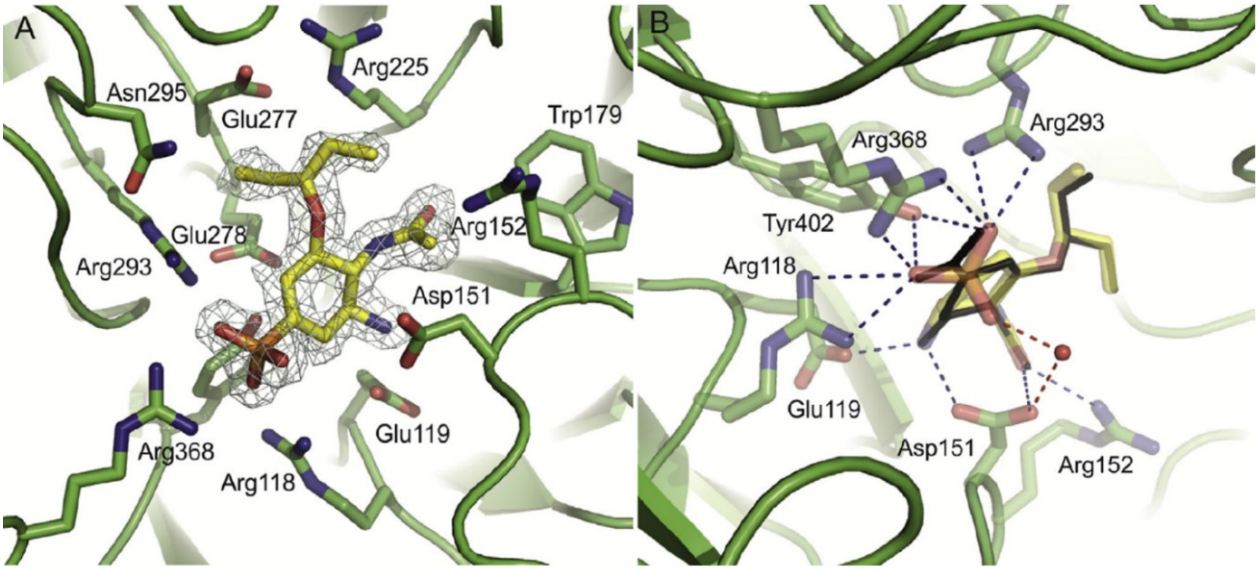


Figure 31. **A**) Detail of the crystal structure of tamiphosphor in complex with NA2009wt; all residues interacting with the inhibitor are shown. The $2F_o$-F_c electron density map for the inhibitor contoured at 1.0 σ is shown. **B**) Hydrogen bonding comparison between tamiphosphor and oseltamivir carboxylate (from PDB entry 3TI6, in black).

The crystal structure of tamiphosphor binding to the catalytic site of neuraminidase showed that tamiphosphor and oseltamivir carboxylate have comparable interactions with the active site which is in concordance with the almost identical binding constants. Only one significant difference was found; the third oxygen of the phosphonate, which is exposed to the solvent, forms a water-mediated contact with Asp151 side chain. The structural information obtained for the interaction of tamiphosphor with NA was used in the design of second-generation inhibitors as well as detection probes (see upcoming chapter).

3.2 Design and synthesis of the Probes

The second part of this Thesis is focused on the synthesis of probes suitable for the development of several screening assays. At first, let us define what is a probe; according to Arrowsmith and co-workers: "*probes are small-molecule modulators of a protein's function that allow the user to ask mechanistic and phenotypic questions about its molecular target*".[114] The probes are indispensable tools for medicinal as well as biological chemists since they enable visualization, advanced imaging, identification and quantification of the enzymes based on their specific activity in vivo and in situ. Furthermore, they could be used for efficient and high-throughput inhibitor testing.

The probes described in this work share the same general structure (Figure 32); a binding head based on the structure of a well-known inhibitor (ligand), a spacer usually made of polyethylene glycol linkers and an anchor group, usually a bioorthogonal functional group (azide) or a small molecule able to conjugate proteins (biotin). These probes have been used for the development of different assays for testing the activity of small molecules against influenza's neuraminidase, PA-endonuclease and PB2 cap-binding domain.

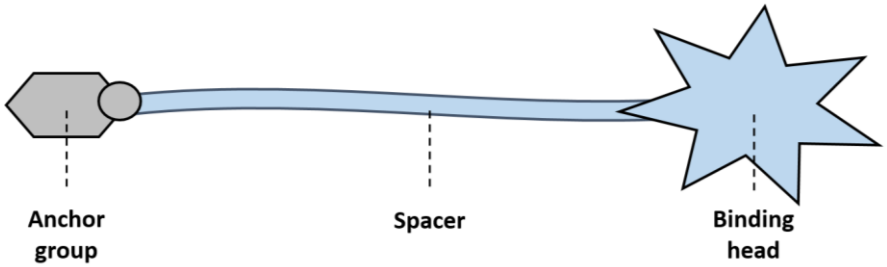


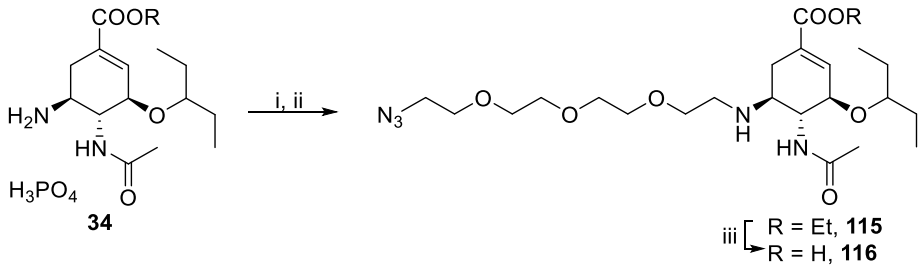
Figure 32. General structure of the detection probes prepared

3.2.1 Oseltamivir-based probes for DIANA assay

One of the fundamental requisites for the design of probes suitable for DIANA assay is that requires separation of the tethered sialylmimetic (the enzyme binding site) from the oligonucleotide part by a linker of sufficient length. The linker should end up with a bioorthogonal functional group capable of ligation to the oligonucleotide. Since the ligation reaction is done by strain-promoted alkyne-azide cycloaddition (SPAAC) the functional group required is an azide (Figure 22).

Oseltamivir was firstly selected as the sialylmimetic part of the detection probe since the nitrogen atom at carbon C-5 of oseltamivir is a highly convenient exit vector. This primary amine could be easily alkylated as we showed in the chapter dealing with C-5 oseltamivir derivatives.

For the synthesis of our first probe, we used commercially available oseltamivir phosphonate as starting material. Standard alkylation with N_3-PEG4-I agent (Scheme 32) under basic conditions gave the compound **115**. The linker was prepared in our lab from tetraethylene glycol following reported procedures. After saponification of the ester functional group, the probe **116** was obtained in moderate yields.



Scheme 32. *Reagents and conditions.* (i) $NaHCO_3$; (ii) 1-azido-2-(2-(2-(2-iodoethoxyl)ethoxy)ethoxy)ethane, Et_3N, 24h, 60% yield; (iii) NaOH 0.5M, 1,4-dioxane, 24 h, 83% yield.

Compound **116** was tested for its use in DIANA assay but provided a deficient sensitivity not suitable for the assay, this could be because the amine at C-5 points towards the 150-cavity and not into the solvent and therefore no space is available to accommodate the tethered oligonucleotide.

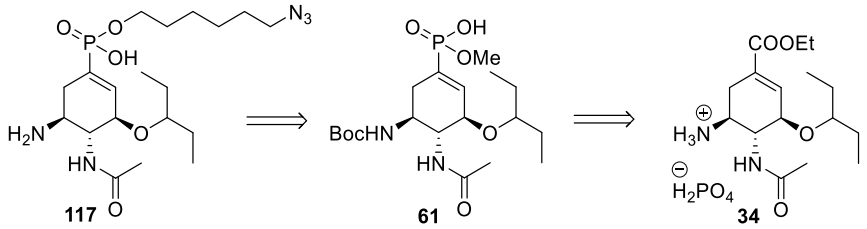
3.2.2 Tamiphosphor-based probes for DIANA assay

Using the amine at the carbon C-5 of oseltamivir structure as an exit vector for installation of the linker gave unsatisfactory results. Consequently, we decided to change our strategy for the synthesis of probes suitable to DIANA assay. Our previously reported crystal structure of the tamiphosphor complex with neuraminidase showed that the third oxygen atom of the phosphonate functionality is heading out of the enzymatic site and is not engaged in any direct interactions with protein residues.[113] We postulated that tamiphosphor could be used as a valid sialylmimetic for the construction of the probe, the phosphonate group would allow us to simultaneously introduce the connection of the linker at position C-1 and keep the fundamental negative charge.

As I described in the introduction and as I noted in previous chapters, a negative charge at carbon C-1 is indispensable for the inhibition activity. Streicher and co-workers firstly reported that monoalkyl phosphonates retain inhibitory potency comparable to oseltamivir.[92] Later on, he reported a solution for tethering of tamiphosphor without impairing its binding potency.[94] The compound synthesized by Streicher was ω-hexylazido tamiphosphor **117**. We decided that there was no need to introduce changes in its structure since it meets all the requirements to be a valid recognition probe for DIANA.

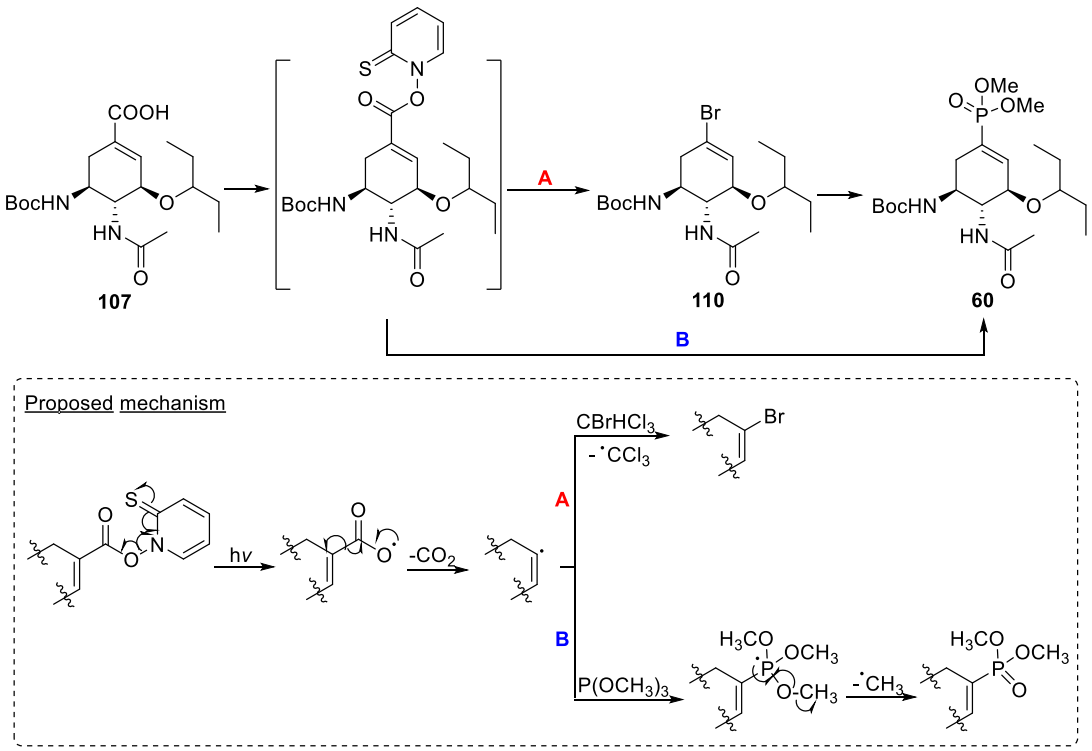
3.2.2.1 Design and synthesis of ω-hexylazido tamiphosphor

For the preparation of ω-hexylazido tamiphosphor I initially used the key mono-demethylated intermediate **61**, which was prepared in the synthesis of tamiphosphor (see the chapter about tamiphosphor) from oseltamivir phosphate (Scheme 33).



Scheme 33. Strategy for the synthesis of a tamiphosphor-based detection probe for DIANA.

In an effort to obtain the dimethyl phosphonate **60** in a more efficient manner I experimented with the direct conversion of the Barton thioester into the phosphonate. Photo-Arbuzov reactions were firstly reported back in 1966; aryl iodides were used as starting material which upon irradiation form an aryl radical that can be trapped with a phosphite which after rearrangement delivers the corresponding phosphonate.[115]

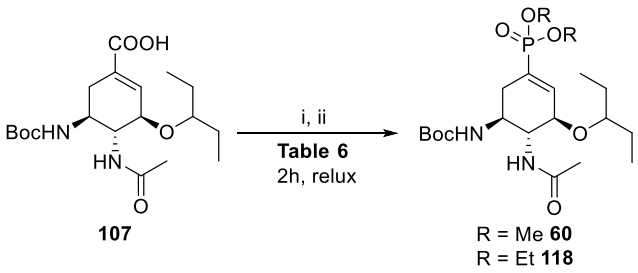


Scheme 34. **A**. Old route for the conversion of carboxylate **107** into phosphonate **60** *via* vinyl bromide formation and palladium catalyzed C-P bond formation; **B**. New one-pot conversion of Barton's thioester into phosphonate *via* photo-Arbuzov reaction. The proposed mechanism is depicted inside the dashed box.

We postulated that by irradiation of the Barton thioester we would form a vinyl radical *via* homolytic cleavage of the thioester and radical decarboxylation. The vinyl radial could be then directly trapped with trimethyl phosphite to form the phosphonate **60** (Scheme 34). This would be an important shortcut in the synthesis of tamiphosphor and would avoid the palladium catalyzed C-P bond formation. Following this approach, we could be able to transform the unsaturated carboxylic acid **107** into the unsaturated phosphonate **60** in *one-pot* procedure. Only one method has been published so far for this specific transformation which uses nickel catalyst for the decarboxylation and C-P cross-coupling step.[116] A recent publication reported a transition-metal-free C-P bond formation using several phosphine oxides but failed to introduce diethyl phosphonate moiety.[117]

The Barton thioester was prepared *in situ* from the free acid **107** using the highest yielding condition developed during my synthesis of tamiphosphor (Scheme 35). The solvent was then evaporated and four different sets of conditions were attempted for the radical decarboxylation and photo-Arbuzov reaction (Table 6). The desired product was formed under all four conditions but in unimpressive yields. Use of radical initiator AIBN and tris(trimethylsilyl)silane did not lead to

a substantial improvement of the yield (run **2** and **4**). Slightly higher yields were obtained when triethylphosphite was used although still were in 30 – 40 % range.



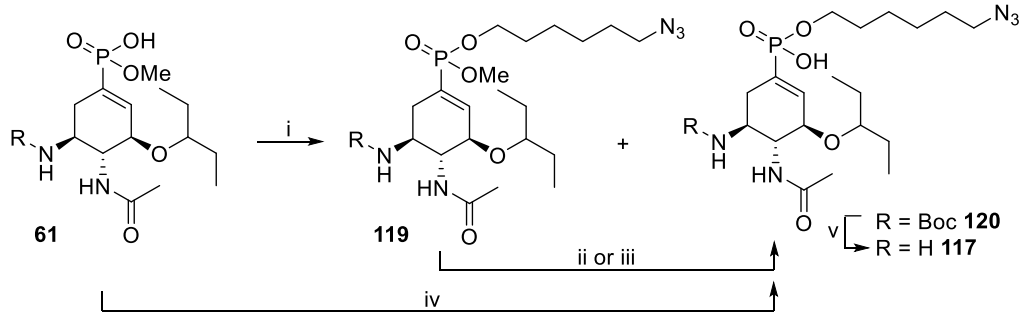
Scheme 35. The thioester was formed as previously described. Conditions for the radical decarboxylation and photo-Arbuzov descrived in **Table 6.** *Reagents and conditions.* (i) HOTT, Et_3N, THF, 1 h, r.t.; (ii) see **Table 6**.

N° Run	Phosphite	Eq. Phosphite	Eq. AIBN	Eq. TTMSS	Solvent	Yield
1	$P(OMe)_3$	15	-	-	Tol/DCM	28 %
2	$P(OMe)_3$	15	0.15	1.3	Tol/DCM	29 %
3	$P(OEt)_3$	15	-	-	Tol/DCM	32 %
4	$P(OEt)_3$	15	0.15	1.3	Tol/DCM	41 %

Table 6. Trimethyphosphite and triethylphosphite were used as radical trapping agents. The importance of using radical initiator AIBN and tris(trimethylsilyl)silane was explored (compare **1** and **2**, **3** and **4**).

Although the reaction produced the desired product and shortened the synthesis in one step, due to the project moving on I could not carry on with the optimization. Although the best yield obtained for the photo-Arbuzov reaction (run **4**) was slightly inferior in comparison to the previous approach (decarboxylation followed by Pd-catalyzed C-P bond formation) proper optimization of the reaction conditions could lead to a novel and straighforward method for synthesis of unsaturated dialkyl phosphonates.

The key intermediate *O*-methyl tamiphosphor **61** (Boc-OMT) was prepared again from derivative **60**. Direct alkylation of the phosphonate **61** always gave **119** in underwhelming yields. More extensive examination of the reaction showed that the alkylated product lacking the methyl ester moiety **120** was formed as a side-product (Scheme 36). This was an exciting surprise since any increasing of the side-product **120** formation would shorten the synthesis in one step and would avoid the use of thiophenol for the second *O*-demethylation step, which is problematic due to its pungent odor.



Scheme 36. *Reagents and conditions.* (i) 1-azido-6-bromohexane, Et_3N, DMF, 40 °C, 25% yield; (ii) PhSH, Et_3N, 3 days; (iii) KOTMS, Et_2O, 16 h, 77% yield; (iv) 1-azido-6-bromohexane, NaI, Et_3N, DMF, 60 °C, 56% yield; (v) TFA, 1h.

We hypothesized that under the applied conditions the desired alkylation was followed with *O*-demethylation by the action of the free bromide anion coming from 1-azido-6-bromohexane. We expected that using catalytic amounts of sodium iodide would facilitate this transformation since iodide is a better leaving group than bromide and has comparable nucleophilicity. Increased temperature and longer reaction times also favored the formation of **120** but *O*-demethylation of **119** has never reached full completion. Although I was able to obtain **120** in around 50 % yields from **61** the reaction was not thoroughly optimized due to lack of time.

The selective second *O*-demethylation of **119** was firstly accomplished by treatment with thiophenolate but underwhelming yields were obtained. Besides, thiophenol is an uncomfortable reagent to work with. Potassium trimethylsilanolate has been reported as a mild and selective *O*-demethylating reagent for phosphonates.[118] When potassium trimethylsilanolate was employed the desired phosphonate **120** was obtained in excellent yield and the reaction set-up was substantially simplified.

After Boc-deprotection of **120** by treatment with trifuoroacetic acid (TFA), a mixture of the diastereomeric monoalkyl esters of **117** was obtained. This is because the phosphorus atom represents an additional stereogenic center. Separation of the diastereomers was not observed on analytical HPLC and therefore they were used as such.

3.2.2.2 Results obtained from ω-hexylazido tamiphosphor

In our previous work we reported the crystal structure of the catalytic domain of $NA2009_{wt}$ with tamiphosphor (Figure 31),[113] this work will be extended in an upcoming publication where we reveal the crystal structures of methyl- (**114**) and ω-hexylazido tamiphosphor (**117**) with the

catalytic domain of neuraminidase (Figure 34). Analysis of the complex of **114** with NA showed that the methyl ester moiety points outside from the active site which is also the case for the more sophisticated compound **117** in which the ligand extension leads directly into the solvent. This could explain the almost identical inhibitory potency obtained for the two compounds.

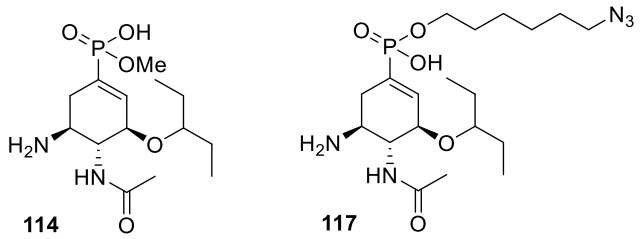


Figure 33. Structure of the inhibitors that were co-crystallized with the catalytic domain of $NA2009_{wt}$

In the following-up publication, we demonstrate that DIANA assay employing compound **117** is a new, reliable and rapid screening method for candidate inhibitors of influenza neuraminidase. The preparation of the detection probe ω-hexylazido tamiphosphor was reported in this publication as well as the crystal structures shown in Figure 34. Furthermore, a comparison of the binding constant for various inhibitors tested by DIANA assay with results from the standard fluorometric assay (which uses MUNANA as a substrate) was also reported (Table 7).

Kožíšek, M.; Navrátil, V.; Rojíková, K.; Pokorná, J.; Albiñana, B.C.; Pachl, P.; Zemanová, J.; Machara, A.; Hudlický, J.; Císařová, I.; Řezáčová, P.; Konvalinka, J. DNA-linked Inhibitor Antibody Assay (DIANA) as a new method for screening of inhibitors of influenza neuraminidase.[119] Manuscript submitted

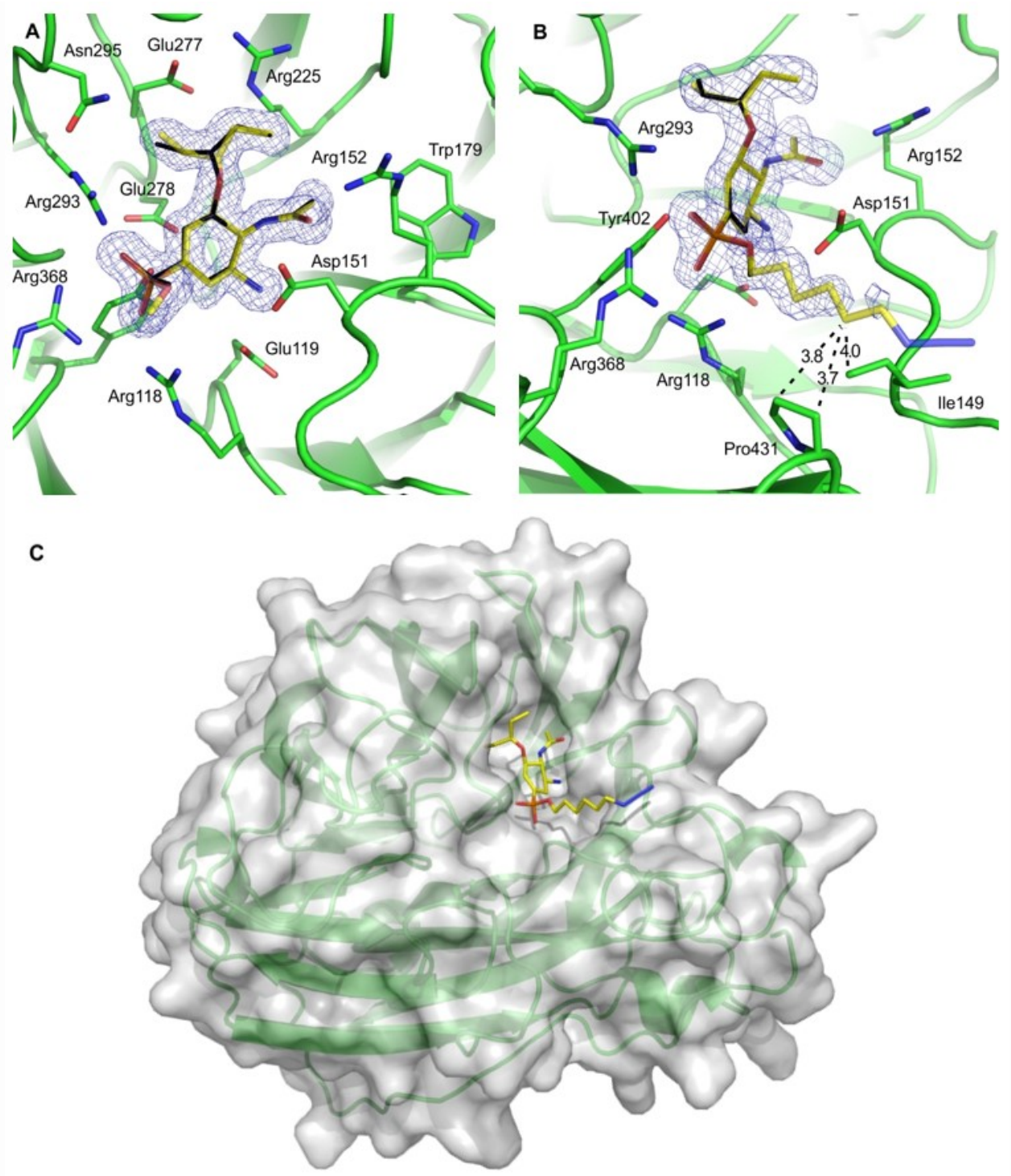


Figure 34. Crystal structure of $NA2009_{wt}$ in complex with compound **114** (**A**) and compound **117** (**B, C**). Compounds are shown as stick models and carbon atoms are yellow, oxygen atoms are red, nitrogen atoms are blue, and phosphorus atoms are orange. In panel **A**, tamiphosphor molecule from the previous paper is overlaid and colored black.[119] In panel **B**, compound **114** is overlaid and colored black.

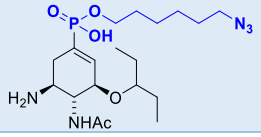
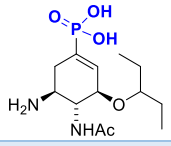
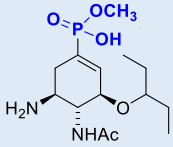
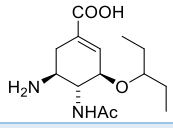
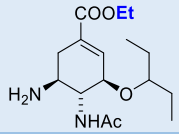
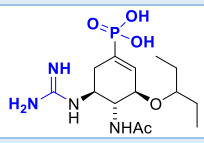
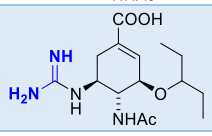
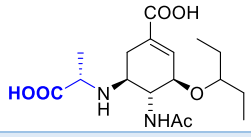
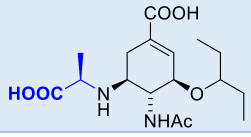
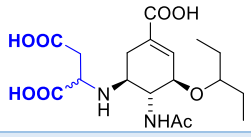
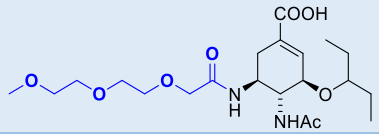
Compound	Chemical formula	K_i (enzyme kinetics) (pH 6.15) [nM]	K_i (DIANA) (pH 7.4) [nM]	fold K_i
117		24 ± 5	33 ± 8	1.4
55		26 ± 4	52 ± 9	2.0
114		39 ± 1	100 ± 20	2.6
35		24 ± 4	31 ± 6	1.3
34		2 100 ± 200	2 000 ± 500	1.0
113		140 ± 30	570 ± 180	4.1
105		46 ± 12	570 ± 200	12
97		15 ± 2	170 ± 70	11
98		250 ± 40	960 ± 260	3.8
101		13 000 ± 1 000	16 000 ± 5 000	1.3
103		43 000 ± 11 000	210 000 ± 110 000	5

Table 7. Results of inhibition constants determined by enzyme kinetics and DIANA assay. Fold K_i parameter represents the ratio K_i (DIANA; pH 7.4)/K_i (enzyme kinetics; pH 6.15).

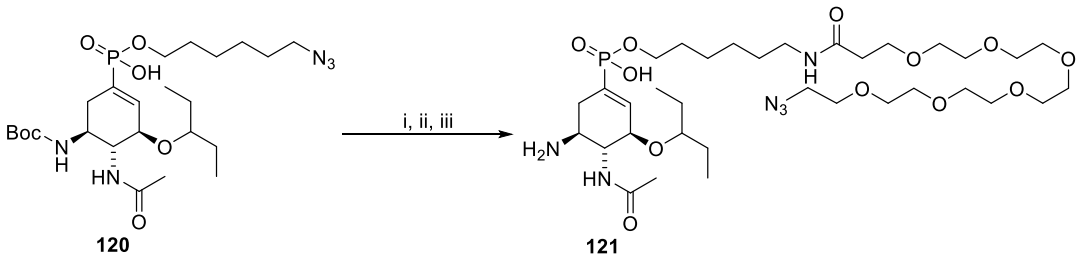
Here a brief summary of the conclusions we extract from the binding potency of the tested compounds is provided: tamiphosphor **55**, methyl tamiphosphor **114** and ω-hexylazido tamiphosphor **117** have comparable potency to oseltamivir carboxylate **34**, which validates our decision of using the phosphonate moiety of tamiphosphor as a way to introduce the linker without disrupting the binding affinity. Contrary to previously published data,[120,109] replacement of the amine at C-5 by a guanidine group did not cause an improvement in the binding affinity. The *S* diastereomer **97** is significantly more potent than the *R* diastereomer **98**, and it's the only inhibitor prepared by us which presents superior inhibition values than oseltamivir carboxylate **35**. The much bulkier substituent in **101** is not well tolerated and obstructs the binding. Unsurprisingly, the presence of a non-basic moiety at C-5 in **103** impaired the binding activity of the inhibitor. The reason behind the synthesis of compound **103** was to validate the DIANA assay for a very weak inhibitor, we observed that even in the micromolar K_i range the data obtained by DIANA fits well with the data provided by standard MUNANA assay. The observed deviation in the K_i data provided by both assays could be explained by the different pH used in both methods.

3.2.2.3 Elongated ω-hexylazido tamiphosphor

Inspired by the promising results obtained with ω-hexylazido tamiphosphor, we decided to extend the application of this probe and use it in a different side-project.

In short, the aim was to decorate a polymeric material with a neuraminidase inhibitor. The inhibitor-containing polymer is supposed to enhance the pharmacological properties of the bounded tamiphospor and the material is intended to be administrated to patients in life-treating stages of influenza's infection.

ω-Hexylazido tamiphosphor was firstly used to coat the polymeric material. Unfortunately, its antiviral activity was unsatisfactory presumably due to the insufficient length of the linker. Likely, the tethered inhibitors remained in close proximity to the polymer which hinders the binding to neuraminidase. Therefore, elongation of the linker was needed for a better antiviral activity. Thus, a facile one-pot elongation of Boc-protected ω-hexylazido tamiphosphor **120** was suggested and eventually accomplished, see Scheme 37.



Scheme 37. *Reagents and conditions.* (i) Lindlar catalyst, H_2 (1 atm), MeOH, 5 h, r.t.; (ii) NHS-PEG6-N_3, Et_3N, DMF, 24 r.t.; (iii) TFA, 1h, r.t., 42% yield.

The synthesis begins with the reduction of the azido moiety in **120** to form the free amine. Catalytic hydrogenation was selected as the right reduction method because it allowed us to produce the free amine without the need for further purification. Lindlar catalyst was used to avoid possible hydrogenation of the cyclohexene ring. Once the reaction was completed, the catalyst was filtered out and the solvent was evaporated. The crude product was dissolved in DMF and treated with the commercially available linker N_3-PEG6-NHS ester. Finally, the reaction mixture was treated with an excess of trifluoroacetic acid to deprotect the amine. Purification *via* preparative HPLC afforded the tamiphosphor with the long linker **121** in 41% overall yield (from **120**).

Compound **121** was then used for the design of potential anti influenza compounds based on active small molecules conjugated to a polymeric scaffold. Preliminary tests of the prepared drug candidates tested on influenza infectivity showed promising antiviral activity.

3.2.3 VX-787-based probe for DIANA assay

Turning a well-known neuraminidase inhibitor into a probe allowed us to develop a simple and reliable assay for screening of low-molecular-weight inhibitor (sialylmimetics). Encouraged by the results obtained from the neuraminidase project, we decided to use the same approach to develop a DIANA assay that allows us to screen low-molecular-weight inhibitors of a different vital enzyme of influenza.

We realized that only limited number of compounds that interact with the cap-binding domain of PB2 in RNA-polymerase have been reported so far. Therefore, we decided to broaden the knowledge in this topic by developing an assay for screening of new inhibitors of the cap-binding domain of PB2. One of the reported inhibitors (VX-787, **28**) showed extreme potency and

high selectivity for PB2 cap-binding domain. Its outstanding properties[51] makes VX-787 (**28**) a perfect candidate for conversion into a detection probe.

Moreover, the crystal structure of **28** binding to the active site of PB2 has been characterized (Figure 14, page 27)[51]. This fact allowed us to meticulously study its binding mode and determinate a suitable position for attachment of the linker (exit vector) without compromising the inhibitory activity. The fluorine atom at the pyrimidine ring points directly outside of the catalytic site of PB2, thus we hypothesized that attachment of the linker at this position would not interfere with the binding potency (Figure 35).

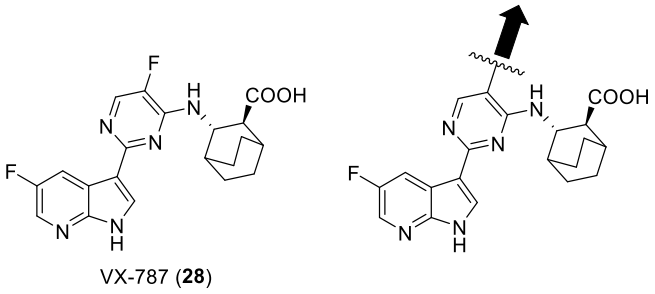


Figure 35. Structure of the VX-787 (on the left) and proposed exit vector for the design of the probe (on the right).

Unlike in the synthesis of ω-hexylazido tamiphosphor, where I started from a commercially available inhibitor and systematically modified its structure, for the conversion of VX-787 to the desired probe I had to synthesize the inhibitor/probe from few commercially available building-blocks.

Not one, but actually two similar probes which differ only in the anchor group at the end of the linker had to be prepared for the development of the assay. One probe had to possess a biotin group which makes it suitable for SPR assay (Surface Plasmon Resonance). The other had to bear a linker ending with an azido moiety required for the ligation to the oligonucleotide. The first probe will be used for testing the binding activity of the molecule to the catalytic site of PB2 and shall confirm that the affinity for the enzyme was not disrupted. The second probe will be used as the actual detection probe in the DIANA assay.

In Figure 36 we proposed a modular synthesis for **122** in which synthons **A**, **B** and **D** were prepared in parallel and then were sequentially introduced to the core fragment **C**, which is commercially available. To sum it up, compound **122** was selected as our target molecule, it was

intended to function as a common intermediate that can be directly converted into both probes in the last step of the synthesis.

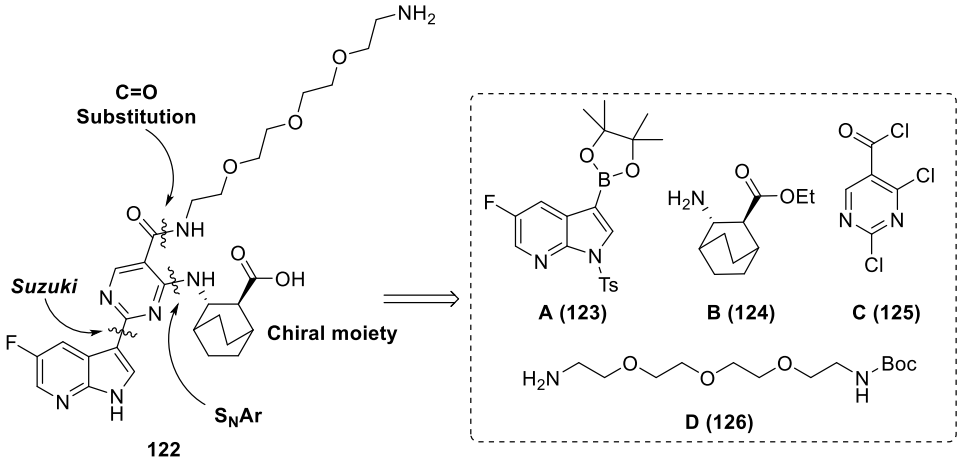


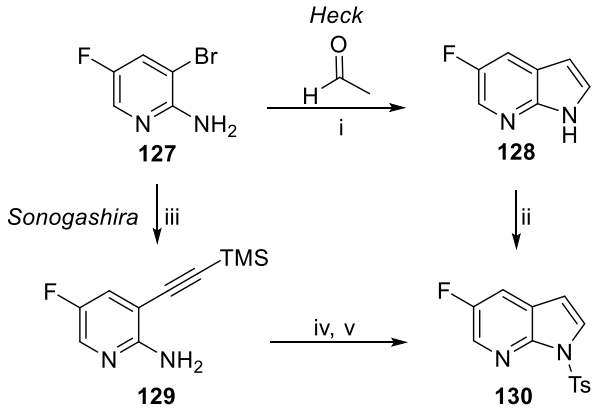
Figure 36. Brief retrosynthetic analysis. On the left, structure of the target molecule **122** showing how the connection of the synthons could be done. On the right, depiction of the structures of the four key synthons needed for the construction of compound **122**. Note, alphabetical descriptors of each synthon.

3.2.3.1 Preparation of synthon A

For the synthesis of synthon A (**123**), I loosely followed a patented procedure developed by Vertex Pharmaceuticals for the large-scale preparation of VX-787.[121] The procedure had to be adapted to the milligram-gram scale with notable differences introduced.

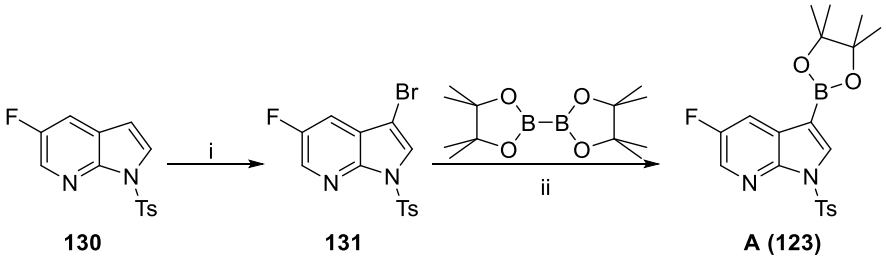
2-Amino-3-bromo-5-fluoropyridine **127** was chosen as the starting material. The synthesis begins with formation of the 7-azaindole derivative **130** (Scheme 38). Two pathways were investigated to get **130**; in both of them a Pd-catalyzed reactions were involved. The *Heck* pathway starts with *one-pot* condensation of the aminopyridine **127** with acetaldehyde to form the respective enamine which under the applied conditions and in the presence of a palladium pre-catalyst, undergoes intramolecular *Heck* reaction to furnish the azaindole **128**. The catalyst and ligand used in the patent, bis(dibenzylideneacetone)palladium(0) and tri-*tert*-butyl phosphine, were not convenient for small scale synthesis. Thus, I decided to replace it by Buchwald precatalysts. Reaction with *t*BuXPhos Pd G1 complex gave **128** in 25 % yield. When the catalyst was changed

to *t*BuXPhos Pd G3 complex the yields rose to 61 %. The azaindole was then tosylated under basic conditions to form compound **130** in a 54 % overall yield for the two steps.



Scheme 38. *Reagents and conditions.* (i) *t*BuXPhos Pd G3, DABCO, TBAB, DMSO/EtOAc, 75 °C, 24h, 63% yield; (ii) NaH, TsCl, 1h, 88% yield; (iii) Ethynyltrimethylsilane, CuI, $Pd(dppf)_2Cl_2$, DCM, 55 °C, 24 h, 83% yield; (iv) *t*BuOK, NMP, 70 °C, 2h; (v) TsCl, NMP, 0 °C, 1h, 31% yield.

A second pathway was explored as an attempt to improve the formation of intermediate **130**. *Sonogashira* coupling of the bromopyridine **127** with ethynyltrimethylsilane gave the desired compound **129** in excellent yields. The following step was ring-closing reaction under basic conditions to produce the azaindole heterocycle. This ring-closing reaction belongs to the 5-*endo*-dig type and therefore is favored according to Baldwin's rules. Subsequent *in situ* protection of the pyrrole nitrogen with tosyl chloride afforded product **130** in a low yield (31%). Although the Sonogashira reaction was more efficient than the intramolecular Heck reaction, the low yields associated with the ring-closing reaction made the Sonogashira pathway significantly inferior than the Heck approach.



Scheme 39. *Reagents and conditions.* (i) NBS, DCM, 24 h, 68% yield; (ii) $Pd(OAc)_2$, PPh_3, KOAc, 1,4-dioxane, 100 °C, 15 min, 76% yield.

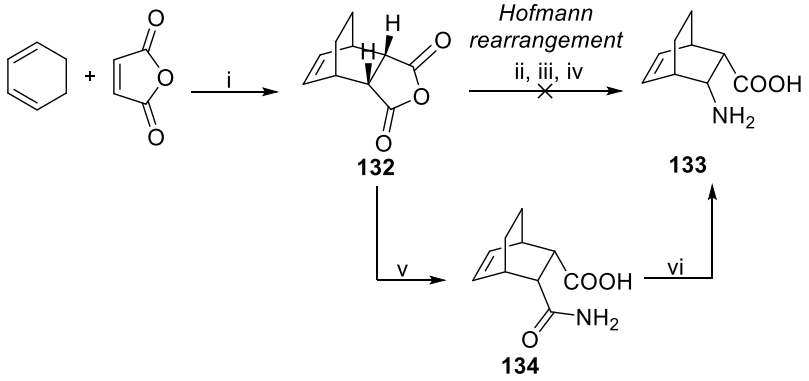
The following step was bromination of the annulated pyrrole by treatment with *N*-bromosuccinimide as the source of electrophilic bromine (Scheme 39). Azaindole **130** reactivity is

analogous to indole and accordingly, the most reactive position for electrophilic aromatic substitution is carbon C-3. A single regioisomer was obtained from the bromination reaction in fair yields (68%). The bromoazaindole **131** had to be then converted into the coupling partner for Suzuki reaction. Miyaura borylation reaction with bis(pinacolato)diboron catalyzed with palladium acetate gave the boronic ester **123** in high yields. Thus, synthesis of synthon A was accomplished. This key compound **123** was introduced to the pyrimidine core *via* Suzuki coupling reaction (*vide infra*).

3.2.3.2 Preparation of synthon B

Preparation of synthon B was synthetically more challenging since it involved several key steps that needed to be addresed by a rational design of the synthetic pathway. The construction of the bicyclo[2.2.2]octane amino acid moiety and the resolution of its racemate were the major obstacles to overcome.

The formation of the bicyclo[2.2.2]octane was done in the first step *via* Diels-Alder reaction between 1,3-cyclohexadiene and freshly submiled maleic anhydride (Scheme 40). The patented conditions used for this reaction gave me exclusively the *endo* product **134** in excellent yields.[122]

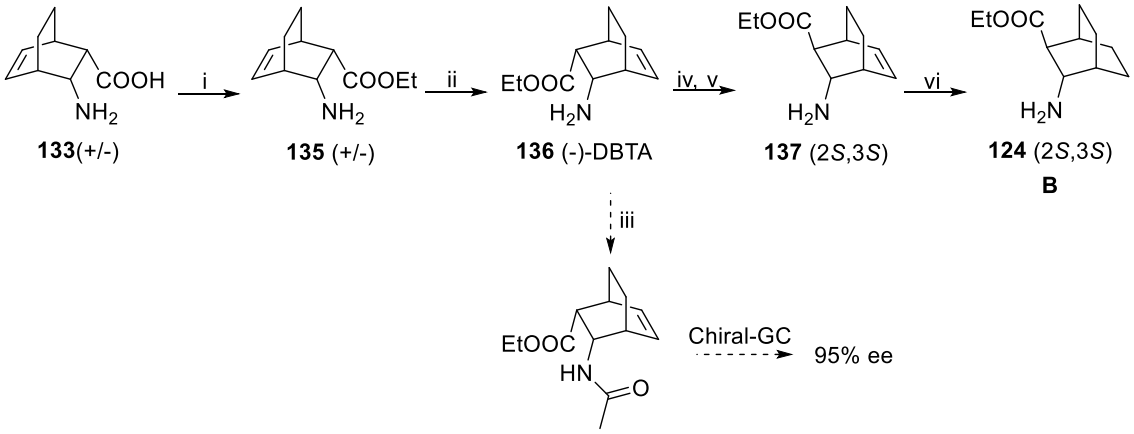


Scheme 40. *Reagents and conditions.* (i) DCM, 0 °C, 24 h, 85% yield; (ii) NH_4Cl 6%, 0 °C, 30 min; (iii). NaOH 2 M, 0 °C, 30 min; (iv) NaClO 1 M, 10 min, 75 °C; (v) NH_4Cl 28%, THF, 5h, r.t., 94% yield; (vi) PIDA, EtOAc/AcCN/H_2O, 24h, r.t., 90% yield.

