
# Synthesis and Biological Evaluation of Purine and Pyrimidine Based Ligands for the $A_3$ and the $P2Y_2$ Purinergic Receptors

**Apr. Liesbet Cosyn**

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**Promoter**
**Prof. dr. apr. Serge Van Calenbergh**

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# Summary

P1-receptors, or adenosine receptors, and P2Y receptors, are G-protein-coupled receptors, belonging to the purinergic receptor family. Adenosine is the natural ligand of the P1 receptors, while P2Y receptors are activated by nucleotide ligands (ATP, ADP, UTP and UDP). The adenosine receptors (P1) consist of 4 receptor subtypes: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. The P2Y receptors are subdivided in 8 subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$. In the first part of this work (chapter 2 and 3), we present the synthesis and biological evalutation of two series of adenosine $A_3$ receptor ligands. In the second part (chapter 4), we discuss the synthesis and biological evaluation of new $P2Y_2$ receptor ligands.

Adenosine receptors are not only the major targets of caffeine, the most commonly consumed drug in the world, they could also be promising therapeutic targets in a wide range of conditions, including cardiovascular, inflammatory and neurodegenerative diseases. As a consequence of their numerous therapeutic possibilities and the ubiquity of the ARs, synthetic ligands need high selectivity with respect to receptor subtype and tissue to be of therapeutic value. The main approach for the discovery of selective $A_3$AR ligands, has been the modification of the natural, non-selective ligand adenosine. Previous investigations showed that introduction of a 3'-amino group improved the selectivity for the human $A_3$AR, while enhancing the water solubility. By introducing extra modifications, we evaluated the possibility of overcoming the $A_3$AR affinity drop caused by this 3'-modification. We synthesized a series of hypermodified adenosine derivatives (chapter 2) and demonstrated that introduction of a 2-phenylethynyl substituent in concert with a $N^6$-methyl group restored the $A_3$AR receptor affinity.

Based on the good results obtained with the 2-phenylethynyl modification, both on $A_3$AR receptor selectivity and affinity, we decided to further explore the modifications at the 2-position. Therefore, we synthesized a series of 4-substituted 2-(1,2,3)-triazol-1-yl $N^6$-methyl adenosine analogues via Huisgen [3+2]cycloaddition (chapter 3). Combined with an unmodified ribose moiety, such 2-(1,2,3-triazol-1-yl) substitution resulted in very potent and selective $A_3$AR antagonists, for example 2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl)-$N^6$-methyl-9-(β-D-ribofuranosyl)adenine (**3.10**, $K_i$ ($A_3$AR) = 1.3 nM). The reduced efficacy resulting from such modifications at the 2-

position were restored by the 4'-ethylcarbamoyl ribose modification, resulting in a series of potent an selective $A_3$AR agonists, with 9-(5-ethylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-(4-pyridin-2-yl-1,2,3-triazol-1-yl) adenine as the most potent one (**3.17b**, $K_i$ ($A_3$AR) = 1.8nM).

$P2Y_2$ agonists are promising potential therapeutics for cystic fibrosis, cancer and dry eye syndrome. $P2Y_2$ antagonists exhibit anti-inflammatory and neuroprotective effects and have been suggested as potential treatment for coronary vasospastic disorders. As for the $A_3$AR receptor ligands, the natural, non-selective ligand uridine 5'-triphosphate was modified to gain access to more selective $P2Y_2$ receptor ligands. Previous investigation demonstrated that the 2-thiouracil modification and the 2'-amino-2'-deoxy modification enhanced both $P2Y_2$ receptor affinity and selectivity. Therefore, we combined these modifications, which resulted in the very potent and selective $P2Y_2$ receptor agonist 2'-amino-2'-deoxy-2-thiouridine 5'-triphosphate (**4.2**, $EC_{50}$ ($P2Y_2$) = 8 nM) (chapter 4).

UTP and its diverse synthesized analogues are sensitive to enzymatic degradation. Replacement of the α-phosphate group by an isosteric phosphonomethyl group should enhance the metabolic stability. However, the resulting uridine 5'-phosphonodiphosphate (**4.7**) suffered from complete chemical hydrolysis to the corresponding 5'-phosphonate (**4.50**) in the buffer storage solution (chapter 4).

In summary, in this thesis we succeeded to identify new selective ligands for both envisaged receptor subtypes: the $A_3$A and the $P2Y_2$ receptors.

# Samenvatting

P1-receptoren of adenosine receptoren en $P2Y_2$ receptoren zijn G-proteïne gekoppelde receptoren die behoren tot de familie van purinerge receptoren. De P1 receptoren herkennen adenosine als natuurlijke agonist, terwijl de P2Y receptoren geactiveerd worden door de nucleotiden ATP, ADP, UTP of UDP. Tot de adenosine receptoren (P1) behoren 4 receptor subtypes: $A_1$, $A_{2A}$, $A_{2B}$ en $A_3$. De P2Y receptoren worden onderverdeeld in 8 subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$. In het eerste deel van dit werk (hoofdstuk 2 en 3) wordt de synthese en de biologische evalutatie van een aantal liganden voor de adenosine $A_3$ receptor voorgesteld. In het tweede deel (hoofdstuk 4), wordt de synthese en de biologische evalutatie van nieuwe $P2Y_2$ receptor liganden besproken.

Adenosine receptoren zijn niet enkel verantwoordelijk voor het effect van caffeïne, de meest gebruikte drug ter wereld, het zijn eveneens beloftevolle doelwitproteïnen voor een brede waaier van therapeutische toepassingen, zoals de behandeling van cardiovasculaire, inflammatoire en neurodegeneratieve aandoeningen. Hun ruime therapeutische mogelijkheden en de wijdverspreide aanwezigheid van de verschillende AR subtypes in ons lichaam zorgen ervoor dat gesynthetiseerde liganden subtype- en weefselselectief moeten zijn om therapeutisch bruikbaar te zijn. Modificatie van de basistructuur van het natuurlijke, niet-selectieve ligand adenosine kan leiden tot $A_3$AR selectieve liganden. Vroeger onderzoek leerde dat introductie van een 3'-amino functie zowel de selectiviteit voor de $A_3$AR, als de wateroplosbaarheid ten goede komt. Deze 3'-modificatie verminderde echter de affintiteit voor de receptor. Om te onderzoeken of extra modificaties dit affiniteitsverlies konden compenseren, synthetiseerden we een aantal hypergemodificeerde adenosine derivaten (hoofstuk 2). Invoering van een 2-fenylethynyl substituent, in combinatie met een $N^6$-methyl groep bleek in staat de affiniteitsdaling veroorzaakt door de 3'-amino modificatie te herstellen.

Op basis van de goede resultaten bekomen met de 2-fenylethynyl modificatie, zowel wat affiniteit als selectiviteit voor de $A_3$AR betreft, werd besloten de 2-positie verder te exploreren. Hiervoor werd via Huisgen [3+2]cycloadditie een reeks 4-gesubstitueerde 2-(1,2,3-triazol-1-yl) $N^6$-methyl adenosine analogen gesynthetiseerd (hoofdstuk 3). Gecombineerd met een ongemodificeerd suikergedeelte, leverde deze

2-(1,2,3)-triazol-1-yl substitutie zeer potente en selectieve $A_3$AR antagonisten op, zoals bv. 2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl)-*N*$^6$-methyl-9-(β-D-ribofuranosyl)-adenine (**3.10**, $K_i$ ($A_3$AR)= 1.3 nM). Door invoering van een 4'-ethylcarbamoyl groep kon de intrinsieke activiteit voor de $A_3$AR teruggewonnen worden. Deze ribose modificatie resulteerde in een reeks potente en selectieve $A_3$AR agonisten, waarvan 9-(5-ethylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyl-2-(4-pyridin-2-yl-1,2,3-triazol-1-yl) het meest potente derivaat was (**3.17b**, $K_i$ ($A_3$AR) = 1.8nM).

$P2Y_2$ agonisten zijn potentiële geneesmiddelen voor de behandeling van mucoviscidose, kanker en het droge ogen-syndroom. $P2Y_2$ antagonisten zijn anti-inflammatoir, neuroprotectief en worden voorgesteld voor de behandeling van coronaire vasospasmen. We onderzochten of modificaties van het natuurlijke, niet selectieve $P2Y_2$ receptor ligand uridine 5'-trifosfaat (UTP) aanleiding kan geven tot meer selectieve $P2Y_2$ receptor liganden. Vroeger onderzoek toonde aan dat de 2-thiouracil modificatie en de 2'-deoxy-2'-amino ribose modificatie elk afzonderlijk de affiniteit en selectiviteit voor de $P2Y_2$ receptor verhoogden. Daarom kozen we voor de combinatie van beide modificaties, wat aanleiding gaf tot de zeer potente en selectieve $P2Y_2$ receptor agonist 2'-amino-2'-deoxy-2-thiouridine 5'-trifosfaat (**4.2**, $EC_{50}$ ($P2Y_2$) = 8 nM) (hoofdstuk 4).

UTP, alsook de diverse reeds gesynthetiseerde UTP analogen zijn onderhevig aan enzymatische degradatie. Vervanging van de α-fosfaat groep door een isostere fosfonomethylgroep werd uitgevoerd om de metabole stabiliteit op te drijven. Het resulterende uridine 5'-fosfonodifosfaat (**4.7**) onderging echter complete chemische hydrolyse tot het overeenkomstige 5'-fosfonaat (**4.50**) in de bufferoplossing voor bewaring (hoofdstuk 4).

Samengevat: tijdens dit onderzoek slaagden we erin om nieuwe selectieve liganden te identificeren voor beide beoogde receptor-subtypes: de $A_3$A en de $P2Y_2$ receptoren.