The following step was opening of the anhydride using ammonia as nucleophile to form the amide **134** which after Hofmann rearrangement would give a racemate of the desired amino acid **133**. A *one-pot* procedure was attempted in which the anhydride was firstly opened with an ammonia hydroxide solution, strongly basic conditions were subsequently applied. Addition of sodium hypochlorite would chlorinate the amide and after a short refluxing period the amino acid

133 should be formed. Unfortunately, only small amounts of product were generated under these conditions. As an effort to understand why the reaction didn't work, I isolated the amide **134** and experimented with different reagents to perform the Hoffman rearrangement. Sodium hypochlorite and [bis(trifluoroacetoxy)iodo]benzene always produced unsatisfactory results. On the other hand, when the amide was treated with (diacetoxyiodo)benzene (PIDA) the reaction proceeded smoothly affording the amine **133** as a racemate in high yields.

To separate the two enantiomers from each other, I used an approach never attempted in the synthesis of VX-787. Firstly, esterification of the carboxylic acid was necessary for a successful resolution of the enantiomers (Scheme 41).[123] The racemate **135** was then treated with half equivalent of the resolving agent (-)-dibenzoyl-*L*-tartaric acid monohydride [also known as (-)-DBTA] which precipitated the desired enantiomer out of the solution as the corresponding diastereomeric salt **136**. The diastereomeric salt was then recrystallized several times to increase the diastereomeric excess, although the yield was reduced. To determine the efficiency of the resolution, an aliquot of **136** was acetylated and the sample was submitted for chiral gas chromatography. The measurement showed that the desired enantiomer was present in a satisfactory 95% enantiomeric excess.



133(+/-) **135** (+/-) **136** (-)-DBTA **137** (2*S*,3*S*) **124** (2*S*,3*S*) **B**

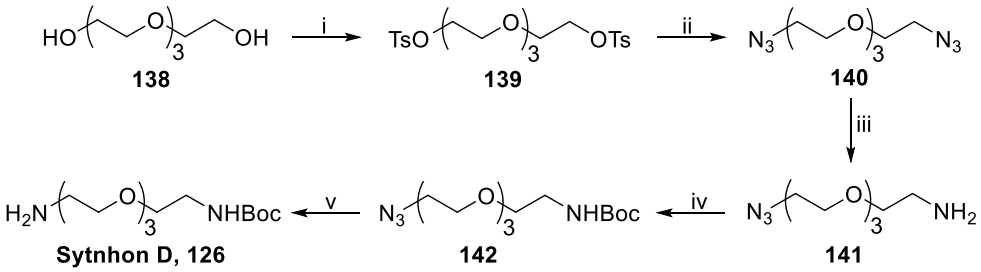
Scheme 41. *Reagents and conditions.* (i) $SOCl_2$, EtOH, 24 h, r.t., 5 h, reflux, 73% yield; (ii) (-)-DBTA (0.5 eq.), EtOH, 75 °C, 5 min, 25% yield; (iii) Ac_2O, DMAP, Py; (iv) $NaHCO_3$; (v) NaOEt, EtOH, 2h, reflux, 32% yield; (vi) H_2 (1 atm), Pd/C 10%, EtOH, 24 h, r.t., 95% yield.

The free base of **136** was treated with sodium ethoxide to epimerize at the alfa carbon related to the ester group. This transformation gave the *trans* stereoisomer **137**. The reaction proceeded in low yields although the unreacted starting material could be recovered. The last step

in the preparation of synthon B was hydrogenation of the double bound which afforded key synthon B (**124**) in quantitative yields.

3.2.3.3 Construction of the Probe based on VX-787

The linker used for tethering the probe, named as synthon D (**126**), was prepared from tetraethylene glycol following procedures developed in our group in 52 % overall yield. A key step involving the selective mono-reduction in a Staudinger-like fashion of tetraethylene bis(azide) **140** was used to break the symmetry in the linker. Compared to standard Staudinger condition, this transformation was done in a two-phase system composed of an acidic aqueous layer and a diethylether layer. The aqueous layer immediately collects the product of the monoreduction and thus prevents compound **141** from overreduction which would happen under standard conditions (one-phase system). The synthesis of synthon D is depicted in Scheme 42.



Scheme 42. *Reagents and conditions.* (i) TsCl, KOH, DCM, 3 h, 0 °C, 98% yield; (ii) NaN_3, DMF, 60 °C, 24 h, 82% yield; (iii) PPh_3, Et_2O, HCl 5%, 24 h, r.t., 81% yield; (iv) Boc_2O, Et_3N, THF, 5h, r.t., 89% yield; (v) PPh_3, THF, 24 h, r.t., water, 60 °C, 2h, 90% yield.

The required synthon C is commercially available and was purchased from a supplier. Synthon C represents the core of the target molecule where the other synthons would be attached. The order in reactivity of the different sites at the pyrimidine derivative (synthon C) defined the order in which the synthons should be introduced. The most reactive site should be the acyl chloride, followed by the carbon C-4 of the pyrimidine ring and carbon C-2 (Figure 37).

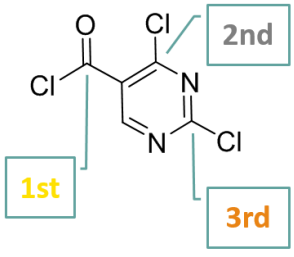
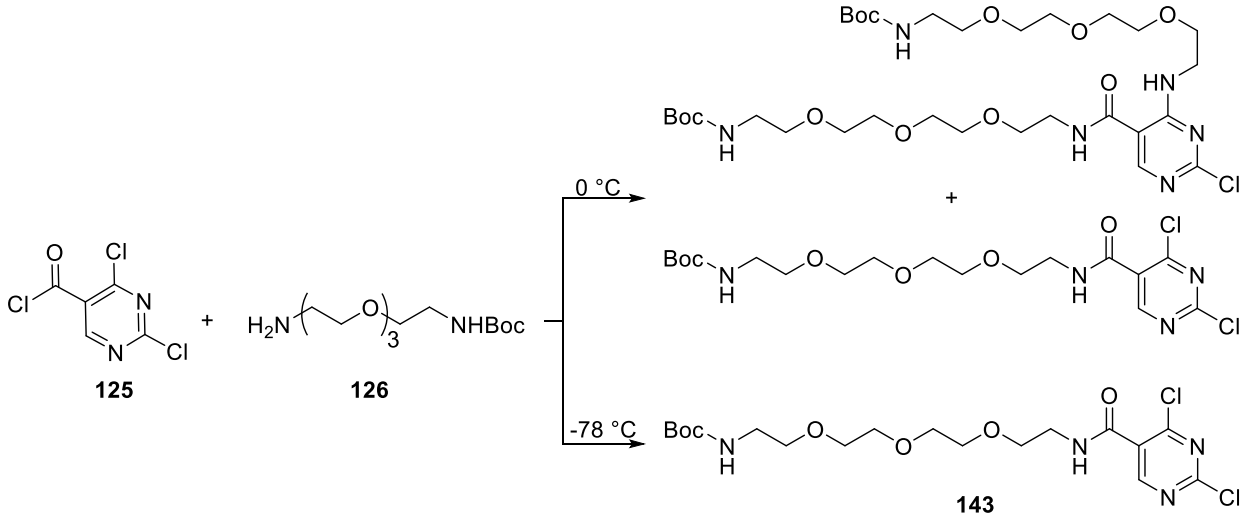


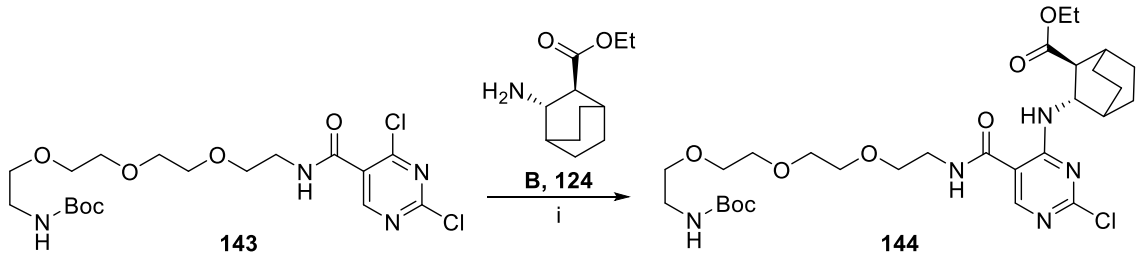
Figure 37. Order of reactivity in Synthon C.

Accordingly, the introduction of the linker (synthon D, **126**) should be done in the first step of the assembly of the probe. In the first experiment, one equivalent of the linker **126** was added to an ice-cold DCM solution of synthon C (**125**) in the presence of DIPEA. After 90 min the reaction was quenched and the worked-up reaction mixture was separated by column chromatography. Surprisingly I observed also substitution at position C-4 with a second equivalent of **126** giving a mixture of products (Scheme 43). Position C-4 in pyrimidines is known for being highly reactive towards nucleophilic aromatic substitution especially in pyrimidines bearing electron-withdrawing groups. Lowering the reaction temperature to -78 °C and slowing down the rate of addition of the linker led to a higher regioselectivity of the reaction. The formation of side-product was thus suppressed.



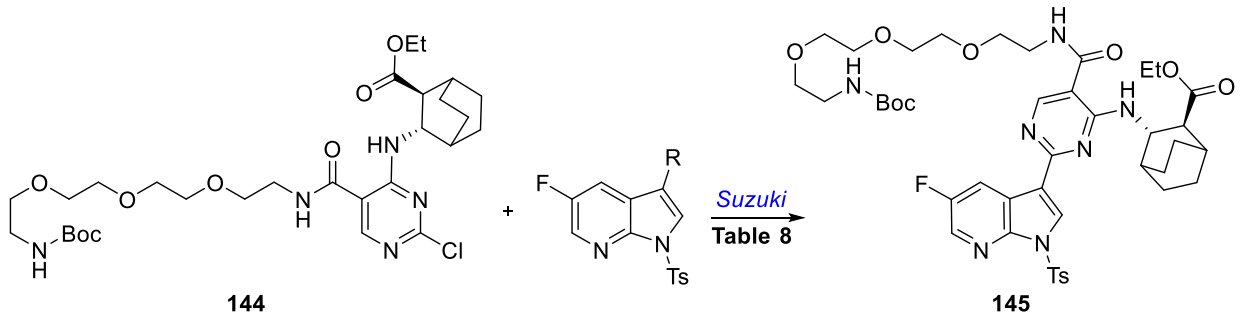
Scheme 43. *Reagents and conditions.* DIPEA, DCM, 90 min. Effect of the temperature on the regioselectivity of the reaction. The reaction conducted at -78 °C gave the desired product in 56% yield.

The high reactivity of position C-4 in the pyrimidine ring turned to be an advantage during the introduction of synthon B, which was done *via* nucleophilic aromatic substitution of the chlorine atom by the free amine of **124** (Scheme 44). The desired intermediate **144** was obtained in high yield and perfect regioselectivity (no substitution at C-2 was observed).



Scheme 44. *Reagents and conditions.* (i) DIPEA, *i*-PrOH, 24 h, r.t., 84% yield.

The last step in the construction of the probe turns to be tricky. The introduction of the synthon A (**123**) to intermediate **144** is in fact a carbon-carbon bond formation reaction between the pyrimidine and the azaindole heterocycles. Therefore, it requires a combination of coupling partners suitable for Suzuki-Miyaura reaction (Scheme 45).



Scheme 45. Conditions showed in Table 8. Azaindole used either as the boronic ester or the trifluoroborate salt.

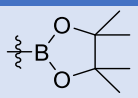
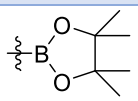
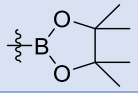
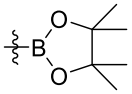
R =	N ° Run	Solvent	Base (eq)	Pd source (eq)	Ligand (eq)	T (°C)	Time (h)	Yield
	**1**	Tol/water (10:1)	K_2CO_3 (4)	*t*BuXPhos Pd G3 (0.05)	-	85	24	traces
	**2**	THF	Na_2CO_3 (3)	$Pd(PPh_3)_4$ (0.1)	-	70	5	traces
	**3**	THF/water (25:1)	K_2CO_3 (4)	*t*BuXPhos Pd G3 (0.05)	-	70	24	traces
	**4**	THF/water (25:1)	K_2CO_3 (4)	SPhos Pd G3 (0.1)	-	70	24	67 %
	**5**	*n*-propanol	Et_3N (3)	$Pd(OAc)_2$ (0.1)	SPhos (0.15)	85	18	< 5 %
	**6**	Tol/water (10:1)	K_2CO_3 (3)	SPhos Pd G3 (0.1)	-	85	24	45 %

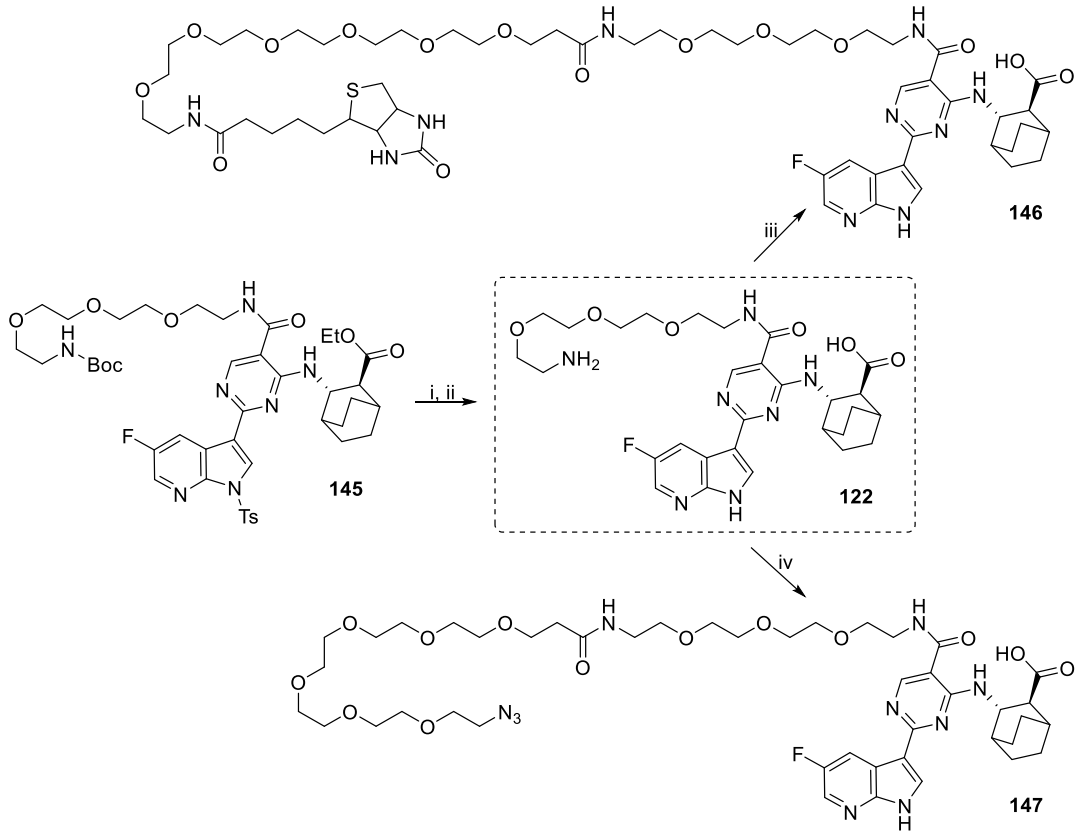
Table 8. Conditions screened for Suzuki coupling; no optimization was done. The boronic ester was transformed into the trifluoroborate salt by treatment with potassium hydrogenflourid. Buchwald pre-catalyst SPhos Pd G3 gave the best results.

The conditions screened for the reaction are summarized in Table 8. The coupling of the boronic ester **123** with the pyrimidine derivative **144** was attempted four times (runs **1**, **2**, **3** and **4**) but only one set of conditions (run **4**) furnished the product in fair yields. Disappointed by the results obtained with the boronic ester I decided to transform the borate **123** into the potassium trifuoroborate salt. These salts are known to be less prone to protodeboronation that usually

complicates Suzuki-Miyaura reactions. Unfortunately, I did not observe an improvement by using the trifluoroborate salts (runs **5** and **6**).

Although the yield obtained by applying conditions **4** for the Suzuki coupling was not spectacular, it represents a viable route to intermediate **145**. In few experiments carried in the same manner like in run **4**, I got enough material to finish the synthesis of the probe. Therefore, no further optimization of the Suzuki-Miyaura reactions was performed.

Treatment of **145** with a solution of lithium hydroxide resulted in the hydrolysis of the ethyl ester moiety and simultaneous removal of the tosyl group (Scheme 46). The solvent was then evaporated and the crude mixture was dissolved in a solution of DCM/TFA to deprotect the Boc-amine. The key intermediate **122** was isolated after preparative HPLC purification in 69 % yield over the two steps.



Scheme 46. *Reagents and conditions*. (i) LiOH 2M, 45 °C, 4 days; (ii) TFA/DCM 1:1, 40 min, r.t., 69% yield; (iii) Biotin-PEG6-NHS ester, Et_3N, AcCN/DMF, 24 h, r.t., 43% yield; (iv) azido-PEG6-NHS ester, Et_3N, AcCN/DMF, 24 h, r.t., 51% yield.

The intermediate **122** presents most of the necessary features of a probe: the inhibitor part is already constructed and is tethered by the linker. Compound **122** was designed to act as a "pre-probe" that would allow us to easily transform it into different probes suitable for SPR and DIANA assay. The free amine group at the linker allows us to introduce different anchor groups in one simple step without having to modify or repeat the synthesis of the probe.

The first probe that I prepared was used to validate the binding of the molecule to the cap-binding domain of the PB2. The method used was Surface Plasmon Resonance (SPR) and this method required a probe linked to a biotin group. The preparation of this probe was done by treatment of **122** with the commercially available biotin-PEG6-NHS ester under the presence of triethylamine. The primary amine is without any doubts more reactive than the sterically hindered amino-pyrimidine group and thus and gave the desired probe **146** in moderate yields (Scheme 46). The probe was then purified by preparative HPLC.

Once the binding of the probe **146** to PB2 was corroborated by SPR, I had to prepare a second probe this time suitable for DIANA assay. We postulated that by separating the binding head and the anchor group with a linker of enough length we could minimize the possible effect in the binding that different anchor groups could have. Therefore, we assumed that the binding activity of probe **146** must be analogous to **147**. Treatment of **122** with azido-PEG6-NHS ester using the previously described conditions afforded the desired probe **147** in moderate yields.

The flexible and modular approach used for the preparation of VX-787-based probes not only allowed us to produce a common intermediate **122** that can be transformed into different detection probes, but also works as a backup plan in the case that further elongation of the linker was needed. Probe **147** was used to develop and optimize a DIANA technology-based screening assay, at the time writing the assay is ready for screening IOCB's vast library of nucleoside-like compounds. Promising results are expected to come since the natural ligand of the cap-binding PB2 domain is a nucleoside (7-methyl GTP).

3.2.4 L-742,001-based probe suitable for AlphaScreen assay

As I described in the introduction chapter, PA-endonuclease domain is an exceptionally promising therapeutic target for the development of new inhibitors since it's essential for the life cycle of the virus. Additionally, the enzyme is highly conserved among all three families of influenza and has no counterpart in human cells.

This last chapter is dedicated to the preparation of chemical tools, specifically to the preparation of probes required for development of an assay suitable for screening low-molecular-weight compounds against the PA-endonuclease. Unfortunately, in this case DIANA technology could not be used to develop the assay due to the endonuclease activity of the enzyme, which would cleave the reporter oligonucleotide part of the detection probe. AlphaScreen technology, which methodology has been described previously in the introduction, was used as a valid alternative method for the development of the screening assay.

From all the reported inhibitors of PA-endonuclease catalytic site, L-742,001 (**15**) stands out as the molecule with the highest potency (IC_{50} = 0.43 μM) and also *in vitro* activity.[41] In addition, L-742,001 has been successfully converted into a fluorescent probe previously, therefore the optimal position where structural modifications could be introduced has been already disclosed (Figure 38).[124]

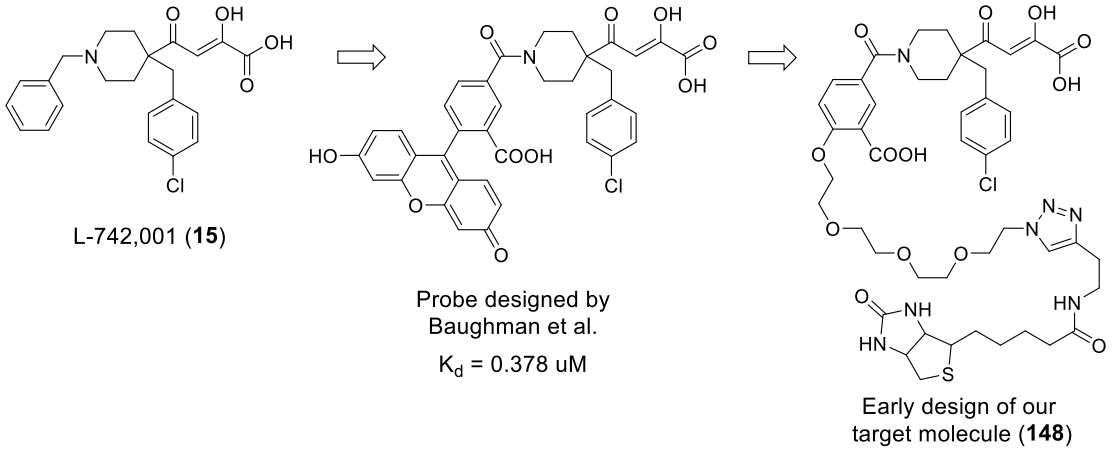


Figure 38. Structure of L-742,001 (left), fluorescent probe reported by Baughman et al. (center),[124] and the early design of our probe (right) which was partially based on the structure of the fluorescent probe.

We proposed that if the substitution of the *N*-benzyl group of L-742,001 by fluorescein did not compromise the binding ability of the molecule, replacement of the fluorescein group by the

much more flexible linker would not have an adverse effect either. Scrupulous study of the reported structure of 2009 H1N1 PA endonuclease in complex with L-742,001 (Figure 39) supported our assumption.

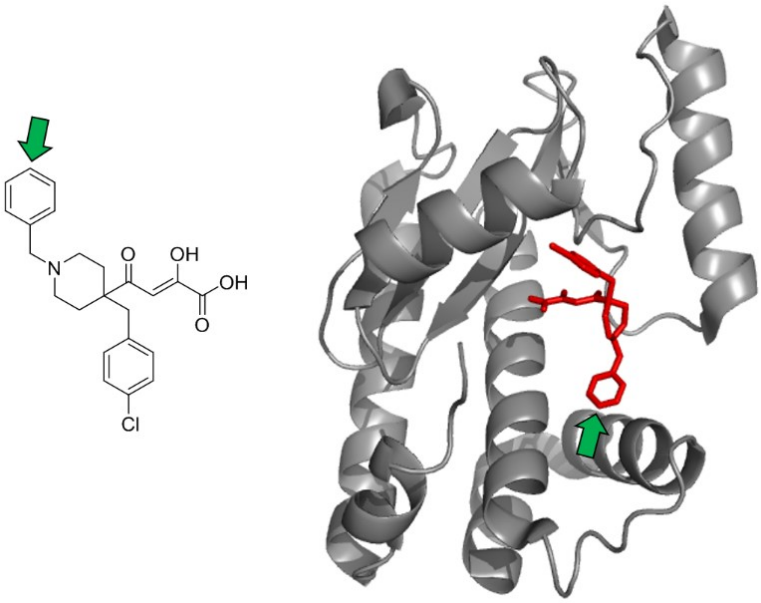


Figure 39. Structure of L-742,001 (left), the green arrow points to the future modification point. Co-crystal structure of L-742,001 (in red) with the catalytic site of PA (right). PDB 5CGV

The structure of the firstly designed probe is depicted in Figure 40. Three synthons can be readily disconnected, synthon **A** would form the binding head of the probe since it contains the β-diketo acid group, synthon **B** would mimic the second benzyl "wing" of L-742,001 and at the same time introduce the tetraethylene glycol linker which would be attached to biotin in the latest stage of the synthesis. Synthon **A** and **B** were prepared from the commercially available ethyl isonipecotate **149** and dimethyl 4-hydroxyisophthalate **150**, respectively.

Synthon **A** and **B** were prepared in parallel and the construction of the probe was done in a modular manner similar to the synthesis of the detection probes for the PB2 cap-binding domain.

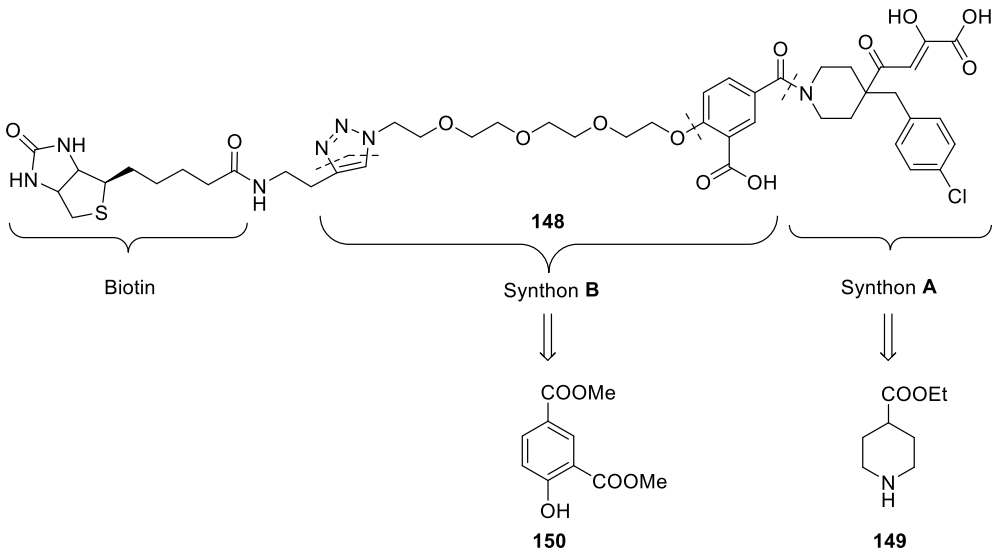
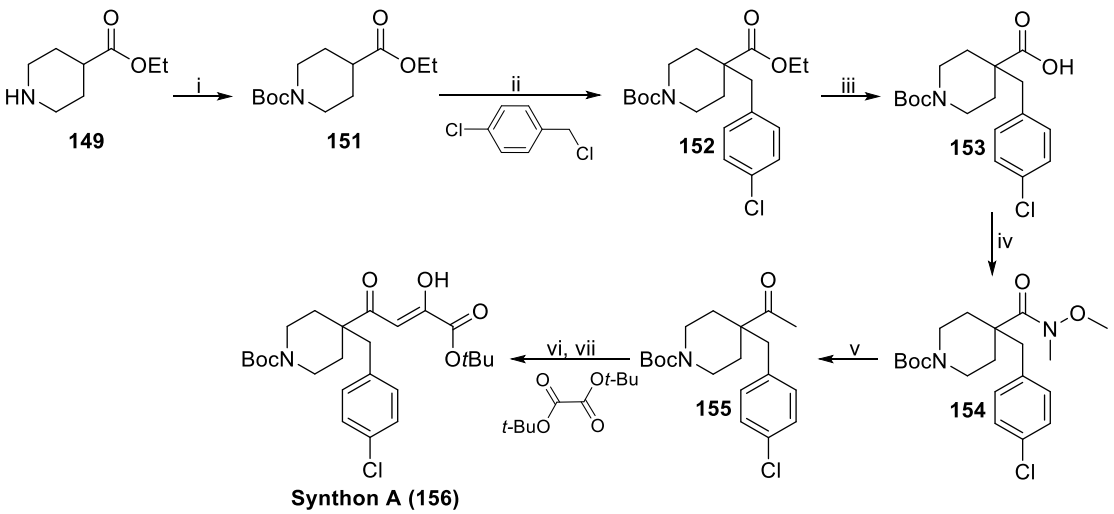


Figure 40. Structure of the first probe synthesized. The disconnection between synthon A, synthon B and biotin is represented, as well as the disconnection between the linker and the aromatic part of synthon B. The structure of the starting compounds used for the preparation of the two synthons is depicted as well.

3.2.4.1 Preparation of Synthon A

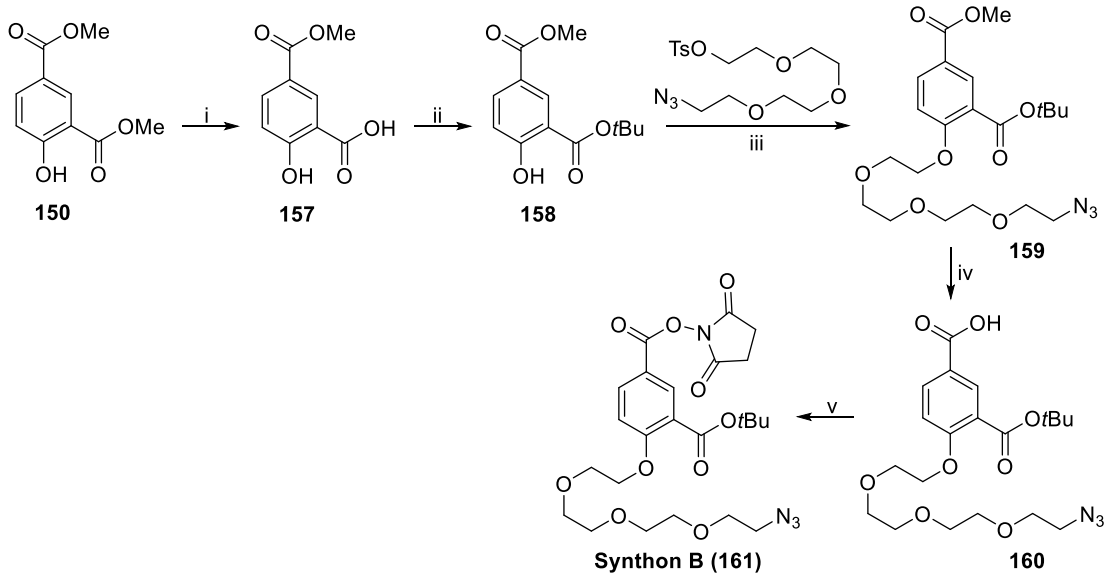
For the preparation of synthon **A** (**156**) I followed a reported procedure for the synthesis of L-742,001.[125] However, several modifications had to be introduced (Scheme 47). The synthesis begins with Boc-protection of the piperidine nitrogen atom in ethyl isonipecotate **149** followed by formation of the enolate by treatment with a strong base. Subsequent alkylation of the enolate with 4-chlorobenzyl chloride formed the first "wing" of the inhibitor. The ethyl ester group of **152** was hydrolyzed and the acid **153** was transformed to methyl ketone **155** *via* formation of the Weinreb amide **154** and its subsequent reaction with methylmagnesium chloride. Addition of one equivalent of the organometallic reagent to the Weinreb amide produces a tetrahedral intermediate that is stabilized and does not collapse to ketone under reaction conditions. The last step involves the formation of the key β-diketo acid moiety which was done *via* Claisen condensation of the ketone **155** with di-*tert*-butyl oxalate. Although di-*tert*-butyl oxalate could be less reactive than other oxalates due to the steric hindrance, the introduction of the *tert*-butyl ester was the most sensate option since Boc-deprotection and hydrolysis of the *tert*-butyl ester could be done simultaneously.



Scheme 47. *Reagents and conditions*. (i) Boc_2O, Et_3N, DCM, 16 h, r.t., 97% yield; (ii) NaHMDS 0.6M, THF, 4-chlorobenzyl chloride, -78 °C, 3h, 80% yield; (iii) KOH, MeOH/Dioxane, reflux, 48 h, 98%; (iv) EDC·HCl, THF, 15 min, r.t. then *N,O*-dimethylhydroxylamine hydrochloride, 48 h, r.t., 66% yield; (v) MeMgBr 3.0 M, THF, reflux, 40 min, 94% yield; (vi) NaHMDS 0.6 M, THF, -78 °C, 20 min; (vii) di-*tert*-butyl oxalate, 24 h, r.t., 78% yield.

3.2.4.2 Preparation of Synthon B

The preparation of synthon **B** (depicted in Scheme 48) commits with the regioselective hydrolysis of the *ortho* ester moiety on dimethyl 4-hydroxyisophthalate **150** using a reported set of conditions.[126] The resulting acid **157** was converted into a *tert*-butyl ester **158** and then the phenol was alkylated with the linker. The azide-PEG4-Ts linker was prepared from tetraethylene glycol

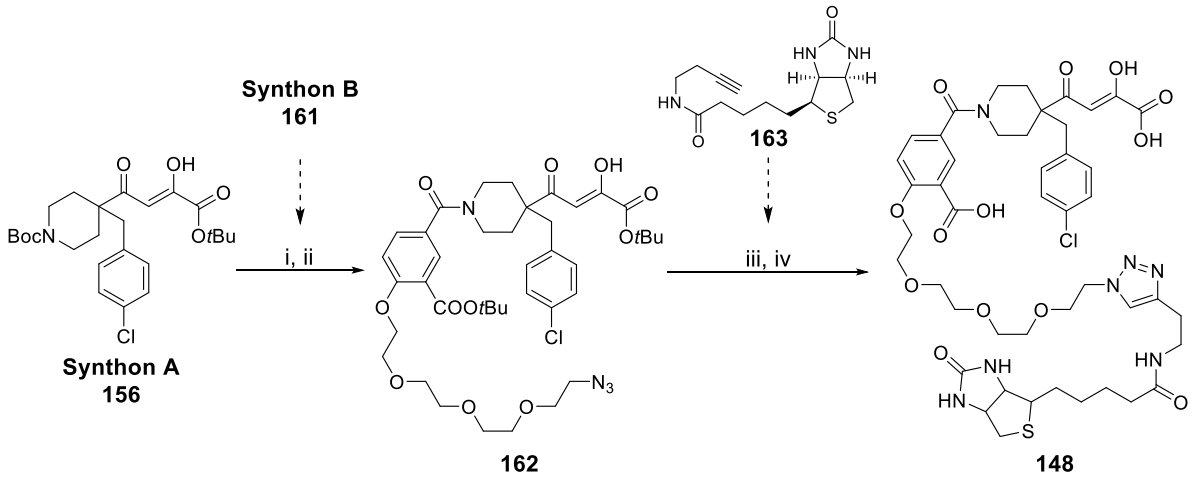


Scheme 48. *Reagents and conditions*. (i) py, reflux, 20 h, 93% yield; (ii) *t*-BuOH, $MgSO_4$, H_2SO_4, DCM, 48 h, 53% yield; (iii) azide-PEG4-Ts, K_2CO_3, DMF, 60 °C, 24 h, 94% yield; (iv) NaOH 0.5 M, 1,4-dioxane, 24 h, r.t., 97% yield; (v) TBTU, THF, Et_3N, *N*-hydroxysuccinimide, 24 h, r.t., 87% yield.

by bis-tosylation of both hydroxyl groups followed by S_N2 reaction with one equivalent of sodium azide. The last two steps involved the hydrolysis of the methyl ester in *para* position and formation of the *N*-hydroxysuccinimide ester (NHS) using TBTU coupling agent.

3.2.4.3 Construction of the probe based on L-742,001

The assembly of the probe is depicted in Scheme 49 and starts with the Boc-deprotection of the piperidine of synthon **A**. Treatment of intermediate **156** with a DCM solution of TFA at 0 °C gave me the expected free amine. Surprisingly when the reaction times were shorter than 1 hour I did not observe the cleavage of the *tert*-butyl ester. Once the deprotection was completed, the solvent was evaporated and the crude mixture re-dissolved in DCM, an excess of triethylamine was added to quench the residual TFA followed by addition of synthon **B** in dichloromethane. The coupled intermediate **162** had to be attached to the anchor group *N*-(but-3-ynyl)biotinamide **163** via CuAAC reaction. Compound **163** was prepared from commercially available biotin through the corresponding NHS ester and then the activated ester was treated with 1-amino-3-butyne. The first attempt for the CuAAC reaction used tetrakis(acetonitrile)copper(I) hexafluorophosphate as catalyst and tris(benzyltriazolyl-methyl)amine. Unfortunately, no formation of product was observed under these conditions. On the other hand, when copper(II) sulfate pentahydrate and sodium L-ascorbate were used instead, the desired product was formed in fair yields. The solvent was then removed and the crude mixture treated with a DCM solution of TFA to cleave the two remaining *tert*-butyl esters. The desired probe **148** was isolated after purification by preparative HPLC.

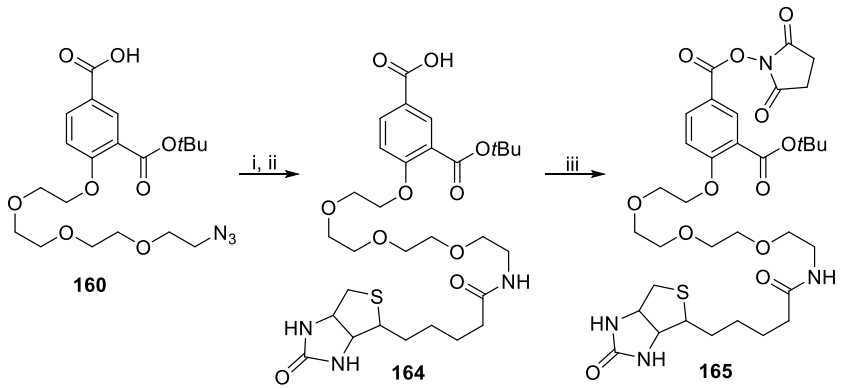


Scheme 49. *Reagents and conditions*. (i) TFA/DCM (1:1), 0 °C, 1 h. (ii) Synthon B, Et_3N, DCM, 24 h, r.t., 30% yield; (iii) **163**, $CuSO_4 \cdot 5H_2O$, sodium L-ascorbate, EtOH/water/THF (2:2:1), 1 h, r.t.; (iv) TFA/DCM (1:1), 1 h, r.t.

The results measured by SPR for this first probe **148** showed high binding activity towards PA endonuclease (K_d < 7.3 nM), but unfortunately the binding was non-stoichiometric which is fundamental for the AlphaScreen assay. Dr. Milan Kožíšek who worked on the development of the assay pointed two possible causes for the non-stoichiometric binding; either the triazole connection is affecting the binding between biotin and Streptavidin or the linker has an insufficient length to separate the donor bead and the PA endonuclease domain.

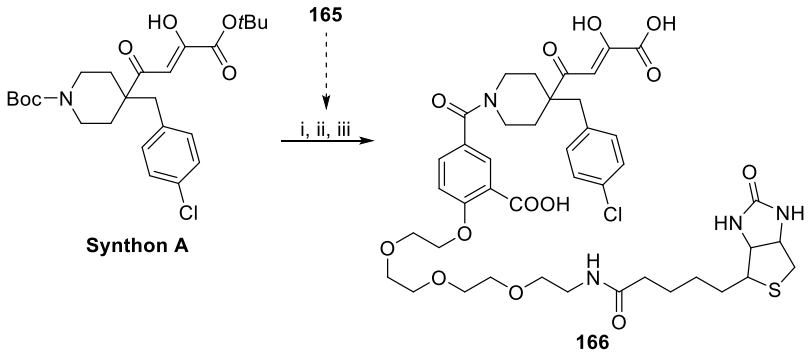
In the case of the second probe, we decided to test if the triazole group is responsible for the unusual binding. Thus, I replaced the triazole connection (between the biotin and the linker) by an amidic bond and study how this modification affects the binding activity of the probe.

To accomplish the synthesis of this second probe only a minor modification in the preparation of synthon **B** was needed (Scheme 50). The azido moiety from intermediate **160** had to be reduced *via* catalytic hydrogenation to form the free amine, the catalyst was then filtered out and the solvent evaporated. The resulting crude mixture was treated with a solution containing the NHS ester of biotin, which was prepared from biotin and *N*-hydroxysuccinimide. The biotinylated product **164** was isolated and converted to the activated NHS ester **165**.



Scheme 50. *Reagents and conditions*. (i) Lindlar catalyst, H_2 (1 atm), MeOH, 90 min, r.t.; (ii) NHS-biotin, Et_3N, DMF, 46 h, r.t., 39% yield; (iii) *N*-hydroxysuccinimide, TBTU, Et_3N, DMF, 24 h, r.t., 70% yield.

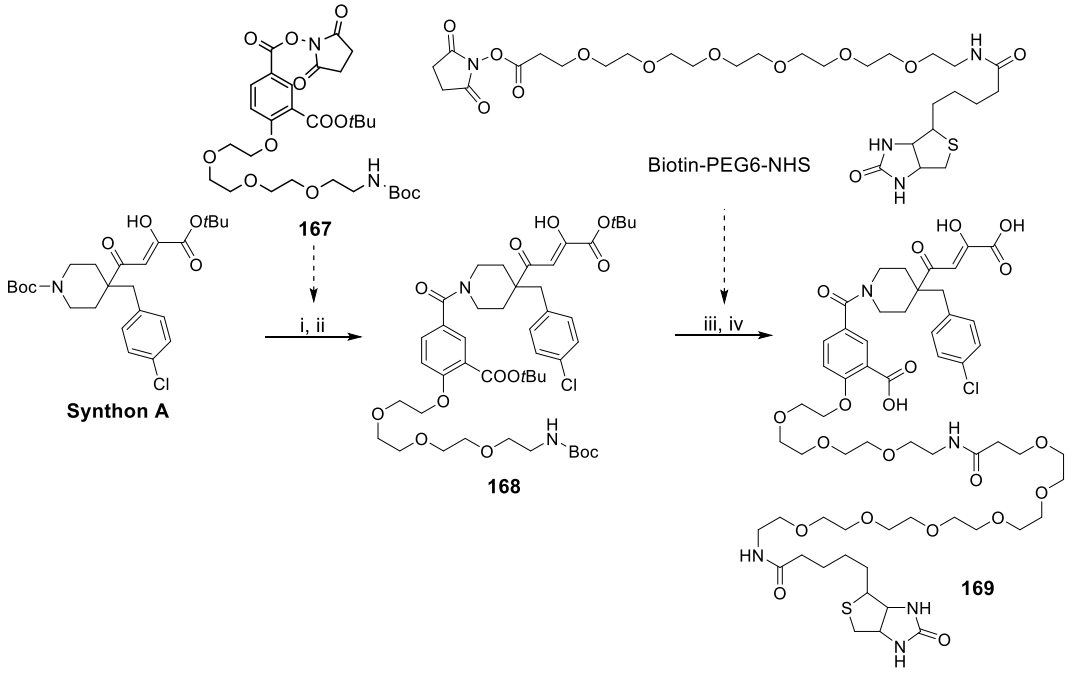
The connection between synthon **A** and the new synthon **B** (**165**) was done in an identical fashion as in the case of the first probe (Scheme 49, page 92). However, in this case, the crude coupled product was directly treated with TFA for the deprotection of the *tert*-butyl esters. Subsequent purification by preparative HPLC afforded the second probe **166**, (Scheme 51).



Scheme 51. *Reagents and conditions*. (i) TFA/DCM (1:1), 0 °C, 30 min; (ii) **165**, Et_3N, DCM, 24 h, r.t.; (iii) TFA/DCM (2:1) 2 h, r.t., 27% yield.

Although the binding activity was similar to probe 1 (**148**), the change in the construction of the probe did not fix the non-stoichiometric binding observed when the probe is used in the AlphaScreen assay. We decided to prepare a third probe in which the spacer between the binding head and the biotin group has been substantially elongated.

To simplify the synthesis, we decided to introduce a biotinylated linker in the latest step of the preparation. As a consequence of this change, I had to transform the azide intermediate **160** into the protected amine **167** *via one-pot* procedure in which the azido moiety of **160** was firstly reduced and the resulting amine was Boc-protected.



Scheme 52. *Reagents and conditions*. (i) TFA/DCM (1:5), 15 min, r.t.; (ii) **167**, Et_3N, DCM, 24 h, r.t., 68% yield; (iii) TFA/DCM (1:5), 15 min, r.t.; (iv) Biotin-PEG6-NHS ester, Et_3N, DCM, 24 h, r.t., 15% yield.

The construction of the probe was performed in a modular fashion by a sequence of Boc-amine deprotection and amide formation reactions (Scheme 52). Synthon **A** was firstly Boc-deprotected and the free amine coupled with the activated ester **167** in *one-pot* procedure. The resulting product **168** contains also a protected amine that was submitted for the same deprotection-coupling procedure, but this time using Biotin-PEG6-NHS ester. Purification by preparative HPLC afforded the desired third probe (**169**) with the elongated linker.

3.2.3.4 Results obtained from the AlphaScreen assay

The third probe (**169**) showed comparable binding activities (K_d < 9.3 nM) to the first and second probe (**148** and **166**) but in this case we observed a stoichiometric binding with a classic Hook effect as well as much higher sensitivity in the AlphaScreen assay (Figure 41). The results obtained verified the assumption that the short length of the linker was compromising the binding between the bead caring the probe and the bead attached to the PA endonuclease domain. These steric effects were minimized by the introduction of a much longer spacer.

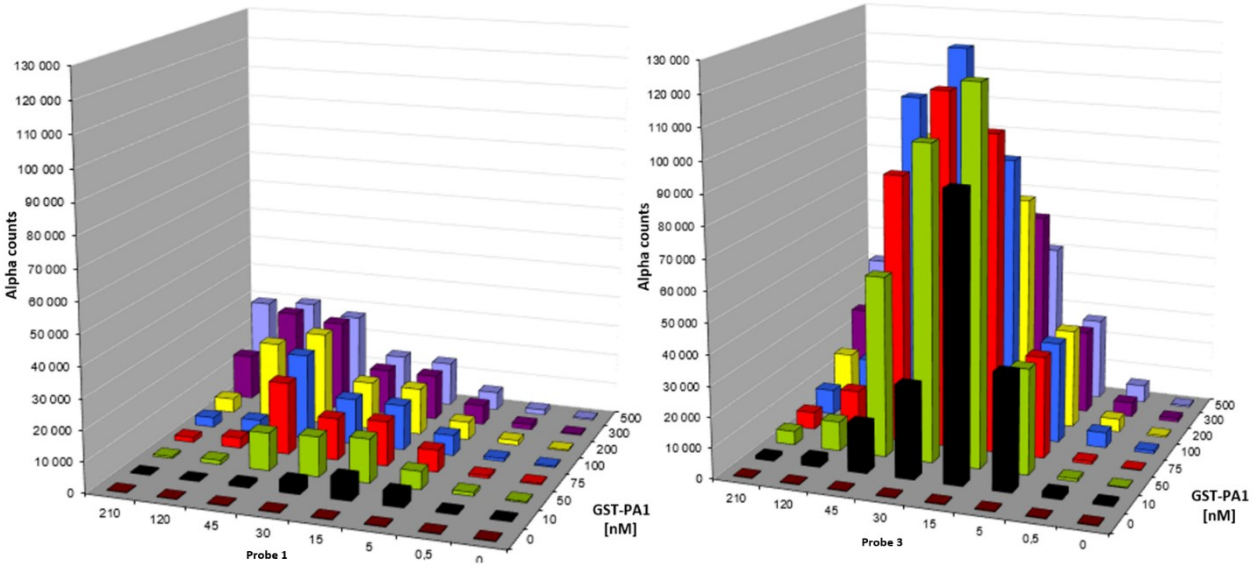


Figure 41. Comparison between the Alpha counts obtained by using 1st Probe (**148**, left) and 3rd Probe (**169**, right), an acute increment in the response of the assay can be easily appreciated for the 3rd Probe as well as a classic Hook effect.

The developed AlphaScreen assay was used to screen a first batch of fifty compounds for binding activity against PA endonuclease. Two integrase inhibitors were found to bind to PA endonuclease, both of them showing IC_{50} in the low micromolar range. Further screening will be done using the described probe.

4. Conclusion and outlook

During my research, I was able to reproduce Shi's and Hudlicky's syntheses of oseltamivir and adapt them for the production of C-3 modified derivatives of oseltamivir. The issues encountered during the key aziridine-formation and aziridine-opening steps were solved and several alcohols were screened for the aziridine opening step. The first set of compounds were prepared, and the results obtained will be published soon. The developed strategy allows a simple and direct preparation of an uncountable number of C-3 modified oseltamivir derivatives from (-)-shikimic acid. This part of my Thesis became the central work of one of my colleagues and further research is being carried out solely by him.

With regards to the modification at C-5 of oseltamivir, a short and effective synthetic strategy was proposed and the first set of derivatives were prepared following this method. A manuscript is being drafted at the time of this writing which will show the activities of the C-5 modified derivatives prepared, as well as the crystal structure of **94** with the catalytic site of neuraminidase. The objective of this publication is to shed light on the binding mode of these inhibitors to the 150-cavity adjacent to the NA catalytic site.

The reported synthesis of tamiphosphor was simplified and shortened, and tamiphosphor and its congeners were prepared. Furthermore, a novel radical-decarboxylation/photo-Arbuzov approach was explored for the conversion of vinyl carboxylic acids to vinyl phosphates in one step. To the best of our knowledge, only one similar reaction has been reported for this transformation. The synthesis and the activities of the prepared inhibitors, as well as the crystal structure of tamiphosphor binding to the neuraminidase active site have been published in the European Journal of Medicinal Chemistry.

In the second part of my Thesis, I pursued the preparation of chemical tools/probes which are required for the development of novel screening assays.

Several detection probes suitable for DIANA assay containing sialylmimetic groups were prepared. In particular, a highly active tamiphosphor-based detection probe was prepared and the crystal structure of the probe binding the catalytic site was furnished. The prepared inhibitors were used to validate DIANA assay as an efficient and reliable screening method and these findings are currently being prepared for publication.

A second tamiphosphor-based probe was also synthesized; this much longer probe was used for the preparation of a polymeric material which presents enhanced pharmacological properties. The polymer is intended to be administrated to patients in life-treating stages of influenza's infection. Unfortunately, due to the possibility of a future patent application, the findings could only be partially disclosed.

We also designed and prepared two detection probes based on the structure of the PB2 inhibitor VX-787. The synthesis had to be highly modified and optimized for the successful preparation of the probes from commercially available building blocks. The synthesized probes present strong binding affinities and are being used to develop a screening assay which aims to find novel inhibitors of PB2 cap-binding domain.

Lastly, a similar strategy was used to design and prepare several probes based on the structure of the PA endonuclease inhibitor L-742,001. The issues associated with the low sensitivity of the first probe were solved and the final probe was used to develop a screening assay based on AlphaScreen technology that has already discovered new inhibitors of PA endonuclease.

All of the detection probes could be used for an extensive period of time in multiple screening campaigns. Therefore, my research will contribute to the discovery of future drug candidates at IOCB against neuraminidase, PB2 cap-binding domain and PA endonuclease by the screening methods partially based on the work described this Thesis.

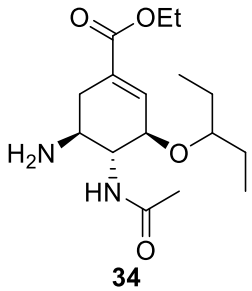
5. Experimental Section

General: Unless otherwise noted, all reactions were carried out under argon in oven-dried glassware. The solvents used for reactions were distilled from the corresponding drying agents indicated and were transferred under argon: THF (Na/benzophenone); toluene (Na); DCM (CaH_2). Chromatography was performed either classical or using HPFC Biotage Isolera One system, using Fluka silica gel 60 (0.040 - 0.063mm) or Merck silica gel 60 RP-18 F_{254} –coated aluminium sheets were used. The spots were detected both in UV and 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%). All starting materials were used as purchased (Sigma Aldrich, Alfa Aesar, Strem Chemicals, Santiago, 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 minutes), with column Waters SunFire C18 OBD Prep Column, 5 μm, 19 x 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 minutes) with column Watrex C18 Analytical Column, 5 μm, 250x5 mm. The final inhibitors were all at least of 95 % purity. The ^{1}H-NMR spectra were measured at 400.13 or 600.13 MHz, the ^{13}C-NMR spectra at 100.61 or 150.90 MHz in $CDCl_3$, MeOH-d_4, D_2O or $(CD_3)_2SO$, with tetramethylsilane or solvent peaks 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 solvent.

General procedure for saponification of the ethyl esters.

To a solution of the desired ethyl ester (1 equivalent) in 1,4-dioxane (0.14 M) was added an aqueous solution of NaOH (2 equivalents, 0.5 M), the reaction mixture was stirred from 2 h to 24 h at room temperature. The consumption of the starting material was monitored by TLC. Once the reaction was complete, the pH of the reaction was adjusted to neutral by addition of amberlite IR 120. The amberlite was filtered out and the beads rinsed with MeOH several times, the filtrate was then evaporated to dryness and the remaining product was purified either by preparative HPLC or flash chromatography to afford the desired free acid.

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

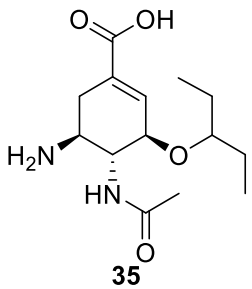


34

To a solution of oseltamivir phosphate (1.0 g, 2.43 mmol) in water (30 mL) was added a saturated solution of sodium bicarbonate (10 mL). The reaction mixture was stirred for 5 min at room temperature. The mixture was extracted with DCM/MeOH mixture (3:1; 4x10 mL). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to furnish the free base of oseltamivir (0.75 g, 98% yield). The product was used without further purification.

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 6.78 (t, *J* = 2.1 Hz, 1H), 6.26 (d, *J* = 8.3 Hz, 1H), 4.34 – 4.10 (m, 3H), 3.55 (dt, *J* = 10.3, 8.4 Hz, 1H), 3.37 (dd, *J* = 17.7, 12.0 Hz, 1H), 3.26 – 3.08 (m, 1H), 2.76 (dd, *J* = 17.7, 5.1 Hz, 1H), 2.26 – 2.08 (m, 1H), 2.04 (s, 3H), 1.58 (s, 2H), 1.55 – 1.44 (m, 4H), 1.29 (t, *J* = 7.1 Hz, 3H), 0.90 (td, *J* = 7.4, 3.8 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 171.1, 166.4, 137.8, 129.5, 81.7, 77.6, 77.2, 76.7, 75.0., 60.8, 58.9, 49.3, 33.7, 26.2, 25.7, 23.6, 14.2, 9.6, 9.3. HR-ESI-MS calculated for $C_{16}H_{29}O_4N_2$ ($M+H^+$) 313.2122, found 313.2122.

Oseltamivir carboxylate



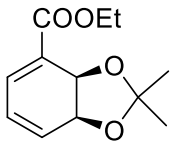
35

The conditions used are described in the general procedure for the saponification of ethyl esters. Purified by preparative HPLC (0.12 g, 88% yield).

^{1}H NMR (400 MHz, Methanol-*d*$_4$) δ 6.64 (s, 1H), 4.11 (d, *J* = 8.5 Hz, 1H), 3.83 (dd, *J* = 11.4, 8.7 Hz, 1H), 3.40 (td, *J* = 10.8, 5.5 Hz, 1H), 3.34 – 3.22

(m, 1H), 3.16 (dt, *J* = 3.3, 1.6 Hz, 1H), 2.80 (dd, *J* = 17.3, 5.1 Hz, 1H), 2.38 – 2.18 (m, 1H), 1.91 (s, 3H), 1.47 – 1.28 (m, 4H), 0.75 (dt, *J* = 12.6, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.9, 169.6, 138.4, 129.8, 83.7, 76.0, 54.5, 50.5, 30.0, 27.2, 26.6, 23.4, 9.8, 9.6. HR-ESI-MS calculated for $C_{14}H_{23}O_4N_2$ ($M\text{-}H^+$) 283.1663, found 283.1656.

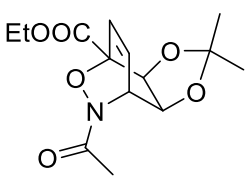
Ethyl (3a*R*,7a*S*)-2,2-dimethyl-3a,7a-dihydrobenzo[*d*][1,3]dioxole-4-carboxylate



63

(3a*S*,7a*S*)-4-Iodo-2,2-dimethyl-3a,7a-dihydro-1,3-benzodioxole (3.5 g, 12.6 mmol) was dissolved in 2,2-dimethoxypropane (10 mL) and *p*-toluenesulfonic acid monohydrate (0.17 g, 5 w%) was added, the reaction mixture was stirred for 5 min, after which the consumption of the starting material was checked by TLC (hexanes/EtOAc 4:1). The reaction was quenched by addition of a saturated solution of sodium bicarbonate (10 mL) and the aqueous phase was extracted with EtOAc (3x 15 mL), the combined organic layers were evaporated under reduced pressure at 20 ºC. The resulting product was dissolved in EtOH (200 mL) followed by addition of triethylamine (3.5 mL, 25.2 mmol), the reaction mixture was purged with carbon monoxide for 15 min. Triphenylphosphine (0.66 g, 2.53 mmol) and palladium(II) acetate (0.28 g, 1.26 mmol) were subsequently added, the reaction mixture was heated to 40 ºC for 2 h under constant bubbling of carbon monoxide through the solution. The solvent was evaporated under reduced pressure and the resulting mixture was purified by flash chromatography (Hexanes/EtOAc 4:1) to yield the desired ethyl ester (2.4 g, 85%). The product was immediately used for the next reaction due to the low stability of the compound.

3-Acetyl-1-ethoxycarbonyl-5,6-O-isopropylidine-2-oxa-3-azabicyclo[2.2.2]oct-7-ene- 5,6-diol

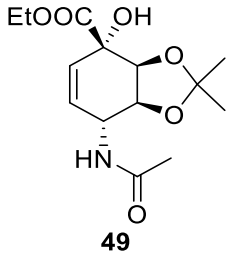


48

Sodium periodate (1.5 g, 7.06 mmol) was added portion wise to a solution of ethyl (3a*R*,7a*S*)-2,2-dimethyl-3a,7a-dihydrobenzo[*d*][1,3]dioxole-4-carboxylate (0.79 g, 3.53 mmol) in aqueous methanol (80 mL, MeOH/water 3:1) at 0 ºC. A solution of acetohydroxamic acid (0.53 g, 7.06 mmol) in methanol (20 mL) was added dropwise to the reaction mixture at 0 ºC and the resulting solution was stirred overnight at room temperature. The reaction mixture was quenched by addition of a saturated solution of sodium bicarbonate (10 mL), the precipitate was filtered out and the filtrate was concentrated down under reduced pressure. The resulting aqueous phase was

extracted with EtOAc (4x 25 ml), the combined organic phases were washed with a saturated solution of sodium bicarbonate (20 mL) and evaporated down. The resulting mixture was purified by flash chromatography (Hexanes/EtOAc 3:1) to yield the cycloaddition product (0.9 g, 88%).
^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.66 – 6.54 (m, 2H), 5.47 (s, 1H), 4.70 (dd, J = 6.9, 0.8 Hz, 1H), 4.54 (ddd, J = 6.9, 4.2, 0.8 Hz, 1H), 4.37 (q, J = 7.1 Hz, 2H), 1.99 (s, 3H), 1.36 (t, J = 7.1 Hz, 3H), 1.31 (s, 3H), 1.29 (s, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 174.0, 166.6, 132.5, 128.5, 111.7, 79.3, 76.2, 72.9, 62.8, 50.1, 25.7, 25.5, 21.8, 14.2.

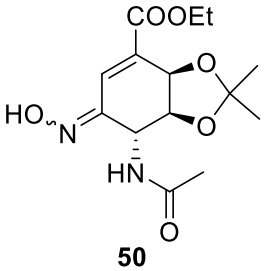
Ethyl (3a*S*,4*S*,7*R*,7a*S*)- -7-acetamido-4-hydroxy-2,2-dimethyl-3a,4,7,7a-tetrahydrobenzo[*d*]-[1,3]dioxole-4-carboxylate



49

Molybdenum hexacarbonyl (0.92 g, 3.48 mmol) was added to a solution of 3-acetyl-1-ethoxycarbonyl-5,6-O-isopropylidine-2-oxa-3-azabicyclo[2.2.2]oct-7-ene-5,6-diol (0.5 g, 1.74 mmol) in aqueous acetonitrile (9 mL, CH_3CN/water 15:1) and the reaction was heated up to 85 ºC for 4 h. The reaction was cooled down to room temperature and the resulting slurry was filtered through a pad of celite, rinsed several times with EtOAc and the combined organic phases were evaporated down under reduced pressure. The resulting mixture was purified by flash chromatography (EtOAc) to afford the reduced alcohol (0.40 g, 77%).
^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.12 (d, J = 8.8 Hz, 1H), 6.06 – 5.91 (m, 2H), 4.81 (dtd, J = 8.8, 3.8, 1.3 Hz, 1H), 4.40 – 4.33 (m, 2H), 4.27 (qd, J = 7.2, 1.4 Hz, 2H), 4.00 (s, 1H), 1.99 (s, 3H), 1.36 (s, 3H), 1.33 (t, J = 7.2 Hz, 3H), 1.29 (s, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 172.8, 169.9, 133.0, 129.7, 109.4, 81.1, 76.5, 74.5, 62.9, 48.9, 26.3, 24.3, 23.6, 14.1.

Ethyl (3a*R*,7*R*,7a*S*)-7-acetamido-6-(hydroxyimino)-2,2-dimethyl-3a,6,7,7atetrahydrobenzo-[*d*][1,3]dioxole-4-carboxylate



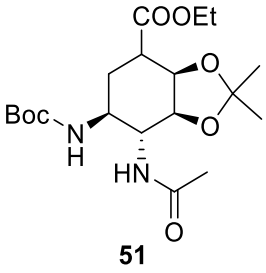
50

Chromium(VI) oxide (0.8 g, 8.0 mmol) was carefully added to a solution of acetic anhydride (2 mL) at 80 ºC, the solution was stirred for 5 min until all the chromium oxide was dissolved. The solution was allowed to cool down to room temperature, the mixture was diluted in DCM (6 mL) and further cooled down to 0 ºC on an ice bath. In a separate flask was prepared a

solution of the alcohol (0.96 g, 3.2 mmol) in DCM (20 mL) and the solution was cooled down to 0 ºC in an ice bath, the chromium oxide solution was added dropwise to the solution of the alcohol and the reaction was stirred for 5 min, the complete consumption of starting material was checked by TLC (EtOAc). The reaction was cooled down to 0 ºC again followed by addition of EtOH (7.5 mL), sodium bicarbonate (1.9 g), pyridine (0.35 mL) and hydroxylamine hydrochloride (2.2 g, 32.0 mmol), the resulting reaction mixture was stirred for 16 h at room temperature. The reaction was carefully quenched by addition of a saturated solution of sodium bicarbonate (10 mL) and the two phases were separated, the aqueous solution was extracted with EtOAc (3x 15 mL). The combined organic layers were evaporated under reduced pressure and the remaining mixture was purified by column chromatography (EtOAc) to furnish the desired oxime (0.71 g, 71%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 9.56 (s, 1H), 7.75 (s, 1H), 6.00 (d, J = 8.6 Hz, 1H), 5.09 – 4.89 (m, 2H), 4.42 – 4.19 (m, 3H), 2.04 (s, 3H), 1.49 (s, 3H), 1.43 (s, 3H), 1.34 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 171.2, 165.5, 149.1, 132.6, 124.9, 111.3, 76.2, 70.8, 61.9, 50.1, 28.0, 26.6, 23.5, 14.3.

(3a*R*,6*S*,7*R*,7a*S*)-7-Acetylamino-6-*tert*-butoxycarbonylamino-2,2-dimethyl-3a,6,7,7a-tetrahydro-benzo[1,3]dioxole-4-carboxylic acid ethyl ester



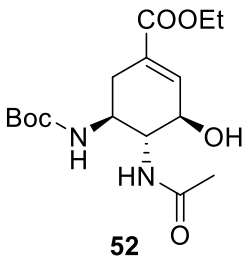
51

A suspension of ethyl (3a*R*,7*R,*7a*S*)-7-acetamido-6-(hydroxyimino)-2,2-dimethyl-3a,6,7,7a-tetrahydrobenzo-[*d*][1,3]dioxole-4-carboxylate (0.43 g, 1.38 mmol), di-*tert*-butyl dicarbonate (1.04 g, 3.17 mmol) and Rh/Al_2O_3 5% (0.17 g, 40 wt%) in aqueous ethanol (10 mL, EtOH/water 100:1) was thoroughly purged with hydrogen followed by hydrogenation in a Parr apparatus (60 pound/inch^2). After 16 h the reaction was filtered, the catalyst was rinsed with ethanol several times and the combined ethanolic phases were concentrated down, the resulting mixture was purified by column chromatography (Hexanes/EtOAc 1:1) to yield the hydrogenated Boc-protected amine (0.38 g, 69%) as a white solid.

^{1}H NMR (300 MHz, Chloroform-d_3) δ 5.67 (d, J = 9.3 Hz, 1H), 4.98 (d, J = 8.8 Hz, 1H), 4.54 (t, J = 4.1 Hz, 1H), 4.31 – 4.04 (m, 2H), 3.97 (dt, J = 11.4, 9.2 Hz, 1H), 3.84 (dd, J = 8.9, 4.4 Hz, 1H), 3.35 (tdd, J = 11.9, 8.8, 3.2 Hz, 1H), 2.80 (dt, J = 13.0, 4.3 Hz, 1H), 2.14 – 2.04 (m, 1H), 1.98 (s, 3H), 1.99 – 1.84 (m, 1H), 1.54 (s, 3H), 1.40 (s, 9H), 1.33 (s, 3H), 1.25 (t, J = 7.1 Hz, 3H). ^{13}C NMR

(75 MHz, Chloroform-d_3) δ 171.3, 170.5, 156.3, 110.0, 79.8, 78.8, 73.9, 61.1, 55.3, 51.0, 41.5, 28.5 (x4), 28.1, 26.3, 23.5, 14.3.

(3*R*,4*R*,5*S*)-4-Acetylamino-5-*tert*-butoxycarbonylamino-3-hydroxy-cyclohex-1-enecarboxylic acid ethyl ester

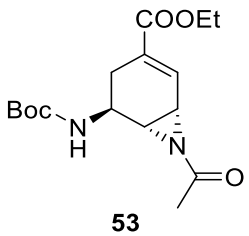


52

A solution of sodium ethoxide in ethanol (6.4 mL, 0.05 M) was added to a flask containing a solution of the (3a*R*,6*S*,7*R*,7a*S*)-7-acetylamino-6-*tert*-butoxycarbonylamino-2,2-dimethyl-3a,6,7,7a-tetrahydro-benzo[1,3]-dioxole-4-carboxylic acid ethyl ester (0.33 g, 0.83 mmol) in absolute ethanol (7.7 mL) and the reaction was stirred for 1 h at room temperature. The mixture was quenched by addition of water (10 mL) and an aqueous solution of citric acid (0.5 mL, 5 w%). The aqueous solution was extracted with EtOAc (3x 10 mL), the combined organic layers were washed with brine (5 mL) and concentrated under reduced pressure. The resulting mixture was purified by column chromatography (DCM/MeOH 20:1) to yield the elimination product (0.247 g, 87%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 7.35 (d, J = 5.9 Hz, 1H), 6.81 (t, J = 2.4 Hz, 1H), 5.02 (broad s, 1H), 4.82 (d, J = 7.8 Hz, 1H), 4.31 (dq, J = 5.8, 1.9 Hz, 1H), 4.20 (qd, J = 7.1, 1.7 Hz, 2H), 3.81 (ddd, J = 10.7, 7.7, 5.4 Hz, 1H), 3.72 (ddd, J = 11.6, 8.0, 5.9 Hz, 1H), 2.91 – 2.75 (m, 1H), 2.27 – 2.09 (m, 1H), 2.01 (s, 3H), 1.46 (s, 9H), 1.28 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 173.8, 166.0, 157.8, 139.1, 127.7, 81.1, 73.9, 61.2, 60.9, 48.1, 31.0, 28.4, 23.2, 14.3.

Ethyl (1*S*,5*S*,6*R*)- 7-acetyl-5-(*tert*-butoxycarbonylamino)-7-azabicyclo[4.1.0]hept-2-ene-3-carboxylate



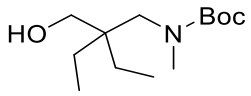
53

Diisopropyl azodicarboxylate (0.14 g, 0.69 mmol) was added dropwise to a solution of dimethylphenylphosphine (0.09 g, 0.69 mmol) in DCM (1.3 mL) at -40 ºC under inert atmosphere. In a separated flask was prepared a solution of (3*R*,4*R*,5*S*)-4-acetylamino-5-*tert*-butoxycarbonylamino-3-hydroxy-cyclohex-1-enecarboxylic acid ethyl ester (0.11 g, 0.32 mmol) and triethylamine (9 µL) in DCM (1.3 mL) under inert atmosphere. The solution of the substrate was

cooled down to 0 ºC and then it was added dropwise to the solution of the phosphine at -40 ºC. The reaction was stirred for 10 min allowing it to gradually warm up to 0 ºC. The solvents were evaporated under reduced pressure and the resulting mixture was purified by column chromatography (hexanes/EtOAc 3:1) to yield the aziridine (0.07 g, 66%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 7.19 (dd, J = 4.3, 3.3 Hz, 1H), 4.55 (m, 1H), 4.44 (m, 1H), 4.21 (q, J = 7.1 Hz, 2H), 3.18 – 3.05 (m, 2H), 2.75 (dt, J = 17.2, 1.9 Hz, 1H), 2.42 – 2.26 (m, 1H), 2.15 (s, 3H), 1.44 (s, 9H), 1.36 – 1.24 (t, J = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 165.6, 155.1, 133.6, 130.3, 80.0, 61.2, 42.1, 41.2, 32.0, 28.5 (3x), 28.2, 26.9, 22.8, 14.3.

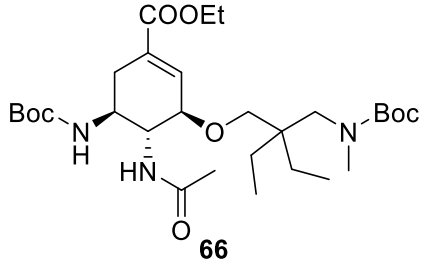
2-Ethyl-2-[(*tert*-butoxycarbonylmethylamino)methyl]butan-1-ol



Di-*tert*-butyl dicarbonate (0.94 g, 4.3 mmol) was added to a solution of 2-ethyl-2-[(methylamino)methyl]butan-1-ol (0.5 g, 3.4 mmol) and triethylamine (1.05 mL, 7.5 mmol) in methanol (10 mL) and the reaction was stirred at room temperature. After 6 h the reaction mixture was concentrated under reduced pressure and the remaining oil was purified by column chromatography (DCM/MeOH 20:1) to afford the protected amine (0.79 g, 95%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 4.55 (t, J = 7.6 Hz, 1H), 3.15 (d, J = 7.6 Hz, 2H), 3.05 (s, 2H), 2.89 (s, 3H), 1.46 (s, 9H), 1.28 (dt, J = 15.0, 7.4 Hz, 2H), 1.14 (dt, J = 14.3, 7.3 Hz, 2H), 0.83 (t, J = 7.5 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 155.2, 80.5, 64.3, 52.6, 43.0, 38.1, 28.5 (x3), 22.6 (x2), 7.1 (x2).

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(2-(((*tert*-butoxycarbonyl)(methyl)amino)methyl)-2-ethylbutoxy)-5-((*tert*-butoxycarbonyl)amino)cyclohex-1-ene-1-carboxylate



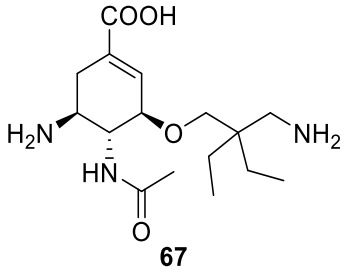
66

An oven-dried flask was charged with a solution of ethyl (1*S*,5*S*,6*R*)- 7-acetyl-5-(*tert*-butoxycarbonylamino)-7-azabicyclo[4.1.0]hept-2-ene-3-carboxylate (42 mg, 0.13 mmol) and 2-ethyl-2-[(*tert*-butoxycarbonylmethylamino)methyl]-butan-1-ol (158 mg, 0.64 mmol) in DCM (1 mL), the flask was purged with argon and the solution was cooled to -40 ºC. Boron trifluoride diethyl etherate (36 mg, 0.26 mmol) was added dropwise to the solution of the aziridine

and the reaction mixture was gradually warmed to 0 ºC. After 1 h stirring at 0 ºC, the solvent was removed under reduced pressure and the resulting mixture was purified by column chromatography (hexanes/EtOAc 1:1) to yield the desired ether (25 mg, 34%).

^{1}H NMR (600 MHz, Chloroform-*d*$_3$) δ 7.83 – 7.61 (m, 1H), 6.87 (s, 1H), 6.05 (d, *J* = 7.3 Hz, 1H), 4.21 (qd, *J* = 7.2, 4.1 Hz, 2H), 3.97 – 3.84 (m, 2H), 3.80 – 3.71 (m, 1H), 3.65 (m, 1H), 3.47 (d, *J* = 8.8 Hz, 1H), 3.01 – 2.91 (m, 2H), 2.88 (s, 3H), 2.46 (d, *J* = 14.3 Hz, 1H), 2.23 – 2.14 (m, 1H), 1.98 (s, 3H), 1.46 (s, 9H), 1.43 (s, 9H), 1.29 (m, 7H), 0.85 (t, *J* = 7.4 Hz, 3H), 0.76 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*$_3$) δ 173.5, 166.4, 157.1, 156.4, 134.9, 130.0, 79.9, 79.2, 78.0, 71.2, 61.1, 54.2, 52.5, 51.9, 42.6, 38.3, 32.4, 28.6 (6x), 23.4, 23.1, 22.2, 14.4, 7.6, 7.1. HR-ESI-MS calculated for $C_{29}H_{51}O_8N_3Na$ $(M+Na)^+$ 592.3568, found 592.3569.

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(2-(aminomethyl)-2-ethylbutoxy)cyclohex-1-ene-1-carboxylic acid

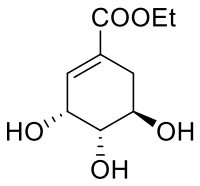


67

The conditions used are described in the general procedure for the saponification of ethyl esters. The solvent was removed under reduced pressure and the remaining solid was dissolved in TFA (3 mL, 100%), the reaction was stirred for 1 h at room temperature. The acid was removed under reduced pressure and the resulting solid was purified by preparative HPLC to yield the desired amino acid.

^{1}H NMR (400 MHz, D_2O) δ 7.00 (t, *J* = 2.1 Hz, 1H), 4.35 – 4.14 (m, 2H), 3.77 (d, *J* = 9.5 Hz, 1H), 3.63 (td, *J* = 10.7, 5.7 Hz, 1H), 3.48 (d, *J* = 9.5 Hz, 1H), 3.05 (s, 2H), 3.03 – 2.94 (m, 1H), 2.72 (s, 3H), 2.59 – 2.47 (m, 1H), 2.12 (s, 3H), 1.48 – 1.30 (m, 4H), 0.85 (td, *J* = 7.5, 5.6 Hz, 6H). ^{13}C NMR (101 MHz, D_2O) δ 175.4, 168.9, 136.1, 128.3, 77.8, 74.3, 55.9, 51.3, 48.6, 39.1, 34.3, 28.4, 23.5, 23.1, 22.3, 6.3, 6.2. HR-ESI-MS calculated for $C_{17}H_{32}O_4N_3$ $(M+H)^+$ 342.2387, found 342.2388.

Ethyl (3*R*,4*S*,5*R*)-(−)-3,4,5-trihydroxy-1-cyclohexenecarboxylate

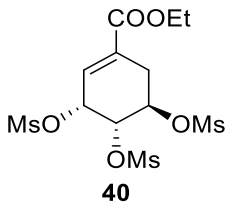


39

(-)-Shikimic acid (1 g, 5.7 mmol) was dissolved in absolute EtOH (10 mL) and thionyl chloride (0.21 mL, 2.8 mmol) was added dropwise to reaction mixture. The solution was then refluxed for 3 h under inert atmosphere. The solvent was evaporated under reduced pressure and the remaining residue was triturated with refluxing *tert*-butyl methyl ether (20 mL). The solution was slowly cooled to 0 ºC and the formed precipitate was filtered and dried under reduced pressure, the desired ethyl ester was obtained as a white solid (1 g, 86%), mp 97.7 - 99.3 ºC (*tert*-butyl methyl ether).

^{1}H NMR (300 MHz, DMSO-d_6) δ 6.60 (s, 1H), 4.22 (d, J = 2.3 Hz, 1H), 4.11 (q, J = 7.1 Hz, 2H), 3.85 (dt, J = 6.1, 4.1 Hz, 1H), 3.65 – 3.54 (m, 1H), 2.46 – 2.35 (m, 1H), 2.13 – 1.98 (m, 1H), 1.21 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.3, 139.6, 127.7, 70.1, 66.9, 65.5, 60.1, 29.7, 14.2.

Ethyl (3*R*,4*S*,5*R*)-3,4,5-trimethanesulfonyl-1-cyclohexenecarboxylate

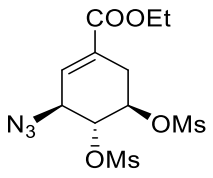


40

To a vigorously stirred solution of ethyl (3*R*,4*S*,5*R*)-(−)-3,4,5-trihydroxy-1-cyclohexenecarboxylate (1.34 g, 6.62 mmol) in EtOAc (17 mL) at 0 ºC was added dropwise methanesulfonyl chloride (2.3 mL, 29.8 mmol) and 4-dimethylaminopyridine (0.24 g, 1.98 mmol). Triethylamine (4.6 mL, 33.1 mmol) was subsequently added over a period of 30 min and the resulting solution was stirred for a further hour at 0 ºC. The reaction mixture was quenched by addition of aqueous HCl 1M (11.5 mL) and the organic phase was separated, washed with an aqueous solution of K_2CO_3, dried over $MgSO_4$ anhydrous and evaporated down. The resulting oil was triturated with aqueous MeOH (MeOH/water 8:2) and the formed precipitate was filtered, rinsed with aqueous MeOH and dried under reduced pressure to afford the mesylated compound as a white solid (2.45 g, 97%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.89 – 6.78 (m, 1H), 5.50 (t, J = 4.4 Hz, 1H), 5.13 (ddd, J = 9.2, 7.2, 5.9 Hz, 1H), 5.04 – 4.93 (m, 1H), 4.25 (q, J = 7.1 Hz, 2H), 3.28 – 3.22 (m, 1H), 3.19 (s, 3H), 3.17 (s, 3H), 3.13 (s, 3H), 2.68 (dddd, J = 18.8, 7.3, 2.5, 1.1 Hz, 1H), 1.31 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 164.4, 133.2, 130.1, 74.6, 72.9, 72.8, 62.0, 39.1 (x2), 39.0, 30.7, 14.2.

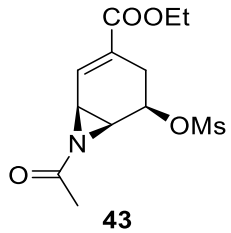
Ethyl (3*S*,4*S*,5*R*)-3-azido-4,5-dimethanesulfonyl-1-cyclohexenecarboxylate



41

A double neck flask equipped with an internal thermometer was charged with ethyl (3*R*,4*S*,5*R*)-3,4,5-O-trimethanesulfonyl-1-cyclohexenecarboxylate (0.5 g, 1.14 mmol) and acetone (4 mL), the solution was cooled down to 0ºC in an ice bath and a solution of sodium azide (0.29 g, 4.5 mmol) in water (0.8 mL) was added dropwise over a period of 10 min, keeping the reaction mixture always under 5 ºC, the reaction was left stirring for 4 h more at 0 ºC. The reaction mixture was diluted in toluene (10 mL) and quenched by water (2.5 mL). The organic phase was separated and washed successively with water (10 mL) and brine (5 mL). The organic phase was dried over anhydrous $MgSO_4$, and the solvents were evaporated under reduced pressure. The crude product was then purified by flash chromatography (Hexanes/EtOAc 1:1) to furnish azide compound (89%).
^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.73 (td, J = 3.5, 2.8, 0.8 Hz, 1H), 4.87 (td, J = 9.6, 6.1 Hz, 1H), 4.74 (dd, J = 10.0, 7.9 Hz, 1H), 4.33 (dq, J = 8.0, 1.9 Hz, 1H), 4.22 (q, J = 7.1 Hz, 2H), 3.21 – 3.15 (m, 1H), 3.20 (s, 3H), 3.13 (s, 3H), 2.77 – 2.52 (m, 1H), 1.29 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 164.2, 132.1, 130.3, 79.1, 74.0, 61.8, 61.2, 39.4, 39.0, 31.2, 14.2.

Ethyl (1*S*,5*R*,6*S*)-7-Acetyl-5-methanesulfonyloxy-7-azabicyclo[4.1.0]hept-2-ene-3-carboxylate



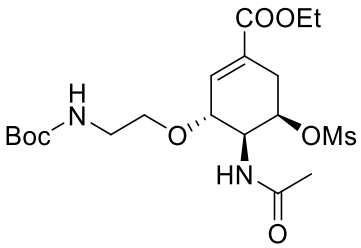
43

To a solution of ethyl (3*R*,4*S*,5*R*)-3-azido-4,5-dimethanesulfonyl-1-cyclohexenecarboxylate (0.8 g, 2.1 mmol) in THF (20 mL) was added triphenylphosphine (0.6 g, 2.3 mmol) in portions, the reaction was stirred for 30 min at room temperature. Triethylamine (0.86 mL, 6.3 mmol) and water (1.6 mL) were added and the reaction mixture was allowed to stir overnight at room temperature. After addition of water (3 mL) the solution was extracted with EtOAc (3x 20 mL), the combined organic layers were washed with brine (5 mL) and evaporated to dryness. The remaining oil was dissolved in EtOAc (28 mL) and cooled down in an ice bath, triethylamine (0.83 mL, 6.3 mmol) was added followed by dropwise addition of acetic anhydride (0.38 mL, 4.2 mmol) over a 10 min period, the reaction mixture was stirred 30 min more. The reaction mixture was quenched by addition of a solution of K_2CO_3 (15 w%, 6 mL), the organic layer was then washed with water (5 mL) and brine (5 mL). The combined organic layers were evaporated to dryness and

the remaining residue was purified by flash chromatography (Hexanes/EtOAc 1:1) to yield the acetylated aziridine (0.32 g, 51%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 7.09 (dd, *J* = 4.6, 3.4 Hz, 1H), 5.01 (ddd, *J* = 10.2, 6.6, 2.3 Hz, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.37 (dt, *J* = 6.1, 2.1 Hz, 1H), 3.23 (dd, *J* = 6.1, 4.6 Hz, 1H), 3.16 (s, 3H), 3.07 (ddd, *J* = 16.2, 6.6, 1.9 Hz, 1H), 2.38 (ddd, *J* = 16.2, 10.2, 3.5 Hz, 1H), 2.20 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 181.8, 165.0, 132.7, 131.2, 75.7, 61.4, 40.5, 39.2, 35.2, 26.7, 23.4, 14.3.

Ethyl (3*R*,4*S*,5*R*)-4-acetamido-3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)-5-((methyl-sulfonyl)oxy)cyclohex-1-ene-1-carboxylate

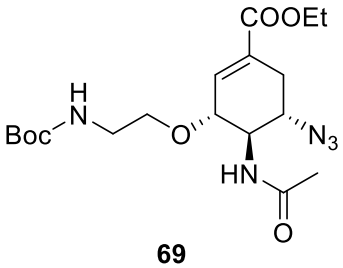


68

Ethyl (1*S*,5*R*,6*S*)-7-Acetyl-5-methanesulfonyloxy-7-azabicyclo-[4.1.0]-hept-2-ene-3-carboxylate (0.13 g, 0.43 mmol) and *N*-(*tert*-Butoxycarbonyl)ethanolamine (0.7 g, 1.3 mmol) were dissolved in DCM (1 mL) and the resulting solution was cooled to -40 ºC. Boron trifluoride diethyl etherate 1.65 M in DCM (0.39 mL, 0.64 mmol) was slowly added dropwise and the reaction mixture was gradually warmed up to room temperature. After 1 h at room temperature, the reaction was quenched by addition of water (2 mL), the pH was adjusted to neutral by addition of a saturated solution of sodium bicarbonate. The aqueous phase was extracted with EtOAc (4x 5 mL), the combined organic layers were washed with brine (3 mL) and evaporated down. The residue was purified by flash chromatography (hexanes/EtOAc 1:1) to afford the desired ether (0.12 g, 60%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 6.90 (q, *J* = 1.9 Hz, 1H), 6.41 (d, *J* = 7.2 Hz, 1H), 5.23 (td, *J* = 3.4, 2.0 Hz, 1H), 4.99 (s, 1H), 4.32 – 4.12 (m, 4H), 3.77 – 3.65 (m, 1H), 3.51 (ddd, *J* = 9.8, 6.8, 3.7 Hz, 1H), 3.35 – 3.23 (m, 2H), 3.03 (s, 3H), 2.79 (dt, *J* = 4.1, 2.0 Hz, 2H), 2.04 (s, 3H), 1.43 (s, 9H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 171.2, 165.6, 156.2, 135.8, 132.2, 79.6, 78.3, 74.6, 68.5, 61.4, 60.5, 51.0, 38.5, 30.5, 28.5 (3x), 23.3, 14.3. HR-ESI-MS calculated for $C_{19}H_{33}O_9N_2S$ $(M+H)^+$ 465.1901, found 465.1902.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)cyclohex-1-ene-1-carboxylate

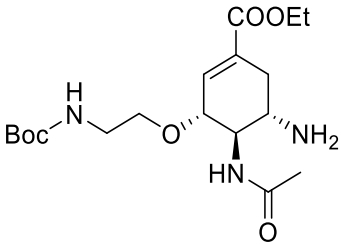


69

Sodium azide (0.08 g, 1.2 mmol) was added to a solution of the ethyl (3*R*,4*S*,5*R*)-4-acetamido-3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)-5-((methylsulfonyl)oxy)cyclohex-1-ene-1-carboxylate (0.14 g, 0.30 mmol) in aqueous EtOH (1,5 mL, EtOH/water 5:1) and the reaction was heated to reflux for 24h. The solvent was evaporated under reduced pressur, the residue was dissolved in EtOAc (5 mL) and the organic phase was washed with water (1.5 mL) and brine (1 mL). The organic layer was evaporated down and the residue was purified by column chromatography (EtOAc) to furnish the desired azide (0.1 g, 81%).

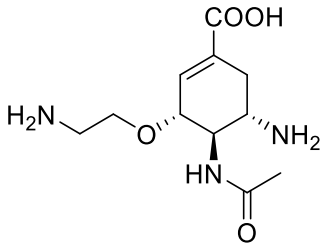
^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 6.81 (t, *J* = 2.6 Hz, 1H), 6.05 (d, *J* = 8.0 Hz, 1H), 4.96 (s, 1H), 4.38 (s, 1H), 4.29 – 4.15 (q, *J* = 7.1, 2H), 4.07 – 3.88 (m, 1H), 3.79 – 3.61 (m, 2H), 3.52 (td, *J* = 6.7, 3.5 Hz, 1H), 3.30 (m, 2H), 2.88 (ddd, *J* = 18.5, 5.9, 1.4 Hz, 1H), 2.43 – 2.18 (m, 1H), 2.07 (s, 3H), 1.44 (s, 9H), 1.30 (t, *J* = 7.1, 3H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 171.46, 165.6, 156.1, 136.3, 132.1, 79.8, 78.4, 68.7, 61.4, 57.7, 55.6, 40.8, 30.6, 28.5 (x3), 23.6, 14.3. HR-ESI-MS calculated for $C_{18}H_{29}O_6N_5Na$ $(M+Na)^+$ 434.2010, found 434.2011.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)cyclohex-1-ene-1-carboxylate



Lindlar catalyst (45 mg, 60 w%) was added to a solution of the ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*tert*-butoxycarbonyl)amino)-ethoxy)cyclohex-1-ene-1-carboxylate (73 mg, 0.17 mmol) in absolute EtOH (2.5 mL) the flask was sealed and purged with hydrogen, the reaction was left stirring overnight at room temperature under 1 atmosphere of hydrogen. The catalyst was filtered out and rinsed several times with EtOH, the filtrate was evaporated to dryness to yield the amine (67 mg, 98%). The product was used in the next step without further purification. HR-ESI-MS calculated for $C_{18}H_{32}O_6N_3$ $(M+H)^+$ 386.2286, found 386.2286.