# List of abbreviations

| | |
|---|---|
| AB-MECA | $N^6$-(4-amino-3-iodobenzyl)-5'-*N*-methylcarbamoyladenosine |
| ADP | adenosine 5'-diphosphate |
| AMP | adenosine 5'-monophosphate |
| AR | adenosine receptor |
| ATP | adenosine 5'-triphosphate |
| BAIB | [bis(acetoxy)iodo]benzene |
| BOM | benzyloxy methyl |
| *t*-BuOH | *tert*-butanol |
| BSA | *N*,*O*-bis(trimethylsilyl)acetamide |
| CaCC | $Ca^{2+}$ regulated channels |
| cAMP | cyclic adenosine monophosphate |
| CCPA | 2-chloro-$N^6$-cyclopentyladenosine |
| CGS 21680 | 2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-*N*-ethylcarbamoyladenosine |
| CF | cystic fibrosis |
| CFTR | cystic fibrosis transmembrane regulator |
| CHO | Chinese hamster ovary |
| Cl-IB-MECA | 2-chloro-$N^6$-(2-iodobenzyl)- 5'-*N*-methylcarbamoyladenosine |
| COSY | COrrelation SpectroscopY |
| DABCO | 1,4-diazabicyclo[2.2.2]octane |
| DBU | 1,8-diazabicyclo[5.4.0]undec-7-ene |
| DEAE | diethylaminoethyl |
| DIEA | *N*,*N*-diisopropylethylamine |
| DMAP | 4-dimethylaminopyridine |
| DMF | dimethyl formamide |
| DMPA | $N^6$-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine |
| DMSO | dimethyl sulfoxide |
| $EC_{50}$ | half maximal effective concentration |
| EDC | 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride |
| EL | extracellular loop |
| ENaC | epithelial $Na^+$ channel |
| $Et_3N$ | triethylamine |
| EtOH | ethanol |
| FAD | flavin adenine dinucleotide |
| GPCR | G-protein-coupled receptor |
| HMDS | 1,1,1,3,3,3-hexamethyldisilazane |
| HSQC | Heteronuclear Single Quantum Coherence |
| HRMS | high resolution mass |

| | |
|---|---|
| I-AB-MECA | $N^6$-(4-amino-3-iodobenzyl)-2-iodo-5'-*N*-methylcarbamoyladenosine |
| IBX | 2-iodoxybenzoic acid |
| $IC_{50}$ | half maximal inhibitory concentration |
| IL | intracellular loop |
| $IP_3$ | (1,4,5)-triphosphate |
| $K_i$ | affinity constant |
| LR | Lawesson reagent |
| MCMM | Monte Carlo Multiple Minimum |
| MD | molecular dynamics |
| MECA | 5'-*N*-methylcarbamoyladenosine |
| MeOH | methanol |
| NAD | nicotinamide adenine dinucleotide |
| NECA | 5'-*N*-ethylcarbamoyladenosine |
| NMDA | *N*-methyl-D-aspartic acid |
| NT | N-terminal |
| NMR | nuclear magnetic resonance |
| *i*PrOH | isopropanol |
| $PPh_3$ | triphenylphosphine |
| $(PPh_3)_2PdCl_2$ | bis(triphenylphosphine)palladium(II) dichloride |
| PLC | phospholipase C |
| RB-2 | reactive blue 2 |
| SAR | structure-activity-relationship |
| sat. aq. | saturated aqueous |
| TBAP | tetrabutylammonium dihydrogenphosphate |
| TBDMSCl | *tert*-butyl dimethyl silyl chloride |
| TCDI | 1,1-thiocarbodiimidazole |
| TEAA | triethylammonium acetate |
| TEAB | tetraethylammonium bicarbonate |
| TFA | trifluoro acetic acid |
| THF | tetrahydrofuran |
| TLC | thin layer chromatography |
| $TIPDSCl_2$ | 1,3-dichloro 1,1,3,3,-tetraisopropyldisiloxane |
| TM | transmembrane |
| TMSBr | trimethylsilyl bromide |
| TMSOTf | trimethylsilyl trifluoromethanesulfonate |
| TNF$\alpha$ | tumor necrosis factor $\alpha$ |
| UDP | uridine 5'-diphosphate |
| UTP | uridine 5'-triphosphate |

# CHAPTER 1:

# INTRODUCTION

# 1 INTRODUCTION

## 1.1 Purinergic Receptors

Extracellular purines [e.g., adenosine, adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP)] and pyrimidines [e.g., uridine 5'-diphosphate (UDP) and uridine 5'-triphosphate (UTP)] are important signalling molecules that mediate diverse biological effects via cell-surface receptors termed purinergic receptors. These receptors were first formally recognized by Burnstock in 1978.[1] He proposed that these receptors can be divided into two classes termed 'P1-purinoceptors', recognizing adenosine as principal natural ligand, and 'P2-purinoceptors', which are activated by ATP and ADP. Although receptor charactarisation is now based on distinct molecular structures of the receptors, this major P1/P2 division remains a fundamental part of purine receptor classification. In addition, receptors for pyrimidines are included within the P2 receptor family.[2]

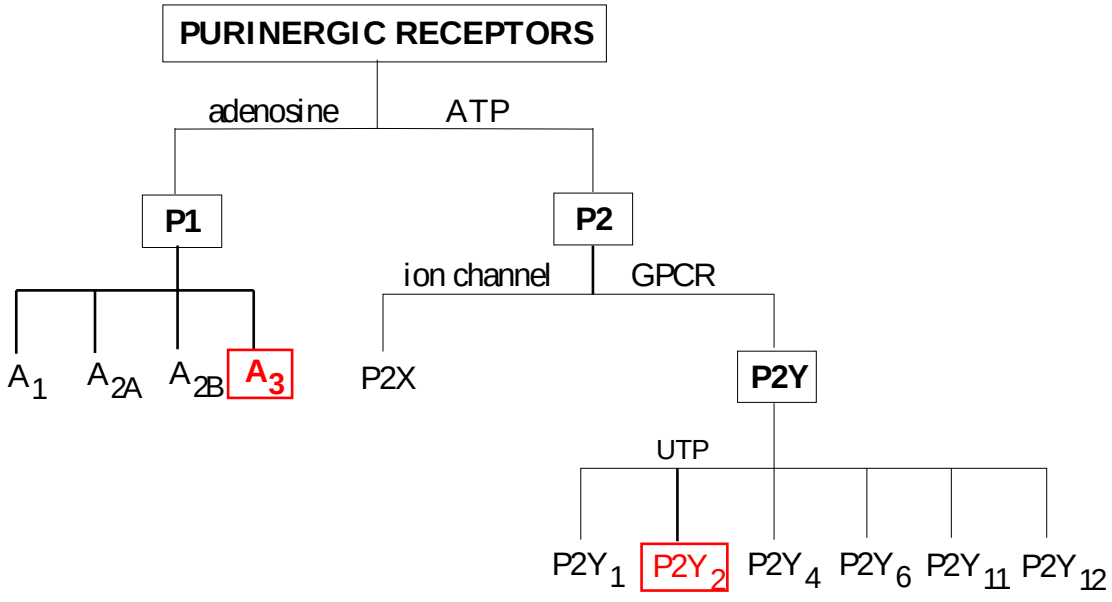



**Figure 1.1**

Classification of the purinergic receptors

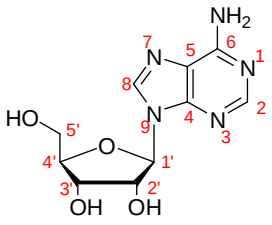
The P1 and P2 receptor families (Figure 1.1) are both further subdivided according to convergent molecular, biochemical, and pharmacological evidence. The P1 receptors

or adenosine receptors consist of 4 subtypes classified as $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. P2 receptors have broad natural ligand specificity, recognizing ATP, ADP, UTP, UDP, and some dinucleotides. The P2 receptors are divided naturally into two separate classes, based on whether they are ligand-gated ion channels[3] (P2X receptors[4,5]) or coupled to G-proteins[6] (P2Y receptors[4,5]). Seven mammalian P2X receptors have been cloned ($P2X_1$-$P2X_7$).[7] At least seven different P2Y subtypes are observed, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$, all of which have been cloned.[8] The recently cloned UDP-glucose (UDPG) receptor appears to belong to the same family and is designated as the $P2Y_{14}$ receptor.[9] In this thesis the synthesis and biological evaluation of a series of $A_3$ adenosine receptor ligands and $P2Y_2$ receptor ligands will be discussed.

## 1.2 Adenosine Analogues and the Adenosine $A_3$ Receptor

### 1.2.1 Adenosine

Adenosine (**1.1**) is a nucleoside that consists of a sugar part, D-ribofuranose, which is linked to the purine base adenine via a β-glycosidic bond. Adenosine is metabolically and structurally related to the bioactive nucleotides adenosine 5'-monophosphate (AMP), ADP, ATP, and cyclic adenosine monophosphate (cAMP) and to the biochemical methylating agent *S*-adenosyl-L-methionine (SAM). The structures of ribonucleic acid (RNA) and the coenzymes nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) and coenzyme A are also derived from adenosine.




**1.1**

**Figure 1.2**

Structure of Adenosine (**1.1**)

Adenosine is continuously formed intracellulary as well as extracellulary (Figure 1.3).

Intracellular adenosine is formed by

- dephosphorylation of AMP mediated by 5'-endonucleotidases,[10, 11] which catalyse the enzymatic breakdown of intracellular ATP to adenosine
- hydrolysis of *S*-adenosyl-homocysteine.[12]

Extracellulary, adenosine formation

- by dephosphorylation of ATP is catalysed by 5'-ectonucleotidases.[13]
- from cAMP, released from neurons, occurs through its conversion to AMP by extracellular phosphodiesterases, followed by 5'-ectonucleotidase catalyzed AMP dephosphorylation.[14]
- can be increased by *N*-methyl-D-aspartic acid (NMDA) receptor stimulation and by activation of the dopamine $D_1$ receptors via a NMDA receptor dependent mechanism.[15]
- can also be controlled by nitric oxide.[16]

When extracellular adenosine levels are high, adenosine is deaminated by adenosine deaminase to inosine or transported into the cells either by active transport or by facilitated diffusion.