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(2-aminoethoxy)cyclohex-1-ene-1-carboxylic acid

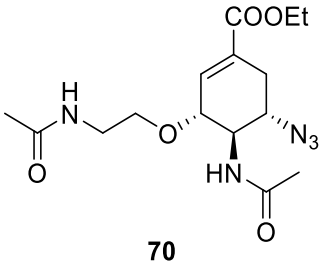


74

The conditions used are described in the general procedure for the saponification of ethyl esters. The solvent was evaporated under reduced pressure and the remaining crude mixture was dissolved in TFA (1 mL, 100%), the reaction was stirred for 30 min at room temperature. The TFA was removed by evaporation under reduced pressure and the remaining solid was purified by preparative HPLC to afford the desired amino acid (30 mg, 34%).

^{1}H NMR (401 MHz, Methanol-d_4) δ 6.87 (t, J = 2.5 Hz, 1H), 4.26 (ddt, J = 8.8, 3.8, 1.8 Hz, 1H), 4.04 (dd, J = 11.1, 8.7 Hz, 1H), 3.87 (ddd, J = 10.6, 6.2, 4.4 Hz, 1H), 3.81 – 3.60 (m, 2H), 3.11 (t, J = 4.7 Hz, 2H), 3.00 – 2.81 (m, 1H), 2.35 – 2.15 (m, 1H), 2.05 (s, 3H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.3, 168.6, 136.9, 131.6, 79.2, 65.5, 59.8, 54.9, 40.9, 31.3, 23.0. HR-ESI-MS calculated for $C_{11}H_{20}O_4N_3$ $(M+H)^+$ 258.1448, found 258.1449.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(2-acetamidoethoxy)-5-azidocyclohex-1-ene-1-carboxylate



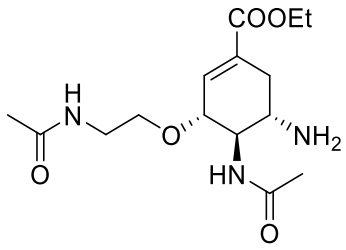
70

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*tert*-butoxycarbonyl)-amino)ethoxy)cyclohex-1-ene-1-carboxylate (0.08 g, 0.19 mmol) was dissolved in DCM (1mL) followed by addition of TFA (0.25 mL), the reaction was stirred for 1 h at room temperature. The solvents were removed under reduced pressure and the dry residue was dissolved in EtOAc (1.5 mL). Triethylamine (0.17 mL, 0.76 mmol) was subsequently added followed by acetic anhydride (0.04 ml, 0.38 mmol) and the reaction was stirred for 3 h at room temperature. The mixture was diluted in EtOAc (15 mL) and the organic phase was washed with diluted solution of potassium carbonate (2 mL) and brine (2 mL). The organic layer was evaporated to dryness and the crude residue purified by flash chromatography (DCM/MeOH 10:1) to yield the acetamide (0.06 g, 88%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.87 – 6.71 (m, 2H), 6.54 (t, J = 5.3 Hz, 1H), 4.21 (m, 3H), 3.95 (dt, J = 11.1, 8.5 Hz, 1H), 3.85 – 3.66 (m, 2H), 3.55 (ddd, J = 12.1, 5.6, 2.9 Hz, 1H), 3.50 – 3.40 (m, 1H), 3.29 (ddt, J = 14.0, 6.3, 3.7 Hz, 1H), 2.97 – 2.78 (m, 1H), 2.30 (ddt, J = 17.7, 10.1, 3.2 Hz, 1H), 2.06 (s, 3H), 2.00 (s, 3H), 1.29 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-

d$_3$) δ 171.7, 170.9, 165.5, 136.1, 129.6, 77.6, 67.7, 61.4, 58.1, 54.2, 39.6, 30.4, 23.6, 23.2, 14.3. HR-ESI-MS calculated for $C_{15}H_{24}O_5N_5$ $(M+H)^+$ 354.1772, found 354.1773.

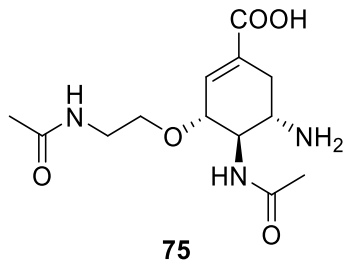
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(2-acetamidoethoxy)-5-aminocyclohex-1-ene-1-carboxylate



Lindlar catalyst (34 mg, 60 w%) was added to a solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(2-acetamidoethoxy)-5-azidocyclohex-1-ene-1-carboxylate (57 mg, 0.16 mmol) in absolute ethanol (2.5 mL), the flask was purged with hydrogen twice and the reaction was left stirring at room temperature for 3 h under hydrogen (1 atm) atmosphere. The catalyst was filtered and rinsed several times with ethanol, the filtrate was evaporated to dryness under reduced pressure. The resulting amine (47 mg, 90%) was used in the following step without further purification.

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 6.79 (d, *J* = 2.6 Hz, 1H), 6.68 (t, *J* = 5.3 Hz, 1H), 6.53 (d, *J* = 8.8 Hz, 1H), 4.20 (q, *J* = 7.0 Hz, 2H), 4.13 – 4.01 (m, 1H), 3.89 (dt, *J* = 10.6, 8.8 Hz, 1H), 3.78 – 3.65 (m, 2H), 3.59 – 3.40 (m, 2H), 3.28 (ddd, *J* = 13.4, 7.0, 3.2 Hz, 1H), 3.08 – 2.85 (m, 1H), 2.89 – 2.72 (m, 1H), 2.21 (broad s, 2H), 2.06 (s, 3H), 2.00 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 172.1, 170.9, 166.0, 136.0, 130.8, 78.4, 67.4, 61.2, 56.0, 49.7, 39.6, 33.9, 23.7, 23.2, 14.3. HR-ESI-MS calculated for $C_{15}H_{25}O_5N_3Na$ $(M+Na)^+$ 350.1686, found 350.1687.

(3*R*,4*R*,5*S*)-4-Acetamido-3-(2-acetamidoethoxy)-5-aminocyclohex-1-ene-1-carboxylic acid

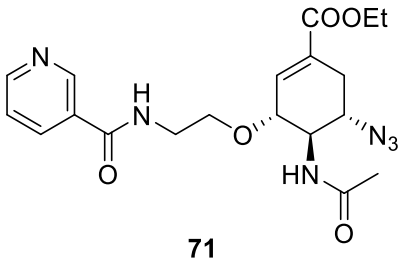


75

The conditions used are described in the general procedure for the saponification of ethyl esters. The product was purified by preparative HPLC to afford the desired amino acid (35 mg, 82%).

^{1}H NMR (400 MHz, D_2O) δ 6.95 (s, 1H), 4.36 – 4.28 (m, 1H), 4.18 (dd, *J* = 11.3, 8.7 Hz, 1H), 3.80 (ddd, *J* = 10.3, 6.0, 4.5 Hz, 1H), 3.72 – 3.57 (m, 2H), 3.36 (td, *J* = 6.7, 6.3, 4.2 Hz, 2H), 3.01 – 2.88 (m, 1H), 2.54 (ddt, *J* = 16.8, 10.3, 3.1 Hz, 1H), 2.09 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (101 MHz, D_2O) δ 175.2, 174.2, 168.8, 136.7, 128.4, 76.6, 67.8, 51.2, 48.8, 39.4, 28.0, 22.2, 21.8. HR-ESI-MS calculated for $C_{13}H_{22}O_5N_3$ $(M+H)^+$ 300.1554, found 300.1556.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-(nicotinamido)ethoxy)cyclohex-1-ene-1-carboxylate

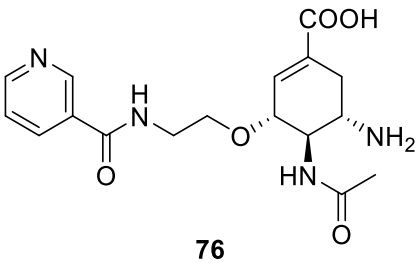


71

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*tert*-butoxy-carbonyl)amino)ethoxy)cyclohex-1-ene-1-carboxylate (0.08 g, 0.19 mmol) was dissolved in DCM (1mL) followed by addition of TFA (0.25 mL), the reaction was stirred for 1 h at room temperature. The solvents were removed under reduced pressure, the dry residue was dissolved in DCM (2 mL) and the solution was cooled to 0 ºC in an ice-bath. Triethylamine (0.13 mL, 0.95 mmol) was added followed by a solution of nicotinoyl chloride hydrochloride (0.053 g, 0.28 mmol) and the reaction was stirred for 4h at room temperature. The solution was diluted in EtOAc (10 mL) and the organic phase was washed with water (2 mL) and brine (1.5 mL), the organic layer was dried under reduced pressure and the remaining mixture purified by chromatography (DCM/MeOH 10:1) to yield the desired amide (0.057 g, 71%).

^{1}H NMR (400 MHz, Chloroform-d_3) δ 9.07 (d, J = 2.3 Hz, 1H), 8.63 (dd, J = 4.9, 1.6 Hz, 1H), 8.21 (dt, J = 7.9, 2.0 Hz, 1H), 7.76 (t, J = 5.1 Hz, 1H), 7.43 – 7.28 (m, 2H), 6.78 (s, 1H), 4.26 (dt, J = 9.0, 1.9 Hz, 1H), 4.17 (q, J = 7.0 Hz, 2H), 3.98 (dt, J = 11.2, 8.7 Hz, 1H), 3.84 (ddd, J = 9.6, 7.2, 3.4 Hz, 1H), 3.77 – 3.60 (m, 3H), 3.57 – 3.46 (m, 1H), 2.92 – 2.80 (m, 1H), 2.26 (ddq, J = 17.2, 10.3, 3.0 Hz, 1H), 1.83 (s, 3H), 1.25 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 171.9, 166.1, 165.4, 151.9, 148.6, 136.1, 135.5, 130.1, 129.6, 123.5, 77.6, 66.9, 61.4, 58.2, 53.8, 40.3, 30.4, 23.2, 14.2. HR-ESI-MS calculated for $C_{19}H_{25}O_5N_6$ $(M+H)^+$ 417.1881, found 417.1882.

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(2-(nicotinamido)ethoxy)cyclohex-1-ene-1-carboxylic acid



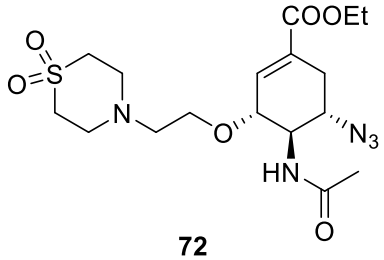
76

Lindlar catalyst (34 mg, 60 w%) was added to a solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-(nicotinamido)ethoxy)-cyclo-hex-1-ene-1-carboxylate (57 mg, 0.16 mmol) in absolute ethanol (2.5 mL), the flask was purged with hydrogen twice and the reaction was left stirring at room temperature for 3 h under

hydrogen (1 atm) atmosphere. The catalyst was filtered and rinsed several times with ethanol, the filtrate was evaporated to dryness under reduced pressure. The resulting amine (45 mg, 89%) was used in the following step without further purification. A solution of NaOH 0.5 M (0.55 mL, 0.23 mmol) was added to a solution of the ethyl ester (45 mg, 0.12 mmol) in 1,4-dioxane (0.55 mL), the reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated under reduced pressure and the remaining solid was purified by preparative HPLC to afford the desired amino acid (34 mg, 79%).

^{1}H NMR (400 MHz, D_2O) δ 9.20 (dd, *J* = 2.0, 0.8 Hz, 1H), 8.97 (ddt, *J* = 5.9, 1.4, 0.7 Hz, 1H), 8.96 – 8.88 (m, 1H), 8.20 (dddd, *J* = 6.7, 5.8, 1.6, 0.8 Hz, 1H), 6.93 (s, 1H), 4.40 – 4.32 (m, 1H), 4.21 (dd, *J* = 11.4, 8.7 Hz, 1H), 3.93 (dt, *J* = 10.4, 5.2 Hz, 1H), 3.83 (dt, *J* = 10.3, 5.0 Hz, 1H), 3.69 – 3.58 (m, 3H), 3.01 – 2.87 (m, 1H), 2.53 (ddt, *J* = 17.4, 10.2, 3.1 Hz, 1H), 1.99 (s, 3H). ^{13}C NMR (101 MHz, D_2O) δ 175.1, 168.8, 166.0, 144.5, 143.9, 141.3, 136.5, 133.2, 128.7, 127.4, 76.7, 67.5, 51.1, 48.8, 40.3, 28.0, 22.1. HR-ESI-MS calculated for $C_{17}H_{23}O_5N_4$ $(M+H)^+$ 363.1663, found 363.1665.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-(1,1-dioxidothiomorpholino)ethoxy)cyclohex-1-ene-1-carboxylate



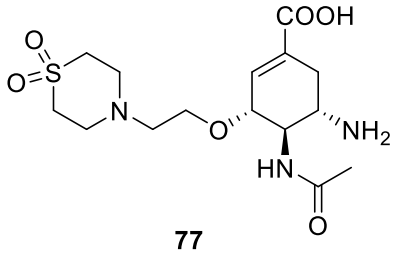
72

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*tert*-butoxy-carbonyl)-amino)ethoxy)cyclohex-1-ene-1-carboxylate (0.066 g, 0.16 mmol) was dissolved in DCM (1mL) followed by addition of TFA (0.25 mL), the reaction was stirred for 1 h at room temperature. The solvents were removed under reduced pressure, the dry residue was dissolved in propan-2-ol (1mL) followed by addition of triethylamine (0.11 mL, 0.8 mmol) and divinyl sulfone (0.022 g, 0.19 mmol), the reaction mixture was then heated to 60 ºC for 3 h. The solvent was evaporated under reduced pressure and the remaining mixture was directly purified by flash chromatography (DCM/MeOH 10:1) to yield the conjugated addition product (0.061 g, 89%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 6.82 (s, 1H), 6.04 (d, *J* = 8.2 Hz, 1H), 4.43 (dt, *J* = 8.7, 2.0 Hz, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 3.98 (td, *J* = 10.4, 5.7 Hz, 1H), 3.80 – 3.52 (m, 3H), 3.16 – 2.98 (m, 8H), 2.88 (ddd, *J* = 17.2, 6.2, 3.3 Hz, 1H), 2.81 – 2.65 (m, 2H), 2.39 – 2.19 (m, 1H), 2.06 (s,

3H), 1.29 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 171.2, 165.5, 136.5, 129.7, 76.5, 66.6, 61.4, 57.8, 56.6, 55.0, 51.5, 51.0, 30.5, 23.7, 14.3. HR-ESI-MS calculated for $C_{17}H_{28}O_6N_5S$ $(M+H)^+$ 430.1755, found 430.1755.

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(2-(1,1-dioxidothiomorpholino)ethoxy)cyclohex-1-ene-1-carboxylic acid

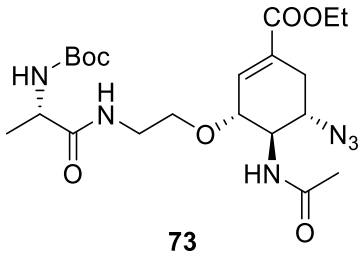


77

Lindlar catalyst (36 mg, 60 w%) was added to a solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-(1,1-dioxidothiomorpholino)-ethoxy)cyclohex-1-ene-1-carboxylate (60 mg, 0.16 mmol) in absolute ethanol (2.5 mL), the flask was purged with hydrogen twice and the reaction was left stirring at room temperature for 3 h under hydrogen (1 atm) atmosphere. The catalyst was filtered and rinsed several times with ethanol, the filtrate was evaporated to dryness under reduced pressure. The resulting amine (52 mg, 93%) was used in the following step without further purification. A solution of NaOH 0.5 M (0.56 mL, 0.28 mmol) was added to a solution of the ethyl ester (45 mg, 0.14 mmol) in 1,4-dioxane (0.56 mL), the reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated under reduced pressure and the remaining solid was purified by preparative HPLC to afford the desired amino acid (46 mg, 88%).

^{1}H NMR (300 MHz, D_2O) δ 6.95 (s, 1H), 4.44 – 4.33 (m, 1H), 4.28 (dd, J = 11.2, 8.7 Hz, 1H), 4.13 (dt, J = 10.8, 4.9 Hz, 1H), 3.98 (dt, J = 5.3, 3.9 Hz, 2H), 3.91 (t, J = 5.5 Hz, 3H), 3.76 – 3.60 (m, J = 5.5 Hz, 5H), 3.53 (dt, J = 16.1, 5.0 Hz, 2H), 2.99 (dd, J = 17.4, 5.7 Hz, 1H), 2.56 (ddt, J = 17.0, 10.4, 3.0 Hz, 1H), 2.12 (s, 3H). ^{13}C NMR (75 MHz, D_2O) δ 175.2, 169.2, 134.8, 129.7, 77.1, 62.5, 55.7, 55.1, 50.9, 50.8, 48.7, 47.5, 47.4, 28.2, 22.2. HR-ESI-MS calculated for $C_{15}H_{26}O_6N_3S$ $(M+H)^+$ 376.1537, found 376.1537.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-propanamido)ethoxy)cyclohex-1-ene-1-carboxylate

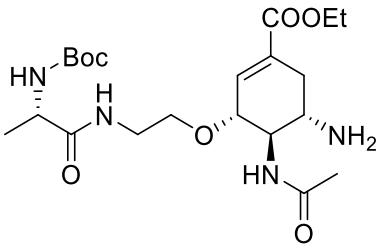


73

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*tert*-butoxy-carbonyl)-amino)ethoxy)cyclohex-1-ene-1-carboxylate (0.07 g, 0.17 mmol) was dissolved in DCM (1 mL) followed by addition of TFA (0.25 mL), the reaction was stirred for 1 h at room temperature. The solvents were removed under reduced pressure and the dry residue was dissolved in DMF (1 mL). In a separate flask was prepared under inert atmosphere a solution of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (0.13 g, 0.41 mmol), Boc-*L*-alanine (0.064 g, 0.32 mmol) and triethylamine (0.23 mL, 1.7 mL) in DMF (1.5 mL). The reaction was stirred 10 min at room temperature followed by addition of the DMF solution containing the amine, the resulting reaction was stirred overnight at room temperature. The following day the reaction was quenched by addition of a saturated solution of sodium bicarbonate (3 mL) and water (3 mL), the aqueous layer was extracted with EtOAc (3x 10 mL) and the combined organic phases were washed with water (2x 3 mL), brine (3 mL) and evaporated down. The remaining mixture was purified by flash chromatography (DCM/MeOH from 20:1 to 10:1) to yield the amide as a white solid (0.047 g, 58%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 6.86 – 6.69 (m, 2H), 5.28 (d, *J* = 7.4 Hz, 1H), 4.34 – 4.19 (m, 3H), 4.16 – 4.06 (m, 1H), 3.91 – 3.77 (m, 2H), 3.68 – 3.56 (m, 2H), 3.41 – 3.16 (m, 1H), 2.97 – 2.83 (m, 1H), 2.41 – 2.23 (m, 1H), 2.10 (s, 3H), 1.88 (s, 2H), 1.45 (s, 9H), 1.43 – 1.35 (m, 3H), 1.32 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 173.2, 171.8, 165.6, 158.1, 136.0, 129.4, 80.8, 68.1, 61.4, 61.3, 58.5, 55.2, 50.6, 39.8, 30.6, 28.5, 23.6, 18.5, 14.3. HR-ESI-MS calculated for $C_{21}H_{34}N_6O_7Na$ $(M + Na)^+$ 505.2381, found 505.2382.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(2-((*S*)-2-((*tert*-butoxycarbonyl)amino)-propanamido)ethoxy)cyclohex-1-ene-1-carboxylate

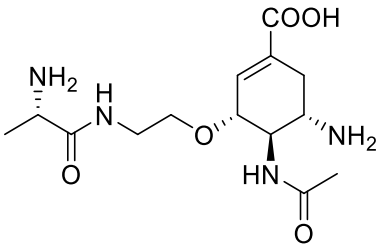


Lindlar catalyst (46 mg, 60 w%) was added to a solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2-((*S*)-2-((*tert*-butoxycarbon-yl)amino)propanamido)ethoxy)cyclohex-1-ene-1-carboxylate (77 mg, 0.16 mmol) in absolute ethanol (2.5 mL), the flask was purged with hydrogen twice and the reaction was left stirring at room

temperature for 3 h under hydrogen (1 atm) atmosphere. The catalyst was filtered and rinsed several times with ethanol. The filtrate was evaporated to dryness under reduced pressure. The remaining solid was purified by flash chromatography (DCM/MeOH 10:1) to yield the desired amine (70 mg, 96%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 7.37 (broad s, 1H), 7.03 (bread s, 1H), 6.78 (s, 1H), 5.48 (broad s, 1H), 4.27 – 4.04 (m, 5H), 3.98 (d, *J* = 9.9 Hz, 2H), 3.76 (dd, *J* = 7.2, 3.3 Hz, 2H), 3.66 – 3.42 (m, 2H), 3.23 (s, 1H), 2.90 (d, *J* = 18.2 Hz, 1H), 2.48 – 2.27 (m, 1H), 2.09 (d, *J* = 2.6 Hz, 3H), 1.41 (s, 9H), 1.37 – 1.31 (m, 3H), 1.30 – 1.18 (m, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 173.2, 172.8, 165.9, 155.8, 136.1, 130.0, 80.1, 78.0, 68.1, 61.3, 55.2, 50.3, 49.9, 39.7, 32.3, 28.5, 23.8, 19.1, 14.3. HR-ESI-MS calculated for $C_{21}H_{36}O_7N_4Na$ $(M+Na)^+$ 479.2476, found 479.2476.

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(2-((*S*)-2-aminopropanamido)ethoxy)cyclohex-1-ene-1-carboxylic acid

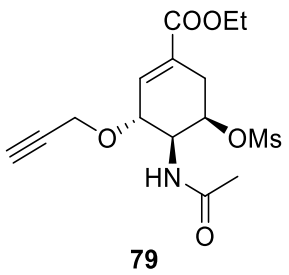


78

The conditions used are described in the general procedure for the saponification of ethyl esters. After removal of the solvent the remaining solid was dissolved in TFA (1 mL) and the reaction was stirred for 1 h at room temperature. The acid was evaporated under reduced pressure and the mixture was purified by preparative HPLC to give the desired aminoacid (38 mg, 85%).

^{1}H NMR (400 MHz, D_2O) δ 6.91 (t, *J* = 2.7 Hz, 1H), 4.33 (dq, *J* = 7.7, 1.8 Hz, 1H), 4.26 – 4.14 (m, 1H), 4.06 (dd, *J* = 8.7, 5.5 Hz, 1H), 3.80 (td, *J* = 6.6, 3.3 Hz, 1H), 3.74 – 3.55 (m, 2H), 3.55 – 3.32 (m, 2H), 3.04 – 2.92 (m, 1H), 2.54 (ddt, *J* = 17.6, 10.2, 3.1 Hz, 1H), 2.10 (s, 3H), 1.53 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, D_2O) δ 175.3, 170.8, 168.8, 136.5, 128.7, 76.6, 67.6, 51.2, 49.1, 48.8, 39.5, 28.0, 22.2, 16.5. HR-ESI-MS calculated for $C_{14}H_{25}O_5N_4$ $(M+H)^+$ 329.1820, found 329.1821.

Ethyl (3*R*,4*S*,5*R*)-4-acetamido-5-((methylsulfonyl)oxy)-3-(prop-2-yn-1-yloxy)cyclohex-1-ene-1-carboxylate

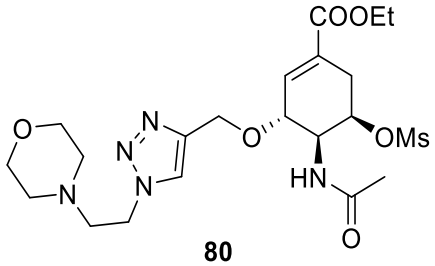


79

Ethyl (1*S*,5*R*,6*S*)-7-acetyl-5-methanesulfonyloxy-7-azabicyclo[4.1.0]-hept-2-ene-3-carboxylate (0.2 g, 0.66 mmol) and propargyl alcohol (0.37 g, 6.6 mmol) were dissolved in DCM (1.5 mL) and the resulting solution was cooled to -40 ºC. Boron trifluoride diethyl etherate 1.65 M in DCM (0.6 mL, 0.99 mmol) was slowly added dropwise and the reaction mixture was gradually warmed up to room temperature. After 1 h at room temperature, the reaction was quenched by addition of water (2 mL), the pH was adjusted to neutral by addition of a saturated solution of sodium bicarbonate. The aqueous phase was extracted with EtOAc (4x 5 mL), the combined organic layers were washed with brine (3 mL) and evaporated down. The residue was purified by flash chromatography (EtOAc) to afford the desired ether (0.14 g, 58%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 6.93 (q, *J* = 2.0 Hz, 1H), 6.15 (d, *J* = 7.4 Hz, 1H), 5.26 (td, *J* = 3.5, 2.2 Hz, 1H), 4.39 – 4.31 (m, 1H), 4.30 (dd, *J* = 11.1, 2.3 Hz, 2H), 4.27 – 4.17 (m, 3H), 3.04 (s, 3H), 2.80 (dt, *J* = 4.5, 2.2 Hz, 2H), 2.54 (s, 1H), 2.03 (s, 3H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 171.0, 165.6, 135.4, 128.4, 79.6, 78.2, 75.7, 73.0, 61.4, 57.0, 51.4, 38.5, 30.3, 23.4, 14.3. HR-ESI-MS calculated for $C_{15}H_{21}O_7NNa$ $(M+Na)^+$ 382.0931, found 382.0932.

Ethyl (3*R*,4*S*,5*R*)-4-acetamido-5-((methylsulfonyl)oxy)-3-((1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)cyclohex-1-ene-1-carboxylate

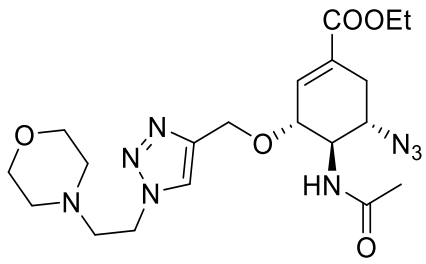


80

An oven dried flask was charged with ethyl (3*R*,4*S*,5*R*)-4-acetamido-5-((methylsulfonyl)oxy)-3-(prop-2-yn-1-yloxy)-cyclohex-1-ene-1-carboxylate (61 mg, 0.17 mmol) tetrakis(acetonitrile)copper(I) hexafluorophosphate (6 mg, 0.017 mmol), tris (1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl amine (10 mg, 0.017 mmol) and distilled THF (1.5 mL). The flask was purged with argon followed by dropwise addition of a solution of 4-(2-azidoethyl)morpholine (32 mg, 0.20 mmol) in THF (0.5 mL), the reaction mixture was stirred overnight at room temperature. The solution was evaporated to dryness and the remaining mixture was purified by flash chromatography (DCM/MeOH 20:1) to yield the triazole (67 mg, 77%).

^{1}H NMR (600 MHz, Chloroform-d_3) δ 7.70 (s, 1H), 7.43 (d, J = 5.7 Hz, 1H), 6.94 (s, 1H), 5.37 (q, J = 3.1 Hz, 1H), 4.88 (d, J = 12.9 Hz, 1H), 4.75 (d, J = 13.0 Hz, 1H), 4.46 (t, J = 6.2 Hz, 2H), 4.40 (d, J = 9.3 Hz, 1H), 4.20 (tt, J = 7.2, 3.4 Hz, 2H), 4.16 – 4.08 (m, 1H), 3.68 (t, J = 4.6 Hz, 3H), 3.00 (s, 3H), 2.89 – 2.70 (m, 3H), 2.49 (t, J = 4.6 Hz, 4H), 2.27 (s, 1H), 2.01 (s, 3H), 1.29 (t, J = 7.0 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-d_3) δ 171.5, 165.7, 144.7, 135.7, 128.1, 123.1, 77.8, 73.6, 66.9 (x2), 62.6, 61.3, 57.9, 53.6 (x2), 52.4, 47.5, 38.1, 30.3, 23.3, 14.3. HR-ESI-MS calculated for $C_{21}H_{33}O_8N_5SNa$ $(M+Na)^+$ 538.1942, found 538.1941.

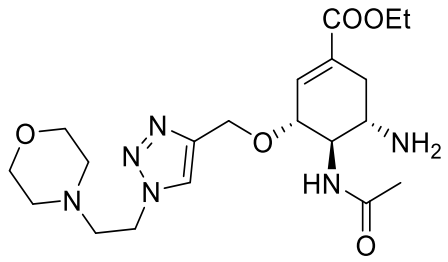
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-((1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)cyclohex-1-ene-1-carboxylate



Sodium azide (82 mg, 1.2 mmol) was added to a solution of ethyl (3*R*,4*S*,5*R*)-4-acetamido-5-((methylsulfonyl)oxy)-3-((1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)cyclohex-1-ene-1-carboxylate (162 mg, 0.32 mmol) in aqueous EtOH (1.5 mL, EtOH/water 5:1), the reaction was refluxed for 24 h. The ethanol was evaporated under reduced pressure and the remaining slurry was dissolved in water (5 mL), the aqueous solution was extracted with EtOAc (3x 10 mL) and the combined organic layers were washed with water (3 mL) and brine (3 ml). The azide was obtained after evaporation of the solvent, no further purification was needed (55 mg, 37%).

^{1}H NMR (400 MHz, Chloroform-d_3) δ 7.70 (s, 1H), 6.80 (t, J = 2.4 Hz, 1H), 6.70 (d, J = 8.1 Hz, 1H), 4.80 (d, J = 12.5 Hz, 1H), 4.68 (d, J = 12.4 Hz, 1H), 4.59 (dt, J = 8.8, 1.9 Hz, 1H), 4.44 (t, J = 6.3 Hz, 2H), 4.18 (t, J = 7.1 Hz, 2H), 4.02 – 3.90 (m, 1H), 3.82 – 3.71 (m, 1H), 3.70 – 3.59 (m, 4H), 2.87 – 2.76 (m, 3H), 2.52 – 2.44 (m, 4H), 2.30 – 2.20 (m, 1H), 1.95 (s, 3H), 1.27 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 171.4, 165.6, 144.6, 136.6, 129.4, 123.6, 75.8, 66.9 (x2), 62.7, 61.3, 58.0, 57.9, 55.6, 53.5 (x2), 47.4, 30.4, 23.5, 14.3. HR-ESI-MS calculated for $C_{20}H_{30}O_5N_8Na$ $(M+Na)^+$ 485.2231, found 485.2231.

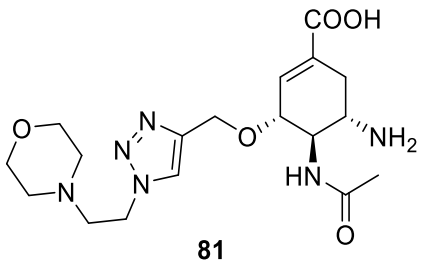
Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-((1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)cyclohex-1-ene-1-carboxylate



Lindlar catalyst (33 mg, 60 w%) was added to a solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-((1-(2-morpholino-ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)cyclohex-1-ene-1-carboxylate (55 mg, 0.12 mmol) in absolute ethanol (1.8 mL), the flask was purged with hydrogen twice and the reaction was left stirring at room temperature for 5 h under hydrogen (1 atm) atmosphere. The catalyst was filtered and rinsed several times with ethanol, the filtrate was evaporated to dryness under reduced pressure. The resulting amine (44 mg, 85%) was used in the following step without further purification.

^{1}H NMR (400 MHz, Methanol-*d*$_4$) δ 7.96 (s, 1H), 6.80 (dt, *J* = 2.6, 1.3 Hz, 1H), 4.73 (d, *J* = 12.2 Hz, 1H), 4.64 (d, *J* = 12.1 Hz, 1H), 4.48 (t, *J* = 6.3 Hz, 2H), 4.32 – 4.22 (m, 1H), 4.15 (q, *J* = 7.1 Hz, 2H), 3.90 (dd, *J* = 10.7, 8.4 Hz, 1H), 3.63 – 3.54 (m, 4H), 3.31 – 3.20 (m, 1H), 2.87 – 2.73 (m, 3H), 2.43 (dd, *J* = 5.5, 3.7 Hz, 4H), 2.28 (ddt, *J* = 17.8, 9.7, 3.0 Hz, 1H), 1.95 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 174.6, 167.0, 145.5, 137.4, 130.3, 125.7, 77.4, 67.8, 63.6, 62.2, 58.8, 58.3, 54.9, 54.5, 50.5, 31.1, 23.1, 14.5. HR-ESI-MS calculated for $C_{20}H_{33}O_5N_6$ $(M+H)^+$ 437.2507, found 437.2508.

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-((1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)cyclohex-1-ene-1-carboxylic acid

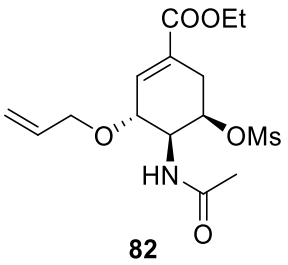


81

The conditions used are described in the general procedure for the saponification of ethyl esters. Purified by preparative HPLC to afford the desired amino acid (24 mg, 57%).

^{1}H NMR (400 MHz, D_2O) δ 8.13 (s, 1H), 6.98 (t, *J* = 2.5 Hz, 1H), 4.97 (t, *J* = 6.1 Hz, 2H), 4.73 (hidden behind the signal of D_2O, d, *J* = 12.2 Hz, 1H), 4.64 (hidden behind the signal of D_2O d, *J* = 12.1 Hz, 1H), 4.52 – 4.43 (m, 1H), 4.17 (dd, *J* = 11.4, 8.7 Hz, 1H), 3.99 (broad s, 4H), 3.85 (t, *J* = 6.1 Hz, 2H), 3.66 (ddd, J = 11.6, 10.2, 5.7 Hz, 1H), 3.47 (broad s, 4H), 3.06 – 2.91 (m, 1H), 2.54 (ddt, *J* = 17.5, 10.2, 3.1 Hz, 1H), 2.04 (s, 3H). ^{13}C NMR (101 MHz, D_2O) δ 175.2, 168.8, 144.3, 136.4, 128.7, 125.6, 76.1, 63.5 (x2), 61.7, 55.4, 52.0 (x2) , 51.3, 48.6, 44.1, 28.1, 22.2. HR-ESI-MS calculated for $C_{18}H_{28}O_5N_6Na$ $(M+Na)^+$ 431.2013, found 431.2014.

Ethyl (3*R*,4*S*,5*R*)-4-acetamido-3-(allyloxy)-5-((methylsulfonyl)oxy)cyclohex-1-ene-1-carboxylate

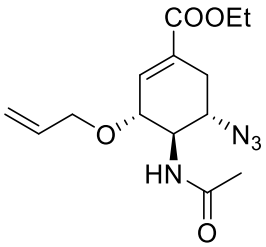


82

Ethyl (1*S*,5*R*,6*S*)-7-acetyl-5-methanesulfonyloxy-7-azabicyclo[4.1.0]-hept-2-ene-3-carboxylate (0.15 g, 0.49 mmol) and propargyl alcohol (0.33 mL, 4.9 mmol) were dissolved in DCM (1.25 mL) and the resulting solution was cooled to -40 ºC. Boron trifluoride diethyl etherate 1.65 M in DCM (0.45 mL, 0.73 mmol) was slowly added dropwise and the reaction mixture was gradually warmed up to room temperature. After 1 h at room temperature, the reaction was quenched by addition of water (2 mL), the pH was adjusted to neutral by addition of a saturated solution of sodium bicarbonate. The aqueous phase was extracted with EtOAc (4x 5 mL), the combined organic layers were washed with brine (3 mL) and evaporated down. The residue was purified by flash chromatography (EtOAc) to afford the desired ether (0.12 g, 64%).

^{1}H NMR (400 MHz, Chloroform-*d3*) δ 6.95 – 6.90 (m, 1H), 6.14 (d, *J* = 7.8 Hz, 1H), 5.87 (ddt, *J* = 17.2, 10.4, 5.6 Hz, 1H), 5.32 – 5.18 (m, 3H), 4.29 (ddd, *J* = 8.8, 7.8, 2.3 Hz, 1H), 4.21 (qd, *J* = 7.1, 1.5 Hz, 2H), 4.18 – 4.12 (m, 2H), 4.02 (ddt, *J* = 12.6, 5.7, 1.4 Hz, 1H), 3.03 (s, 3H), 2.89 – 2.73 (m, 2H), 2.01 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 170.9, 165.6, 136.1, 134.2, 128.3, 118.0, 78.3, 73.5, 70.1, 61.4, 51.0, 38.5, 30.2, 23.3, 14.3. HR-ESI-MS calculated for $C_{15}H_{23}O_7NSNa$ $(M+Na)^+$ 384.1087, found 384.1089.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(allyloxy)-5-azidocyclohex-1-ene-1-carboxylate

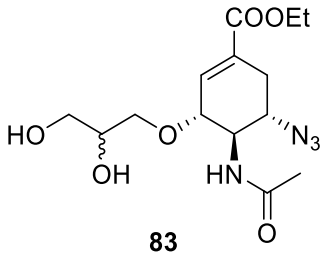


Sodium azide (150 mg, 1.6 mmol) was added to a solution of ethyl (3*R*,4*S*,5*R*)-4-acetamido-3-(allyloxy)-5-((methylsulfonyl)oxy)cyclohex-1-ene-1-carboxylate (150 mg, 0.41 mmol) in aqueous EtOH (1.8 mL, EtOH/water 5:1), the reaction was refluxed for 24 h. The ethanol was evaporated under reduced pressure and the remaining slurry was dissolved in water (5 mL), the aqueous solution was extracted with EtOAc (3x 10 mL) and the combined organic layers were washed with water (3 mL) and brine (3 ml). The organic phase was evaporated

down and the resulting residue purified by flash chromatography (Hexanes/EtOAc 1:1) to yield the desired azide (55 mg, 37%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 6.84 (s, 1H), 5.96 – 5.81 (m, 1H), 5.75 (d, *J* = 7.8 Hz, 1H), 5.29 (d, *J* = 17.2 Hz, 1H), 5.21 (d, *J* = 10.3 Hz, 1H), 4.57 – 4.49 (m, 1H), 4.22 (qd, *J* = 7.2, 1.0 Hz, 2H), 4.21 – 4.11 (m, 2H), 4.09 – 3.99 (m, 1H), 3.53 (dt, *J* = 11.1, 8.3 Hz, 1H), 2.88 (dd, *J* = 18.0, 5.6 Hz, 1H), 2.27 (ddt, *J* = 17.3, 10.3, 3.1 Hz, 1H), 2.05 (s, 3H), 1.30 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 171.1, 165.7, 136.8, 134.3, 129.2, 118.1, 75.1, 70.7, 61.3, 57.5, 56.7, 30.6, 23.7, 14.3. HR-ESI-MS calculated for $C_{14}H_{20}O_4N_4Na$ $(M+Na)^+$ 331.1377, found 331.1377.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(2,3-dihydroxypropoxy)cyclohex-1-ene-1-carboxylate

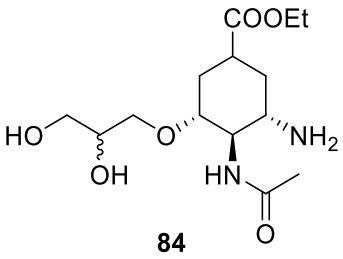


83

AD-mix-α (0.35 g, 1.4 g per 1 mmol of alkene) was dissolved in a mixture of *tert*-butanol and water (2.6 mL, 1:1) at room temperature, the solution was cooled down to 0 ºC and ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(allyloxy)-5-azidocyclohex-1-ene-1-carboxylate (0.078 g, 0.25 mmol) was added all at once, the reaction mixture was stirred for 24 h at 0 ºC. The reaction was quenched by addition of sodium sulfite (0.36 g) to the ice-cold solution, the bath was removed and the reaction was stirred for 30 min at room temperature. The aqueous reaction mixture was extracted with EtOAc (3x 5 mL) and the combiner organic layers were washed with brine (3 mL). The organic phase was evaporated down under reduced pressure and the resulting mixture purified by flash chromatography (DCM/MeOH 20:1) to yield the dehydroxylated product (0.053 g, 61%) as a mixture of diastereomers.

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 7.26 (d, *J* = 8.4 Hz, 1H), 6.81 (s, 1H), 4.40 – 4.26 (m, 1H), 4.20 (q, *J* = 7.1 Hz, 2H), 4.06 (broad s, 1H), 3.94 – 3.82 (m, 2H), 3.82 – 3.74 (m, 2H), 3.74 – 3.44 (m, 4H), 2.86 (dd, *J* = 17.4, 5.4 Hz, 1H), 2.27 (dd, *J* = 17.6, 9.9 Hz, 1H), 2.06 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H). Major diastereomer ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 172.5, 165.7, 136.6, 129.6, 77.6, 71.0, 70.1, 63.6, 61.5, 58.3, 54.5, 30.6, 23.5, 14.3. Minor diastereomer ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 172.5, 165.7, 136.6, 129.5, 77.7, 71.1, 70.5, 63.5, 61.5, 58.3, 54.6, 30.6, 23.4, 14.3. HR-ESI-MS calculated for $C_{14}H_{22}O_6N_4Na$ $(M+Na)^+$ 365.1432, found 365.1432.

Ethyl (3*S*,4*R*,5*R*)-4-acetamido-3-amino-5-(2,3-dihydroxypropoxy)cyclohexane-1-carboxylate

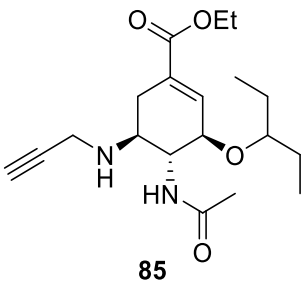


84

Lindlar catalyst (46 mg, 60 w%) was added to a solution of the azide (77 mg, 0.23 mmol) in absolute ethanol (3.5 mL), the flask was purged with hydrogen twice and the reaction was left stirring at room temperature overnight under hydrogen (1 atm) atmosphere. The catalyst was filtered and rinsed several times with ethanol, the filtrate was evaporated to dryness under reduced pressure. The resulting amine (68 mg, 95%) was used in the following step without further purification.

1H NMR (400 MHz, Methanol-*d4*) δ 4.08 (q, *J* = 7.1 Hz, 2H), 3.66 – 3.34 (m, 6H), 3.23 – 3.17 (m, 1H), 2.71 – 2.62 (m, 1H), 2.47 – 2.33 (m, 2H), 2.16 – 2.07 (m, 1H), 1.97 (s, 3H), 1.45 – 1.28 (m, 2H), 1.19 (t, *J* = 7.1 Hz, 3H). Major diastereomer 13C NMR (101 MHz, Methanol-*d4*) δ 175.3, 167.5, 80.1, 72.3, 71.9, 64.4, 61.9, 60.0, 53.5, 39.7, 35.8, 34.1, 23.1, 14.5. Minor diastereomer ^{13}C NMR (101 MHz, Methanol-*d4*) δ 174.8, 167.5, 79.4, 72.5, 71.9, 64.3, 62.1, 60.0, 53.5, 39.7, 35.8, 34.1, 23.1, 14.5. HR-ESI-MS calculated for $C_{14}H_{26}O_6N_2Na$ $(M+Na)^+$ 341.1683, found 341.1684.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(prop-2-yn-1-ylamino)cyclohex-1-ene-1-carboxylate



85

Oseltamivir phosphate (1.5 g, 3.6 mmol) and potassium carbonate (2.5 g, 18.2 mmol) were dissolved in acetonitrile (60 mL) under inert atmosphere, the resulting solution was cooled down to 0 ºC in an ice bath followed by dropwise addition of propargyl bromide (80%, 0.47 g, 4.32 mmol). The reaction mixture was stirred 16 h at room temperature and then heated to 50 ºC for 4 h more. The solvent was evaporated under reduced pressure and the remaining residue was dissolved in water (50 mL), the aqueous layer was extracted with DCM (3x 30 mL), the combined organic layers were washed with brine (10 mL) and evaporated to dryness. The resulting product was purified by flash chromatography (DCM/MeOH/NH_3 80:20:1) to furnish the propargylated product (0.83 g, 65%).

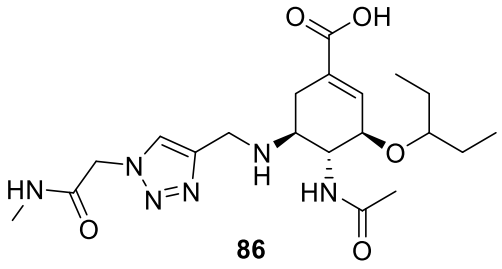
^{1}H NMR (401 MHz, Chloroform-*d3*) δ 6.80 (td, *J* = 2.5, 1.1 Hz, 1H), 5.50 (d, *J* = 8.2 Hz, 1H), 4.27 – 4.19 (m, 1H), 4.19 (q, *J* = 7.1 Hz, 2H), 3.68 (dt, *J* = 9.9, 8.0 Hz, 1H), 3.58 – 3.38 (m, 2H), 3.40

– 3.25 (m, 2H), 2.79 – 2.68 (m, 1H), 2.20 (t, *J* = 2.4 Hz, 1H), 2.19 – 2.12 (m, 1H), 2.03 (s, 3H), 1.77 (bs, 1H), 1.56 – 1.46 (m, 4H), 1.29 (t, *J* = 7.1 Hz, 3H), 0.90 (td, *J* = 7.4, 3.0 Hz, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 170.8, 166.6, 137.3, 129.3, 82.0, 81.8, 74.6, 71.7, 61.0, 56.1, 52.8, 35.6, 30.2, 26.3, 25.8, 23.9, 14.4, 9.6, 9.5. HR-ESI-MS calculated for $C_{19}H_{31}O_4N_2$ $(M+H)^+$ 351.2278, found 351.2281.

General procedure for CuAAC reaction between azides and propargylated oseltamivir.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(pentan-3-yloxy)-5-(prop-2-yn-1-ylamino)cyclohex-1-ene-1-carboxylate (0.14 mmol) was dissolved in a THF/water mixture (2:1, 3 mL), the corresponding azide (0.17 mmol) and (+)-sodium *L*-ascorbate (0.7 mmol) were added and the reaction was purged with argon, copper(II) sulfate pentahydrate (0.28 mmol) was added and the reaction was heated to 45 ºC for 2 h. The reaction mixture was cooled down to room temperature and a saturated solution of $NaHCO_3$ (4 mL) was added, the aqueous layer was extracted with EtOAc (3x 5mL) and the combined organic layers were washed with brine (2 mL) and evaporated to dryness under reduced pressure. The products were used in the next step without further purification.

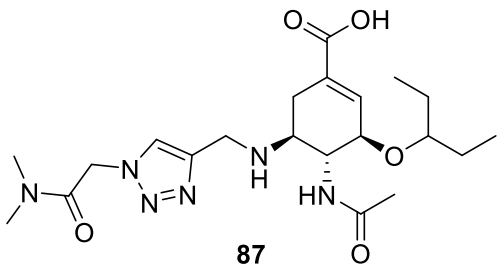
(3*R*,4*R*,5*S*)-4-Acetamido-5-(((1-(2-(methylamino)-2-oxoethyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid



86

The conditions used are described in the general procedure for the saponification of ethyl esters. The product was purified by preparative HPLC. (33 mg, 55%). ^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 8.16 (s, 1H), 6.90 (s, 1H), 5.31 (s, 2H), 4.50 (q, *J* = 14.2 Hz, 2H), 4.27 – 4.11 (m, 2H), 3.67 – 3.54 (m, 1H), 3.46 (t, *J* = 5.6 Hz, 1H), 3.22 – 3.07 (m, 1H), 2.81 (s, 3H), 2.68 – 2.51 (m, 1H), 2.05 (d, *J* = 1.9 Hz, 3H), 1.66 – 1.43 (m, 4H), 0.93 (dt, *J* = 12.4, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 175.0, 170.2, 168.5, 138.9, 138.7, 128.6, 128.1, 83.8, 75.8, 55.7, 53.1, 52.1, 39.9, 27.2, 27.1, 26.6, 26.5, 23.3, 9.8, 9.6. HR-ESI-MS calculated for $C_{20}H_{33}O_5N_6$ $(M+H)^+$ 437.2507, found 437.2507.

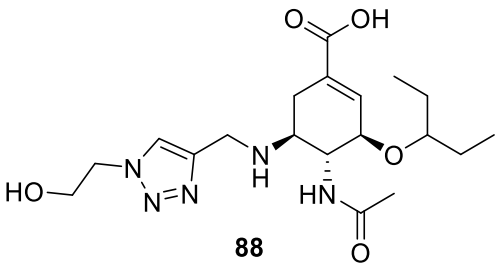
(3*R*,4*R*,5*S*)-4-Acetamido-5-(((1-(2-(dimethylamino)-2-oxoethyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid



87

The conditions used are described in the general procedure for the saponification of ethyl esters. The product was purified by preparative HPLC (13 mg, 21%).^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 8.07 (s 1H), 6.91 – 6.80 (m, 1H), 5.52 (s, 2H), 4.49 (q, *J* = 14.3, 2H), 4.16 (m, 2H), 3.62 – 3.50 (m, 1H), 3.45 (m, 1H), 3.20 – 3.10 (m, 1H), 3.15 (s, 3H), 3.00 (s, 3H), 2.66 – 2.54 (m, 1H), 2.06 – 1.99 (s, 3H), 1.54 (ddt, *J* = 9.7, 5.5, 2.1 Hz, 4H), 0.91 (dt, *J* = 12.5, 7.2, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 175.0, 168.5, 167.7, 138.8, 138.7, 128.6 (2x), 83.8, 75.8, 55.7, 53.0, 52.1, 39.9, 36.7, 36.1, 27.2, 27.1, 26.6, 23.3, 9.8, 9.5. HR-ESI-MS calculated for $C_{21}H_{33}O_5N_6$ $(M-H)^-$ 449.2518, found 449.2521.

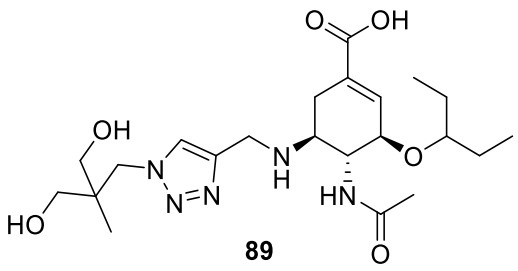
(3*R*,4*R*,5*S*)-4-Acetamido-5-(((1-(2-hydroxyethyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid.



88

The conditions used are described in the general procedure for the saponification of ethyl esters The product was purified by preparative HPLC (27 mg, 48 %).^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 8.12 (s, 1H), 6.89 (d, *J* = 1.1 Hz, 1H), 4.57 – 4.39 (m, 4H), 4.24 – 4.16 (m, 1H), 4.12 (dd, *J* = 10.8, 8.1 Hz, 1H), 3.94 (dd, *J* = 5.6, 4.7 Hz, 2H), 3.67 – 3.55 (m, 1H), 3.44 (t, *J* = 5.6 Hz, 1H), 3.22 – 3.08 (m, 1H), 2.61 (d, *J* = 9.7 Hz, 1H), 2.03 (s, 3H), 1.59 – 1.47 (m, 4H), 0.91 (dt, *J* = 12.3, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 175.0, 168.5, 138.7 (2x), 128.5, 127.3, 83.7, 75.6, 61.5, 55.5, 54.0, 53.2, 40.0, 27.1, 27.1, 26.6, 23.2, 9.8, 9.5. HR-ESI-MS calculated for $C_{19}H_{30}O_5N_5$ $(M-H)^-$ 408.2252, found 408.2254.

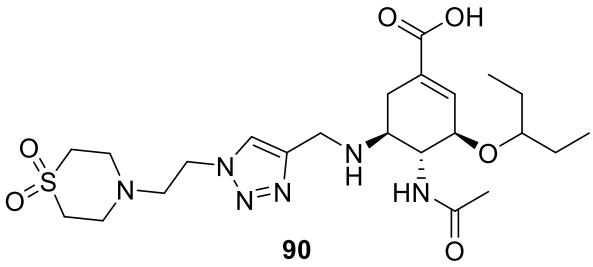
(3*R*,4*R*,5*S*)-4-Acetamido-5-(((1-(3-hydroxy-2-(hydroxymethyl)-2-methylpropyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid



89

The conditions used are described in the general procedure for the saponification of ethyl esters. The product was purified by preparative HPLC (29 mg, 45 %).[1]H NMR (401 MHz, Methanol-*d*$_4$) δ 8.08 (s, 1H), 6.89 (q, *J* = 1.4 Hz, 1H), 4.53 – 4.39 (m, 4H), 4.20 (dd, *J* = 6.3, 3.5 Hz, 1H), 4.13 (dd, *J* = 10.7, 8.0 Hz, 1H), 3.66 – 3.55 (m, 1H), 3.48 – 3.40 (m, 3H), 3.34 (dd, *J* = 11.1, 2.9 Hz, 2H), 3.11 (dd, *J* = 17.2, 5.8 Hz, 1H), 2.59 (ddt, *J* = 12.3, 9.6, 4.8 Hz, 1H), 2.04 (s, 3H), 1.54 (m, 4H), 0.91 (dt, *J* = 11.3, 7.4 Hz, 6H), 0.85 (s, 3H). [13]C NMR (101 MHz, Methanol-*d*$_4$) δ 174.9, 168.5, 138.6, 138.5, 128.5, 128.2, 83.7, 75.5, 65.8 (2x), 55.7, 54.2, 53.1, 43.0, 40.1, 27.1 (2x), 26.6, 23.2, 17.7, 9.8, 9.5. HR-ESI-MS calculated for $C_{22}H_{36}O_6N_5$ $(M-H)^-$ 466.2671, found 466.2673.

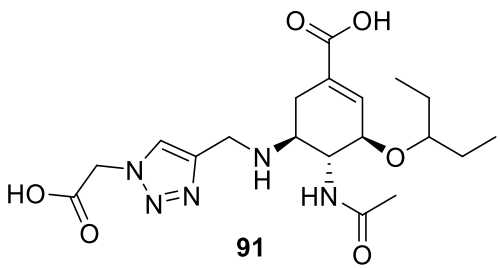
(3*R*,4*R*,5*S*)-4-Acetamido-5-(((1-(2-(1,1-dioxidothiomorpholino)ethyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid.



90

The conditions used are described in the general procedure for the saponification of ethyl esters. The product was purified by preparative HPLC (22 mg, 30 %). [1]H NMR (401 MHz, Methanol-*d*$_4$) δ 8.17 (s, 1H), 6.89 (s, 1H), 4.58 (dq, *J* = 6.8, 3.6 Hz, 2H), 4.48 (q, *J* = 14.3 Hz, 2H), 4.23 (m, 1H), 4.10 (dd, *J* = 10.8, 8.1 Hz, 1H), 3.67 – 3.56 (m, 1H), 3.45 (p, *J* = 5.7 Hz, 1H), 3.07 (m, 11H), 2.65 – 2.52 (m, 1H), 2.04 (s, 3H), 1.53 (m, 4H), 0.91 (dt, *J* = 11.3, 7.4 Hz, 6H). [13]C NMR (101 MHz, Methanol-*d*$_4$) δ 174.9, 168.5, 138.7 (x2), 128.5, 127.2, 83.7, 75.5, 56.4 (2x), 55.5, 53.3, 51.9 (2x), 51.8 (2x), 40.0, 27.1 (2x), 26.6, 23.3, 9.8, 9.5. HR-ESI-MS calculated for $C_{23}H_{39}O_6N_6S$ $(M+H)^+$ 527.2646, found 527.2647.

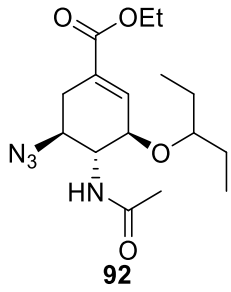
(3*R*,4*R*,5*S*)-4-Acetamido-5-(((1-(carboxymethyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid.



91

The conditions used are described in the general procedure for the saponification of ethyl esters. The product was purified by preparative HPLC (7 mg, 12 %). ^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 8.14 (s, 1H), 6.88 (s, 1H), 5.27 (s, 2H), 4.57 – 4.39 (m, 2H), 4.24 – 4.05 (m, 2H), 3.59 (m, 1H), 3.44 (m, 1H), 3.15 (dd, *J* = 17.4, 5.6 Hz, 1H), 2.70 – 2.50 (m, 1H), 2.03 (s, 3H), 1.62 – 1.37 (m, 4H), 0.91 (dt, *J* = 12.8, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 175.0, 170.3, 168.5, 139.0, 138.7, 128.6, 128.1, 83.8, 75.8, 55.7, 53.1, 52.2, 39.9, 27.2, 27.1, 26.6, 23.3, 9.8, 9.5. HR-ESI-MS calculated for $C_{19}H_{28}O_6N_5$ $(M-H)^-$ 422.2045, found 422.2043.

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

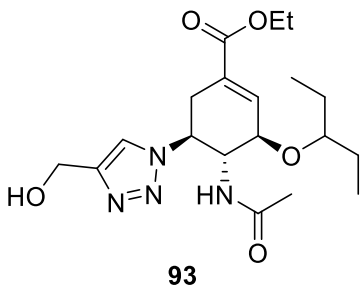


92

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 x 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 (0.66 g, 80%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) 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).

E**thyl (3*R*,4*R*,5*S*)-4-acetamido-5-(4-(hydroxymethyl)-1*H*-1,2,3-triazol-1-yl)-3-(ethylpropoxy)-1-cyclohexene-1-carboxylate**

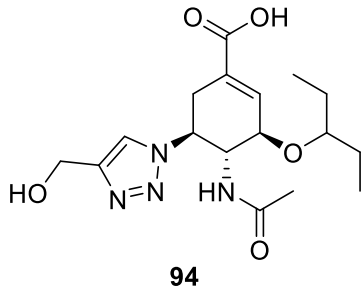


93

CuI (0.054 g, 0.29 mmol) was added to a stirred solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(3-pentyloxy)cyclohex-1-ene-carboxylate (0.3 g, 0.89 mmol), propargyl alcohol (0.15 g, 2.6 mmol) and *N,N*-diisopropylethylamine (0.46 mL, 2.6 mmol) in toluene (6 mL) under inert atmosphere. The reaction mixture was refluxed for 5 min and then stirred at room temperature for 2 h more. The resulting yellow solution was dried under reduced pressure and purified by flash chromatography (DCM/MeOH 90:10) to furnish a solid product that was recrystallized from MeOH (0.32 g, 91%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 7.72 (s, 1H), 6.89 (s, 1H), 6.49 (d, *J* = 8.0 Hz, 1H), 5.38 (td, *J* = 10.9, 6.4 Hz, 1H), 4.75 (s, 2H), 4.68 (dd, *J* = 8.8, 2.2 Hz, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 4.06 (dt, *J* = 11.8, 8.5 Hz, 1H), 3.38 (m, *J* = 5.7 Hz, 1H), 3.10 – 2.88 (m, 1H), 1.76 (s, 3H), 1.51 (dd, *J* = 11.8, 6.4 Hz, 4H), 1.29 (t, *J* = 7.1 Hz, 3H), 0.89 (dt, *J* = 18.8, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 171.7, 165.7, 147.5, 138.6, 128.6, 123.6, 83.0, 74.6, 61.3, 57.4, 56.1, 31.3, 29.8, 26.5, 25.7, 23.2, 14.3, 9.6, 9.4. HR-ESI-MS calculated for $C_{19}H_{31}O_5N_4$ $(M+H)^+$ 395.2289, found 395.2290.

(3*R*,4*R*,5*S*)-4-Acetamido-5-(4-(hydroxymethyl)-1*H*-1,2,3-triazol-1-yl)-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid

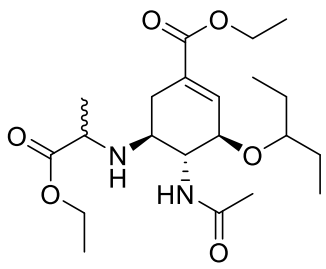


94

The conditions used are described in the general procedure for the saponification of ethyl esters. The purification was done by column chromatography (DCM/MeOH 80:20) to furnish the free carboxylic acid derivative (0.17 g, 85%).

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 8.42 (s, 1H), 6.98 – 6.85 (m, 1H), 5.21 – 5.09 (m, 1H), 4.78 (s, 2H), 4.44 (dt, *J* = 7.5, 2.3 Hz, 1H), 4.34 (dd, *J* = 11.5, 8.7 Hz, 1H), 3.45 (p, *J* = 5.6 Hz, 1H), 3.14 – 3.04 (m, 2H), 1.78 (s, 3H), 1.59 – 1.43 (m, 4H), 0.90 (dt, *J* = 30.9, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 173.6, 168.5, 146.8, 139.2, 129.2, 125.8, 83.9, 76.3, 62.4, 55.9, 55.1, 31.4, 27.3, 26.7, 22.6, 9.9, 9.5. HR-ESI-MS calculated for $C_{17}H_{27}O_5N_4$ $(M+H)^+$ 367.1976, found 367.1978.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(1-ethoxy-1-oxopropan-2-yl)amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate.



To a solution of oseltamivir phosphate (0.2 g, 0.49 mmol) and ethyl 2-bromopropionate (0.11 g, 0.58 mmol) in DMF (3 mL) was added $NaHCO_3$ (0.25 g, 2.9 mmol) portionwise. The reaction mixture was stirred at 80 ºC overnight. The mixture was then quenched by addition of water (5 mL) and extracted with EtOAc (3x 10 mL). The combined organic phase was washed with water (2x 5 mL) and brine (3 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness. The resulting residue was purified by flash chromatography (EtOAc) to yield 70 mg of the A diastereomer and 77 mg of the B diastereomer, (73 % yield altogether).

A diastereomer, less polar (*S*) **95**

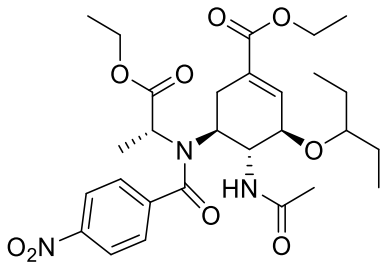
^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.82 – 6.70 (m, 1H), 5.66 (d, J = 7.9 Hz, 1H), 4.29 (ddd, J = 5.9, 2.9, 1.5 Hz, 1H), 4.17 (tt, J = 8.1, 7.1 Hz, 4H), 3.55 (dt, J = 9.7, 7.8 Hz, 1H), 3.46 (q, J = 7.0 Hz, 1H), 3.33 (q, J = 5.7 Hz, 1H), 3.15 (ddd, J = 9.8, 8.8, 5.3 Hz, 1H), 2.75 – 2.60 (m, 1H), 2.18 (ddt, J = 11.7, 5.5, 2.8 Hz, 1H), 2.13 – 2.02 (m, 1H), 1.99 (s, 3H), 1.57 – 1.40 (m, 4H), 1.32 – 1.19 (m, 9H), 0.88 (t, J = 7.4 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 176.0, 170.7, 166.5, 137.4, 129.5, 81.9, 74.3, 60.9 (2x O**C**$H_2$$CH_3$), 57.0, 55.1, 52.9, 31.5, 26.3, 25.9, 23.8, 19.4, 14.3 (2x OCH_2**C**H_3), 9.6, 9.5.

B diastereomer, more polar (*R*) **96**

^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.73 (t, J = 2.5 Hz, 1H), 5.80 (d, J = 6.6 Hz, 1H), 4.15 (dtd, J = 8.6, 7.0, 3.3 Hz, 4H), 4.07 – 3.99 (m, 1H), 3.77 (dt, J = 10.7, 8.7 Hz, 1H), 3.49 – 3.38 (m, 1H), 3.35 – 3.24 (m, 1H), 2.83 – 2.70 (m, 1H), 2.70 – 2.60 (m, 1H), 2.09 (q, J = 5.2, 4.8 Hz, 2H), 2.03 (s, 3H), 1.56 – 1.38 (m, 4H), 1.25 (td, J = 6.8, 4.3 Hz, 9H), 0.95 – 0.76 (m, 6H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 176.4, 170.8, 166.4, 137.7, 129.0, 81.8, 75.8, 60.8 (2x O**C**$H_2$$CH_3$), 55.4, 53.8, 53.5, 31.4, 26.1, 25.6, 23.7, 19.8, 14.2 (2x OCH_2**C**H_3), 9.5, 9.2.

HR-ESI-MS calculated for $C_{21}H_{36}N_2O_6Na$ $(M+Na)^+$ 435.2466, found 435.2466.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[*N*-((*R*)-1-ethoxy-1-oxopropan-2-yl)-4-nitrobenzamido]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate.



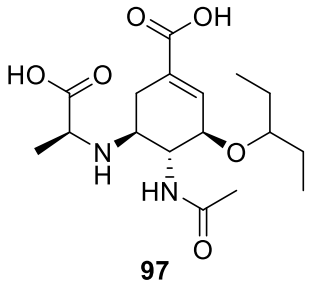
99

4-Nitrobenzoyl chloride (0.086 g, 0.46 mmol) was added to a solution of the B diastereomer (0.16 g, 0.39 mmol) and trimethylamine (0.1 mL, 0.77 mmol) in DCM (3 mL). The reaction mixture was left stirring overnight at room temperature. The reaction was quenched by addition of water (1.5 mL) and the resulting mixture was extracted with EtOAc (3x 10mL). The combined organic layers were washed with water, brine and were evaporated to dryness. The residue was purified by flash chromatography (Hex/EtOAc 1:2) which was followed with crystallization (EtOH) to furnish the desired compound (79 mg, 36% yield).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 8.26 (d, *J* = 8.7 Hz, 2H), 7.48 (d, *J* = 8.7 Hz, 2H), 7.13 (d, *J* = 6.1 Hz, 1H), 6.75 (t, *J* = 2.4 Hz, 1H), 5.10 (dd, *J* = 7.3, 3.5 Hz, 1H), 4.63 (td, *J* = 10.9, 6.2 Hz, 1H), 4.35 – 4.16 (m, 4H), 3.79 (q, *J* = 6.8 Hz, 1H), 3.25 (p, *J* = 5.7 Hz, 1H), 3.10 (ddd, *J* = 11.3, 8.5, 6.1 Hz, 1H), 2.89 – 2.77 (m, 1H), 2.56 – 2.43 (m, 1H), 1.96 (d, *J* = 6.3 Hz, 3H), 1.67 (d, *J* = 6.8 Hz, 3H), 1.55 – 1.40 (m, 4H), 1.39 – 1.27 (m, 6H), 0.95 – 0.80 (m, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 171.7, 171.1, 169.8, 166.0, 148.8, 141.2, 138.9, 128.3, 127.9, 126.9, 124.1, 124.0, 82.1, 71.2, 62.2, 61.3, 56.7, 55.6, 52.6, 28.4, 26.5, 25.7, 24.3, 16.3, 14.4, 14.2, 9.8, 9.3. HR-ESI-MS calculated for $C_{28}H_{39}N_3O_9Na$ $(M+Na)^+$ 584.2579, found 584.2578.

X-ray analysis of the crystals determinates that the product obtained was the *R* diastereomer.

(3*R*,4*R*,5*S*)-4-Acetamido-5-{[(*S*)-1-carboxyethyl]amino}-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid

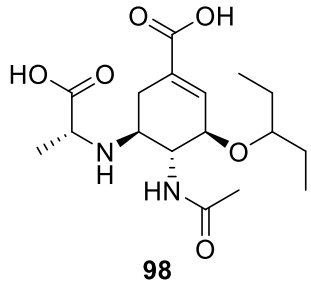


97

A solution of aqueous NaOH (1 mL, 0.5 M) was added dropwise to a solution of the A diastereomer (*S*) (0.025 g, 0.06 mmol) in THF (1 mL) and the reaction mixture was stirred overnight at room temperature. The pH was adjusted to neutral by addition of Amberlite IR 120 hydrogen form. Amberlite was filtered out and the filtrate was concentrated under reduced pressure. The residue was purified by preparative HPLC.

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 6.90 (d, *J* = 1.4 Hz, 1H), 4.26 – 4.11 (m, 2H), 3.77 (dd, *J* = 13.9, 7.1 Hz, 1H), 3.64 – 3.58 (m, 1H), 3.48 – 3.39 (m, 1H), 2.99 – 2.90 (m, 1H), 2.67 – 2.55 (m, 1H), 2.04 (s, 3H), 1.64 – 1.42 (m, 7H), 0.99 – 0.84 (m, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 174.7, 173.8, 168.7, 137.7, 129.3, 83.6, 75.2, 57.3, 55.1, 53.2, 27.7, 27.1, 26.6, 23.1, 15.9, 9.9, 9.5. HR-ESI-MS calculated for $C_{17}H_{28}N_2O_6Na$ $(M+Na)^+$ 379.1840, found 379.1839.

(3*R*,4*R*,5*S*)-4-Acetamido-5-{[(*R*)-1-carboxyethyl]amino}-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid

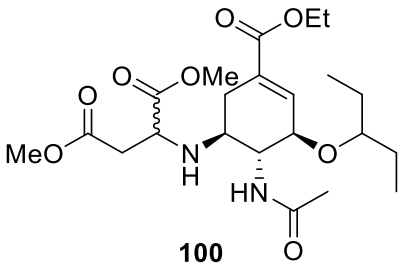


98

A solution of aqueous NaOH (2 mL, 0.5 M) was added dropwise to a solution of the diastereomer B (*R*) (0.05 g, 0.12 mmol) in THF (2 mL) and the reaction mixture was stirred overnight at room temperature. The pH was adjusted to neutral by addition of Amberlite IR 120 hydrogen form. Amberlite was filtered out and the filtrate was concentrated under reduced pressure. The residue was purified by preparative HPLC.

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 6.92 – 6.80 (m, 1H), 4.21 – 4.05 (m, 2H), 3.82 (q, *J* = 7.2 Hz, 1H), 3.52 – 3.39 (m, 2H), 3.08 – 2.95 (m, 1H), 2.52 – 2.39 (m, 1H), 2.09 (s, 3H), 1.59 – 1.47 (m, 7H), 0.91 (dt, *J* = 11.2, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 175.1, 173.6, 168.5, 138.8, 128.7, 83.9, 76.1, 57.3, 57.2, 53.5, 27.8, 27.2, 26.6, 23.2, 16.9, 9.8, 9.6. HR-ESI-MS calculated for $C_{17}H_{28}N_2O_6Na$ $(M+Na)^+$ 379.1840, found 379.1839.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[N-[dimethyl aspartyl]methyl]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate

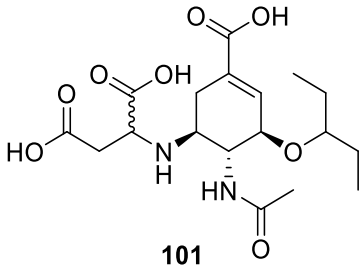


100

Dimethyl maleate (0.57 g, 4.0 mmol) was added to a stirred solution of oseltamivir free base (0.22 g, 0.73 mmol) in methanol (3 mL) under argon atmosphere, the mixture was refluxed overnight. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (DCM/MeOH 90:10) to yield the mixture of diastereomers (0.25 g, 75%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.68 (s, 1H), 6.03 (d, J = 7.5 Hz, 1H), 4.26 (d, J = 8.3 Hz, 1H), 4.19 – 4.01 (m, 2H), 3.64 (s, 3H), 3.67 – 3.63 (m, 1H), 3.60 (s, 3H), 3.51 – 3.36 (m, 1H), 3.35 – 3.20 (m, 1H), 3.08 (dt, J = 10.1, 5.0 Hz, 1H), 2.96 – 2.86 (m, 1H), 2.72 – 2.52 (m, 3H), 2.10 – 1.95 (m, 1H), 1.92 (s, 3H), 1.42 (ddt, J = 7.4, 5.7, 1.9 Hz, 4H), 1.26 – 1.13 (m, 3H), 0.90 – 0.75 (m, 6H).^{13}C NMR (75 MHz, Chloroform-d_3) of major diastereomer δ 174.2, 171.4, 170.9, 166.3, 137.7, 129.0, 81.7, 74.4, 60.7, 57.4, 56.1, 53.4, 52.3, 51.8, 37.8, 31.5, 26.2, 25.6, 23.6, 14.2, 9.5, 9.3. ^{13}C NMR (75 MHz, Chloroform-d_3) of minor diastereomer δ 174.8, 171.3, 171.0, 166.5, 137.8, 129.1, 82.0, 75.9, 60.9, 55.9, 55.2, 53.8, 52.4, 52.0, 38.7, 31.7, 26.2, 25.7, 23.8, 14.3, 9.7, 9.4. HR-ESI-MS calculated for $C_{22}H_{36}N_2O_8Na$ $(M+Na)^+$ 479.2364, found 479.2364.

((1*S*,5*R*,6*R*)-6-Acetamido-3-carboxy-5-(pentan-3-yloxy)cyclohex-3-en-1-yl)aspartic acid

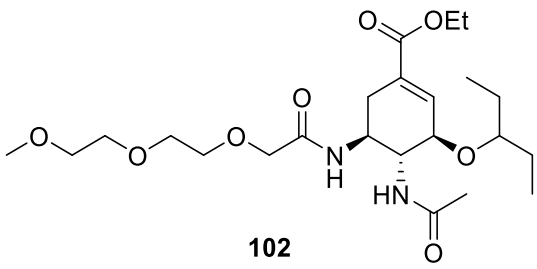


101

An aqueous solution of NaOH (0.5 M; 7.2 mL) was added dropwise to a stirred solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[N-[dimethyl aspartyl]methyl]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate (0.09 g, 0.19 mmol) in THF (3 mL). The reaction was stirred for 24 h at room temperature. The pH was adjusted to neutral by addition of Amberlite IR 120 hydrogen form. Amberlite was filtered out; the filtrate was concentrated under reduced pressure and purified by flash chromatography (DCM/MeOH 80:20) to furnish the free carboxylic acid derivative (0.053 g, 70%).

^{1}H NMR (401 MHz, Methanol-d_4) δ 6.87 (t, J = 1.2 Hz, 1H), 4.28 (dd, J = 8.1, 2.0 Hz, 1H), 4.07 (dd, J = 10.7, 8.1 Hz, 1H), 3.99 (t, J = 5.1 Hz, 1H), 3.67 (td, J = 10.2, 5.6 Hz, 1H), 3.48 – 3.39 (m, 1H), 3.05 – 2.91 (m, 3H), 2.62 – 2.52 (m, 1H), 2.04 (s, 3H), 1.60 – 1.49 (m, 4H), 0.92 (dt, J = 10.4, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-d_4) for the major diastereomer δ 175.4, 175.0, 172.1, 168.7, 138.4, 129.1, 83.6, 75.3, 58.0, 55.5, 54.1, 33.6, 28.0, 27.2, 26.6, 23.2, 9.9, 9.5. ^{13}C NMR (101 MHz, Methanol-d_4) for the minor diastereomer δ 175.4, 175.0, 173.9, 168.7, 138.9, 129.0, 83.9, 76.3, 57.8, 55.5, 53.9, 35.9, 28.0, 27.2, 26.7, 23.2, 9.8, 9.6. HR-ESI-MS calculated for $C_{18}H_{27}N_2O_8$ $(M-H)^-$ 399.1769, found 399.1773.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-{2-[2-(2-methoxyethoxy)ethoxy]acetamido}-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate.

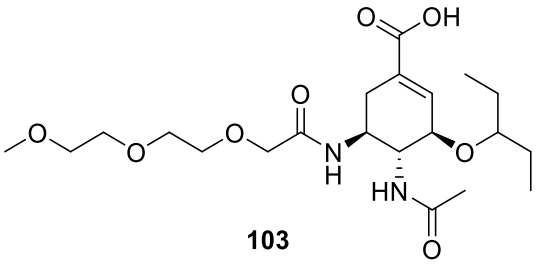


102

To a solution of and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (0.28 g, 0.87 mmol) and 2-[2-(2-methoxyethoxy)ethoxy]acetic acid (0.13 g, 0.72 mmol) in DMF (3 mL) was added triethylamine (0.5 ml, 3.6 mmol) under argon atmosphere. The reaction mixture was stirred for 5 min at room temperature followed by addition of Oseltamivir phosphate (0.15 g, 0.36 mmol) and further stirring for 16 h. The reaction mixture was quenched by addition of water (10 mL) and $NaHCO_3$ saturated solution (5 mL) until the pH was slightly basic. The aqueous solution was extracted with DCM and the combined organic phases were washed with water and brine, dried over Na_2SO_4, and evaporated to dryness under reduced pressure. Purification of crude product by flash column chromatography (eluent DCM/MeOH 90:10) furnished the amide (0.16 g, 93%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 7.33 (d, *J* = 8.6 Hz, 1H), 6.82 – 6.74 (m, 1H), 6.06 (d, *J* = 8.8 Hz, 1H), 4.19 (qd, *J* = 7.1, 1.0 Hz, 3H), 4.13 – 4.06 (m, 1H), 4.04 – 3.99 (m, 1H), 3.69 – 3.61 (m, 8H), 3.59 – 3.52 (m, 2H), 3.37 - 3.31 (m, 1H), 3.35 (s, 3H), 2.85 – 2.69 (m, 1H), 2.37 (dd, *J* = 17.7, 9.8 Hz, 1H), 1.92 (s, 3H), 1.56 – 1.43 (m, 4H), 1.27 (t, *J* = 7.1 Hz, 3H), 0.87 (dt, *J* = 10.4, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 170.8, 170.7, 166.1, 137.9, 129.3, 82.4, 75.7, 72.0, 71.1, 70.7, 70.4, 70.4, 61.0, 59.1, 54.5, 47.5, 30.6, 26.3, 25.8, 23.4, 14.3, 9.7, 9.4. HR-ESI-MS calculated for $C_{23}H_{40}O_8N_2$ $(M+H)^+$ 473.2865, found 473.2857.

(3*R*,4*R*,5*S*)-4-Acetamido-5-{2-[2-(2-methoxyethoxy)ethoxy]acetamido}-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid



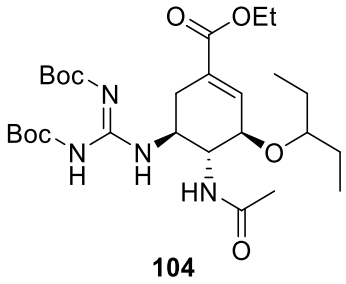
103

The conditions used are described in the general procedure for the saponification of ethyl esters. The product was purified by column chromatography (DCM/MeOH 80:20) to furnish the free carboxylic acid derivative (0.069 g, 70% yield).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 7.73 (d, *J* = 9.2 Hz, 1H), 6.92 (dt, *J* = 9.1, 4.6 Hz, 1H), 6.85 (d, *J* = 2.5 Hz, 1H), 5.74 (bs, 1H), 4.24 – 4.16 (m, 1H), 4.15 – 4.10 (m, 1H), 4.07 – 4.00 (m, 1H), 3.71 – 3.61 (m, 8H), 3.59 – 3.53 (m, 2H), 3.40 – 3.31 (m, 1H), 3.36 (s, 3H), 2.71 (dd, *J* = 18.0, 5.2 Hz, 1H), 2.47 – 2.32 (m, 1H), 1.95 (d, *J* = 1.7 Hz,

3H), 1.59 – 1.40 (m, 4H), 0.87 (m, 6H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 177.1, 176.7, 174.0, 144.6, 134.5, 87.9, 81.2, 77.3, 76.5, 76.1, 75.9, 64.5, 60.1, 56.0, 53.2, 35.9, 31.8, 31.1, 28.6, 15.2, 14.7. HR-ESI-MS calculated for $C_{21}H_{36}O_8N_2$ $(M+Na)^+$ 467.2364, found 467.2363.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[*N*^{2},*N*^{3}-bis(*tert*-butoxycarbonyl)guanidino]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate

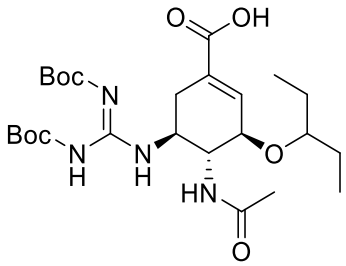


104

Mercury(II) chloride (0.11 g, 0.40 mmol) was added portionwise to a solution of oseltamivir base (0.10 g, 0.32 mmol), *N,N′*−di-(*tert*-butoxycarbonyl)thiourea (0.11 g, 0.40 mmol) and triethylamine (0.11 mL, 0.80 mmol) in DMF (15 mL) at 0 °C. The reaction mixture was stirred for 24 h at room temperature. The mixture was diluted with EtOAc (10 mL), filtered through a pad of Celite and concentrated under reduced pressure. The residue was diluted with water (30 mL) and extracted with EtOAc (3x10 mL). The combined organic phase was washed with water, brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash column chromatography (eluent Hexanes/EtOAc, 2:1) to afford the Boc-protected guanidine derivative (0.16 g, 89%).

TLC (Hexanes/EtOAc 1:1) R_f = 0.4. ^{1}H NMR (300 MHz, Chloroform-d_3) δ 11.33 (s, 1H), 8.57 (d, J = 8.1 Hz, 1H), 6.76 (s, 1H), 6.16 (d, J = 8.9 Hz, 1H), 4.43 – 4.23 (m, 1H), 4.20 – 4.01 (m, 3H), 3.96 (d, J = 7.7 Hz, 1H), 3.36 – 3.21 (m, 1H), 2.72 (dd, J = 17.6, 5.3 Hz, 1H), 2.42 – 2.23 (m, 1H), 1.85 (s, 3H), 1.55 – 1.49 (m, J = 8.4, 4.7 Hz, 22H), 1.22 (dd, J = 12.6, 5.5 Hz, 3H), 0.82 (dt, J = 10.3, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 170.3, 166.0, 163.3, 157.0, 152.7, 138.1, 128.7, 83.6, 82.8, 79.6, 76.3, 61.1, 54.5, 48.2, 30.6, 28.4, 28.2, 26.2, 25.9, 23.4, 14.3, 9.7, 9.4. HR-ESI-MS calculated for $C_{27}H_{47}O_8N_4$ $(M+H)^+$ 555.3388, found 555.3389.

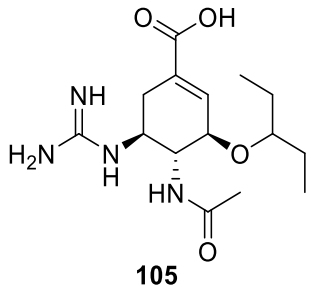
(3*R*,4*R*,5*S*)-4-Acetamido-5-[*N*^{2},*N*^{3}-bis(*tert*-butoxycarbonyl)guanidino]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid



The conditions used are described in the general procedure for the saponification of ethyl esters. The residue was purified by flash column chromatography (Toluene/EtOAc/AcOH 1:1:0.5%) to afford the free acid (0.07 g, 48%).

TLC (Toluene/EtOAc/AcOH 1:1:0.5%) R_f = 0.2. ^{1}H NMR (300 MHz, Chloroform-d_3) δ 8.64 (d, J = 8.1 Hz, 1H), 6.82 (s, 1H), 6.23 (d, J = 8.9 Hz, 1H), 4.39 (dd, J = 8.3, 6.0 Hz, 1H), 4.26 – 4.16 (m, 2H), 4.16 – 4.08 (m, 1H), 4.06 – 3.98 (m, 1H), 3.41 – 3.29 (m, 1H), 2.78 (dd, J = 17.6, 5.3 Hz, 1H), 2.47 – 2.32 (m, 1H), 1.91 (s, 3H), 1.61 – 1.41 (m, 22H), 1.27 (dt, J = 10.9, 7.1 Hz, 4H), 0.89 (dt, J = 10.3, 7.4 Hz, 6H). ^{13}C NMR (75 MHz, Methanol-d_4) δ 173.6, 169.3, 164.4, 157.7, 153.7, 138.7, 130.4, 84.6, 84.0, 80.5, 76.6, 54.6, 50.0, 31.4, 28.5, 28.2, 27.3, 26.9, 22.7, 9.9, 9.7. HR-ESI-MS calculated for $C_{25}H_{43}O_8N_4$ $(M+H)^+$ 527.3075, found 527.3076.

(3*R*,4*R*,5*S*)-4-Acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid

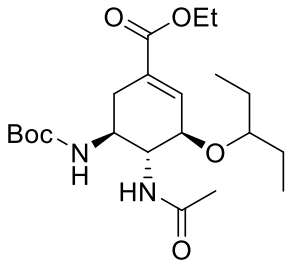


105

(3*R*,4*R*,5*S*)-4-Acetamido-5-[N^2,N^3-bis(*tert*-butoxycarbonyl)guanidino]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid (0.07 g, 0.13 mmol) was stirred with trifluoroacetic acid (50% in water, 5 mL) at room temperature for 1 h. The solution was evaporated under reduced pressure and purified by preparative HPLC to furnish the guanidine derivative (0.02 g, 43%).

^{1}H NMR (400 MHz, Methanol-d_4) δ 7.28 (d, J = 9.0 Hz, 1H), 6.79 (s, 1H), 4.15 (dd, J = 5.3, 2.1 Hz, 1H), 3.93 – 3.73 (m, 2H), 3.42 – 3.31 (m, 1H), 2.77 (dd, J = 17.6, 5.0 Hz, 1H), 2.37 – 2.20 (m, 1H), 1.94 (s, 3H), 1.48 (tdd, J = 12.9, 6.2, 4.5 Hz, 4H), 0.86 (dt, J = 12.8, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.2, 169.0, 158.6, 138.8, 129.8, 83.8, 76.1, 55.8, 51.7, 31.3, 27.2, 26.8, 22.8, 9.8, 9.7. HR-ESI-MS calculated for $C_{15}H_{27}O_4N_4$ $(M+H)^+$ 327.2027, found 327.2028.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate

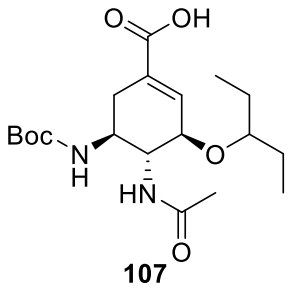


106

To a solution of oseltamivir free base (0.38 g, 1.21 mmol) in DCM (3 mL) was added triethylamine (0.61 g, 6.08 mmol) followed by addition of di-*tert*-butyl dicarbonate (0.53 g, 2.42 mmol) and then the reaction mixture was stirred for 4 h at room temperature. The mixture was diluted with water (10 mL) and then extracted with DCM (3x10 mL). The combined organic phase was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (eluent DCM to DCM/MeOH, 20:1) to afford the protected amine (0.49 g, 98% yield) as a white solid.

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 6.78 (s, 1H), 5.80 (d, *J* = 8.2 Hz, 1H), 5.11 (d, *J* = 9.0 Hz, 1H), 4.20 (q, *J* = 7.1 Hz, 2H), 4.13 – 4.00 (m, 1H), 3.97 (s, 1H), 3.79 (dd, *J* = 9.7, 5.1 Hz, 1H), 3.52 – 3.28 (m, 1H), 2.74 (dd, *J* = 18.1, 4.9 Hz, 1H), 2.29 (dd, *J* = 17.7, 9.6 Hz, 1H), 1.98 (s, 3H), 1.51 (dd, *J* = 5.5, 4.1 Hz, 4H), 1.42 (s, 9H), 1.28 (t, *J* = 7.1 Hz, 3H), 0.88 (dd, *J* = 13.6, 7.3 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 170.9, 166.1, 156.4, 137.7, 129.5, 82.3, 79.8, 76.0, 61.1, 54.5, 49.2, 31.1, 28.5, 26.3, 25.8, 23.5, 14.3, 9.6, 9.4. HR-ESI-MS calculated for $C_{21}H_{37}O_6N_2$ $(M+H)^+$ 413.2646, found 413.2648.

(3*R*,4*R*,5*S*)-4-Acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid

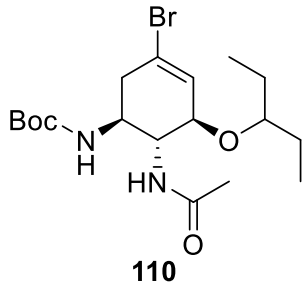


107

The conditions used are described in the general procedure for the saponification of ethyl esters. Purified by flash column chromatography (eluent DCM/MeOH gradient 10:1 to 10:3) to furnish the free acid (1.28 g, 95% yield) as a white solid.

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 6.81 (s, 1H), 6.69 (s, 1H), 5.72 (d, *J* = 9.3 Hz, 1H), 4.02 (d, *J* = 6.8 Hz, 2H), 3.75 (dd, *J* = 13.1, 8.4 Hz, 1H), 3.39 – 3.26 (m, 1H), 2.70 (dd, *J* = 17.5, 5.0 Hz, 1H), 2.25 (dd, *J* = 17.7, 11.1 Hz, 1H), 1.99 (s, 3H), 1.49 (dd, *J* = 14.0, 6.7 Hz, 4H), 1.41 (s, 9H), 0.86 (dd, *J* = 16.5, 7.4 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 171.5, 169.1, 156.9, 139.2, 129.2, 82.3, 79.8, 76.2, 55.2, 49.6, 31.0, 28.5, 26.3, 25.7, 23.4, 9.8, 9.2. HR-ESI-MS calculated for $C_{19}H_{33}O_6N_2$ $(M+H)^+$ 385.2333, found 385.2335.