Intracellular adenosine is either deaminated to inosine or phosphorylated to AMP by adenosine kinase (Figure 1.3).[17]

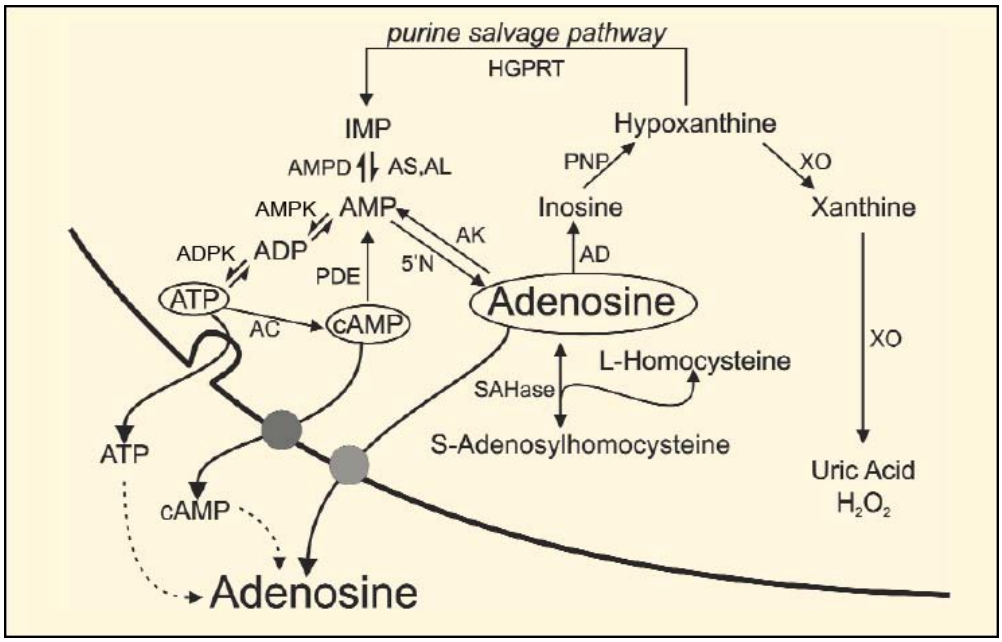



**Figure 1.3**

Intracellular and extracellular formation of adenosine and adenosine metabolism.[18] AC = adenylate cyclase; AMPD: adenosine monophosphate deaminase; AK = adenosine kinase, AMPK = adenosine monophosphate kinase; ADPK = adenosine diphosphate kinase; AL = adenylosuccinate lyase; AS = adenylosuccinate synthase; HGPRT = hypoxanthine guanine phosphoribosyl transferase; 5'N = 5'-endonucleotidase; PDE = phosphodiesterase; PNP = purine nucleoside phosphorylase; SAHase = S-adenosylhomocysteinase; XO = xanthine oxidase

### 1.2.2 The Adenosine Receptors: G-protein-Coupled Receptors

G-protein-coupled receptors (GPCRs) constitute a large family of heptahelical, integral membrane proteins that mediate a wide variety of physiological processes, ranging from the transmission of light and odorant signals to the mediation of neurotransmission and hormonal action.[19] Based on amino acid sequence similarity, the superfamily of GPCRs can be subdivided into five main families of receptors, named the glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin family (GRAFS classification). The adenosine receptors, as well as the majority of GPCRs identified to date, belong to the rhodopsin family (family A), which is also the best studied family, both structurally and functionally. The rhodopsin family consists of four main groups with 13 subbranches.[20]

The different GPCR families are characterized by seven transmembrane (TM) domains of hydrophobic amino acids, constituting an $\alpha$-helix of 21 to 28 amino acids. The N-terminal of the protein lies on the extracellular side and the C-terminal on the cytoplasmic side of the membrane. The TM domains are connected by three extracellular (EL1, EL2 and EL3) and three cytoplasmic hydrophilic loops (IL1, IL2 and IL3). Two cysteine residues, one in TM3 and one in EL3, which are conserved in most GPCRs, form an essential disulfide link responsible for the packing and stabilization of a restricted number of conformations of these seven TM domains. A pocket for the ligand binding site is formed by the three-dimensional arrangement of the $\alpha$-helical TM domains, and in the rhodopsin family the ligand is believed to bind with the upper half of this pore.[2] An experimentally determined 3D-structure is still lacking for most GPCRs, due to the technical difficulties regarding X-ray crystallography and NMR experiments on membrane associated receptors. The best known GPCR structure, the high resolution X-ray structure of bovine rhodopsin has proven to be a suitable template for the resting state (resembling an antagonist-like state) of most family A GPCRs, including the $A_3$AR. However, it is questionable whether this rhodopsin structure also represents an appropriate template for the active state (agonist-like state).[21,22]

Recently, Stevens et al.[23] reported the crystal structure of a human $\beta_2$-adrenergic receptor–T4 lysozyme fusion protein bound to the partial inverse agonist carazolol at

2.4 Å resolution. The engineered $\beta_2$-adrenergic receptor included lysozyme in place of one of the intracellular loops, which reduced conformational heterogeneity and facilitated crystal nucleation. Although the location of carazolol in the $\beta_2$-adrenergic receptor is very similar to that of retinal in rhodopsin, structural differences in the ligand binding site and other regions highlight the challenges in using rhodopsin as a template model for the large GPCR receptor family.

G proteins initiate the receptor signalling cascade, responsible for the receptor mediated effect. There are two main classes of G proteins: heterotrimeric G proteins and small cytoplasmic G proteins. Heterotrimeric G proteins associate with G protein coupled receptors (GPCRs) and consist of an $\alpha$-subunit and a tightly associated $\beta\gamma$-complex (Figure 1.4). In the inactive state, the $\alpha$-subunit is associated with the $\beta\gamma$-complex and guanosine diphosphate (GDP) is bound to the $\alpha$-subunit. Agonist binding on the GPCR results in coupling of the receptor to one or more G proteins followed by exchange of GDP for guanosine triphosphate (GTP) on the $\alpha$-subunit. This results in conformational changes and subsequent dissociation of the subunits. Both the $\alpha$-GTP and $\beta\gamma$ subunits interact with downstream effectors and regulate their activity. The intrinsic GTP hydrolase activity of the $\alpha$-subunit returns the protein to the GDP-bound state and thereby restores the association of the subunits. $G_\alpha$ subunits are commonly classified into four subfamilies based on their amino acid sequence and function: $G_s$, $G_i$, $G_q$ and $G_{12}$ family.[22,24]

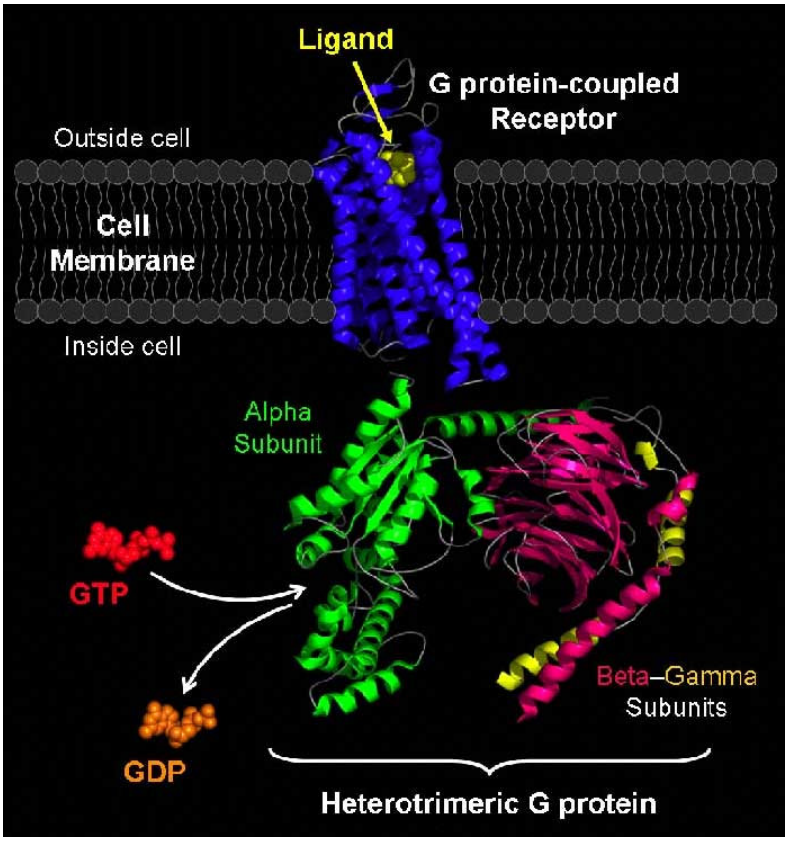



**Figure 1.4**

Structure of a GPCR and its coupled heterotrimeric G-protein[25]

### 1.2.3 Adenosine Receptor Subtypes and Their Signalling

Adenosine receptors (AR) consist of four subtypes classified as $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. Receptors from each of these four distinct subtypes have been cloned from a variety of species and characterized following functional expression in mammalian cells or *Xenopus* oocytes.[2]

The adenosine $A_1/A_2$ receptor classification was initially based on their inhibiting and stimulating activity on adenylate cyclase.[26,27] The $A_1$ and $A_2$AR (both $A_{2A}$ and $A_{2B}$AR) are indeed coupled to $G_i$ (inhibiting) and $G_s$ (stimulating) proteins, respectively. The $A_3$AR is the most recently identified adenosine receptor[28] and is also $G_i$ protein-coupled. As further described, the adenosine receptor signalling pathways also include other G proteins. After the activation of the G proteins, enzymes and ion channels are affected and the resulting signalling cascade produces the specific receptor-mediated effect (Figure 1.5).

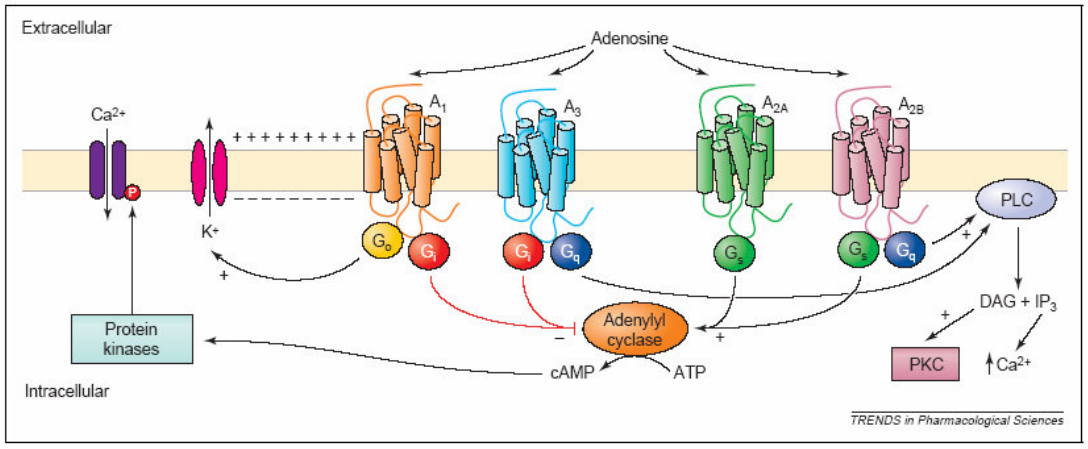



**Figure 1.5**

Signal transduction pathways associated with the human adenosine receptors[22]

Activation of $A_1$ receptors by adenosine inhibits adenylate cyclase activity through activation of pertussis toxin-sensitive $G_i$ proteins.[27] In cardiac muscles and neurons, $A_1$AR activation leads to activation of the $G_0$ family, which leads directly to the stabilisation of the neuronal membrane potential by activating the $K^+$ efflux from inside to outside the cell.[22] Coupling to $K^+$ channels in supraventricular tissue is responsible for the brachycardic effect of adenosine on heart function.[29] Activation of

the $A_1$AR results also in increased activity of phospholipase C (PLC)[30,31] and in inhibiting Q, P and N-type $Ca^{2+}$ channels.[5]

$A_{2A}$ARs seem to be mainly associated with $G_s$ proteins whereby $A_{2A}$AR activation increases adenylate cyclase activity. $A_{2A}$AR stimulation also induces formation of inositol phosphates under certain circumstances.[32] In the heart, $A_1$AR and $A_{2A}$AR agonist induced preconditioning has been suggested to occur via modulation of p44/42 extracellular signal-regulated protein kinase (ERK) signalling.[33]

The $A_{2B}$AR is positively coupled to both adenylate cyclase and PLC. The PLC activation, through $G_q$ proteins, possibly mediates many of the important $A_{2B}$AR functions.[34]

$A_3$ receptors couple negatively to adenylate cyclase through $G_{i2,3}$, and couple to $G_{q/11}$ family, leading to stimulation of PLC.[28,35] In cardiac cells, $A_3$AR agonists induce protection through the activation of $K_{ATP}$ channels.[36] $A_3$AR stimulation can also lead to activation of phospholipase D.

PLC catalyses the formation of diacylglycerol (DAG) and inositol (1,4,5)-triphosphate ($IP_3$) from phosphatidylinositol-4-5-biphosphate ($PIP_2$). DAG is implicated in the regulation of protein kinase C activity and $IP_3$ increases the intracellular concentration of $Ca^{2+}$ ions. In the brain, by inhibiting neuronal $Ca^{2+}$ influx (through inhibition of adenylate cyclase), adenosine counteracts the presynaptic release of the potentially excitotoxic neurotransmitters glutamate and aspartate, which can impair intracellular $Ca^{2+}$ homeostasis.[22]

## 1.2.4 The Adenosine $A_3$ Receptor

The adenosine $A_3$ receptor is the most recently identified adenosine receptor and was first cloned, expressed and functionally characterised from rat striatum by Zhou et al. in 1992.[28] The receptor was previously isolated from a rat testis cDNA library by Meyerhof et al.,[37] but without ligand identification. Homologs of the rat striatal $A_3$AR have been cloned from sheep part tuberalis (pituitary tissue),[38] human heart[39] and striatum.[40] The interspecies differences in $A_3$ receptor structure are large. There is only 74% homology of the rat $A_3$ receptor with both the sheep and human $A_3$ receptor. Sheep and human $A_3$ receptors show 85% homology. This is reflected in the very different pharmacological profiles of the species homologs, particulary with respect to antagonist binding.

### 1.2.4.1 Adenosine $A_3$ Receptor Agonists

Adenosine itself is a useful therapeutic agent when a short acting response is sufficient to achieve the desired tissue state. It is used clinically in the treatment of paroxysmal supraventricular tachycardia (Adenocard®).[17] The adverse effects are rapidly self-limiting because the half-life of adenosine in blood after peripheral intravenous injection is only 10 seconds. Adenosine is translocated by nucleoside transporters and degraded by adenosine deaminase located on the extracellular surface of endothelial cells of small coronary arteries.[41] The main approach for the discovery of more stable and more selective AR agonists has been modification of adenosine. Most of the useful analogues are modified in the $N^6$- or 2-position of the adenine moiety and in the 3'-, 4'- or 5'- position of the ribose moiety. A summary of the performed adenosine modifications is given in Figure 1.6

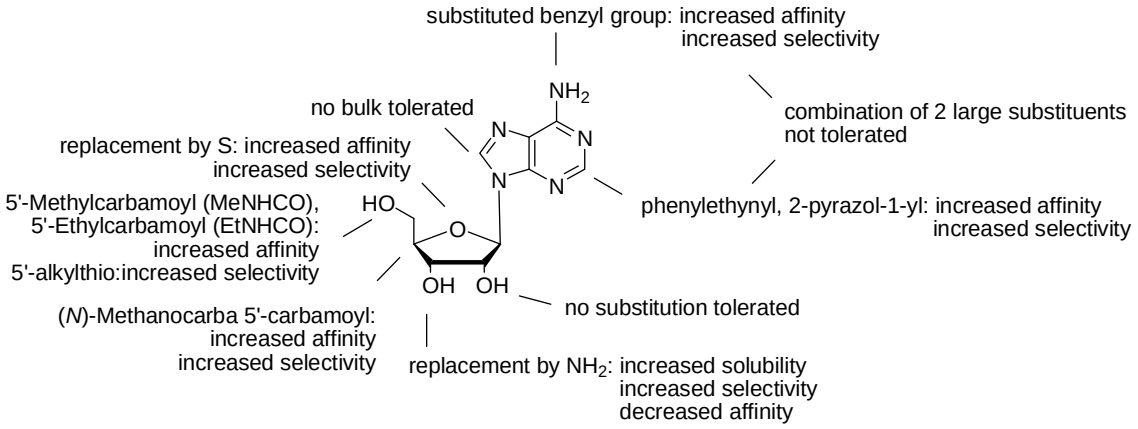



**Figure 1.6**

Summary of the adenosine modifications, resulting in $A_3$AR agonists

## Ribose Modifications

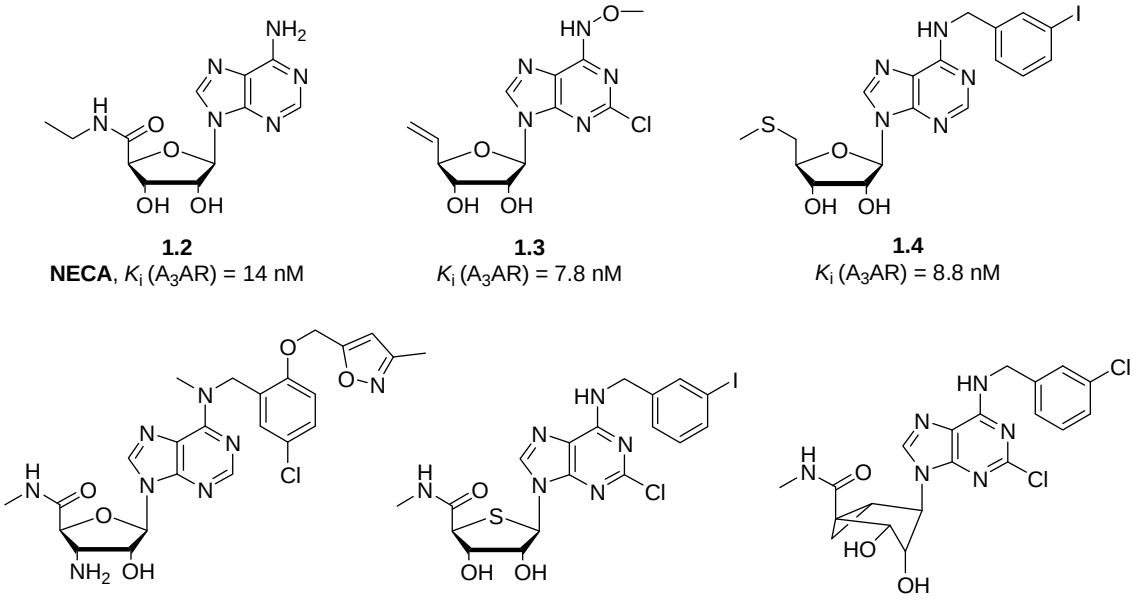
The most potent adenosine agonists with modified ribose moiety are depicted in Figure 1.7. 5'-Methyl- and 5'-ethylcarbamoyl modifications usually lead to an increase in $A_3$AR affinity.[42] For example, 5'-*N*-ethylcarbamoyladenosine (NECA, **1.2**), a non-selective AR agonist, which was the first high-affinity ligand for the $A_{2A}$AR ($K_i$ = 20 nM), displayed even higher affinity for the $A_3$AR ($K_i$ = 14 nM).[43] 5'-*N*-propylcarbamoyladenosine showed good $A_3$AR affinity and selectivity ($K_i$ = 44 nM).[44] *N*-cyclopentyl-carbamoyl substitution reduced the $A_3$AR affinity,[42] suggesting there is only limited space in the ribose binding domain of the $A_3$ adenosine receptor.

Bioisosteric replacement of the alkylcarbamoyl moiety by a vinyl group in combination with $N^6$-methoxy adenine substitution resulted in the highly active and selective $A_3$AR ligand **1.3**.[45] 5'-Alkylthio substitution led to an increase in $A_3$ selectivity. 5'-alkylthioadenosine derivatives with $N^6$-benzyl substituents showed high $A_3$AR affinity and partial agonist activity, with 5'-methylthio analogue **1.4** as the best analogue in this series ($K_i$ = 8.8 nM).[46]

Substitution of the 5'-hydroxylgroup by a chlorine atom is tolerated by $A_1$ as well as $A_3$ARs,[45] while 2'- and 3'- hydroxyl groups are generally required for affinity and/or ability to fully activate the receptor.[47] Replacement of the 3'-hydroxyl with 3'-amino caused decreased $A_3$AR affinity, but improved the $A_3$AR selectivity and water solubility. By combining 3'-amino modification with large the $N^6$-substituents as in **1.5** the $A_3$AR affinity could be restored.[48]

Replacement of the ribose furane ring by the corresponding tetrahydrothiophene ring yielded several highly potent and selective $A_3$AR agonists, such as **1.6** ($K_i$ = 0.38 nM).[49] Conformational studies of the ribose moiety and its equivalents indicated that the ring oxygen is not required. However, replacement of the ribose furane ring by a cyclopentane ring resulted in a large decrease in AR affinity.[50] That the North (N) ring conformation is preferred in binding to the $A_3$AR is illustrated by the highly potent and selective $A_3$AR agonists recently reported in the series of (*N*)-methanocarba-5′-uronamide derivatives (**1.7**, $K_i$ = 0.29 nM).[51]