(3*R*,4*R*,5*S*)-4-Acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-bromocyclohexene

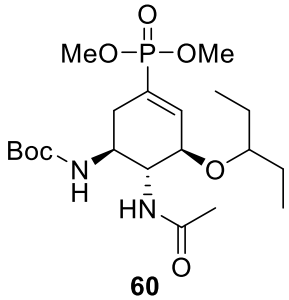


110

S-(1-Oxido-2-pyridyl)-*N,N,N′,N′*-tetramethylthiuronium hexafluorophosphate (0.26 g, 0.70 mmol) was added to a solution of (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cy-clohexene-1-carboxylic acid (0.21 g, 0.54 mmol), triethylamine (0.22 mL, 1.61 mmol) and 4-(dimethylamino)pyridine (0.007 g, 0.054 mmol) in dry THF (4 mL). The reaction was stirred in the dark for 40 min at room temperature. The solvent was removed by evaporation under reduced pressure. The remaining green oil was dissolved in DCM (2 mL) and bromotrichloromethane (2 mL). The formed solution was irradiated (refluxed) with a flood lamp for 90 min. The mixture was concentrated and purified by flash column chromatography (eluent Toluene/EtOAc gradient 2:1 to 1:1) to afford the vinyl bromide (0.18 g, 78% yield).

TLC (Toluene/EtOAc 1:1) R_f = 0.45. ^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.07 (s, 1H), 5.52 (d, J = 9.4 Hz, 1H), 5.35 (d, J = 9.0 Hz, 1H), 4.09 (dd, J = 9.1, 6.8 Hz, 1H), 3.88 (dd, J = 7.9, 5.5 Hz, 1H), 3.83 (s, 1H), 3.39 – 3.25 (m, 1H), 2.68 (m, J = 26.0, 18.0, 6.8 Hz, 2H), 1.99 (s, 3H), 1.59 (s, 1H), 1.49 (dd, J = 7.3, 6.0 Hz, 3H), 1.42 (s, 9H), 0.88 (t, J = 7.4 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 171.5, 156.9, 139.2, 129.2, 82.3, 79.8, 76.2, 55.2, 50.9, 49.6, 28.5, 26.3, 25.7, 23.4, 9.7, 9.2. HR-ESI-MS calculated for $C_{18}H_{31}BrO_4N_2Na$ $(M+Na)^+$ 441.1359, found 441.1360.

Dimethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate



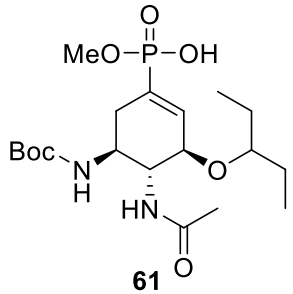
60

To a solution of (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-bromocyclohexene (0.18 g, 0.39 mmol) in toluene (10 mL) was added tetrakis(triphenylphosphine)palladium (0.07 g, 0.06 mmol), triethylamine (0.21 mL, 1.54 mmol) and dimethyl phosphite (0.14 mL, 1.54 mmol). The reaction mixture was stirred at 80 °C for 90 min. The reaction was quenched with a saturated solution of NH_4Cl (6 mL) and later was diluted with DCM (30 mL). The organic phase was washed with a saturated solution of NH_4Cl (6 mL) and brine (2x5 mL), dried over

$MgSO_4$ and concentrated. The residue was purified by flash column chromatography (eluent EtOAc gradient to EtOAc/MeOH 6:1) to afford the phosphonate (0.16 g, 86% yield).

TLC (EtOAc/MeOH 6:1) R_f = 0.42. ^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.59 (d, J = 21.8 Hz, 1H), 6.02 (d, J = 8.8 Hz, 1H), 5.17 (d, J = 9.0 Hz, 1H), 4.11 – 3.99 (m, 1H), 3.94 (s, 1H), 3.77 (d, J = 11.9 Hz, 1H), 3.72 (d, J = 2.4 Hz, 3H), 3.69 (d, J = 2.4 Hz, 3H), 3.39 – 3.27 (m, 1H), 2.67 – 2.49 (m, 1H), 2.28 – 2.11 (m, 1H), 1.97 (s, 3H), 1.56 – 1.44 (m, 4H), 1.40 (s, 9H), 0.86 (td, J = 7.4, 5.1 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 171.2, 156.3, 141.9 (d, J = 6.8 Hz), 129.3 (d, J = 180.4 Hz), 81.8, 79.8, 76.0 (d, J = 20.2Hz), 54.1, 52.8 (d), 52.7 (d), 49.8 (d), 30.7 (d), 28.2, 25.9, 25.6, 23.1, 9.7, 9.3. HR-ESI-MS calculated for $C_{20}H_{37}O_7NaN_2P$ $(M+Na)^+$ 471.2231, found 471.2232.

Methyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (OMT)

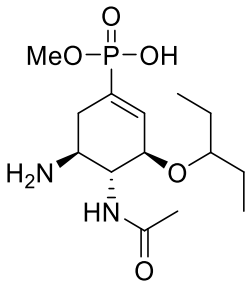


61

An aqueous solution of NaOH (0.5M; 2.8 mL) was added to a stirred solution of the dimethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (0.31 g, 0.698 mmol) in 1,4-dioxane (2.8 mL). The reaction mixture was stirred for 18 h at room temperature. The pH was adjusted to neutral by addition of Amberlite IR 120 hydrogen form. Amberlite was filtered out, rinsed with methanol several times and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (eluent EtOAc/MeOH gradient 6:1 to 1:2) to furnish phosphonic acid (0.27 g, 91% yield) as a white solid.

TLC (DCM/MeOH 2:1), R_f = 0.2. ^{1}H NMR (600 MHz, D_2O) δ 6.32 (d, J = 19.7 Hz, 1H), 4.25 (d, J = 8.6 Hz, 1H), 3.83 (t, J = 10.0 Hz, 1H), 3.75 (s, 1H), 3.52 (d, J = 10.7 Hz, 3H), 2.45 – 2.43 (m, 2H), 2.24 (s, 3H), 1.57 (dd, J = 13.6, 6.7 Hz, 4H), 1.51 – 1.34 (m, 9H), 0.88 (dt, J = 34.7, 7.3 Hz, 6H).^{13}C NMR (151 MHz, D_2O) δ 174.2, 157.6, 137.0, 131.6, 130.5, 84.2, 80.9, 76.9, 76.7, 55.6, 51.6, 51.6, 49.3, 49.2, 31.1, 27.6, 25.6, 25.3, 22.2, 8.6, 8.5. ^{31}P NMR (121 MHz, D_2O) δ 15.27. HR-ESI-MS calculated for $C_{19}H_{36}O_7N_2P$ $(M+H)^+$ 435.2255, found 435.2256.

Methyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate

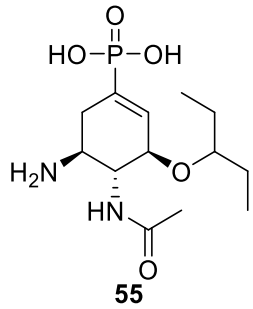


114

Methyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (0.05 g, 1.14 mmol) was stirred with trifluoroacetic acid (50% in water, 4 mL) at room temperature for 1 h. The solution was evaporated under reduced pressure and later residue was purified by preparative HPLC to furnish the title compound (0.02 g, 43% yield).

^{1}H NMR (600 MHz, D_2O) δ 6.38 (d, *J* = 19.4 Hz, 1H), 4.28 (d, *J* = 7.5 Hz, 1H), 4.14 – 4.01 (m, 1H), 3.65 – 3.55 (m, 2H), 3.53 (d, *J* = 10.8 Hz, 3H), 2.82 – 2.72 (m, 1H), 2.49 – 2.40 (m, 1H), 2.09 (s, 3H), 1.60 – 1.45 (m, 4H), 0.90 (t, *J* = 7.2 Hz, 3H), 0.85 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (151 MHz, D_2O) δ 175.8, 137.9 (d, *J* = 6.8 Hz), 129.7 (d, *J* = 174.4 Hz), 84.9, 76.5 (d, *J* = 19.4 Hz), 53.5, 52.5 (d, *J* = 5.1 Hz), 50.3 (d, *J* = 14.1 Hz), 29.8 (d, *J* = 11.0 Hz), 26.1, 25.8, 22.9, 9.2, 9.1. HR-ESI-MS calculated for $C_{14}H_{28}O_5N_2P$ $(M+H)^+$ 335.1730, found 335.1732.

(3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate

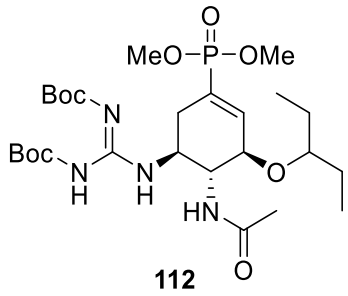


55

Trimethylsilyl bromide (0.41 mL, 3.18 mmol) and 2,6-lutidine (0.44 mL, 3.81 mmol) were added to a solution of methyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (0.14 g, 0.318 mmol) in DCM (10 mL) and the reaction was allowed to stir for 9 h at room temperature. The solvent was evaporated and TFA (50% in water, 10 mL) was added. Then the reaction was allowed to stir for 1 h and the solvent was evaporated under reduced pressure. The residue was purified by preparative HPLC.

HR-ESI-MS calculated for $C_{13}H_{25}O_5N_2NaP$ $(M+Na)^+$ 343.1393, found 343.1395.

Dimethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[*N*2,*N*3-bis(*tert*-butoxycarbonyl)guanidino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate

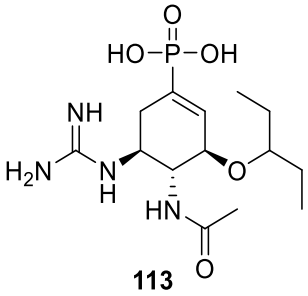


112

Dimethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (0.12 g, 0.259 mmol) was treated with neat TFA (1 mL) for 1 h and then the acid was removed by evaporation under reduced pressure. The residue was dissolved in acetonitrile (1.5 mL) and triethylamine (0.18 mL, 1.29 mmol) was added dropwise followed by addition of *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine (0.08 g, 0.26 mmol). The reaction mixture was stirred for 18 h at room temperature and then was evaporated to dryness. The residue was purified by flash column chromatography (eluent EtOAc gradient to EtOAc/MeOH 6:1) to afford the guanidine phosphonate (0.07 g, 47% yield).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 11.39 (s, 1H), 8.60 (d, J = 7.3 Hz, 1H), 6.64 (d, J = 22.5 Hz, 1H), 6.35 (d, J = 9.2 Hz, 1H), 4.41 (d, J = 8.2 Hz, 1H), 4.23 – 4.04 (m, 1H), 3.99 (s, 1H), 3.73 (s, 3H), 3.70 (s, 3H), 3.39 – 3.27 (m, 1H), 2.72 – 2.58 (m, 1H), 2.36 – 2.22 (m, 1H), 1.92 (s, 3H), 1.58 – 1.41 (m, 22H), 0.88 (dd, J = 16.2, 7.4 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 170.6, 163.2, 157.0, 152.7, 143.2, 143.1, 132.2, 132.1, 126.6, 124.1, 83.7, 82.8, 79.8, 76.4, 76.1, 54.3, 52.8, 52.7, 52.6, 52.6, 48.4, 48.2, 30.9, 30.8, 28.3, 28.1, 26.0, 25.7, 23.3, 9.6, 9.3. HR-ESI-MS calculated for $C_{26}H_{48}O_9N_4P$ $(M+H)^+$ 591.3153, found 591.3153.

(3*R*,4*R*,5*S*)-4-Acetamido-5-guanidino-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate



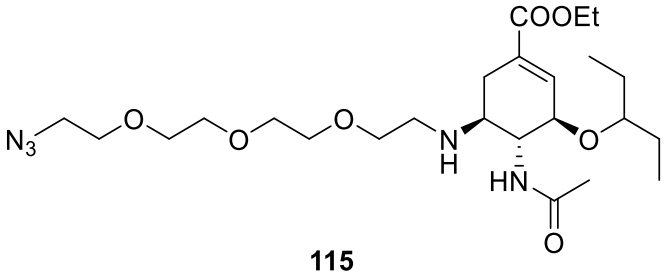
113

Neat bromotrimethylsilane (1.49 g, 9.68 mmol) was added to a solution of dimethyl (3*R*,4*R*,5*S*)-4-acetamido-5-[*N*2,*N*3-bis(*tert*-butoxycarbonyl)-guanidino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (0.07 g, 0.121 mmol) in DCM (5 mL) at 0 °C and the reaction was allowed to stir for 24 h at room temperature. The solvent was evaporated under reduced pressure; the remaining residue was quenched with water (4 mL) and then the mixture was stirred for 2 h. The solution was evaporated to dryness and later was purified by preparative HPLC to afford the free guanidine (0.09 g, 21% yield).

^{1}H NMR (400 MHz, D_2O) δ 6.31 (d, J = 19.8 Hz, 1H), 4.26 (d, J = 8.5 Hz, 1H), 4.01 – 3.88 (m, 1H), 3.80 (td, J = 10.4, 5.2 Hz, 1H), 3.60 – 3.47 (m, 1H), 2.85 – 2.66 (m, 1H), 2.46 – 2.31 (m, 1H), 2.04 (s, 3H), 1.65 – 1.50 (m, 3H), 1.45 (dt, J = 14.4, 7.2 Hz, 1H), 0.87 (dt, J = 19.5, 7.4 Hz, 6H).

^{13}C NMR (151 MHz, H_2O +D_2O + *tert*-butyl alcohol) δ 175.3, 157.6, 157.5, 135.3, 135.3, 134.0, 132.8, 85.0, 77.0, 76.9, 70.5 (C), 55.9, 55.9, 51.8, 51.7, 31.5, 31.4, 30.2 (CH_3), 26.3, 26.0, 22.7, 22.6, 9.3, 9.2. ^{31}P NMR (162 MHz, D_2O) δ 11.13. HR-ESI-MS calculated for $C_{14}H_{28}O_5N_4P$ $(M+H)^+$ 363.1792, found 363.1794.

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-((2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)amino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylate

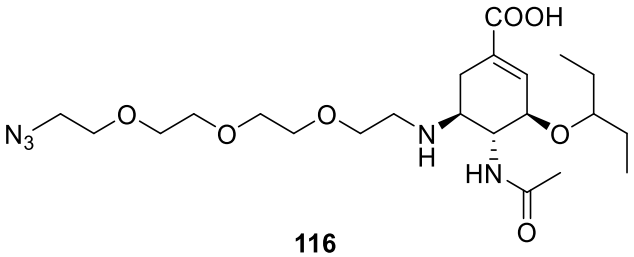


115

1-Azido-2-(2-(2-(2-iodoethoxyl)ethoxy)-ethoxy)ethane (0.1 g, 0.30 mmol) was added dropwise to a solution of the free base of oseltamivir (0.11 g, 0.33 mmol) and triethylamine (0.12 mL, 0.91 mmol) in acetonitrile (3 mL), the reaction was then stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure and the remaining oil was dissolved in water (5 mL), the aqueous solution was then extracted with DCM (3x 10 mL) and the combined organic layers were washed with brine (5 mL), dried over anhydrous $MgSO_4$ and evaporated down. The remaining mixture was purified by flash chromatography (DCM/MeOH 9:1) to yield the alkylated oseltamivir (0.093 g, 60%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 6.77 (d, *J* = 2.3 Hz, 1H), 6.09 (d, *J* = 8.2 Hz, 1H), 4.30 (d, *J* = 8.5 Hz, 1H), 4.19 (q, *J* = 7.2 Hz, 2H), 3.75 – 3.48 (m, 12H), 3.45 – 3.30 (m, 3H), 3.31 – 3.19 (m, 1H), 2.92 (ddd, *J* = 12.2, 6.2, 3.9 Hz, 1H), 2.76 (ddd, *J* = 17.0, 10.5, 3.9 Hz, 4H), 2.22 (dt, *J* = 10.4, 3.4 Hz, 1H), 2.03 (s, 3H), 1.57 – 1.39 (m, 4H), 1.28 (t, *J* = 7.1 Hz, 3H), 0.88 (td, *J* = 7.4, 5.3 Hz, 6H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 171.3, 166.4, 137.9, 129.0, 81.9, 75.0, 70.6, 70.6, 70.5, 70.1, 70.0, 70.0, 60.9, 56.0, 54.1, 50.7, 45.7, 30.3, 26.2, 25.7, 23.7, 14.3, 9.6, 9.3. HR-ESI-MS calculated for $C_{24}H_{44}O_7N_5$ $(M+H)^+$ 514.3235, found 514.3235.

(3*R*,4*R*,5*S*)-4-Acetamido-5-((2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)amino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-carboxylic acid

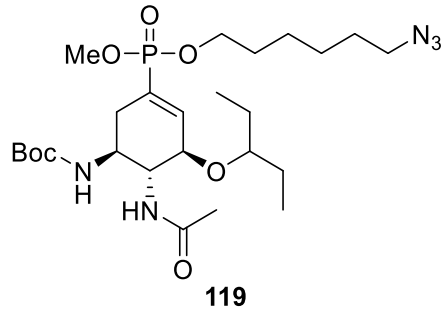


116

The conditions used are described in the general procedure for the saponification of ethyl esters. The resulting product was purified by flash chromatography (DCM/MeOH 80:20) to furnish the free carboxylic acid derivative (0.055 g, 83% yield).

^{1}H NMR (401 MHz, Methanol-d_4) δ 6.85 (t, J = 2.0 Hz, 1H), 4.31 – 4.19 (m, 1H), 4.11 (dd, J = 10.9, 8.0 Hz, 1H), 3.77 (ddd, J = 7.9, 6.2, 3.7 Hz, 2H), 3.72 – 3.59 (m, 12H), 3.46 (d, J = 5.6 Hz, 1H), 3.41 – 3.37 (m, 2H), 3.37 – 3.32 (m, 1H), 2.98 (dd, J = 17.4, 5.6 Hz, 1H), 2.55 (ddt, J = 17.4, 9.7, 2.8 Hz, 1H), 2.05 (s, 3H), 1.54 (tq, J = 9.9, 4.4, 3.4 Hz, 4H), 0.91 (dt, J = 10.9, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 174.7, 169.0, 138.0, 129.3, 83.7, 75.9, 71.6, 71.5, 71.4, 71.40, 71.1, 66.7, 56.1, 52.8, 51.7, 45.3, 27.3, 27.1, 26.6, 23.3, 9.8, 9.5. HR-ESI-MS calculated for $C_{22}H_{40}O_7N_5$ $(M+H)^+$ 486.2922, found 486.2923.

1-(6-Azidohexyl)-1-methyl-(3*R*,4*R*,5*S*)-4-acetylamino-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate



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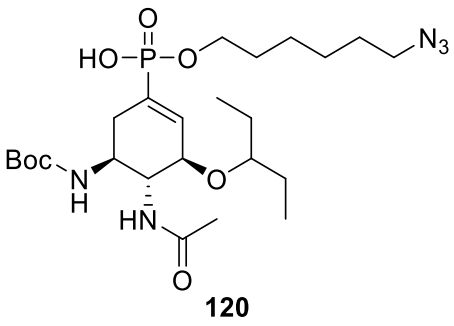
To a solution of methyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (0.05 g, 0.11 mmol) and triethylamine (0.12 ml, 0.88 mmol) in DMF (2 mL) under argon was added 1-azido-6-bromohexane (0.052 g, 0.25 mmol), the reaction mixture was stirred at 40 ºC for 48 h. The DMF was evaporated under reduced pressure and the residue was purified by flash chromatography (EtOAc) to yield the alkylated phosphonate (0.015 g, 25%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 6.60 (dd, J = 21.6, 2.2 Hz, 1H), 5.77 (dd, J = 9.2, 5.4 Hz, 1H), 5.06 (d, J = 9.1 Hz, 1H), 4.10 – 3.95 (m, 3H), 3.92 (m, 1H), 3.79 (dd, J = 9.8, 5.2 Hz, 1H), 3.70 (dd, J = 11.0, 2.3 Hz, 3H), 3.33 (t, J = 5.6 Hz, 1H), 3.27 (td, J = 6.9, 1.1 Hz, 2H), 2.69 – 2.51 (m, 1H), 2.21 (m, 1H), 1.98 (s, 3H), 1.68 -1.61 (m, 4H), 1.56 – 1.31 (m, 17H), 0.96 – 0.74 (m, 6H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 170.9, 156.3, 142.2, 126.7 (d, J = 188.9 Hz), 82.2, 79.7, 76.3, 76.2, 66.5 – 65.01 (m), 54.5, 52.5 (d, J = 5.9 Hz), 51.3, 49.2, 31.3, 30.3 (d, J = 6.2 Hz), 28.7,

28.3, 28.3, 26.3, 26.1, 25.5, 25.1, 23.3, 9.6, 9.1. HR-ESI-MS calculated for $C_{25}H_{47}O_7N_5P$ $(M+H)^+$ 560.3207, found 560.3209.

(6-Azidohexyl) (3*R*,4*R*,5*S*)-4-acetylamino-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonic acid



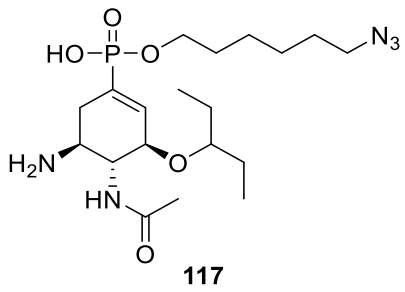
120

Methyl (3*R*,4*R*,5*S*)-4-acetamido-5-[(tert-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (100 mg, 0.23 mmol) was dissolved in DMF (1.5 mL) followed by addition of DIPEA (0.16 mL, 0.92 mmol), 1-azido-6-bromohexane (120 mg, 0.57 mmol) and sodium iodide (5 mg, 0.034 mmol). The reaction was then purged with argon and heated to 60 ºC for 48 h. The solvent was evaporated under reduced pressure and the crude mixture purified by flash chromatography (from EtOAc to EtOAc/MeOH 80:20) to yield the alkylated phosphonic acid (72 mg, 56%).

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 6.36 (d, *J* = 19.0 Hz, 1H), 4.15 – 4.00 (m, 1H), 3.88 – 3.78 (m, 1H), 3.77 (t, *J* = 6.4 Hz, 1H), 3.70 (dd, *J* = 10.0, 4.4 Hz, 1H), 3.55 – 3.44 (m, 1H), 3.41 (t, *J* = 5.4 Hz, 1H), 3.28 (d, *J* = 6.8 Hz, 2H), 2.62 (d, *J* = 17.1 Hz, 1H), 2.22 (t, *J* = 14.2 Hz, 1H), 1.97 (s, 3H), 1.61 (m, 3H), 1.50 (ddd, *J* = 16.4, 7.6, 6.1 Hz, 4H), 1.43 (s, 12H), 1.34 (d, *J* = 6.6 Hz, 2H), 0.89 (dt, *J* = 15.1, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 172.4, 156.6, 136.0, 132.6 (d, J = 211.8 Hz), 82.1, 78.8, 76.5, 64.2, 55.2, 54.5, 51.0, 30.4, 28.5 (x3), 27.4, 26.2, 26.0, 25.4, 25.2, 21.7, 18.0, 8.6, 8.3. ^{31}P NMR (162 MHz, Methanol-*d*$_4$) δ 13.12. HR-ESI-MS calculated for $C_{24}H_{43}O_7N_5P$ $(M-H)^-$ = 544.2906, found 544.2906.

Method 2. 1-(6-Azidohexyl)-1-methyl-(3R,4R,5S)-4-acetylamino-5-[(tert-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate (40 mg, 0.071 mmol) was dissolved in diethyl ether (0.5 mL) under argon atmosphere. Potassium trimethylsilanolate (11 mg, 0.086 mmol) was added and the resulting solution was left stirring overnight at room temperature. The solvent was removed under reduced pressure and the resulting oil triturated in ice-cold diethyl ether. The white solid formed filtered and dried under high vacuum to yield the phosphonic acid (30 mg, 77%).

(6-Azidohexyl) (3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonic acid



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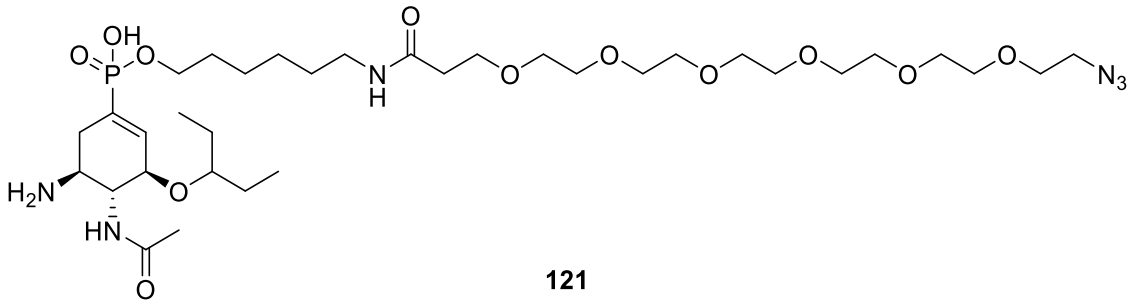
The resulting mixture was dissolved in trifluoro acetic acid (3 mL) and stirred for 2 h at room temperature. The solution was dried under reduced pressure and the resulting residue was purified by preparative HPLC to afford the deprotected phosphonic acid.

^{1}H NMR (500 MHz, Methanol-*d*$_4$) δ 6.42 (dd, *J* = 19.4, 1.0 Hz, 1H), 4.14 – 4.06 (m, 1H), 3.95 (dd, *J* = 11.2, 8.3 Hz, 1H), 3.83 (q, *J* = 6.5 Hz, 2H), 3.50 – 3.36 (m, 2H), 3.31 – 3.23 (m, 2H), 2.82 – 2.69 (m, 1H), 2.39 (ddd, *J* = 17.0, 10.1, 2.9 Hz, 1H), 2.03 (s, 3H), 1.69 – 1.56 (m, 4H), 1.55 – 1.46 (m, 4H), 1.45 – 1.37 (m, 4H), 0.91 (dt, *J* = 15.0, 7.4 Hz, 6H). ^{13}C NMR (126 MHz, Methanol-*d*$_4$) δ 174.7, 137.1, 131.6 (d, *J* = 175.9 Hz), 83.4, 76.3 (d, *J* = 18.9 Hz), 65.7 (d, *J* = 5.4 Hz), 54.6, 52.4, 51.4, 31.8 (d, *J* = 6.8 Hz), 30.4 (d, *J* = 10.4 Hz), 29.9, 27.5, 27.3, 26.6, 26.5, 23.1, 9.9, 9.6. HR-ESI-MS calculated for $C_{19}H_{35}O_5N_5P$ $(M+H)^+$ = 444.2383, found 444.2373.

(1-Azido-21-oxo-3,6,9,12,15,18-hexaoxa-22-azaoctacosan-28-yl) hydrogen (3*R*,4*R*,5*S*)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonic acid



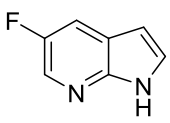
121

(6-Azidohexyl) (3*R*,4*R*,5*S*)-4-acetylamino-5-[(*tert*-butoxycarbonyl)-amino]-3-(1-ethylpropoxy)-1-cyclohexene-1-phosphonic acid (22 mg, 0.04 mmol) was dissolved in MeOH/water (1 mL, 3:1) followed by addition of Lindlar catalyst (22 mg, 100 w%), the flask was sealed and purged with hydrogen, the reaction mixture was left stirring for 5 h at room temperature under 1 atm of hydrogen. The catalyst was filtered out and rinsed with MeOH several times, the filtrate was evaporated to dryness under reduced pressure. The resulting oil was dissolved in DMF (1 mL)

under inert atmosphere, followed by addition of triethylamine (17 µl, 0.12 mmol) and a solution of azido-PEG6-NHS ester (16 mg, 0.05 mmol) in DMF (0.3 mL), the reaction mixture was stirred 16h at room temperature. The reaction mixture was dried under reduced pressure, the remaining oil was dissolved in TFA (1 mL, 100%) and stirred for 1 h at room temperature. The reaction was evaporated to dryness and the resulting mixture was purified by semi-preparative HPLC to yield the desired probe. (13 mg, 42%)

^{1}H NMR (401 MHz, Methanol-d_4) δ 6.51 (d, J = 20.2 Hz, 1H), 4.17 (d, J = 8.7 Hz, 1H), 4.01 – 3.86 (m, 2H), 3.72 (t, J = 6.0 Hz, 2H), 3.70 – 3.53 (m, 24H), 3.43 (t, J = 5.7 Hz, 1H), 3.38 (t, J = 5.0 Hz, 2H), 3.19 (t, J = 7.1 Hz, 2H), 2.91 (dd, J = 12.4, 6.3 Hz, 1H), 2.84 – 2.73 (m, 1H), 2.45 (td, J = 5.9, 0.9 Hz, 2H), 2.04 (s, 3H), 1.66 (p, J = 6.6 Hz, 2H), 1.59 – 1.47 (m, 6H), 1.47 – 1.31 (m, 4H), 0.91 (dt, J = 11.8, 7.4 Hz, 6H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 173.3, 172.5, 136.5 (d, J = 6.3 Hz), 129.7 (d, J = 176.7 Hz), 82.0, 74.9 (d, J = 19.2 Hz), 70.2 (x2), 70.1, 70.1, 70.1, 70.1 (x2), 70.1 70.0 (x2), 69.9, 69.7, 66.9, 64.5 (d, J = 5.5 Hz), 53.2, 50.4, 49.9, 38.7, 36.2, 30.3 (d, J = 6.8 Hz), 29.0 (d, J = 10.7 Hz), 28.8, 25.9 (d, J = 12.6 Hz), 25.2 (d, J = 17.6 Hz), 22.8, 21.8, 8.5, 8.2. ^{31}P NMR (202 MHz, Methanol-d_4) δ 12.22. HR-ESI-MS calculated for $C_{34}H_{66}O_{12}N_6P$ $(M+H)^+$ = 781.4471, found 781.4473.

5-Fluoro-1*H*-pyrrolo[2,3-b]pyridine

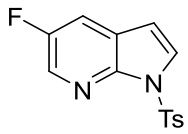


128

In an oven dried flask was dissolved 2-amino-3-bromo-5-fluoropyridine (0.2 g, 1.04 mmol), tetrabutylammonium bromide (0.033 g, 0.10 mmol) and DABCO (0.17 g, 1.56 mmol) in a mixture of DMSO (0.4 mL) and EtOAc (1.2 mL). The reaction mixture was purged with argon over a 30 min period followed by addition of *t*BuXPhos Pd G3 (0.04 g, 0.05 mmol), the flask was sealed and purged again with argon for 10 min. Acetaldehyde was added (0.11 mL, 2.08 mmol) and the mixture was stirred for 1 h at room temperature and subsequently heated to 75 ºC overnight. The reaction was quenched by addition of water (10 mL) and extracted with EtOAc (3x 20 mL), the combined organic layers were washed with water (5 mL), brine (5 mL) and evaporated to dryness under reduced pressure. The remaining residue was purified by flash chromatography (Hex/EtOAc 1:1) to yield the azaindole (0.086 g, 61%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 10.54 (bs, 1H), 8.21 (t, *J* = 2.3 Hz, 1H), 7.66 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.42 (dd, *J* = 3.4, 2.5 Hz, 1H), 6.50 (dd, *J* = 3.5, 2.0 Hz, 1H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 155.8 (d, *J* = 240.9 Hz), 145.6, 131.3 (d, *J* = 29.9 Hz), 127.4, 120.6 (d, *J* = 7.2 Hz), 114.7 (d, *J* = 20.7 Hz), 101.q (d, *J* = 4.3 Hz).

5-Fluoro-1-tosyl-1*H*-pyrrolo[2,3-b]pyridine

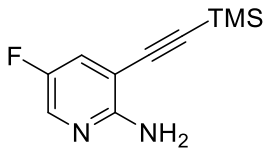


130

Method 1. Sodium hydride (0.07 g, 1.76 mmol) was added portionwise to an ice-cold solution of the azaindole (0.2 g, 1.47 mmol) in dry THF (5 mL), the reaction was stirred for 10 min at 0 ºC followed by dropwise addition of a solution of *p*-toluenesulfonyl chloride (0.31 g, 1.61 mmol) in THF (2 mL). The resulting solution was left stirring for 1 h at room temperature and then quenched by addition of $NaHCO_3$ saturated solution (5 mL), the aqueous layer was extracted with EtOAc (3x 10 mL), the combined organic layers were washed with brine (5 mL), dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. The resulting solid was used in the next step without further purification (0.37 g, 88%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 8.28 (ddd, *J* = 2.3, 1.5, 0.7 Hz, 1H), 8.04 (d, *J* = 8.4 Hz, 2H), 7.78 (dd, *J* = 4.0, 0.5 Hz, 1H), 7.52 (dd, *J* = 8.3, 2.7 Hz, 1H), 7.28 (dd, *J* = 8.7, 0.7 Hz, 2H), 6.56 (d, *J* = 4.0 Hz, 1H), 2.38 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 157.1 (d, *J* = 247.4 Hz), 145.5, 135.3, 133.5 (d, *J* = 29.2 Hz), 130.3, 129.8 (x2), 128.7, 128.2 (x2), 127.2, 115.5 (d, *J* = 21.1 Hz), 105.1 (d, *J* = 3.7 Hz), 21.8.

5-Fluoro-3-((trimethylsilyl)ethynyl)pyridin-2-amine



129

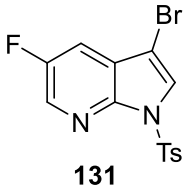
A THF solution of 2-amino-3-bromo-5-fluoropyridine (0.5 g, 2.6 mmol) copper(I) iodide (0.074 g, 0.39 mmol) and triethylamine (8.8 mL) in THF (9.3 mL) was purged with argon over 30 min period followed by addition of [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) complex with dichloromethane (0.134 g, 0.112 mmol). The mixture was purged for 10 min and ethynyltrimethylsilane (0.54 mL, 3.9 mmol) was added. The reaction mixture was heated to 55 ºC overnight. The chilled solution was poured into water (10 mL) and extracted with EtOAc (3x 10 mL), the combiner organic layers were washed with water (5 mL), brine (5 mL) and evaporated

down. The remaining residue was purified by column chromatography (DCM/MeOH 20:1) to afford the desired product (0.45 g, 83%).

^{1}H NMR (401 MHz, Chloroform-d_3) δ 7.90 (s, 1H), 7.30 (dd, J = 8.2, 2.8 Hz, 1H), 5.12 (bs, 2H), 0.27 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 156.2, 152.76 (d, J = 242.8 Hz), 135.5 (d, J = 25.2 Hz), 127.2 (d, J = 21.3 Hz), 102.5 (x2), 99.2 (d, J = 2.1 Hz), -0.1 (x3).

Method 2. A solution of 5-fluoro-3-((triemethylsilyl)ethynyl)pyridin-2-amine (0.4 g, 1.92 mmol) in NMP (1 mL) was added to a solution of potassium *tert*-butoxide (0.26 g, 2.3 mmol) in NMP (4 mL). The reaction mixture was heated to 70 ºC for 2 h. When the LC-MS showed total consumption of the starting material the reaction flask was removed from and oil bath. The reaction was chilled to 0 ºC and a solution of *p*-toluenesulfonyl chloride (0.4 g, 2.11 mmol) in NMP (1 mL) was added dropwise, the reaction was left stirring at room temperature for 1 h. The mixture was quenched by addition of water (5 mL) and the aqueous layer was extracted with DCM (3x 10 mL), the combined organic layers were washed with water (5 mL), brine (5 mL) and concentrated under reduced pressure. The remaining residue was purified by flash chromatography (Hexanes/EtOAc 1:1) to yield the desired azaindole (0.17 g, 31 %).

3-Bromo-5-fluoro-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine



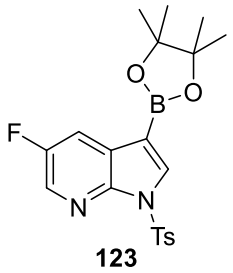
131

A solution of 5-fluoro-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (0.46 g, 1.47 mmol) in DCM (4 mL) was added dropwise to an ice-cold suspension of *N*-bromosuccinimide (0.3 g, 1.69 mmol) in DCM (2 mL) and the reaction was allowed to stir overnight at room temperature. The solution was diluted with DCM (15 mL) and quenched by addition of $NaHCO_3$ saturated solution (15 mL). The aqueous layer was extracted with DCM (3x 20mL) and the combined organic layers were washed with brine (10 mL), concentrated under reduced pressure and purified by flash chromatography (hexanes/EtOAc 1:1) to furnish the brominated azaindole (0.37 g, 68%).

^{1}H NMR (401 MHz, Chloroform-d_3) δ 8.33 (dd, J = 2.8, 1.4 Hz, 1H), 8.12 – 8.02 (m, 2H), 7.85 (s, 1H), 7.50 (dd, J = 7.8, 2.7 Hz, 1H), 7.37 – 7.28 (m, 2H), 2.40 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 157.4 (d, J = 249.7 Hz), 145.9, 142.3, 134.9 (d, J = 29.4 Hz), 134.8, 129.9 (2x),

128.3 (2x), 127.2, 123.2 (d, J = 7.2 Hz), 114.6 (d, J = 22.1 Hz), 94.7 (d, J = 3.6 Hz), 21.8. HR-ESI-MS calculated for $C_{14}H_{10}O_2N_2SBrF$ (M) 367.9630, found 367.9632.

5-Fluoro-1-tosyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyrrolo[2,3-*b*]pyridine

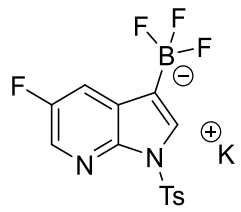


123

A solution of 3-bromo-5-fluoro-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine (0.35 g, 0.95 mmol) in 1,4-dioxane (2.5 mL) was purged with argon for 15 min followed by addition of bis(pinacolato)diboron (0.27 g, 1.04 mmol), potassium acetate (0.25 g, 2.59 mmol), palladium(II) acetate (0.021 g, 0.09 mmol) and triphenylphosphine (0.076 g, 0.29 mmol). The reaction mixture was purged with argon for 10 min, sealed and heated to reflux for 15h. The reaction was diluted in EtOAc (60 mL) and the organic layer was washed with water (5 mL) and brine (3 mL). The solvent was evaporated under reduced pressure and the remaining residue was purified by chromatography (hexanes/EtOAc 10:1 to 8:1). The oil obtained after chromatography was triturated with hexanes to produce the boronic ester as a white solid that was filtered and dried (0.30 g, 76%).

^{1}H NMR (401 MHz, Chloroform-d_3) δ 8.28 (dd, J = 2.8, 1.4 Hz, 1H), 8.20 (s, 1H), 8.09 (d, J = 8.3 Hz, 2H), 7.90 (dd, J = 8.6, 2.8 Hz, 1H), 7.32 – 7.26 (m, 2H), 2.39 (s, 3H), 1.37 (s, 12H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 157.3 (d, J = 247.3 Hz), 145.5, 144.3, 136.9, 135.0, 133.3 (d, J = 29.4 Hz), 129.7 (2x), 128.2 (2x), 128.0, 126.9 (d, J = 7.3 Hz), 117.3 (d, J = 20.9 Hz), 83.9 (2x), 24.9 (4x), 21.7. HR-ESI-MS calculated for $C_{20}H_{23}O_4N_2SBF$ $(M+H)^+$ 417.1450, found 417.1452.

Potassium (5-fluoro-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)trifluoroborate

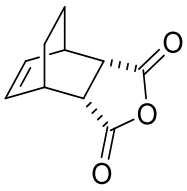


Potassium hydrogenfluoride (0.1 g, 1.3 mmol) was added to a suspension of the boronic ester (0.09 g, 0.21 mmol) in a mixture of acetone (2.5 mL) and water (1 mL). The resulting solution was stirred for 5 h at room temperature. The solvent was evaporated under reduced pressure, the remaining solid was triturated with refluxing acetone and the solvent was decanted and saved, the trituration was repeated two times more and the combined aliquots of solvent were concentrated to approximately 1 mL. Et_2O (0.3 mL) was added dropwise and the solution was left overnight at 4

ºC without stirring. The formed crystals were filtered and dried under reduced pressure to yield the trifluoroborate salt (0.082 g, 97%).

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 8.09 (dd, *J* = 2.9, 1.6 Hz, 1H), 7.97 – 7.87 (m, 2H), 7.75 (dd, *J* = 9.0, 2.8 Hz, 1H), 7.56 (d, *J* = 1.3 Hz, 1H), 7.36 – 7.25 (m, 2H), 2.36 (s, 3H). ^{19}F NMR (377 MHz, Methanol-*d*$_4$) δ -138.33 (dd, *J* = 8.7, 2.7 Hz), -140.78. HR-ESI-MS calculated for $C_{14}H_{10}O_2N_2SBF_4$ $(M)^-$ 357.0498, found 357.0493.

Meso-*endo*-tetrahydro-4,7-ethanoisobenzofuran-1,3-dione

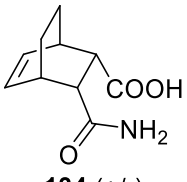


132

Cyclohexa-1,3-diene (1.01 mL, 10.6 mmol) was added dropwise to an ice-cold solution of maleic anhydride (1.0 g, 9.7 mmols) in DCM (12 mL). The mixture was left stirring overnight in the dark at room temperature. The solvent was evaporated down under reduced pressure and the remaining white solid was dissolved in anhydrous MeOH (10 mL) and refluxed for 5 min. The solution was cooled down to room temperature and the white crystals were filtrated and dried under reduced pressure to furnish 1.46 g (85 %) of the cycloaddition product.

^{1}H NMR (401 MHz, chloroform-*d*$_3$) δ 6.30 (dd, *J* = 4.5, 3.1 Hz, 2H), 3.22 (dp, *J* = 4.7, 1.5 Hz, 2H), 3.12 (dd, *J* = 2.0, 1.4 Hz, 2H), 1.63 – 1.55 (m, 2H), 1.44 – 1.33 (m, 2H). ^{13}C NMR (101 MHz, chloroform-*d*$_3$) δ 172.9, 133.1, 44.9, 31.7, 23.1. HR-ESI-MS calculated for $C_{10}H_{10}O_3$ (M) 178.0630, found 178.0628.

Di-*endo*-3-carbamoylbicyclo[2.2.2]oct-5-ene-2-carboxylic acid

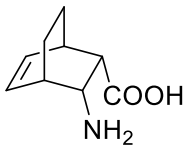


134 (+/-)

Meso-*endo*-tetrahydro-4,7-ethanoisobenzofuran-1,3-dione (1.0 g, 5.71 mmol) was slowly added portion-wise to a solution of ammonium hydroxide solution (28%, 5 mL) in THF (5 mL) at 0 ºC. The resulting slurry was left stirring at room temperature for 5 h. The formed solid was filtered, rinsed with cold THF and dried under reduced pressure to yield the corresponding amide as a racemate (1.03 g, 94 %).

^{1}H NMR (401 MHz, D_2O) δ 6.46 (ddd, *J* = 8.0, 6.5, 1.3 Hz, 2H), 6.28 (ddd, *J* = 8.0, 6.4, 1.2 Hz, 2H), 3.07 – 2.88 (m, 2H), 2.76 (dddd, *J* = 11.9, 5.0, 3.5, 1.7 Hz, 2H), 1.65 – 1.46 (m, 2H), 1.34 – 1.07 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 181.5, 180.3, 134.8, 131.3, 52.1, 49.8, 33.2, 32.6, 24.5, 24.4. HR-ESI-MS calculated for $C_{10}H_{13}NO_3Na$ $(M+Na)^+$ 218.0789, found 218.0788.

Di-*endo*-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylic acid

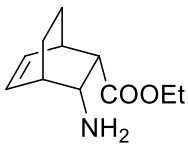


133 (+/-)

The racemic di-*endo*-3-carbamoylbicyclo[2.2.2]oct-5-ene-2-carboxylic acid (2.6 g, 13.3 mmol) was dissolved in a mixture of EtOAc (17 mL), acetonitrile (17 mL) and water (8.5 mL) and was cooled down to 0 ºC on an ice bath. (Diacetoxyiodo)benzene (5.6 g, 17.3 mmol) was added portionwise under vigorous stirring and the reaction mixture was stirred overnight at room temperature. The reaction mixture was acidified by addition of concentrated HCl and extracted with Et_2O (3x 15 mL), the combined ethereal layers were washed with water (5 mL) and brine (5 mL) and evaporated under reduced pressure to furnish the corresponding racemic ammonium salt (2.4 g, 90%). The product was used in the next step without further purification.