In addition to its beneficial effect on the $A_3$AR affinity, 5'-modification of the ribose moiety also increases metabolic stability against adenosine deaminase, because the 5'-hydroxyl group is essential for binding to the enzyme.[52]



**1.2**
**NECA**, $K_i$ ($A_3$AR) = 14 nM

**1.3**
$K_i$ ($A_3$AR) = 7.8 nM

**1.4**
$K_i$ ($A_3$AR) = 8.8 nM

**1.5**
$K_i$ ($A_3$AR) = 5.8 nM

**1.6**
$K_i$ ($A_3$AR) = 0.38 nM

**1.7**
$K_i$ ($A_3$AR) = 0.29 nM

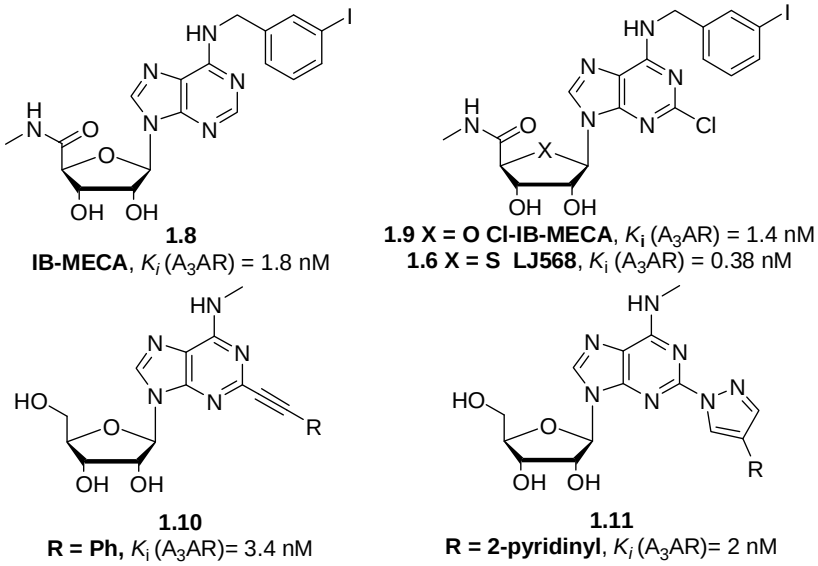
**Figure 1.7**

Examples of the most potent ribose-modified adenosine $A_3$ agonists

## Adenine Modifications

Figure 1.8 includes the most potent adenosine agonists with modified adenine moiety. Large $N^6$-substituents, such as substituted benzyl groups, tend to enhance the $A_3$AR selectivity, but reduce the maximal efficacy resulting in partial agonists. The reduction of efficacy can be overcome by the combination of the large $N^6$-substitution with 5'-alkylcarbamoyl ribose modification. The preferred $N^6$-benzyl substituent turned out to be a meta-iodine. Only small substituents at C-2, such as 2-Cl and 2-I, are tolerated in combination with $N^6$-iodobenzyl. IB-MECA (**1.8**) and the more $A_3$AR selective Cl-IB-MECA (**1.9**) are very potent $A_3$AR agonists containing a $N^6$-iodobenzyl modification. These compounds have been widely used as pharmacological probes in the elucidation of the physiological role of the $A_3$AR.[53] Introduction of a small $N^6$-substituent, such as a methyl group, and a large 2-substituent resulted in very potent and selective $A_3$AR ligands. Examples of large 2-substituents exhibiting very good $A_3$AR affinity and selectivity are 2-phenylethynyl[54] (**1.10**) and some 4-substituted 2-pyrazol-1-yl derivatives[55] (**1.11**). Combination of both large $N^6$- and 2-substitution does not improve the $A_3$AR affinity.[54,55]

Substitution at the 8-position of the ring is not well tolerated by any AR subtype.[50,56] Evaluation of the appropriate deaza analogues indicated that the nitrogen atoms at positions 3 and 7 are required for high affinity of adenosine at all subtypes. [50,56,57]




**1.8**
**IB-MECA**, $K_i$ ($A_3$AR) = 1.8 nM

**1.9 X = O Cl-IB-MECA**, $K_i$ ($A_3$AR) = 1.4 nM
**1.6 X = S LJ568**, $K_i$ ($A_3$AR) = 0.38 nM

**1.10**
**R = Ph,** $K_i$ ($A_3$AR)= 3.4 nM

**1.11**
**R = 2-pyridinyl**, $K_i$ ($A_3$AR)= 2 nM

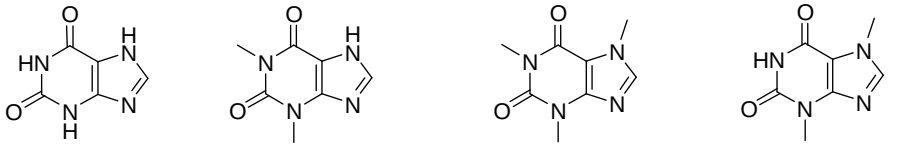
**Figure 1.8**

Examples of potent adenine-modified adenosine agonists

### 1.2.4.2 Adenosine $A_3$ Receptor Antagonists

**Purines**

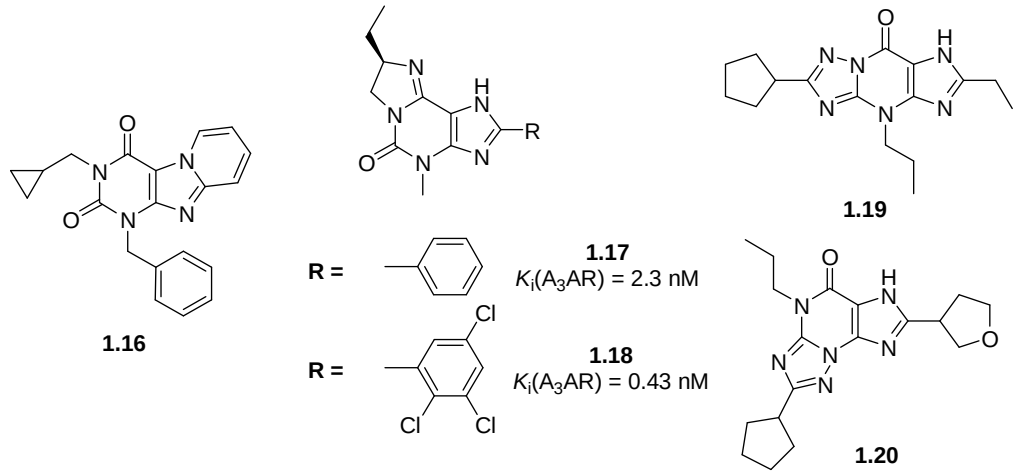
The discovery of $A_3$AR antagonists started with the investigation of xanthines, such as caffeine and theophylline (Figure 1.9). These classical $A_1$, $A_{2A}$ and $A_{2B}$ adenosine receptor antagonists exhibited low $A_3$AR binding affinities. [28]



**1.12 xanthine** **1.13 caffeine** **1.14 theophylline** **1.15 theobromine**

**Figure 1.9**

A series of synthetical xanthine derivatives is depicted in Figure 1.10. Cyclization between the 7- and 8-position, resulted in $A_3$AR selective pyridopurine-2,4-dione derivatives such as **1.16**.[58] Exploration of 2-phenylimidazolepurin-5-ones as water-soluble xanthine derivatives yielded the highly potent human $A_3$AR selective PSB-11 (**1.17**, $K_i$ = 2.3 nM), that was tritiated for characterization of this receptor.[59] The corresponding 2,3,5-trichloro derivative PSB-10 (**1.18**) even showed higher affinity and selectivity for the $A_3$AR ($K_i$ = 0.43 nM). Several classes of extended xanthine structures (e.g., **1.19** and **1.20**) were reported as $A_3$AR antagonists. [60, 61]

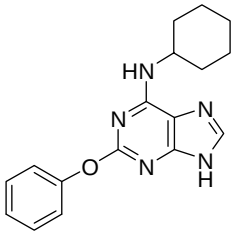



**1.16** **1.19** **1.20**

**Figure 1.10**

Synthetical xanthine derivatives

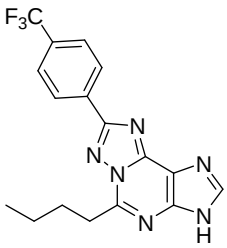
Numerous adenine derivatives have been studied as selective antagonists for $A_1$ or $A_{2A}$ARs, but the adenine derivative **1.21** (Figure 1.11) was reported to be highly selective for the human $A_3$AR.[62]



**1.21**
$K_i$ ($A_3$AR) = 47 nM

**Figure 1.11**

Triazolopurines have been reported as $A_3$ antagonists that are applied topically to the eye for use in the treatment of glaucoma. Compound **1.22** (Figure 1.12) displayed a $K_i$ = 0.61 nM at the human $A_3$AR and >10,000-fold selectivity in comparison to the three other subtypes.[63,64]



**1.22**
$K_i$ ($A_3$AR)=0.61 nM

**Figure 1.12**

## Nonpurines

The most potent nonpurine $A_3$AR antagonists are represented in Figure 1.13 and Figure 1.14. The triazoloquinazoline derivative CGS15943 (**1.23**), previously known as a nonselective adenosine antagonist at the human subtypes, served as template for designed related nonpurine heterocyclic antagonists of the human $A_3$AR. Acylation of the $N^5$-amino group led to **1.24**, which is potent and selective for human, but not rat $A_3$ARs. [65] In a related series of heterocyclic derivatives, the pyrazolopyrimidine derivative **1.25** was found to be a highly selective antagonist at the human $A_3$ receptor and was radiolabeled to provide a hydrophobic, useful

radiotracer with a $K_d$ = 0.80 nM.[66] Analogue **1.26** in the same series displayed a $K_i$ of 0.16 nM at the $A_3$AR.[67] A pyridine moiety was introduced to enhance water solubility (**1.27**).[68]

In search for other heterocyclic systems, chemical library screening resulted in the identification of new high-affinity hits for the human $A_3$AR, including flavonoids (**1.28**), pyridines (**1.29**) and 1,4-dihydropyridines (**1.30**), triazoloquinazolines (**1.24**), isoquinolines and quinazolines (**1.31**), pyrazolo-triazolo-pyrimidines (**1.25-1.27**), and various other classes (**1.32**), which were then optimized, typically by substitution of aromatic rings. The dihydropyridine derivative **1.30** and the pyridinylquinazoline **1.31** are both releatively potent $A_3$AR antagonists. The pyridine derivative **1.29** is a selective $A_3$AR antagonist in both rat and human.[69]

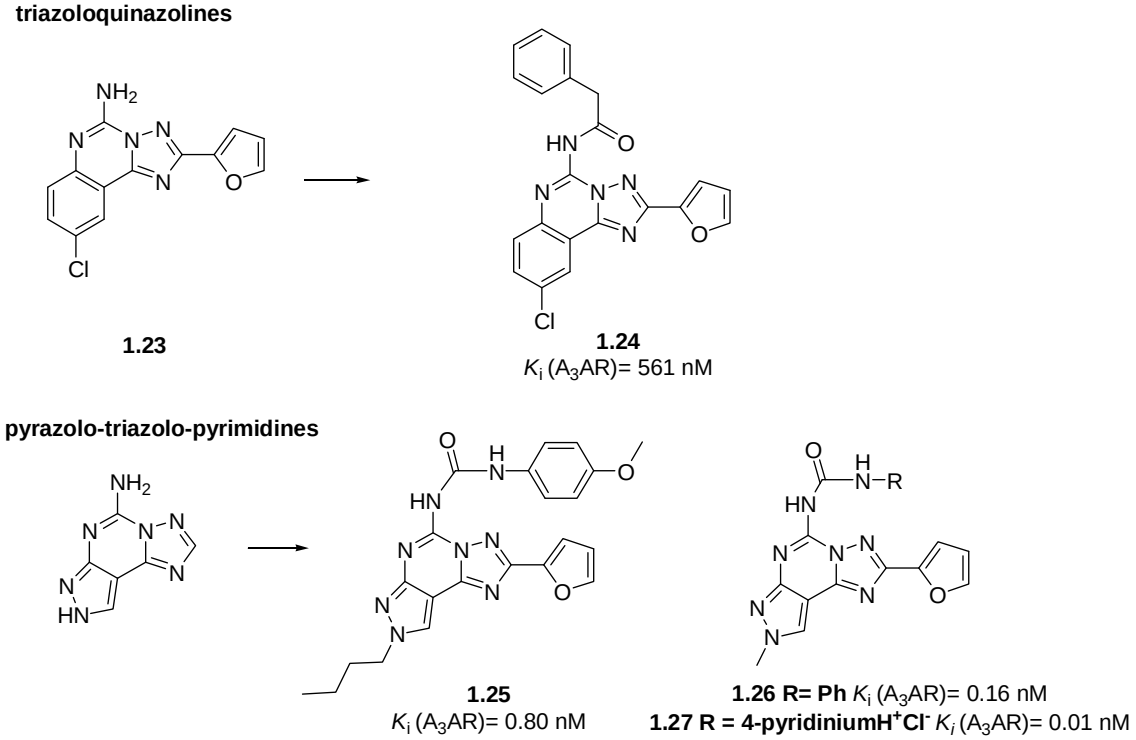



**Figure 1.13**

Triazoloquinazolines and pyrazolo-triazolo-pyrimidines as potent nonpurine $A_3$AR agonists

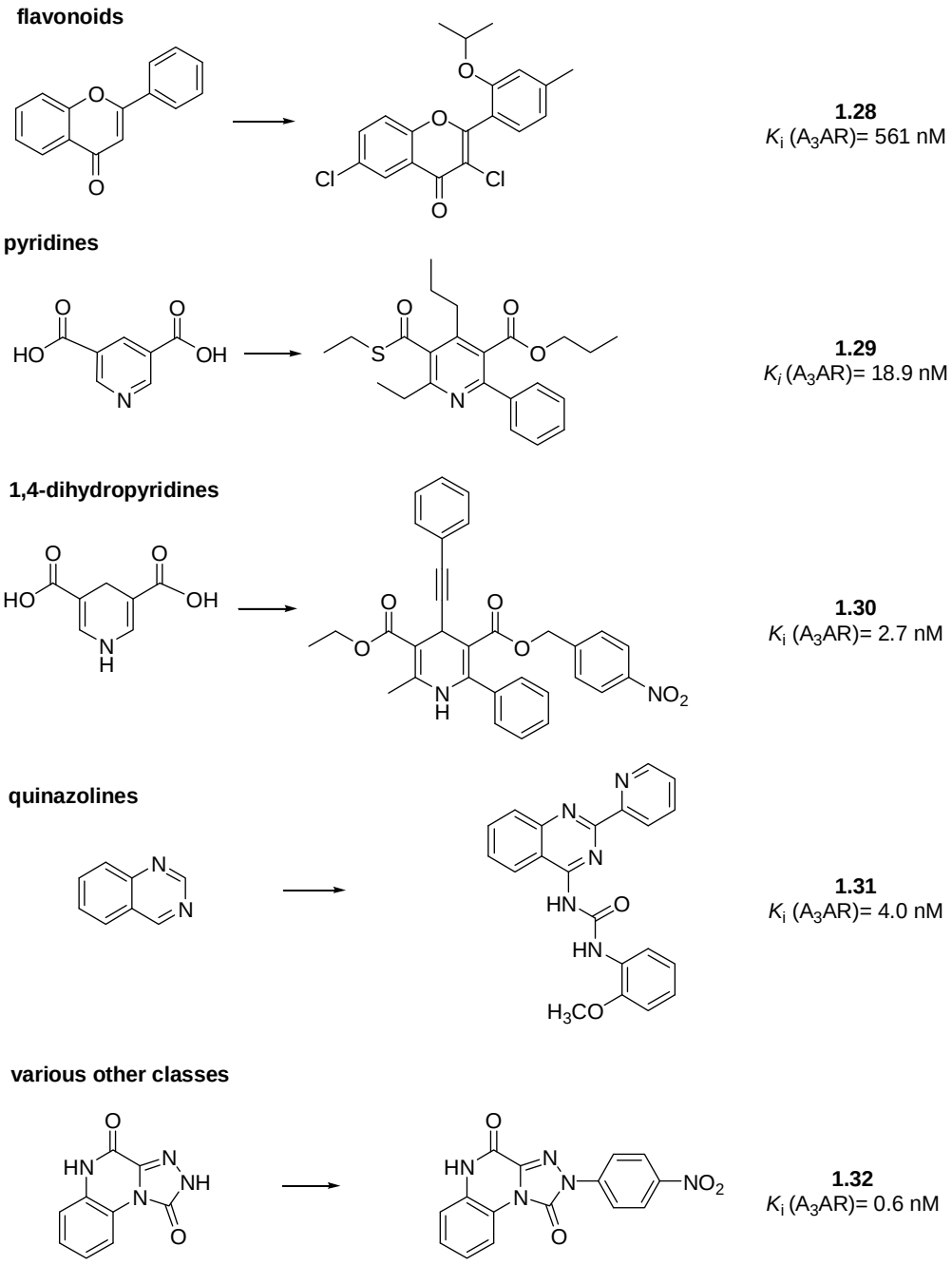



**Figure 1.14**

Summary of various classes nonpurine $A_3$AR antagonists

## Adenosine analogues

Alternatively, antagonists were also obtained starting from high-affinity adenosine derivatives that were modified to remove the capacity to activate the receptor without compromising obtained binding affinity. Substitution or rigidification of adenosine derivatives often reduces their intrinsic affintity.[70] Remarkably, the influence of structural modifications on the receptor efficacy is generally larger at the $A_3$AR than it is at the other AR subtypes.[70, 71] A few examples of adenosine-based $A_3$AR antagonists are depicted in Figure 1.15. The large $N^6$-substituents of 2-chloro-$N^6$-cyclopentyladenosine (CCPA, **1.33**, $K_i$ = 38 nM) and $N^6$-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl)] adenosine (DPMA, **1.34**, $K_i$ = 106 nM) - respectively, full $A_1$ and $A_{2A}$ AR agonists - resulted in $A_3$AR antagonist activity. This structural insight was used advantageously to obtain the conformationally constrained nucleoside **1.35** ($K_i$ = 29.3 nM), which proved to be a selective antagonist for both rat and human $A_3$AR.[70,72] Other adenosine analogues were reported as $A_3$AR antagonists by Cristalli et al.,[73] with a series of 8-alkynyladenosine derivatives that exhibited $A_3$AR selectivity, but suffered from weak $A_3$AR affinity (**1.36**, $K_i$ = 650 nM).

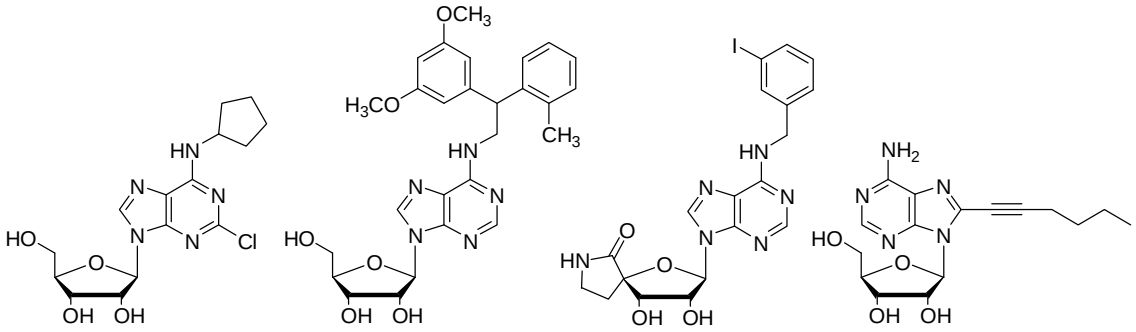
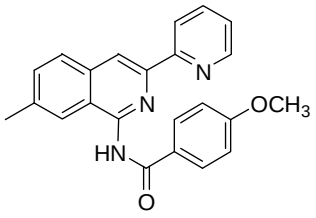



**Figure 1.15**

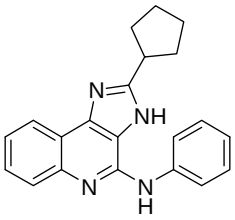
Examples of adenosine-based $A_3$AR antagonists

### 1.2.4.3 Allosteric Modulation

$A_3$AR activity can also be modulated by certain agents that bind to an allosteric site, distinct from adenosine binding site. Pyridinylquinolines (**1.37**) and imidazoloquinolines (**1.38**) were shown to positively modulate $A_3$ binding and/or action of agonists, although both substances retain antagonistic properties as well. Compound **1.37** and **1.38** significantly enhanced the agonist response on forskolin-induced cAMP production and slowed the dissociation of the agonist radioligand [$^{125}$I]I-AB-MECA in a concentration-dependent manner, suggesting an allosteric interaction. The compounds had no effect on the dissociation of the radiolabeled antagonist [$^3$H]PSB-11 (**[$^3$H]1.17**) from the $A_3$ adenosine receptor, suggesting a selective enhancement of agonist binding. [74, 75]




**1.37**




**1.38**

**Figure 1.16**

### 1.2.4.4 Molecular Modeling of the Adenosine $A_3$ Receptor

As we mentioned in 1.2.2, the structure of bovine rhodopsine served as a template to construct a homology model of the $A_3$AR receptor in its resting state (antagonist-like state). This rhodopsine-based $A_3$AR receptor model was recently refined by Moro et al.[22] Similar to rhodopsin, the $A_3$ receptor model reveals a seven-helix bundle with a central cavity surrounded by helices 1-7 and 5-6. The second extracellular loop (EL2), which has been described in bovine rhodopsin to fold back over transmembrane helices, limits the size of the recognition cavity. TM4 is not part of the cavity wall and makes contacts only with TM3. The hairpin (EL2) between TM4 and TM5 lies between the helices, roughly parallel to the membrane surface, and has contacts with side-chains of most of the helices. The most prominent contact is a disulfide bridge to helix 3 (TM3). Ligand recognition seems to occur in the upper region of the TM helical bundle, and TMs 3, 5, 6 and 7 appear to be crucial for the recognition of both agonists and antagonists.