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 6.53 (ddd, *J* = 8.0, 6.6, 1.2 Hz, 1H), 6.37 – 6.21 (m, 1H), 3.73 (dd, *J* = 9.2, 2.6 Hz, 1H), 3.08 (d, *J* = 9.6 Hz, 2H), 2.96 – 2.80 (m, 1H), 1.77 – 1.55 (m, 2H), 1.49 – 1.25 (m, 2H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 174.5, 137.0, 130.4, 52.6, 46.2, 35.4, 35.0, 25.2, 23.0. HR-ESI-MS calculated for $C_9H_{12}O_2$ $(M-H)^-$ 166.0874, found 166.0874.

Ethyl di-*endo*-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylate



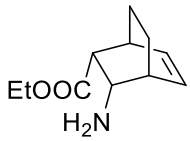
135 (+/-)

Thionyl chloride (0.72 mL, 9.7 mmol) was added dropwise to absolute EtOH (11 mL) at 0 ºC, followed by addition of di-*endo*-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylic acid (0.78 g, 3.9 mmol). The reaction mixture was stirred overnight at room temperature and subsequently refluxed for 5h. The solvent was evaporated under reduced pressure, the remaining slurry was dissolved in water (5 mL) and the pH was adjusted to basic by addition of $NaHCO_3$ saturated solution. The aqueous layer was extracted with DCM (3x 15 mL), the combined organic layers were washed with brine (5 mL) and evaporated to dryness. The residue was purified by chromatography (DCM/MeOH/NH_3 90:10:1) to furnish the racemic aminoester (0.56 g, 73%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 6.56 – 6.42 (m, 1H), 6.19 – 6.06 (m, 1H), 4.10 (dt, *J* = 7.1, 3.5 Hz, 2H), 3.41 – 3.26 (m, 1H), 2.81 (d, *J* = 8.9 Hz, 1H), 2.74 (ddd, *J* = 6.8, 3.0, 1.6 Hz, 1H), 2.64 (d, *J* = 5.7 Hz, 1H), 1.36 (bs, 2H), 1.62 – 1.37 (m, 2H), 1.33 – 1.10 (m, 5H). ^{13}C NMR (101

MHz, Chloroform-d_3) δ 173.1, 135.7, 129.8, 60.3, 53.9, 52.3, 38.3, 32.4, 25.2, 22.5, 14.5. HR-ESI-MS calculated for $C_{11}H_{18}O_2N$ $(M + H)^+$ 196.1332, found 196.1332.

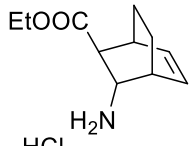
Ethyl (1*S*,2*R*,3*S*,4*R*)-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylate (-)-DBTA



136 (-)-DBTA

To a refluxing solution of the racemic ethyl di-*endo*-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylate (0.63 g, 3.23 mmol) in EtOH (5.8 mL) was added portionwise (-)-dibenzoyl-*L*-tartaric acid monohydrate (0.6 g, 1.61 mmol). The mixture was vigorously stirred at reflux until complete dissolution of all solids, the solution was cooled to room temperature over a 30 min period. The slurry was filtered and rinsed with Et_2O, the white solid was recrystallized from ethanol to afford the pure diastereomeric salt (0.43 g, 25%). ^{1}H NMR (401 MHz, Methanol-d_4) δ 8.20 – 8.06 (m, 4H), 7.67 – 7.54 (m, 2H), 7.49 (dd, J = 8.4, 7.0 Hz, 4H), 6.45 (ddd, J = 8.1, 6.5, 1.3 Hz, 1H), 6.24 (t, J = 7.3 Hz, 1H), 5.91 (s, 2H), 4.26 – 4.00 (m, 2H), 3.69 (dd, J = 9.4, 2.7 Hz, 1H), 3.07 (dd, J = 9.3, 1.9 Hz, 1H), 2.99 (ddd, J = 6.6, 3.2, 1.5 Hz, 1H), 2.90 – 2.76 (m, 1H), 1.70 – 1.54 (m, 2H), 1.27 (t, J = 7.2 Hz, 5H). ^{13}C NMR (101 MHz, Methanol-$_{d4}$) δ 172.9, 171.5, 167.3, 136.7, 134.4, 131.2, 131.0, 130.6, 129.5, 75.0, 62.4, 52.6, 46.5, 35.3, 35.0, 25.2, 22.9, 14.3. $[\alpha]_D$ = -81.4 (c = 0.5, EtOH).

Ethyl (1*S*,2*S*,3*S*,4*R*)-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylate hydrochloride

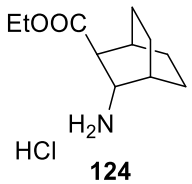


137

The diastereomeric salt of ethyl (1*S*,2*R*,3*S*,4*R*)-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylate (-)-DBTA (1.13 g, 2.04 mmol) was dissolved in water (15 mL) and the solution was basified by addition of aqueous $NaHCO_3$ saturated solution (7.5 mL). The aqueous layer was extracted with DCM (3x 20mL) and the combined organic layers were washed with brine (5 mL), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. The free base was dissolved in absolute ethanol (12 mL) and freshly prepared sodium ethoxide (0.29 g, 4.08 mmol) was added. The reaction mixture was refluxed for 2h. The reaction was evaporated down, the residue was dissolved in DCM (90 mL) and the organic layer was washed with water (2x 20 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The remaining oil was dissolved in a hydrogen chloride solution (2.45 M in dioxane, 5 mL) and evaporated under reduced pressure to furnish the corresponding product as hydrochlorid (0.15 g, 32%).

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 6.63 (ddd, *J* = 8.1, 6.7, 1.3 Hz, 1H), 6.36 – 6.17 (m, 1H), 4.25 (qd, *J* = 7.1, 4.1 Hz, 2H), 3.90 (t, *J* = 3.4 Hz, 1H), 3.04 (ddd, *J* = 6.7, 2.8, 1.1 Hz, 1H), 2.91 – 2.79 (m, 1H), 2.38 – 2.26 (m, 1H), 1.73 – 1.57 (m, 1H), 1.52 – 1.35 (m, 2H), 1.36 – 1.18 (m, 3H), 1.18 (ddd, *J* = 3.9, 2.0, 1.0 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-*d4*) δ 173.0, 137.7, 130.7, 62.6, 53.1, 50.1, 34.5, 34.2, 23.3, 20.2, 14.5. $[\alpha]_D$ = + 38.4 (c = 0.5, EtOH).

Ethyl (2*S*,3*S*)-3-aminobicyclo[2.2.2]octane-2-carboxylate hydrochloride

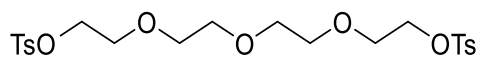


124

Palladium on carbon 10% w/w (0.09 g, 0.38 mmol) was added to a solution of ethyl (1*S*,2*S*,3*S*,4*R*)-3-aminobicyclo[2.2.2]oct-5-ene-2-carboxylate hydrochloride (0.44 g, 1.9 mmol) in ethanol (13 mL) under argon atmosphere. The vessel was purged with hydrogen twice and then it was left stirring overnight at room temperature under hydrogen atmosphere (1 atm). The catalyst was filtered out and rinsed with EtOH, the filtrate was evaporated to dryness and the resulting oil was dissolved in a hydrogen chloride solution (2.45 M in dioxane, 5 mL) and evaporated again to yield the reduced aminoester as a white solid (0.43 g, 95%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 8.49 (bs, 2H), 4.22 (q, *J* = 7.1 Hz, 2H), 3.94 – 3.81 (m, 1H), 3.50 – 3.34 (m, 1H), 2.74 (dd, *J* = 6.0, 2.3 Hz, 1H), 2.18 – 1.97 (m, 2H), 1.88 (m, 1H), 1.66 – 1.39 (m, 6H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 172.5, 90.4, 61.3, 59.4, 51.4, 47.8, 27.8, 25.0, 24.0, 20.7, 18.8, 14.3. $[\alpha]_D$ = - 12.2 (c = 1, MeOH). HR-ESI-MS calculated for $C_{11}H_{19}O_2N$ $(M)^+$ 197.1416, found 197.1418.

Tetraethylene glycol ditosylate



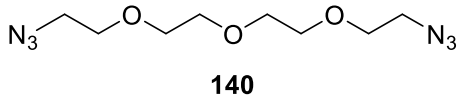
139

To a solution of tetraethylene glycol (8.8 mL, 51.4 mmol) and *p*-toluenesulfonyl chloride (19.6 g, 102.8 mmol) in DCM (50 mL) at 0 ºC was portionwise added powdered KOH (23.8 g, 411.2 mmol) keeping the temperature always below 4 ºC. The resulting slurry was stirred a further 3h at 0 ºC. The reaction mixture was diluted with DCM (50 mL) and quenched by addition of ice-cold water (100 mL). The organic layer was separated and saved, the aqueous layer was extracted with DCM (2x 50 mL) and the combined organic layers were washed with water (30 mL), dried over anhydrous $MgSO_4$, filtered and evaporated to dryness under reduced pressure to

yield the bis-tosylated tetraethylene glycol (23.8 g, 98%). The product was used without further purification.

^{1}H NMR (300 MHz, Chloroform-d_3) δ 7.82 – 7.75 (m, 4H), 7.38 – 7.28 (m, 4H), 4.20 – 4.10 (m, 4H), 3.80 – 3.59 (m, 4H), 3.55 (d, J = 1.1 Hz, 8H), 2.44 (s, 6H).

1,11-Diazido-3,6,9-trioxaundecane

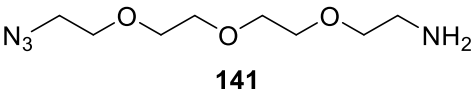


140

Sodium azide (0.38 g, 6.0 mmol) was added to a solution of tetraethylene glycol ditosylate (1.0 g, 2.0 mmol) in DMF (5 mL) and the mixture was heated to 60 ºC overnight. The reaction was quenched by addition of water (10 mL) and the aqueous layer was extracted with EtOAc (3x 20 mL). The combined organic phases were washed with water (2x 5mL) and brine (5 mL) and then evaporated to dryness. The remaining oil was purified by flash chromatography (hexanes/EtOAc 1:1) to yield the bis-azido linker (0.4 g, 82%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 3.74 – 3.61 (m, 12H), 3.38 (t, J = 5.1 Hz, 4H).

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethylamine

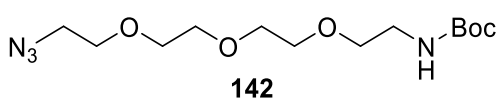


141

A solution of triphenylphosphine (0.64 g, 2.45 mmol) in Et_2O (5 mL) was added dropwise over a period of 10 min to a solution of 1,11-diazido-3,6,9-trioxaundecane (0.70 g, 2.89 mmol) in aqueous HCl (5%, 7 mL). The biphasic mixture was stirred overnight at room temperature. The two phases were then separated and the organic layer was discarded, the aqueous layer was further washed with DCM (3x 2 mL). The aqueous phase was cooled to 0 ºC and the pH was adjusted to basic by addition of KOH pellets. The basic aqueous layer was extracted with DCM (5x 10 mL) and the combined organic layers were washed with brine (2 mL), dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to yield the desired product as an oil (0.43 g, 81%).

^{1}H NMR (401 MHz, Chloroform-d_3) δ 3.72 – 3.57 (m, 10H), 3.52 (t, J = 5.2 Hz, 2H), 3.39 (d, J = 5.0 Hz, 2H), 2.87 (t, J = 5.2 Hz, 2H), 1.72 (bs, 2H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 73.5, 70.8, 70.8, 70.7, 70.4, 70.2, 50.8, 41.9.

***tert*-Butyl (2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)carbamate**

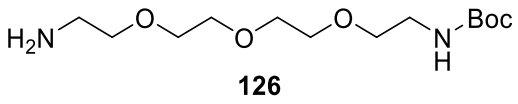


142

A solution of di-*tert*-butyl dicarbonate (2 g, 9.16 mmol) in THF was added to an ice-cold solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethylamine (1.0 g, 4.58 mmol) and triethylamine (1.9 mL, 13.7 mmol) in THF (30 mL), the solution was warmed to room temperature and stirred for 5 h. The solvent was evaporated under reduced pressure and the remaining oil was dissolved in EtOAc (100 mL), the organic phase was washed with a diluted aqueous solution of NH_4Cl (2x 10 mL) and a saturated aqueous solution of $NaHCO_3$ (5 mL). The organic layer was evaporated to dryness under reduced pressure and the remaining oil was purified by chromatography (EtOAc) to yield the boc-protected amine (1.3 g, 89%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 5.04 (bs, 1H), 3.77 – 3.60 (m, 10H), 3.56 (t, *J* = 5.2 Hz, 2H), 3.47 – 3.37 (m, 2H), 3.33 (q, *J* = 5.4 Hz, 2H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 156.0, 79.7, 70.2, 70.7, 70.6, 70.3, 70.2, 70.1, 50.7, 40.4, 28.4.

1-Boc-amine-11-amino-3,6,9-trioxaundecane

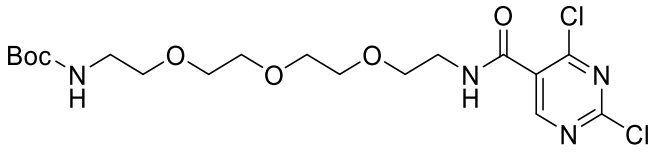


126

tert-Butyl (2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-ethyl)-carbamate (0.7 g, 2.2 mmol) was dissolved in THF (10 mL) and triphenylphosphine (0.86 g, 3.3 mmol) was added portionwise, the reaction mixture was left stirring overnight at room temperature. Subsequently, water (3 mL) was added and the reaction mixture was heated to 60 ºC for 2 h. The solvent was evaporated and the remaining residue was purified by flash chromatography (DCM/MeOH 5:1) to yield the reduced linker (0.57 g, 90%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 5.30 (bs, 1H), 3.71 – 3.60 (m, 8H), 3.59 – 3.49 (m, 4H), 3.32 (d, *J* = 5.4 Hz, 2H), 2.94 – 2.80 (m, 2H), 2.28 – 2.13 (m, 2H), 1.45 (s, 3H).

***tert*-Butyl (1-(2,4-dichloropyrimidin-5-yl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-yl)carbamate**

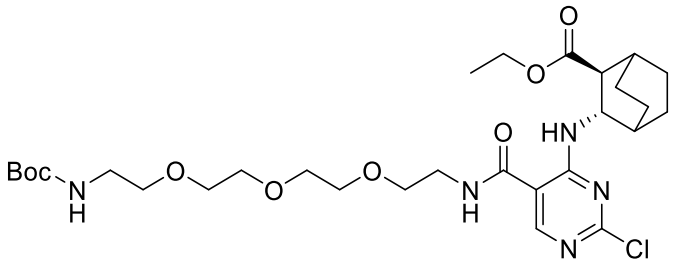


143

A solution of the linker (0.14, 0.47 mmol) in DCM (1 mL) was added dropwise to a solution of 2,4-dichloropyrimidine-5-carbonyl chloride (0.1 g, 0.47 mmol) in DCM (2 mL) at -78 ºC, followed by dropwise addition of DIPEA (0.16 mL, 0.95 mmol). The reaction mixture was slowly warmed up to room temperature over a 40 min period. The solvent was then evaporated down, and the remaining residue was purified by column chromatography (DCM/MeOH 100:5) to furnish the desired compound (0.12 g, 56%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 8.85 (s, 1H), 7.42 (s, 1H), 5.04 (s, 1H), 3.71 – 3.61 (m, 8H), 3.60 – 3.54 (m, 4H), 3.48 (t, *J* = 5.2 Hz, 2H), 3.26 – 3.17 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 162.7, 161.7, 161.2, 161.1, 156.1, 127.6, 79.3, 70.4, 70.3, 70.2, 70.0, 69.2 (2x), 40.3, 40.1, 28.4 (3x). HR-ESI-MS calculated for $C_{18}H_{29}O_6N_4Cl_2$ $(M + H)^+$ 467.1459, found 467.1458.

Ethyl (2*S*,3*S*)-3-((2-chloro-5-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)carbamoyl)pyrimidin-4-yl)amino)bicyclo[2.2.2]octane-2-carboxylate



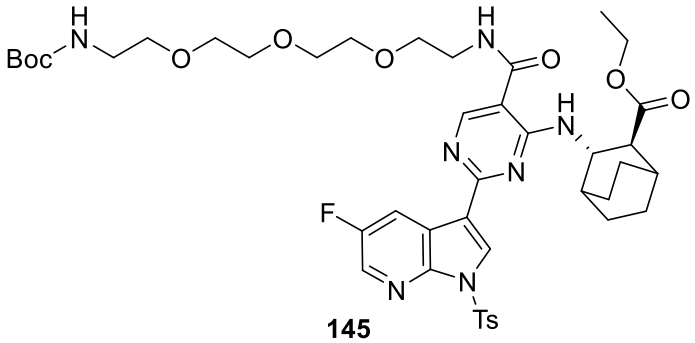
144

To an ice-cool solution of the *tert*-butyl (1-(2,4-dichloropyrimidin-5-yl)-1-oxo-5,8,11-trioxa-2-azatridecan-13-yl)carbamate (52 mg, 0.12 mmol) in isopropyl alcohol (1.5 mL) was added dropwise a solution of ethyl (2*S*,3*S*)-3-aminobicyclo[2.2.2]octane-2-carboxylate hydrochloride (23 mg, 0.12 mmol) in isopropyl alcohol (0.5 mL) followed by addition of DIPEA (31 mg, 0.26 mmol) and the reaction mixture was left stirring at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by column chromatography (DCM/*i*-PrOH 20:1) to yield the substituted pyrimidine (63 mg, 83%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 9.23 (d, *J* = 6.4 Hz, 1H), 8.39 (s, 1H), 7.35 (bs, 1H), 5.35 – 5.19 (bs, 1H), 4.55 – 4.45 (m, 1H), 4.22 (q, *J* = 7.2 Hz, 2H), 3.72 – 3.57 (m, 12H), 3.54 (t, *J* = 5.2

Hz, 2H), 3.29 (d, *J* = 6.4 Hz, 2H), 2.42 – 2.33 (m, 1H), 2.31 (s, 1H), 1.97 (d, *J* = 2.8 Hz, 1H), 1.87 – 1.81 (m, 1H), 1.83 – 1.72 (m, 2H), 1.71 – 1.52 (m, 5H), 1.43 (s, 9H), 1.27 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 174.3, 166.3, 162.3, 161.4, 156.1, 155.5, 106.8, 79.2, 70.4, 70.4, 70.3, 70.2, 70.0, 69.5, 60.6, 60.4, 51.4, 50.7, 40.3, 39.6, 28.5, 28.4 (3x), 25.9, 24.0, 20.8, 19.6, 14.2. HR-ESI-MS calculated for $C_{29}H_{47}O_8N_5Cl$ $(M + H)^+$ 628.3108, found 628.3109.

Ethyl (2*S*,3*S*)-3-((5-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)carbamoyl)-2-(5-fluoro-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pyrimidin-4-yl)amino)bicyclo[2.2.2]octane-2-carboxylate



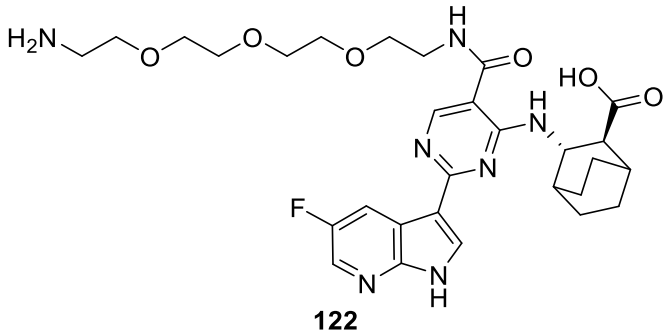
145

Ethyl (2*S*,3*S*)-3-((2-chloro-5-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-aza-hexadecan-16-yl)carbamoyl) pyrimidin-4-yl)amino)bicyclo[2.2.2]octane-2-carbo-xylate (28 mg, 0.044 mmol) was dissolved in a mixture of THF/water (0.2 mL, 25:1) and the flask was purged with argon for 15 min. 5-Fluoro-1-tosyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyrrolo[2,3-b]pyridine (20 mg, 0.049 mmol), K_2CO_3 (24 mg, 0.18 mmol) and SPhos Pd G3 (4 mg, 0.004 mmol) were added and the reaction mixture was purged with argon for 10 min more, the reaction mixture was then heated to 70 ºC overnight. The solvent was evaporated under reduced pressure and the black residue was purified by column chromatography (DCM/MeOH 20:1) to furnish the coupled product (26 mg, 67%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 9.10 (d, *J* = 7.4 Hz, 1H), 8.69 (s, 1H), 8.61 (dd, *J* = 9.0, 2.8 Hz, 1H), 8.54 (s, 1H), 8.30 (dd, *J* = 2.8, 1.1 Hz, 1H), 8.12 (d, *J* = 8.4 Hz, 2H), 7.28 (d, *J* = 8.2 Hz, 2H), 7.05 (bs, 1H), 5.16 (bs, 1H), 4.85 (d, *J* = 6.8 Hz, 1H), 4.16 – 4.05 (m, 2H), 3.74 – 3.58 (m, 12H), 3.55 (t, *J* = 5.1 Hz, 2H), 3.29 (t, *J* = 5.5 Hz, 2H), 2.44 (dt, *J* = 5.7, 1.8 Hz, 1H), 2.37 (s, 3H), 2.07 – 2.05 (m, 1H), 1.96 (q, *J* = 2.9 Hz, 1H), 1.93 – 1.59 (m, 6H), 1.60 – 1.44 (m, 2H), 1.39 (s, 9H), 1.15 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 174.4, 167.0, 162.5, 160.3, 157.5 (d, *J* = 247.6 Hz), 156.1, 154.1, 145.6, 143.9, 134.9, 133.8, 133.7, 131.4 (d, *J* = 6.6 Hz), 129.7, 129.7, 128.4, 128.3, 122.2 (d, *J* = 8.4 Hz), 118.2, 105.7, 79.2, 70.5, 70.3, 70.3, 70.2, 70.1,

69.6, 60.6, 51.4, 51.3, 49.8, 40.3, 39.5, 28.8, 28.6, 28.4, 28.3, 25.9, 24.1, 21.6, 21.0, 19.6, 14.3. ^{19}F NMR (377 MHz, Chloroform-*d*$_3$) δ -133.55. HR-ESI-MS calculated for $C_{43}H_{57}O_{10}N_7FS$ $(M + H)^+$ 882.3866, found 882.3868.

(2*S*,3*S*)-3-((5-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)carbamoyl)-2-(5-fluoro-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pyrimidin-4-yl)amino)bicyclo[2.2.2]octane-2-carboxylic acid

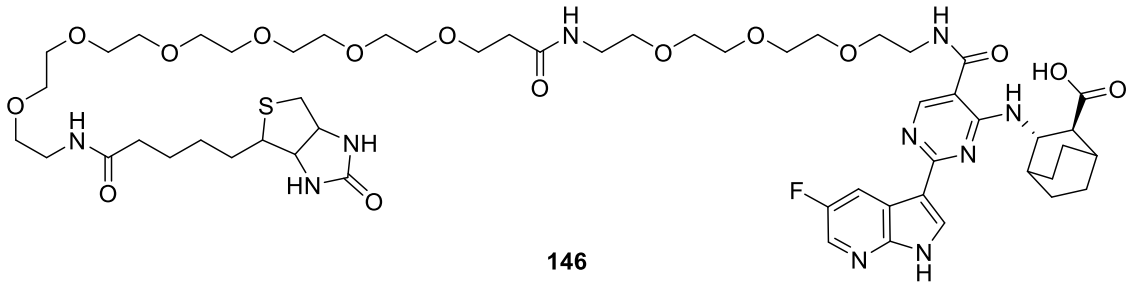


122

An aqueous soliton of LiOH 2M (0.15 mL) was added to solution of ethyl (2*S*,3*S*)-3-((5-((2,2-dimethyl-4-oxo-3,8, 11,14-tetraoxa-5-aza-hexadecan-16-yl) carbamoyl)-2-(5-fluoro-1-tosyl-1H-py-rrolo[2,3-*b*]pyridin-3-yl)pyrimi-din-4-yl)amino)bicyclo[2.2.2]-octane-2-carboxylate (26 mg, 0.029 mmol) in THF (1mL) and the reaction was stirred at 45 ºC for 4 days. When the LC-MS analysis showed total consumption of the starting material the reaction mixture was evaporated to dryness under reduced pressure. The resulting product was dissolved in a TFA/DCM (1 mL, 1:1) mixture and the reaction was stirred at room temperature for 40 min. The solvent was evaporated under reduced pressure and the remaining solid was purified by preparative HPLC to afford the desired product (12 mg, 69%).

^{1}H NMR (401 MHz, Methanol-*d*$_4$) δ 8.66 (dd, *J* = 2.9 Hz, *J* = 1.0 Hz 1H), 8.64 (s, 1H), 8.59 (s, 1H), 8.31 (dd, *J* = 2.8, 1.6 Hz, 1H), 5.16 (dt, *J* = 5.9, 1.9 Hz, 1H), 3.73 – 3.64 (m, 12H), 3.64 – 3.55 (m, 2H), 3.12 (t, *J* = 4.1, 2H), 2.65 – 2.56 (m, 1H), 2.18 (q, *J* = 2.8 Hz, 1H), 2.03 (m, 1H), 1.91 (m, 1H), 1.86 – 1.67 (m, 5H), 1.66 – 1.53 (m, 2H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 175.5, 165.4, 160.2, 156.9 (d, *J* = 244.2 Hz), 156., 145.8, 145.0, 133.9, 133.1 (d, *J* = 30.0 Hz), 130.2, 118.6 (d, *J* = 8.9 Hz), 116.1 (d, *J* = 22.4 Hz), 104.5, 70.1 (2x), 69.8, 69.8, 68.9, 66.4, 51.1, 50.2, 47.9, 39.2, 28.9, 28.5, 25.3, 23.7, 20.5, 19.0. ^{19}F NMR (377 MHz, Methanol-*d*$_4$) δ -137.44. HR-ESI-MS calculated for $C_{29}H_{39}O_6N_7F$ $(M + H)^+$ 600.2940, found 600.2942.

(2*S*,3*S*)-3-((5-((13,35-dioxo-39-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9,16,19,22,25,28,31-nonaoxa-12,34-diazanonatriacontyl)carbamoyl)-2-(5-fluoro-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pyrimidin-4-yl)amino)bicyclo[2.2.2]octane-2-carboxylic acid

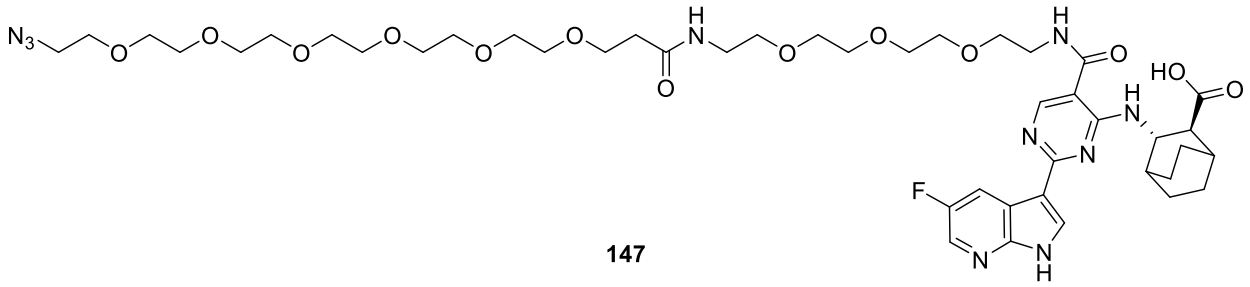


146

To an ice-cool solution of (2*S*,3*S*)-3-((5-((2-(2-(2-(2-aminoethoxy)ethoxy)-ethoxy)-ethyl)carbamoyl)-2-(5-fluoro-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pyrimidin-4-yl)amino)bicycle-[2.2.2]octane-2-carboxylic acid (3 mg, 0.005 mmol) in a mixture of AcCN/DMF (2:1, 1.5 mL) was added triethylamine (1.5 mg, 0.015 mmol) followed by a dropwise addition of a solution of biotin-PEG6-NHS ester (4 mg, 0.0055 mmol) in AcCN (0.5 mL) over a 10 min period, the reaction was stirred for 24 h at room temperature. The solvents were removed under reduced pressure and the remaining oil was purified by preparative HPLC to afford the desired probe (2.5 mg, 43 %).

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.69 – 8.64 (m, 1H), 8.66 (s, 1H), 8.63 (s, 1H), 8.35 (dd, J = 2.8, 1.4 Hz, 1H), 5.25 – 5.18 (m, 1H), 4.49 (ddd, J = 7.9, 5.0, 1.0 Hz, 1H), 4.29 (dd, J = 7.9, 4.4 Hz, 1H), 3.69 – 3.55 (m, 34H), 3.55 – 3.49 (m, 4H), 3.34 (q, J = 5.8 Hz, 4H), 3.19 (ddd, J = 8.8, 5.8, 4.4 Hz, 1H), 2.91 (dd, J = 12.7, 5.0 Hz, 1H), 2.69 (d, J = 12.7 Hz, 1H), 2.64 (dt, J = 5.9, 1.9 Hz, 1H), 2.44 (t, J = 6.1 Hz, 2H), 2.20 (t, J = 7.4 Hz, 2H), 2.19 (s, 1H), 2.03 (m, 1H), 1.91 (m, 1H), 1.88 – 1.53 (m, 11H), 1.43 (m, 2H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 175.3, 174.7, 172.7, 164.8, 164.7, 160.2, 157.0 (d, J = 244.6 Hz), 155.7, 148.6, 145.8, 134.2, 133.5 (d, J = 30.0 Hz), 131.7, 118.6, 116.1 (d, J = 22.7 Hz), 104.6, 70.2, 70.2, 70.1 (x2), 70.13(x4), 70.1, 70.0, 69.9, 69.9, 69.8, 69.8, 69.2, 69.0, 68.9, 66.8, 62.0, 60.2, 55.6, 51.3, 50.0, 39.6, 39.5, 39.0, 38.9, 36.1, 35.3, 29.0, 28.4, 28.3, 28.1, 25.4, 25.2, 23.7, 20.4, 19.0. ^{19}F NMR (470 MHz, Methanol-d_4) δ -136.95. HR-ESI-MS calculated for $C_{54}H_{82}O_{15}N_{10}FS$ $(M + H)^+$ 1,161.5660, found 1,161.5651.

(2*S*,3*S*)-3-((5-((1-azido-21-oxo-3,6,9,12,15,18,25,28,31-nonaoxa-22-azatritriacontan-33-yl)carbamoyl)-2-(5-fluoro-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pyrimidin-4-yl)amino)bicyclo[2.2.2]octane-2-carboxylic acid

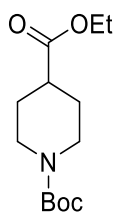


147

To an ice-cool solution of (2*S*,3*S*)-3-((5-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-ethyl)carbamoyl)-2-(5-fluoro-1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pyrimidin-4-yl)amino)bicycle-[2.2.2]-octane-2-carboxylic acid (6 mg, 0.01 mmol) in a mixture of AcCN/DMF (2:1, 2 mL) was added triethylamine (3 mg, 0.03 mmol) followed by a dropwise addition of a solution of azido-PEG6-NHS ester (5 mg, 0.011 mmol) in AcCN (0.5 mL) over a 10 min period, the reaction was stirred for 24 h at room temperature. The solvents were removed under reduced pressure and the remaining oil was purified by preparative HPLC to afford the desired probe. (4.8 mg, 51 %).

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.68 – 8.64 (m, 1H), 8.65 (s,1H), 8.59 (s, 1H), 8.32 (s, 1H), 5.16 (d, J = 4.7 Hz, 1H), 3.72 – 3.55 (m, 36H), 3.53 (t, J = 5.6 Hz, 2H), 3.37 – 3.33 (m, 4H), 2.64 – 2.57 (m, 1H), 2.43 (t, J = 6.1 Hz, 2H), 2.17 (q, J = 2.8 Hz, 1H), 2.02 (q, J = 2.9 Hz, 1H), 1.96 – 1.88 (m, 1H), 1.87 – 1.54 (m, 7H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 176.9, 174.1, 166.7, 161.6, 158.3 (d, J = 243.8 Hz), 158.2 (detected by HMBC), 148.7 (detected by HMBC), 147.2, 135.2, 134.5 (d, J = 29.3 Hz), 131.7 (detected by HMBC), 120.1 (d, J = 8.2 Hz), 117.5, 106.0. 71.64, 71.6 (x2), 71.5 (x8), 71.4, 71.3, 71.3, 71.1, 70.4, 70.3, 68.2, 52.4, 51.7, 51.6, 40.8, 40.4, 37.5, 30.3, 29.9, 26.7, 25.1, 21.9, 20.4. ^{19}F NMR (470 MHz, Methanol-d_4) δ -137.40. HR-ESI-MS calculated for $C_{44}H_{66}O_{13}N_{10}F$ $(M + H)^+$ 961.4789, found 961.4791.

Ethyl 1-*tert*-butyloxycarbonyl-4-piperidinecarboxylate

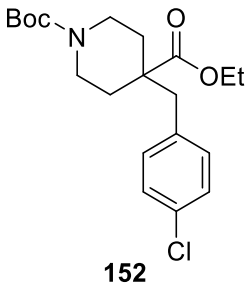


151

Ethyl isonipecotate (1.5 g, 9.5 mmol) was dissolved in DCM (7.5 mL) and the solution was cooled on an ice bath, triethylamine (2.0 mL, 14.3 mmol) was added follower by a solution of di-*tert*-butyl dicarbonate (2.7 g, 12.4 mmol) in DCM (7.5 mL) and the reaction mixture was stirred for 16 h at room temperature. The reaction was quenched by addition of water (20 mL), the phases were separated and the organic layer was washed with a saturated solution of NH_4Cl (2x 10 mL) and brine (5 mL), the organic phase was dried over $MgSO_4$, filtered and evaporated to dryness. The product was used in the next step without further purification (2.38 g, 97%).

^{1}H NMR (400 MHz, Chloroform-d_3) δ 4.08 (q, J = 7.1 Hz, 2H), 4.01 – 3.88 (m, 2H), 2.86 – 2.72 (m, 2H), 2.43 – 2.29 (m, 1H), 1.81 (dd, J = 13.5, 3.8 Hz, 2H), 1.56 (dtd, J = 13.4, 11.3, 4.3 Hz, 2H), 1.47 (s, 3H), 1.40 (s, 6H), 1.20 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 174.5, 154.6, 79.4, 60.4, 43.1 (br), 41.1 (2x), 28.4 (3x), 28.0, 27.4, 14.2. HR-ESI-MS calculated for $C_{13}H_{23}NO_4Na$ $(M + Na)^+$ 280.1519, found 280.1520.

1-(*tert*-Butyl) 4-ethyl 4-(4-chlorobenzyl)piperidine-1,4-dicarboxylate



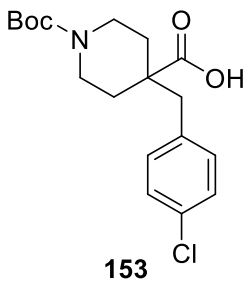
152

Ethyl 1-*tert*-butyloxycarbonyl-4-piperidinecarboxylate (2.9 g, 11.2 mmol) was dissolved in freshly distilled THF (29 mL) and the solution was cooled to -78 ºC under inert atmosphere. A solution of sodium bis(trimethylsilyl)amide 0.6 M in toluene (28 mL, 16.8 mmol) was added dropwise and the mixture was stirred for 15 min at -78 ºC. A 4-chlorobenzyl chloride (1.9 g, 12.3 mmol) solution in distilled THF (17 mL) was added and the reaction mixture was stirred for a further 3 h at -78 ºC followed by 1.5 h at room temperature. The reaction was then quenched by addition of water (55 mL) and the aqueous phase was extracted with EtOAc (3x 40 mL), the combined organic layers were washed with water (10 mL) and brine (7 mL) and evaporated to dryness under reduced pressure. The remaining crude mixture was purified by flash chromatography (hexanes/EtOAc 10:1) to furnish the desired alkylated product (3.4 g, 80%).

^{1}H NMR (400 MHz, Chloroform-d_3) δ 7.25 – 7.19 (m, 2H), 6.99 – 6.92 (m, 2H), 4.10 (q, J = 7.2 Hz, 2H), 3.92 (bs, 2H), 2.83 (bs, 2H), 2.78 (s, 2H), 2.09 – 1.96 (m, 2H), 1.62 (s, 2H), 1.44 (s, 9H),

1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 174.6, 154.8, 134.8, 132.7, 131.2 (2x), 128.2 (2x), 79.5, 60.6, 47.2 (2x), 45.9, 41.0 (br), 33.2 (2x), 28.4 (3x), 14.2.

1-(*tert*-Butoxycarbonyl)-4-(4-chlorobenzyl)piperidine-4-carboxylic acid

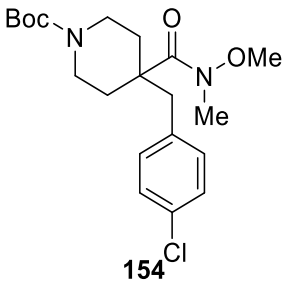


153

1-(*tert*-butyl) 4-ethyl 4-(4-chlorobenzyl)piperidine-1,4-dicarboxylate (2.6 g, 6.7 mmol) was dissolved in a mixture of 1,4-dioxane (36 mL) and MeOH (66 mL) at room temperature, KOH in pellets (3.8 g, 67 mmol) was added and the mixture was heated to reflux for 48 h. The solvents were evaporated under reduced pressure and the remaining residue was dissolved in water (50 mL), the aqueous solution was acidified by addition of aqueous HCl 5% and then it was extracted with EtOAc (3x 40 mL), the combiner organic layers were washed with brine (15 mL), dried over $MgSO_4$ and evaporated to dryness under reduced pressure to furnish the free acid (2.3 g, 98%). The product was used in the next step without further purification.

^{1}H NMR (400 MHz, Chloroform-d_3) δ 7.26 – 7.18 (m, 2H), 7.11 – 6.98 (m, 2H), 3.95 (bs, 2H), 2.88 (bs, 2H), 2.84 (s, 2H), 2.05 (d, J = 13.5 Hz, 2H), 1.45 (m, 11H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 180.2, 155.0, 134.5, 133.1, 131.3 (2x), 128.5 (2x), 79.9, 47.3 (2x), 45.7, 41.0 (br), 33.1 (2x), 28.6 (3x). HR-ESI-MS calculated for $C_{18}H_{24}NO_4Na$ $(M + Na)^+$ 376.1286, found 376.1287.

***tert*-Butyl 4-(4-chlorobenzyl)-4-(methoxy(methyl)carbamoyl)piperidine-1-carboxylate**



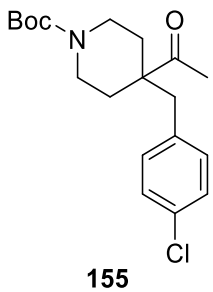
154

1-(*tert*-Butoxycarbonyl)-4-(4-chlorobenzyl)piperidine-4-carboxylic acid (1.1 g, 3.06 mmol) was dissolved in distilled THF (14 mL) under inert atmosphere, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.81 g, 4.3 mmol) was added all at once and the reaction mixture was stirred for 15 min at room temperature. *N,O*-Dimethylhydroxylamine hydrochloride (0.44 g, 4.6 mmol) was subsequently added and the reaction mixture was stirred for 48h at room temperature. The reaction was then quenched by addition of a saturated solution of sodium bicarbonate (10 mL) and the aqueous layer was extracted with DCM (3x 20 mL), the combined organic layers were washed with brine (10 mL) and evaporated to dryness. The resulting product was purified by column

chromatography (Hexanes/EtOAc form 2:1 to 1:1) to furnish the desired Weinreb amide (0.8 g, 66%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 7.23 – 7.13 (m, 2H), 7.01 – 6.89 (m, 2H), 3.86 (bs, 2H), 3.68 (s, 3H), 3.15 (s, 3H), 2.97 (bs, 4H), 2.18 (d, *J* = 13.4 Hz, 2H), 1.48 – 1.26 (m, 11H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 175.0, 155.0, 135.5, 132.6, 131.3 (2x), 128.3 (2x), 79.4, 60.6, 47.9, 42.8 (2x), 41.0 (bs), 34.1 (2x), 33.8, 28.5 (3x).

tert-Butyl 4-acetyl-4-(4-chlorobenzyl)piperidine-1-carboxylate

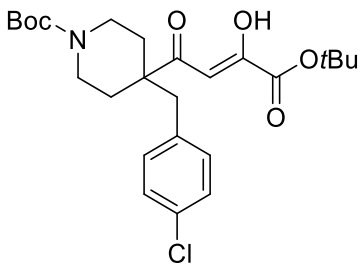


155

To a solution of *tert*-butyl 4-(4-chlorobenzyl)-4-(methoxy(methyl)-carbamoyl)piperidine-1-carboxylate (0.64 g, 1.62 mmol) in distilled THF (7.5 mL) under argon atmosphere was added dropwise a solution of methylmagnesium chloride 3.0 M in THF (6.4 mL, 19.1 mmol) and the reaction was heated to reflux for 40 min. The reaction was quenched by dropwise addition of $NaHCO_3$ saturated solution (25 mL) and the aqueous layer was extracted with EtOAc (3x 20 mL), the combined organic phases were washed with water (10 mL) and brine (5 mL) and then evaporated under reduced pressure. The resulting mixture was purified by flash chromatography (hexanes/EtOAc 1:1) to afford the desired ketone (0.53, 94%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 7.24 – 7.16 (m, 2H), 6.97 – 6.87 (m, 2H), 3.81 (bs, 2H), 2.96 – 2.79 (m, 2H), 2.75 (s, 2H), 2.08 (s, 3H), 2.03 – 1.91 (m, 2H), 1.45 – 1.40 (m, 11H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 211.4, 154.8, 134.4, 132.9, 131.2 (2x), 128.5 (2x), 79.7, 52.0, 44.3 (2x), 41.0 (bs), 32.4 (2x), 28.4 (3x), 26.7. HR-ESI-MS calculated for $C_{19}H_{26}NO_3Na$ $(M + Na)^+$ 374.1493, found 374.1494.

tert-Butyl (_Z_)-4-(4-(tert-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)piperidine-1-carboxylate

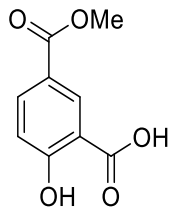


156

A solution of sodium bis(trimethylsilyl)amide 0.6 M in toluene (0.9 mL, 0.55 mmol) was added dropwise to a solution of *tert*-butyl 4-acetyl-4-(4-chlorobenzyl)piperidine-1-carboxylate (0.13 g, 0.37 mmol) in distilled THF (1.5 mL) at -78 ºC under argon atmosphere. The reaction was left stirring on the bath for 20 min followed by addition of di-*tert*-butyl oxalate (0.09 g, 0.44 mmol), the dry-ice bath was removed and the reaction was stirred overnight at room temperature. The reaction mixture was quenched by addition of water (2 mL) and the THF was removed under reduced pressure, the remaining aqueous solution was acidified by addition of an aqueous citric acid solution (5%, 10 mL) and the aqueous layer was extracted with DCM (3x 15 mL). The combined organic layer was washed with brine (5mL) and evaporated to dryness, the resulting mixture was purified by flash chromatography (from DCM to DCM/MeOH 100:2) to yield the β-diketo ester (0.14 g, 78%).

^{1}H NMR (400 MHz, Chloroform-d_3) δ 14.81 (bs, 1H), 7.26 7.08 (m, 2H), 6.90 (dd, J = 8.4, 2.1 Hz, 2H), 6.34 (s, 1H), 3.94 – 3.71 (m, 2H), 2.88 –2.81 (m, 2H), 2.81 (s, 2H), 2.05 (d, J = 13.8 Hz, 2H), 1.71 –1.37 (m, 2H), 1.56 (s, 9H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 205.5, 169.2, 161.1, 154.9, 134.1, 133.1, 131.4 (2x), 128.5 (2x), 99.0, 84.2, 79.8, 49.3, 45.5 (2x), 40.5 (bs), 32.6 (2x), 28.5 (3x), 27.9 (3x). HR-ESI-MS calculated for $C_{25}H_{35}NO_6Cl$ $(M + H)^+$ 480.2147, found 480.2147.