The side-chains of some crucial important residues in proximity (≤ 5Å) to the antagonist binding cavity are shown in Figure 1.17. His95 (TM3) and Ser247 (TM6) seem crucial for the recognition of the antagonist structures. Another strong hydrogen bonding interaction is possible with Asn250 (TM6). This asparagine residue, conserved among all adenosine receptor subtypes, was found to be important for ligand binding. A hydrophobic pocket, delimited by non-polar amino acids, Leu90 (TM3), Leu246 (TM6) and Ile268 (TM7) and a highly conserved region, probably stabilized by $\pi$-$\pi$ interactions, located between Phe168 and Phe182 are important for pharmacophore binding. Another crucial region is mostly hydrophobic and characterized by three non-polar amino acids: Ile98 (TM3), Ile186 (TM5) and Leu244 (TM6).

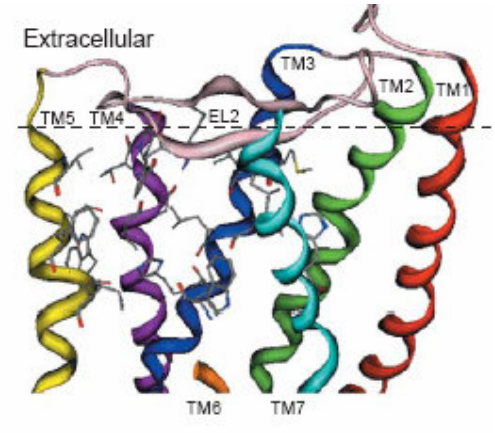


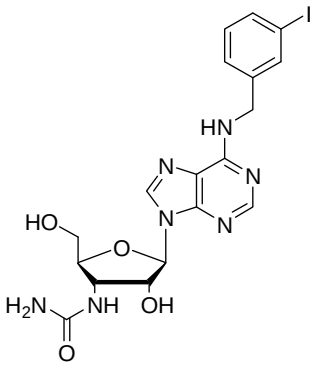

**Figure 1.17**

Rhodopsin-based homology modeling of the human $A_3$AR. The side-chains of some crucial important residues in proximity (≤ 5Å) to the antagonist binding cavity are shown: Leu90 (TM3), Phe185 (TM5), Ile186 (TM5), Trp243 (TM6), Ser247 (TM6), Asn250 (TM6), Ser271 (TM7), His272 (TM7) and Ser275 (TM7).[22]

### 1.2.4.5 The Neoceptor concept

Adenosine receptors are ubiquitously distributed throughout the body, which inherently results in nonselective activation. To adress this issue, efforts have been made to reengineer the GPCRs and their agonists. Rhodopsine-based molecular modeling was used to pinpoint adenosine receptor mutations for selective affinity enhancement, while retaining its capacity for signal transduction. Complementary modifications of adenosine were performed to design novel agonists (neoligands) that activate the reengineered receptor (neoceptor), but are not effective at the native receptor.

The H272E mutant $A_3$ adenosine receptor was found to have decreased affinity for classical ligands, such as NECA and Cl-IB-MECA (20-50-fold), but an enhanced affinity for $N^6$-(3-iodobenzyl)-3'-ureidoadenosine compared to the wild type $A_3$AR (**1.39**, >100-fold).[76]




**1.39**

**Figure 1.18**

The neoceptor-neoligand pairs could be important to validate adenosine receptor agonists docking models. While theoretically the neoceptor concept could be an important therapeutic approach for tissue-specific GPCR activation, given successful targeted delivery of the neoceptor gene to a specific organ or tissue.

### 1.2.4.6 Therapeutic Potential of $A_3$AR Agonists

*Cardiac ischemia*

During myocardial ischemia adenosine is released in large amounts, resulting in protection of the cardiomyocytes. This protection afforded by a brief hypoxic period is termed 'preconditioning' and is mediated through the activation of $A_1$ and $A_3$ ARs.[77,78,79,80] The protection mediated by prior activation of $A_3$ receptors exhibits a significantly longer duration than that produced by activation of the adenosine $A_1$ receptor. The $A_3$ receptor-mediated protection persisted for at least 45 min after the initial exposure to the $A_3$ receptor agonist while the $A_1$ receptor-mediated effect dissipated within 30 minutes.[81] $A_3$AR-mediated cardioprotection is therapeutically more promising because it is obtained in the absence of haemodynamic side effects, such as hypotensive effects. A second advantage of stimulation of the $A_3$ARs over $A_1$AR activation is that $A_3$AR receptor stimulation is less likely to induce brachycardia.[82]

*Cerebral Ischemia/Stroke*

Generally, antagonists of $A_{2A}$ and $A_3$AR receptors are protective when given acutely, whereas agonists are harmful, but the situation reverses with chronic pre-treatment of animals.[83,84] Repeated systemic administration of $A_3$AR agonist Cl-IB-MECA reduced cerebral infarction in stroke rats[85,86] while acute administration of the same $A_3$AR agonist during the ischemia exacerbated histological and functional damage.[86] Due its cerebroprotective effects, chronic treatment with $A_3$AR agonists has been proposed for the prevention of stroke.

*Inflammation, allergies, asthma*

Inhaled adenosine causes bronchoconstriction in asthmatics. $A_3$ARs as well as $A_1$ and $A_{2B}$ ARs may be involved.[87] $A_3$ receptor activation facilitates the release of allergic mediators, such as histamine and stimulates mast cell degranulation.[88] On the other hand, $A_3$AR agonists inhibit lipopolysaccharide-induced stimulation of TNF$\alpha$ production[89] and the release of other inflammatory mediators from human macrophages and eosinophils.[90,91,92] The $A_3$ receptor agonist IB-MECA showed beneficial effects in phase II clinical trials for the treatment of rheumatoid arthritis. IB-

MECA resulted in improvement in signs and symptoms of rheumatoid arthritis that did not achieve statistical significance, and was safe and well tolerated. [93]

*Cancer*

$A_3$AR agonists can induce or attenuate apoptosis depending on the range of agonist concentrations used. High Cl-IB-MECA concentrations induce apoptosis and relatively low concentrations block apoptosis in human leukemia cells.[94] This might have important implications for therapeutic use in disorders such as cancer, in which induction of apoptosis is desired, and such as arthritis, in which the aim is to attenuate apoptosis.

$A_3$AR is more highly expressed in tumour than in normal cells, which justifies the $A_3$AR as potential target for tumour growth inhibition. [95] The apoptotic effect of adenosine or its analogues takes place at micromolar level, while at low nanomolar concentrations reduced cell growth not due to apoptosis was also observed. Cl-IB-MECA, at nanomolar concentrations, inhibited tumor cell growth through a cytostatic pathway, i.e., induced an increase in the number of cells in the $G_0/G_1$ phase of the cell cycle and decreased the telomeric signal. Interestingly, Cl-IB-MECA stimulates murine bone marrow cell proliferation through the induction of granulocyte-colony stimulating factor. Thus, the $A_3$ adenosine receptor agonist Cl-IB-MECA exhibits systemic anticancer and chemoprotective effects.[96]

Recently, Fishman and coworkers proposed the therapeutic treatment of cancer by a combined administration of methotrexate and an agonist of the $A_3$AR (Cl-IB-MECA or IB-MECA).[97]

### 1.2.4.7 Therapeutic Potential of $A_3$AR Antagonists

*Cerebroprotection*

Acute treatment with $A_3$AR antagonists after the ischemia event may exhibit cerebroprotective effects.[86]

*Inflammation and asthma*

Activation of the $A_3$ARs in rodents results in histamine release from mast cells, and also leads to hypotension. Adenosine also plays a role in lung inflammation through the adenosine $A_3$ receptor. On the other hand, adenosine also exhibits anti-inflammatory effects. Therefore, $A_3$AR agonists as well as antagonists have been proposed for the treatment of inflammation and asthma.

*Cancer*

$A_3$AR antagonists seem to enhance anticancer treatment by counteracting P-glycoprotein efflux in multidrug resistance.[98]

*Glaucoma*

Application of $A_3$AR antagonist externally to the eye lowers intraocular pressure in mice and monkeys. Therefore, $A_3$AR antagonists have been proposed for the treatment of glaucoma.[99,100,101]

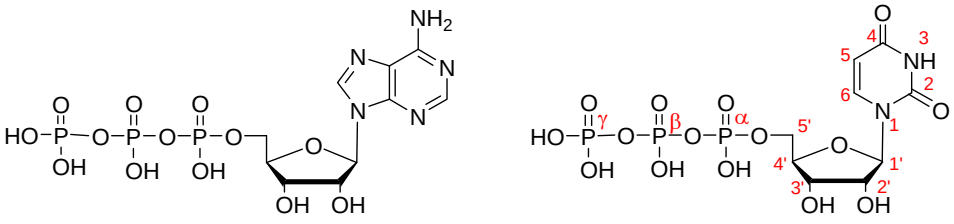
# 1.3 Pyrimidine Nucleotides And The $P2Y_2$ Receptor

## 1.3.1 Uracil And Adenine Nucleotides

Adenosine 5'-triphosphate (**1.40**, ATP) and uridine 5'-triphosphate (**1.42**, UTP) are nucleotides consisting of 5'-triphosphorylated D-ribofuranose, which is linked via a β-glycosidic bond to an adenine or a uridine base, respectively.