2-Hydroxy-5-(methoxycarbonyl)benzoic acid

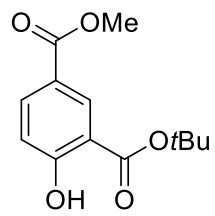


157

Dimethyl 4-hydroxyisophthalate (1.5 g, 7.6 mmol) was dissolved in pyridine (21 mL) under argon atmosphere and the reaction mixture was heated to reflux for 20 h. Pyridine was evaporated under reduced pressure and the remaining mixture was dissolved in an aqueous solution of HCl 1M (8.2 mL), the formed precipitate was filtered and washed with ice-cold water, the solid was dried under reduced pressure overnight (1.3 g, 93 %). The free acid was used in the next step without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.38 (d, J = 2.3 Hz, 1H), 8.03 (dd, J = 8.7, 2.3 Hz, 1H), 7.05 (d, J = 8.7 Hz, 1H), 3.82 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.1, 165.4, 164.8, 135.9, 132.2, 120.5, 117.8, 113.6, 52.1.

3-(*tert*-Butyl) 1-methyl 4-hydroxyisophthalate

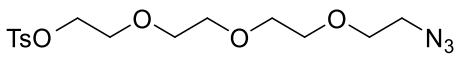


158

2-Hydroxy-5-(methoxycarbonyl)benzoic acid (1.45 g, 7.39 mmol) was dissolved in dry DCM (72 mL) followed by addition of anhydrous $MgSO_4$ (7.7 g, 62.0 mmol) to the solution. *tert*-Butanol (7.6 mL) was added followed by dropwise addition of concentrated sulfuric acid (0.85 mL, 15.3 mmol), the reaction was stirred for 48 h. The $MgSO_4$ was filtered out and the filtrate was mixed with $NaHCO_3$ saturated solution to adjust the pH to neutral, the phases were separated and the organic layer was dried over $MgSO_4$ and evaporated to dryness to afford the tert-butyl ester (0.95 g, 53%). The product was used in the next step without further purification.

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 11.53 (s, 1H), 8.46 (d, *J* = 2.2 Hz, 1H), 8.07 (dd, *J* = 8.7, 2.2 Hz, 1H), 6.97 (d, *J* = 8.8 Hz, 1H), 3.90 (s, 3H), 1.63 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-*d*$_3$) δ 169.5, 166.4, 165.5, 136.2, 132.7, 121.2, 117.8, 113.7, 83.9, 52.2, 28.3 (3x).

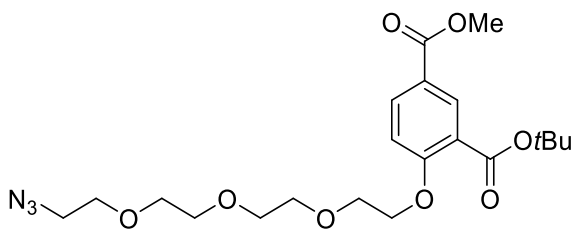
11-Azido-3,6,9-trioxa-undecanyl *p*-toluenesulfonate



Sodium azide (0.64 g, 10.0 mmol) was added to solution of tetraethylene glycol di(*p*-toluenesulfonate) (5 g, 10.0 mmol) in DMF (17 mL) under inert atmosphere and the reaction was heated to 60 ºC for 24h. Water (50 mL) was added to the reaction and the aqueous solution was extracted with EtOAc (40 mL), the combiner organic phase was washed with water (2x 20 mL) and brine (10 mL) a then evaporated down under reduced pressure. The resulting oil was purified by flash chromatography (Hex/EtOAc 1:1) to furnish the mono-azide (1.5 g, 40%).

^{1}H NMR (401 MHz, Chloroform-*d*$_3$) δ 7.85 – 7.73 (m, 2H), 7.35 (d, *J* = 8.0 Hz, 2H), 4.17 (t, *J* = 4.5 Hz, 2H), 3.71 – 3.64 (m, 12H), 3.39 (t, *J* = 5.1 Hz, 2H), 2.46 (s, 3H).

3-(*tert*-Butyl) 1-methyl 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)isophthalate

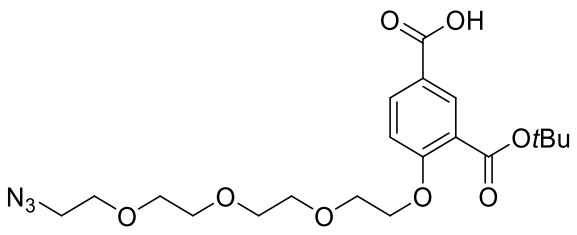


159

The linker 11-azido-3,6,9-trioxa-undecanyl *p*-toluenesulfonate (0.93 g, 2.5 mmol) was added to a solution of 3-(*tert*-butyl) 1-methyl 4-hydroxyisophthalate (0.57 g, 2.2 mmol) and K_2CO_3 (0.62 g, 5.0 mmol) in DMF (10 mL), the reaction was heated to 60 ºC for 24 h. The reaction mixture was concentrated under reduced pressure and the remaining oil was dissolved in water (20 mL), the aqueous solution was extracted with EtOAc (3x 30mL) and the combined organic layers were washed with water (2x 15 mL) and brine (10 mL), evaporated to dryness and purified by flash chromatography (hexanes/EtOAc 1:1) to yield the alkylated phenol (0.96, 94%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 8.32 (d, *J* = 2.3 Hz, 1H), 8.06 (dd, *J* = 8.8, 2.3 Hz, 1H), 6.96 (d, *J* = 8.8 Hz, 1H), 4.23 (dd, *J* = 5.6, 4.4 Hz, 2H), 3.93 – 3.84 (m, 2H), 3.88 (s, 3H), 3.77 – 3.69 (m, 2H), 3.68 – 3.61 (m, 8H), 3.36 (dd, *J* = 5.6, 4.6 Hz, 2H), 1.57 (s, 9H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 166.3, 165.2, 161.3, 134.2, 132.9, 129.9, 128.1, 122.4, 112.7, 81.7, 71.1, 70.9, 70.8, 70.2, 69.5, 68.7, 52.2, 50.8, 28.3 (3x). HR-ESI-MS calculated for $C_{21}H_{31}N_3O_8Na$ $(M + Na)^+$ 476.2003, found 476.2003.

4-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethoxy)-3-(tert-butoxycarbonyl)benzoic acid

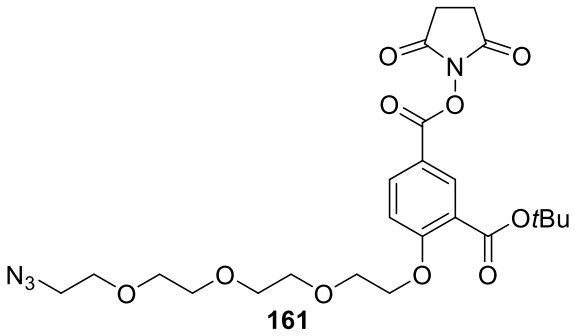


160

The conditions used are described in the general procedure for the saponification of ethyl esters. The free acid was used in the following step without further purification (0.9 g, 97%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 8.38 (d, *J* = 2.3 Hz, 1H), 8.11 (dd, *J* = 8.8, 2.3 Hz, 1H), 6.98 (d, *J* = 8.9 Hz, 1H), 4.24 (t, *J* = 4.9 Hz, 2H), 3.90 (dd, *J* = 5.6, 4.2 Hz, 2H), 3.73 (dt, *J* = 5.2, 2.0 Hz, 2H), 3.69 – 3.58 (m, 8H), 3.35 (t, *J* = 5.0 Hz, 2H), 1.57 (s, 9H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 170.7, 164.9, 161.9, 134.8, 133.6, 123.0, 121.5, 112.7, 81.7, 71.0, 70.8, 70.7, 70.7, 70.1, 69.4, 68.7, 50.7, 28.3. HR-ESI-MS calculated for $C_{20}H_{29}N_3O_8Na$ $(M + Na)^+$ 462.1847, found 462.1848.

3-(*tert*-Butyl) 1-(2,5-dioxopyrrolidin-1-yl) 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-isophthalate

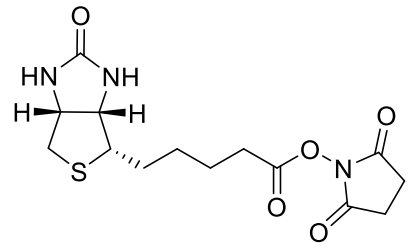


161

4-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethoxy)-3-(*tert*-butoxycarbonyl)benzoic acid (0.15 g, 0.34 mmol) was dissolved in distilled THF (3 mL) under argon atmosphere followed by addition of triethylamine (0.1 mL, 0.68 mmol) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (0.14 g, 0.44 mmol), the reaction was stirred for 10 min and subsequently *N*-hydroxysuccinimide (0.05 g, 0.44 mmol) was added and the mixture was stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure and the remaining mixture was purified by flash chromatography (from hexanes/EtOAc 1:1 to EtOAc) to yield the NHS-ester (0.16 g, 87%).

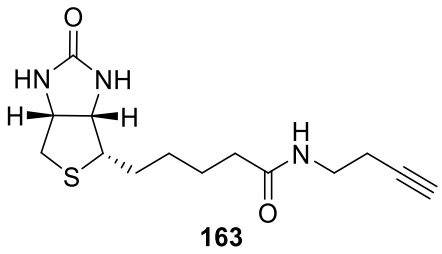
^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 8.44 (d, *J* = 2.3 Hz, 1H), 8.16 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.04 (d, *J* = 8.9 Hz, 1H), 4.27 (t, *J* = 4.9 Hz, 2H), 3.92 (t, *J* = 4.9 Hz, 2H), 3.77 – 3.70 (m, 2H), 3.70 – 3.59 (m, 8H), 3.37 (t, *J* = 5.1 Hz, 2H), 2.89 (s, 4H), 1.57 (s, 9H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 169.4 (2x), 164.3, 162.9, 161.1, 135.3, 134.2, 123.4, 116.9, 113.1, 82.1, 71.1, 70.8, 70.8, 70.8, 70.1, 69.4, 68.9, 50.8, 28.3 (3x), 25.8 (2x). HR-ESI-MS calculated for $C_{24}H_{32}N_4O_{10}Na$ $(M + Na)^+$ 559.2011, found 559.2011.

Biotin *N*-hydroxysuccinimide ester



Biotin (0.15 g, 0.61 mmol) was dissolved in warm DMF (3.5 mL) under inert atmosphere and *N,N*′-dicyclohexylcarbodiimide (0.14 g, 0.67 mmol) was added. The reaction was allowed to stir for 15 min at room temperature. *N*-Hydroxysuccinimide (0.09 g, 0.76 mmol) was subsequently added and the reaction mixture was further stirred for 24 h at room temperature. The resulting suspension was filtered, the filtrate was evaporated to dryness under reduced pressure and the remaining oil was triturated in Et_2O. The formed precipitate was filtrate and dried under high vacuum to yield the NHS-ester (0.19 g, 93%). The compound was used without further purification.

***N*-(but-3-ynyl)biotinamide**



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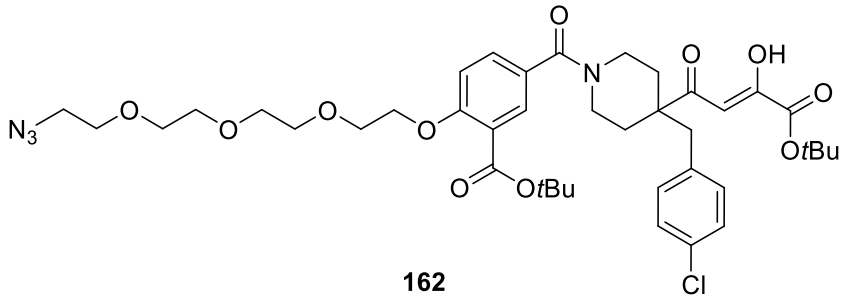
Biotin *N*-hydroxysuccinimide ester (0.09 g, 0.26 mmol) was dissolved in warm DMF (2.5 mL) under inert atmosphere followed by addition of 1-amino-3-butyne (0.02 g, 0.29 mmol), the reaction was left stirring overnight at room temperature. The following day the solvent was evaporated under reduced pressure and the remaining mixture was purified by flash chromatography (DCM/MeOH 10:1) to yield the desired amide (0.045 g, 58%).

^{1}H NMR (300 MHz, Chloroform-d_3 + a drop of methanol-d_4) δ 4.29 (dd, J = 7.9, 4.8 Hz, 1H), 4.09 (dd, J = 7.8, 4.4 Hz, 1H), 3.12 (dd, J = 7.5, 4.9 Hz, 2H), 2.99 – 2.86 (m, 1H), 2.70 (dd, J = 12.9, 4.9 Hz, 1H), 2.51 (d, J = 12.3 Hz, 1H), 2.17 (td, J = 6.8, 2.6 Hz, 2H), 1.99 (t, J = 7.3 Hz, 2H), 1.85 (t, J = 2.7 Hz, 1H), 1.45 (tt, J = 15.2, 7.5 Hz, 4H), 1.30 – 1.15 (m, 2H).

***tert*-Butyl (*Z*)-2-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-5-(4-(4-(*tert*-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)piperidine-1-carbonyl)benzoate**



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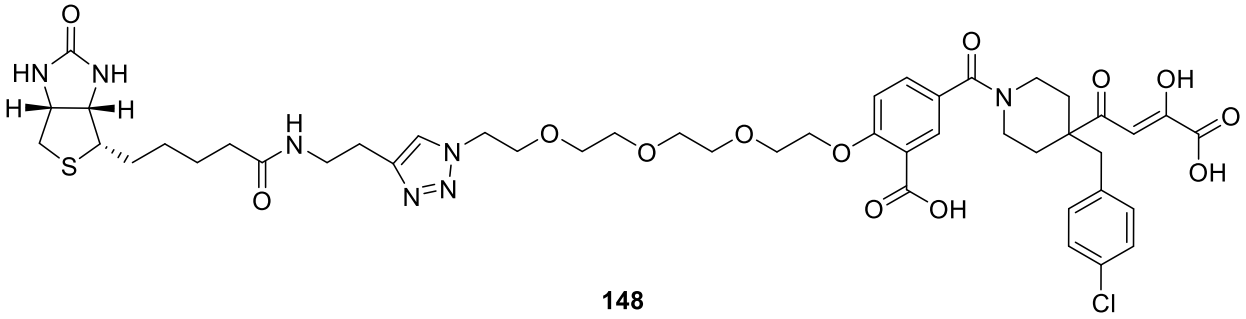
tert-Butyl (Z)-4-(4-(tert-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)piperidine-1-carboxylate (synthon A, 35 mg, 0.073 mmol) was dissolved in a solution of TFA in DCM (1:1, 1mL) at 0 ºC. The reaction was stirred for 1h and then the solvent was removed under reduced pressure. The dried residue was dissolved again in DCM (1.5 mL) and triethylamine (0.5 mL) was added followed by a solution of 3-(*tert*-butyl) 1-(2,5-dioxopyrrolidin-1-yl) 4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)isophthalate (synthon B, 39 mg, 0.073 mmol) in DCM (0.5 mL), the reaction was stirred overnight period at room temperature. The solvent was evaporated,

and the remaining residue was purified by flash chromatography (DCM/MeOH 20:1) afford the connected product (18 mg, 30%).

^{1}H NMR (400 MHz, Chloroform-d_3) δ 14.74 (s, 1H), 7.72 (d, J = 2.3 Hz, 1H), 7.44 (dd, J = 8.6, 2.3 Hz, 1H), 7.20 (d, J = 8.3 Hz, 2H), 6.95 (d, J = 8.6 Hz, 1H), 6.89 (d, J = 8.4 Hz, 2H), 6.36 (s, 1H), 4.38 (m, 2H), 4.20 (t, J = 5.0 Hz, 2H), 3.89 (t, J = 5.0 Hz, 2H), 3.73 (dt, J = 4.8, 3.2 Hz, 2H), 3.66 (q, J = 3.3 Hz, 8H), 3.37 (t, J = 5.1 Hz, 2H), 3.03 (d, J = 3.4 Hz, 2H), 2.84 (s, 2H), 2.23 – 2.01 (m, 2H), 1.56 (d, J = 2.3 Hz, 18H), 1.47 – 1.33 (m, 2H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 205.2, 169.6, 169.1, 165.2, 161.0, 159.1, 133.8, 133.2, 132.0, 131.3 (2x), 130.7, 128.6 (2x), 127.6, 122.8, 113.3, 98.9, 84.3, 81.7, 71.1, 70.9, 70.8, 70.8, 70.2, 69.6, 68.8, 50.8, 49.7, 45.7 (2x), 40.0, 33.0, 28.3 (3x), 28.2, 27.9 (3x). HR-ESI-MS calculated for $C_{40}H_{53}ClN_4O_{11}$ $(M + H)^+$ 801.347, found 801.3477.

***tert*-Butyl 5-(4-((*Z*)-4-(tert-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)-piperidine-1-carbonyl)-2-(2-(2-(2-(2-(4-(2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-*d*]imidazol-4-yl)pentanamido)ethyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethoxy)benzoate**



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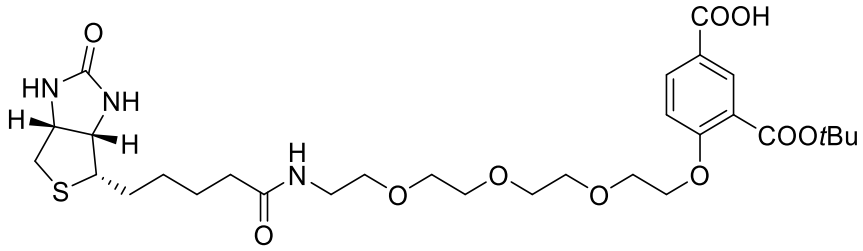
tert-Butyl (*Z*)-2-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)-5-(4-(4-(tert-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)piperidine-1-carbonyl)benzoate (23 mg, 0.028 mmol) was dissolved in a mixture of ethanol, water and THF (EtOH/water/THF 2:2:1, 2.5 mL) and the resulting solution was purged with argon for 10 min. Copper (II) sulfate pentahydrate (14 mg, 0.057 mg) was added followed by *N*-(but-3-ynyl)biotinamide (12 mg, 0.042 mmol) and (+)-sodium *L*-ascorbate (56 mg, 0.28 mmol). The reaction was stirred for 1 h at room temperature under inert atmosphere. The reaction mixture was then filtered and evaporated to dryness under reduced pressure. The crude mixture was then dissolved in a TFA (33% in DCM, 1.5 mL) and the reaction

was stirred for one more hour at room temperature. The solvents were removed under reduced pressure and the remaining mixture was purified by preparative HPLC.

^{1}H NMR (600 MHz, Methanol-d_4) δ 7.87 (d, J = 2.3 Hz, 1H), 7.83 (d, J = 2.4 Hz, 1H), 7.58 (dd, J = 8.6, 2.4 Hz, 1H), 7.28 – 7.20 (m, 4H), 7.06 – 7.00 (m, 2H), 4.56 – 4.49 (m, 2H), 4.49 – 4.43 (m, 1H), 4.35 – 4.24 (m, 4H), 3.88 (dt, J = 24.6, 4.5 Hz, 4H), 3.72 (dt, J = 7.8, 3.1 Hz, 2H), 3.63 – 3.53 (m, 6H), 3.45 (t, J = 7.0 Hz, 2H), 3.19 (dt, J = 3.3, 1.7 Hz, 1H), 3.09 – 3.04 (m, 1H), 2.96 (s, 2H), 2.93 – 2.90 (m, 1H), 2.87 (t, J = 7.0 Hz, 2H), 2.69 (d, J = 12.7 Hz, 1H), 2.22 – 2.13 (m, 2H), 2.21 (bs, 2H), 1.74 – 1.45 (m, 8H), 1.39 (t, J = 7.6 Hz, 2H). ^{13}C NMR (151 MHz, Methanol-d_4) δ 207.2, 176.1, 175.8, 171.3, 168.5, 167.9, 164.9, 160.7, 146.1, 135.9, 133.9, 133.9, 133.1, 132.7 (x2), 132.0, 129.2 (x2), 129.1, 124.6, 121.9, 114.9, 71.9, 71.6, 71.5, 71.5, 70.4, 70.4, 70.3, 63.3, 61.6, 57.0, 55.3, 51.3, 51.1, 49.6, 45.7, 41.0, 40.1, 40.0, 36.8, 36.5, 30.8, 29.8, 29.4, 26.5. HR-ESI-MS calculated for $C_{46}H_{59}ClN_7O_{13}S$ $(M+H)^+$ 984.3575, found 984.3573.

3-(*tert*-butoxycarbonyl)-4-((13-oxo-17-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-3,6,9-trioxa-12-azaheptadecyl)oxy)benzoic acid



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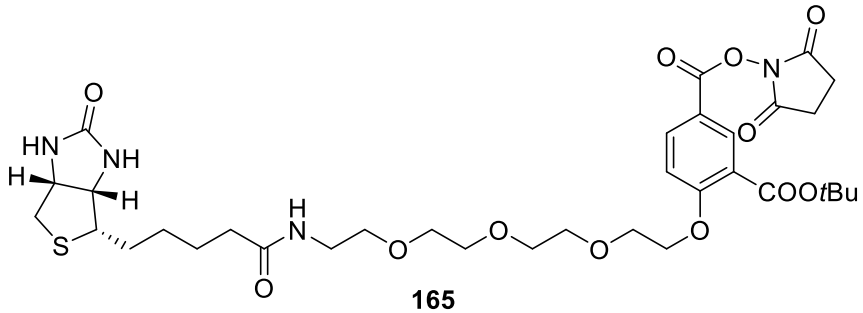
4-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethoxy)-3-(tert-butoxycarbonyl)benzoic acid (77 mg, 0.17 mmol) and Lindlar catalyst (23 mg, 30 w%) were suspended in MeOH (2 mL), the flask was purged with argon and then purged with hydrogen gas and the reaction mixture was stirred for 90 min at room temperature under hydrogen atmosphere (1 atm). Once the TLC showed complete reduction of the starting material the catalyst was filtered out and the filtrate was evaporated to dryness. The resulting crude mixture was dissolved in DMF (2 mL) followed by addition of triethylamine (50 µl, 0.36 mmol) and (+)-biotin *N*-hydroxysuccinimide ester (60 mg, 0.17 mmol). The reaction mixture was stirred for 48 h at room temperature followed by removal of the solvent under reduced pressure. The remaining mixture was diluted in DCM (25 mL) and the organic phase was washed with an aqueous solution of citric acid (5%, 3 mL) and brine (3 mL). The organic layer

was evaporated down and purified by flash chromatography (DCM/MeOH from 9:1 to 8:2) to yield the biotinylated product (43 mg, 39%).

^{1}H NMR (400 MHz, Methanol-*d*$_4$) δ 8.24 (d, *J* = 2.3 Hz, 1H), 8.11 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.18 (d, *J* = 8.8 Hz, 1H), 4.48 (ddd, *J* = 7.9, 5.8, 5.0, 1H), 4.36 – 4.24 (m, 3H), 3.94 – 3.87 (m, 2H), 3.76 – 3.70 (m, 2H), 3.70 – 3.56 (m, 6H), 3.52 (t, *J* = 5.5 Hz, 2H), 3.34 (d, *J* = 5.5 Hz, 1H), 3.26 – 3.12 (m, 1H), 2.92 (ddd, *J* = 12.6, 7.5, 5.0 Hz, 1H), 2.75 – 2.65 (m, 1H), 2.31 (t, *J* = 7.4 Hz, 1H), 2.19 (t, *J* = 7.4 Hz, 1H), 1.79 – 1.61 (m, 4H), 1.60 (s, 9H), 1.52 – 1.35 (m, 3H). ^{13}C NMR (101 MHz, Methanol-*d*$_4$) δ 176.1, 167.1, 166.1, 162.5, 135.6, 133.5, 129.8, 127.0, 123.9, 114.0, 83.0, 71.9, 71.6, 71.6, 71.2, 70.6, 70.6, 70.0, 63.3, 61.6, 57.0, 41.0, 40.3, 36.7, 29.7, 29.5, 28.5, 26.8. HR-ESI-MS calculated for $C_{30}H_{46}N_3O_{10}S$ $(M+H)^+$ 640.2898, found 640.2899.

3-(*tert*-Butyl) 1-(2,5-dioxopyrrolidin-1-yl) 4-((13-oxo-17-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9-trioxa-12-azaheptadecyl)oxy)isophthalate



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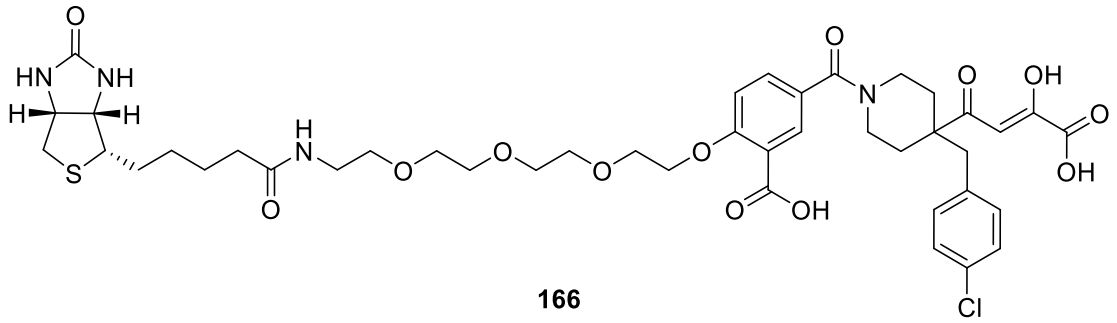
3-(*tert*-Butoxycarbonyl)-4-((13-oxo-17-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazole-4-yl)-3,6,9-trioxa-12-azaheptadecyl)oxy)benzoic acid (70 mg, 0.11 mmol) was dissolved in DMF (2 mL) under inert atmosphere followed by addition of O-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (45 mg, 0.14 mmol) and triethylamine (30 µl, 0.22 mmol), the reaction was stirred for 10 min at room temperature and *N*-hydroxysuccinimide (16 mg, 0.14 mmol) was subsequently added. After stirring for 16 h at room temperature the solvent was evaporated under reduced pressure and the resulting mixture was purified by flash chromatography (DCM/MeOH 9:1) to afford the activated ester (56 mg, 70%).

^{1}H NMR (400 MHz, Chloroform-*d*$_3$) δ 8.40 (d, *J* = 2.4 Hz, 1H), 8.16 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.07 (d, *J* = 8.9 Hz, 1H), 6.66 (t, *J* = 5.6 Hz, 1H), 6.25 (s, 1H), 5.64 (s, 1H), 4.47 (dd, *J* = 7.9, 4.8 Hz,

1H), 4.27 (t, *J* = 4.7 Hz, 3H), 3.94 – 3.84 (m, 2H), 3.72 (dd, *J* = 6.1, 3.2 Hz, 2H), 3.67 – 3.55 (m, 6H), 3.52 (t, *J* = 5.2 Hz, 2H), 3.42 – 3.30 (m, 2H), 3.09 (td, *J* = 7.3, 4.4 Hz, 1H), 2.89 (s, 4H), 2.84 (d, *J* = 4.8 Hz, 1H), 2.72 (d, *J* = 12.9 Hz, 1H), 2.17 (t, *J* = 7.4 Hz, 2H), 1.74 – 1.57 (m, 4H), 1.55 (s, 9H), 1.38 (d, *J* = 7.6 Hz, 2H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 173.6, 169.5 (x2), 164.5, 164.2, 162.8, 161.0, 135.4, 134.0, 123.3, 116.8, 113.2, 82.2, 71.0, 70.5, 70.5, 70.1, 69.9, 69.4, 68.9, 61.9, 60.4, 55.6, 40.6, 39.2, 35.9, 28.3 (x3), 28.2, 28.1, 25.8 (x2), 25.6. HR-ESI-MS calculated for $C_{34}H_{48}N_4O_{12}SNa$ $(M+Na)^+$ 759.2882, found 759.2883.

5-(4-((*Z*)-3-Carboxy-3-hydroxyacryloyl)-4-(4-chlorobenzyl)piperidine-1-carbonyl)-2-((13-oxo-17-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9-trioxa-12-azaheptadecyl)oxy)benzoic acid



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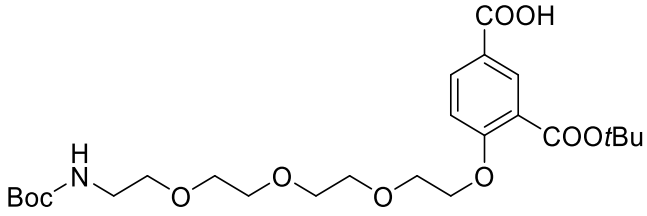
tert-Butyl (*Z*)-4-(4-(*tert*-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)piperidine-1-carboxylate (40 mg, 0.083 mmol) was dissolved in DCM (1 mL) and the resulting solution was cooled down on an ice bath, TFA (100%, 0.5 mL) was added and the reaction was stirred for 30 min at room temperature. Once the TLC showed total consumption of the starting material the solvents were evaporated under reduced pressure and the resulting oil was further dried under high vacuum for 2 h. The dried mixture was then dissolved in DCM (1 mL) followed by addition of triethylamine (0.3 mL) and 3-(*tert*-butyl) 1-(2,5-dioxopyrrolidin-1-yl) 4-((13-oxo-17-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9-trioxa-12-azaheptadecyl)-oxy)-isophthalate (56 mg, 0.076 mmol). After being stirred for 24 h at room temperature, the reaction mixture was evaporated down and dried under high vacuum. The resulting oil was dissolved in a mixture of DCM/TFA (2:1, 1.5 mL) and was stirred for 2 h at room temperature. After complete removal of the solvent under reduced pressure the mixture was purified by preparative HPLC to yield the final probe (18 mg, 27%).

^{1}H NMR (600 MHz, Methanol-*d*$_4$) δ 7.87 (d, *J* = 2.3 Hz, 1H), 7.59 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.24 (m, 4H), 7.03 (d, *J* = 8.3 Hz, 2H), 4.48 (dd, *J* = 7.9, 4.9 Hz, 1H), 4.35 – 4.30 (m, 2H), 4.31 – 4.25 (m, 1H), 3.95 – 3.88 (m, 2H), 3.79 – 3.72 (m, 2H), 3.69 – 3.61 (m, 6H), 3.60 (dd, *J* = 5.6, 2.9 Hz, 2H), 3.52 (t, *J* = 5.5 Hz, 2H), 3.34 (t, *J* = 5.5 Hz, 2H), 3.19 (ddd, *J* = 9.2, 6.0, 4.6 Hz, 1H), 3.07 (bs, 2H), 2.96 (s, 2H), 2.91 (dd, *J* = 12.7, 5.0 Hz, 1H), 2.69 (d, *J* = 12.7 Hz, 1H), 2.21 (bs, 2H), 2.22 – 2.17 (m, 2H), 1.78 – 1.54 (m, 6H), 1.46 – 1.38 (m, 2H). ^{13}C NMR (151 MHz, Methanol-*d*$_4$) δ 207.2, 176.1, 171.3, 168.6, 167.8, 166.1, 164.7, 160.7, 135.9, 133.9, 133.9, 133.1, 132.7, 132.0, 129.2, 129.1, 121.9, 114.9, 71.9, 71.6, 71.6, 71.3, 70.6, 70.4, 70.4, 63.4, 61.6, 57.0, 51.1, 49.6, 46.6, 45.7, 41.0 (x2), 40.4, 36.7 (x2), 29.7, 29.5, 26.8. HR-ESI-MS calculated for $C_{42}H_{52}ClN_4O_{13}S$ $(M-H)^-$ 887.2946, found 887.2943.

3-(*tert*-Butoxycarbonyl)-4-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)oxy)benzoic acid

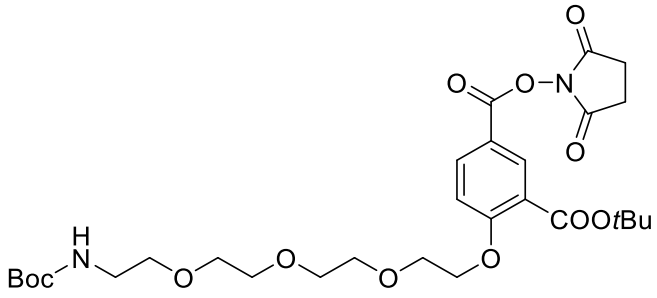


4-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)-ethoxy)-3-(tert-butoxycarbonyl)benzoic acid (0.64 g, 1.46 mmol) and Lindlar catalyst (0.19 g, 30 w%) were suspended in MeOH (15 mL), the flask was first purged with argon and then hydrogen gas was bubbled through the solution for 1 h at room temperature. The catalyst was filtered out and the filtrate was evaporated to dryness under reduced pressure. The resulting crude product was dissolved in DCM (15 mL), the reaction was cooled down in an ice bath followed by addition of triethylamine (0.4 mL, 2.92 mmol) and di-*tert*-butyl dicarbonate (0.38 g, 1.75 mmol). After stirring the reaction for 24 h at room temperature the mixture was diluted in DCM (20 mL) and the organic phase was washed with a saturated solution of ammonium chloride (2x 5 mL) and brine (5 mL), the organic layer was then evaporated to dryness and purified by flash chromatography (DCM/MeOH 20:1) to yield the Boc-protected amine (0.48 g, 64%).

^{1}H NMR (300 MHz, Chloroform-*d*$_3$) δ 8.36 (d, *J* = 2.3 Hz, 1H), 8.09 (dd, *J* = 8.8, 2.3 Hz, 1H), 6.96 (d, *J* = 8.9 Hz, 1H), 5.11 (s, 1H), 4.24 (dd, *J* = 5.7, 4.2 Hz, 2H), 3.90 (dd, *J* = 5.5, 4.3 Hz, 2H), 3.74 (dt, *J* = 4.5, 3.1 Hz, 2H), 3.68 – 3.55 (m, 6H), 3.52 (t, *J* = 5.2 Hz, 2H), 3.36 – 3.19 (m, 2H), 1.57 (s, 9H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, Chloroform-*d*$_3$) δ 170.1, 165.0, 161.7, 149.5, 134.8,

133.5, 122.9, 122.0, 112.6, 81.7, 79.3, 71.0, 70.7, 70.6, 70.3 (x2), 69.5, 68.7, 40.5, 28.5 (x3), 28.3 (x3). HR-ESI-MS calculated for $C_{25}H_{40}NO_{10}$ $(M + H)^+$ 514.2647, found 514.2646.

3-(*tert*-Butyl) 1-(2,5-dioxopyrrolidin-1-yl) 4-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)oxy)isophthalate



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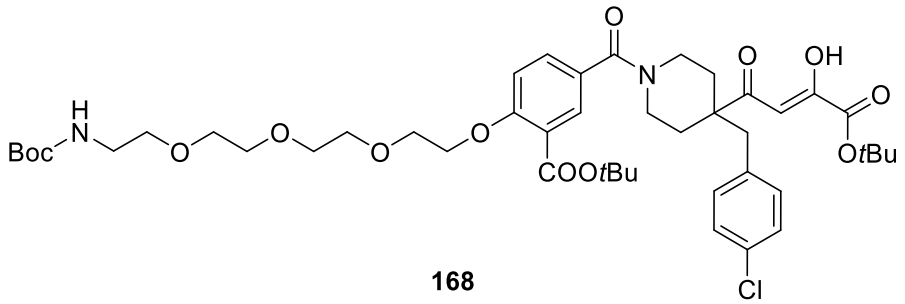
3-(*tert*-Butoxycarbonyl)-4-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)oxy)benzoic acid (0.48 g, 0.94 mmol) was dissolved in THF (17 mL) under inert atmosphere followed by addition of O-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyl-uronium tetrafluoroborate (0.39 g, 1.22 mmol) and triethylamine (0.26 ml, 0.22 mmol), the reaction was stirred for 10 min at room temperature and *N*-hydroxysuccinimide (16 mg, 1.88 mmol) was subsequently added. After stirring for 16 h at room temperature the solvent was evaporated under reduced pressure and the resulting mixture was purified by flash chromatography (DCM/MeOH 20:1) to afford the activated ester (0.47 g, 81%).

^{1}H NMR (300 MHz, Chloroform-d_3) δ 8.40 (d, J = 2.3 Hz, 1H), 8.13 (dd, J = 8.8, 2.2 Hz, 1H), 7.02 (d, J = 8.9 Hz, 1H), 5.01 (bs, 1H), 4.24 (t, J = 5.0 Hz, 2H), 3.88 (q, J = 5.1 Hz, 2H), 3.71 (dd, J = 6.1, 3.5 Hz, 2H), 3.60 (dd, J = 14.6, 3.6 Hz, 6H), 3.49 (t, J = 5.3 Hz, 2H), 3.26 (q, J = 5.5 Hz, 2H), 2.86 (s, 4H), 1.54 (s, 9H), 1.39 (s, 9H). ^{13}C NMR (75 MHz, Chloroform-d_3) δ 169.4 (x2), 164.3, 162.8, 161.0, 156.0, 135.2, 134.0, 123.4, 116.8, 113.0, 81.9, 79.1, 71.0, 70.6, 70.6, 70.2, 70.2, 69.3, 68.8, 40.4, 28.45 (x3), 28.2 (x3), 25.7 (x2). HR-ESI-MS calculated for $C_{29}H_{42}N_2O_{12}Na$ $(M+Na)^+$ 633.2630, found 633.2630.

***tert*-Butyl (*Z*)-5-(4-(4-(*tert*-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)-piperidine-1-carbonyl)-2-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)oxy)benzoate**

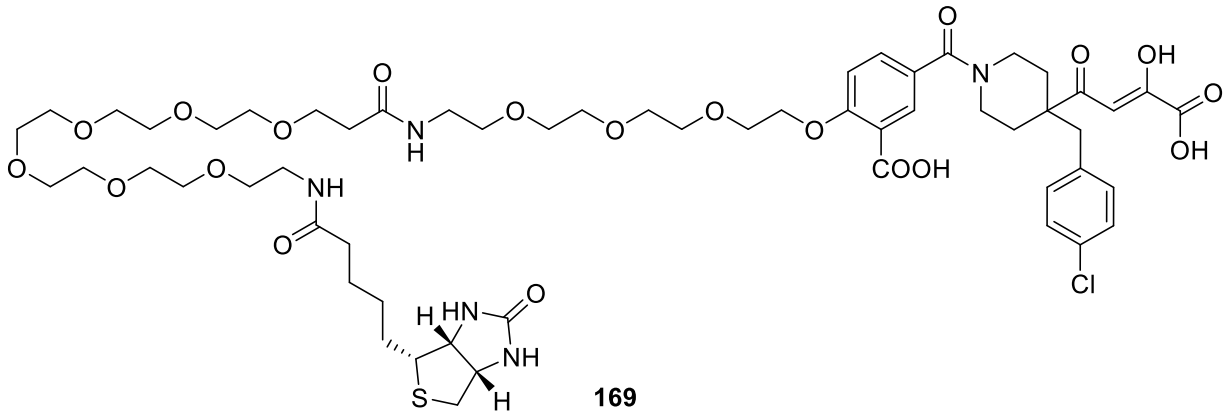


168

tert-Butyl (*Z*)-4-(4-(*tert*-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)piperidine-1-carboxylate (65 mg, 0.136 mmol) was dissolved in DCM (1 mL) and the resulting solution was cooled down on an ice bath, TFA (100%, 0.2 mL) was added and the reaction was stirred for 15 min at room temperature. Once the TLC showed total consumption of the starting material the solvents were evaporated under reduced pressure and the resulting oil was further dried under high vacuum for 2 h. The dried mixture was then dissolved in DCM (1.5 mL) followed by addition of triethylamine (0.3 mL) and 3-(*tert*-butyl) 1-(2,5-dioxopyrrolidin-1-yl) 4-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)oxy)isophthalate (82 mg, 0.136 mmol), the reaction mixture was left stirring at room temperature overnight period. Later, the solvents were removed under reduced pressure and the remaining residue was purified by flash chromatography (DCM/MeOH 20:1) to yield the desired amide (80 mg, 68%).

^{1}H NMR (400 MHz, Chloroform-d_3) δ 14.76 (s, 1H), 7.73 (d, J = 2.3 Hz, 1H), 7.44 (dd, J = 8.6, 2.3 Hz, 1H), 7.20 (d, J = 8.4 Hz, 2H), 6.95 (d, J = 8.6 Hz, 1H), 6.93 – 6.86 (m, 2H), 6.37 (s, 1H), 5.02 (s, 1H), 4.20 (t, J = 5.0 Hz, 2H), 3.90 (t, J = 5.0 Hz, 2H), 3.78 – 3.69 (m, 2H), 3.69 – 3.57 (m, 8H), 3.53 (t, J = 5.1 Hz, 2H), 3.34 – 3.25 (m, 2H), 3.05 (bs, 2H), 2.84 (s, 2H), 2.25 – 2.02 (bs, 2H), 1.72 (bs, 2H), 1.56 (s, 9H), 1.56 (s, 9H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-d_3) δ 205.2, 169.6, 169.1, 165.1, 161.0, 159.1, 134.6, 133.8, 133.4, 133.2, 132.0, 131.3 (x2), 130.7, 128.6 (x2), 127.6, 122.8, 113.3, 99.0, 84.3, 81.7, 71.1, 70.8, 70.7, 70.3, 70.3, 69.6, 68.8, 49.7 (x2), 45.7, 40.5, 38.7 (x2), 32.9, 28.6 (x3), 28.4 (x3), 27.9 (x3).

5-(4-((*Z*)-3-Carboxy-3-hydroxyacryloyl)-4-(4-chlorobenzyl)piperidine-1-carbonyl)-2-((13,35-dioxo-39-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9,16,19,22,25,28,31-nonaoxa-12,34-diazanonatriacontyl)oxy)benzoic acid



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tert-Butyl (*Z*)-5-(4-(4-(*tert*-butoxy)-3-hydroxy-4-oxobut-2-enoyl)-4-(4-chlorobenzyl)- piperidine-1-carbonyl)-2-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)oxy)benzoate (20 mg, 0.023 mmol) was dissolved in DCM (1 mL) and the solution was cooled down on an ice bath. Neat TFA (0.2 mL) was added dropwise and the reaction was stirred for 15 min. Once the TLC showed complete consumption of the starting material, the solvent was evaporated under reduced pressure and the remaining oil was further dried for 3 h under high vacuum. The dried product was dissolved in DCM (1 mL) followed by addition of triethylamine (0.3 mL) and biotin-PEG6-NHS ester (16 mg, 0.023 mmol). After stirring the reaction for 24 h at room temperature, the solvent was removed under reduced pressure and the remaining mixture dried under high vacuum for 2 h more. The crude mixture was dissolved in DCM (1 mL) again and TFA (0.5 mL) was added and the reaction was stirred for 1 h at room temperature. After removing the solvents under reduced pressure, the mixture was purified by preparative HPLC to yield the desired probe (4 mg, 15%).

^{1}H NMR (600 MHz, Methanol-*d*$_4$) δ 7.87 (d, *J* = 2.3 Hz, 1H), 7.59 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.29 – 7.20 (m, 4H), 7.07 – 6.99 (m, 2H), 4.49 (ddd, *J* = 7.9, 5.0, 1.0 Hz, 1H), 4.39 – 4.25 (m, 4H), 3.94 – 3.88 (m, 2H), 3.79 – 3.73 (m, 2H), 3.71 (t, *J* = 6.2 Hz, 3H), 3.69 – 3.55 (m, 26H), 3.53 (td, *J* = 5.5, 4.1 Hz, 4H), 3.35 (td, *J* = 5.5, 2.5 Hz, 4H), 3.20 (ddd, *J* = 8.9, 6.0, 4.6 Hz, 1H), 3.06 (bs, 2H), 2.96 (s, 2H), 2.92 (dd, *J* = 12.7, 5.0 Hz, 1H), 2.70 (d, *J* = 12.7 Hz, 1H), 2.44 (t, *J* = 6.2 Hz, 2H), 2.27 – 2.12 (m, 4H), 1.78 – 1.55 (m, 6H), 1.49 – 1.38 (m, 2H). ^{13}C NMR (151 MHz, Methanol-*d*$_4$) δ 207.3, 176.1, 174.0, 171.2, 168.6, 167.7, 166.1, 164.7, 160.7, 135.9, 133.9, 133.9, 133.1, 132.7 (x2), 132.0, 129.2 (x2), 129.2, 129.0, 121.9, 114.9, 71.9, 71.6, 71.6 (x7), 71.5, 71.5, 71.4, 71.3, 71.3, 70.6, 70.5, 70.4, 70.3, 68.3, 63.4, 61.6, 57.0, 51.1, 46.6, 45.7, 41.1, 40.4, 40.4, 37.6, 36.7, 34.0, 33.4, 29.8, 29.5, 26.9. HR-ESI-MS calculated for $C_{57}H_{82}ClN_5O_{20}S$ $(M+H)^+$ 1224.5035, found 1224.5035.

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