ATP was discovered in 1929 by Lohmann,[102] and was proposed to be the main energy-transfer molecule in the cell by Lipmann in 1941.[103] In signal transduction pathways, ATP is used as a substrate by kinases that phosphorylate proteins and lipids, as well as by adenylate cyclase, which converts ATP to cAMP. ATP is also incorporated into nucleic acids.

UTP is used as a substrate for the synthesis of RNA during transcription. UTP also occurs as energy source or activator of substrates in metabolic reactions. Glucose is activated by UTP while inorganic phosphate is released and the resulting UDP-glucose enters the glycogen synthesis. UDP glucuronate is formed by oxidation of UDP-glucose. Hydrophobic molecules such as bilirubin, steroid hormones and many drugs are conjugated with glucuronate by UDP glucuronyl transferase to form a water-soluble glucuronide derivative before excretion by the kidney.[104]




**1.40** **1.41**

**Figure 1.19**

Structures of the natural $P2Y_2$ receptor ligands ATP and UTP

## 1.3.2 The P2Y Receptors

### 1.3.2.1 The P2 Receptor Family

Based on whether they are ligand-gated ion channels (P2X receptors) or G-protein-coupled receptors (P2Y receptors) P2 receptors are subdivided into two main classes. The P2X receptors consist of seven subtypes ($P2X_1$ – $P2X_7$) and the P2Y receptors include the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors.[7,8] The missing P2Y sequence numbers represent species homologues of other receptors, e.g., p2y3,[105] or receptors that have been misassigned to the P2Y family, e.g., p2y7,[106] which was subsequently cloned as a leukotriene $B_4$ receptor.[107]

The receptor proteins of the defined P2Y-receptor subtypes contain the typical features of G-protein-coupled receptors including 7 predicted hydrophobic transmembrane regions (TMs) connected by 3 extracellular loops (ELs) and 3 intracellular loops. The proteins of the human receptors consist of 328 ($P2Y_6$) to 377 ($P2Y_4$) amino acids corresponding to a predicted molecular mass of 41–53 kDa of the glycosylated proteins. The biochemical analysis of the P2Y-receptor proteins has shown that P2Y-receptors expressed at the level of the cell membrane are in fact modified by N-linked glycosylation.[108,109] For the $P2Y_{12}$-receptor (Figure 1.20), it has recently been demonstrated that N-linked glycosylation is essential for signal transduction, but not for ligand binding or cell surface expression.[109] All known P2Y-receptor subtypes possess at their extracellular domains 4 cysteine residues, which are likely to form 2 disulfide bridges: the first one between the N-terminal domain and EL3 and the second bridge between EL1 and EL2. [110,111]

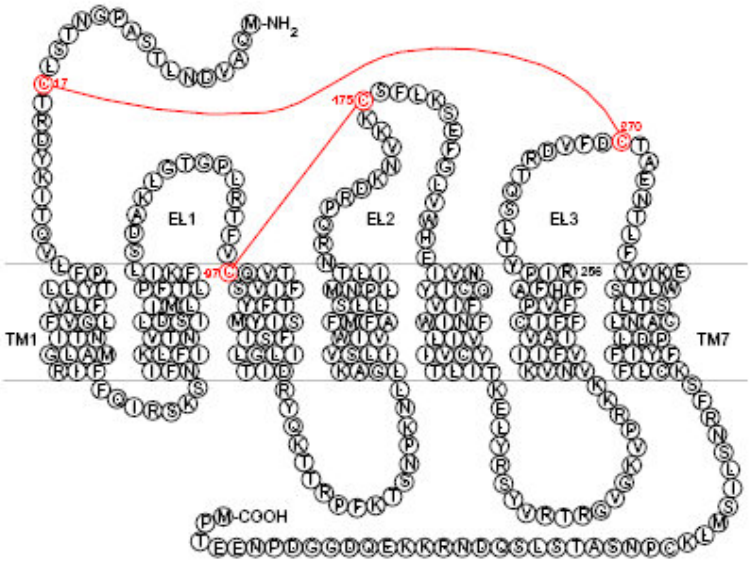



**Figure 1.20**

Predicted secondary structure of the human $P2Y_{12}$-receptor. The red lines show predicted disulfide bridges.[111] Potential sites for N-linked glycosylation are not indicated. TM, transmembrane region; EL, extracellular loop.

### 1.3.2.2 The P2Y Receptor Subtypes and their Signalling

Most P2Y receptors act via G protein coupling to activate phospholipase C leading to the formation of $IP_3$ and the mobilization of intracellular $Ca^{2+}$, which will directly control cellular function. In addition and secondary to the activation of PLC, multiple signal transduction pathways including protein kinase C, phospholipase $A_2$, $Ca^{2+}$-sensitive ion channels and the formation of endothelium-derived relaxing factors have been shown to be involved in the responses to activation of native P2Y-receptors. Coupling to adenylate cyclase by some P2Y receptors has also been described.[2]

The specific downstream signal transduction pathway seems to depend not only on the P2Y-subtype but also on the cell type expressing the receptor.[2] The third intracellular loop and the C-terminus, regions implicated in specific G-protein coupling in other G-protein-coupled receptors,[112] vary greatly between the sequences of the cloned and functionally defined P2Y receptors. This suggests a coupling of different P2Y-subtypes to diverse subsets of G-proteins.

On the basis of their structural similarities, P2Y receptors have been divided into two distinct groups:

group I:

- consists of the $P2Y_1$-, $P2Y_2$-, $P2Y_4$-, $P2Y_6$-, and $P2Y_{11}$-receptors
- couple via $G_q$ proteins resulting in phospholipase C stimulation followed by increased inositol phosphates and $Ca^{2+}$ mobilization
- in addition, the $P2Y_{11}$-receptor mediates an increase in adenylate cyclase activity

group II:

- consists of the $P2Y_{13}$, $P2Y_{12}$ and $P2Y_{14}$ receptors[113]
- group II receptors couple via $G_i$-proteins leading to adenylate cyclase inhibition followed by a decrease in intracellular cAMP levels[114]

The response time of P2Y receptors is longer than that of the rapid responses mediated by P2X receptors because it involves second-messenger systems and/or ionic conductances mediated by G protein coupling.[2]

The $P2Y_2$ receptor couples via $G_q$ proteins to mediate phospholipid breakdown and $IP_3$ formation as well as $Ca^{2+}$ mobilization via PLC.[115] The specific downstream involvement of a given signalling pathway seems to be partially dependent on the cell type in which the $P2Y_2$ receptor is expressed. In airway epithelial cell lines, biliary epithelial cell lines, and avian exocrine salt gland cells, $P2Y_2$ receptor activation leads to opening of $Ca^{2+}$-sensitive $Cl^-$-channels that are involved in epithelial fluid secretion.[116]

## 1.3.3 The $P2Y_2$ Receptor

The $P2Y_2$ receptor has been cloned from mouse,[117] rodent,[118] and human[119] tissue or cells. $P2Y_2$ mRNA has a wide tissue distribution and is found at highest levels in cells in the lung, heart, skeletal muscle, spleen, kidney, liver, and epithelia.[119, 120] The human receptor exhibits 89% identity in the amino acid sequence to the mouse receptor.[119] The $P2Y_2$ receptor is activated by both endogenous ligands UTP and ATP. Desensitization of $P2Y_2$ receptors occurs after ca. 5 minutes of exposure to UTP probably by phosphorylation of their C-terminus by protein kinases. Recovery was observed after 5-10 minutes after removal of the agonist.[121]

### 1.3.3.1 $P2Y_2$ Receptor Agonists

UTP and ATP are full agonists at the $P2Y_2$ receptor. UTP also activates the $P2Y_4$ receptor while ATP acts as a potent competitive antagonist at the human $P2Y_4$ receptor.[122] In several other species, $P2Y_4$ receptors are activated by both UTP, and ATP. Consequently to the high selectivity of the human $P2Y_4$ receptor for UTP it is difficult to find UTP analogues that are $P2Y_2$ receptor selective. However, in controlled clinical studies, UTP is used in preference to ATP as a $P2Y_2$ receptor agonist because of the expected higher selectivity of pyrimidine nucleotides versus the other P2 receptors and because active ATP metabolites such as adenosine may interact with the P1 receptors and lead to unwanted side effects.

When tested under conditions excluding enzymatic conversion of nucleotides, UDP and ADP did not activate the receptor,[123] indicating that at least 3 phosphate residues are required for the activation of the receptor. It has been suggested that the $P2Y_2$ receptor is preferentially activated by the fully ionized forms of ATP or UTP. The UTP and ATP responses correlated with the concentration of the fully ionized form.[124]

In vitro pharmacological data for the most potent $P2Y_2$ receptor agonists currently known are represented in Table 1.1. $EC_{50}$ values represent the half maximal effective concentration in an assay of $P2Y_2$ and $P2Y_4$ receptor-stimulated phospholipase C activity. [125] The structure-activity relationship of the different ribose, uracil and phosphate chain UTP modifications is summarized in Figure 1.21.

| compound | name | $EC_{50}$ ($P2Y_2$) (nM) | $EC_{50}$ ($P2Y_4$) (nM) |
|---|---|---|---|
| **1.40** | ATP | 85 | antagonist |
| **1.41** | UTP | 49 | 73 |
| **1.42** | 2'-amino-2-'deoxy UTP | 62 | 1200 |
| **1.43** | *arabino*UTP | 87 | 710 |
| **1.44** | (N)-methanocarba-UTP | 85 | 91 |
| **1.45** | 2-thioUTP | 35 | 350 |
| **1.46** | 4-thioUTP | 26 | 23 |
| **1.49** | UTP$\gamma$S | 240 | 1600 |
| **1.50** | ATP$\gamma$S | 1720 | no data |
| **1.52** | Ap4A | 180 | inactive |
| **1.53** | $UP_4U$ (diquafosol) | 60 | 200 |
| **1.54** | $UP_4dC$ (denufosol) | 220 | 800 |

**Table 1.1**

In vitro pharmacological data for the most potent $P2Y_2$ receptor agonists currently known

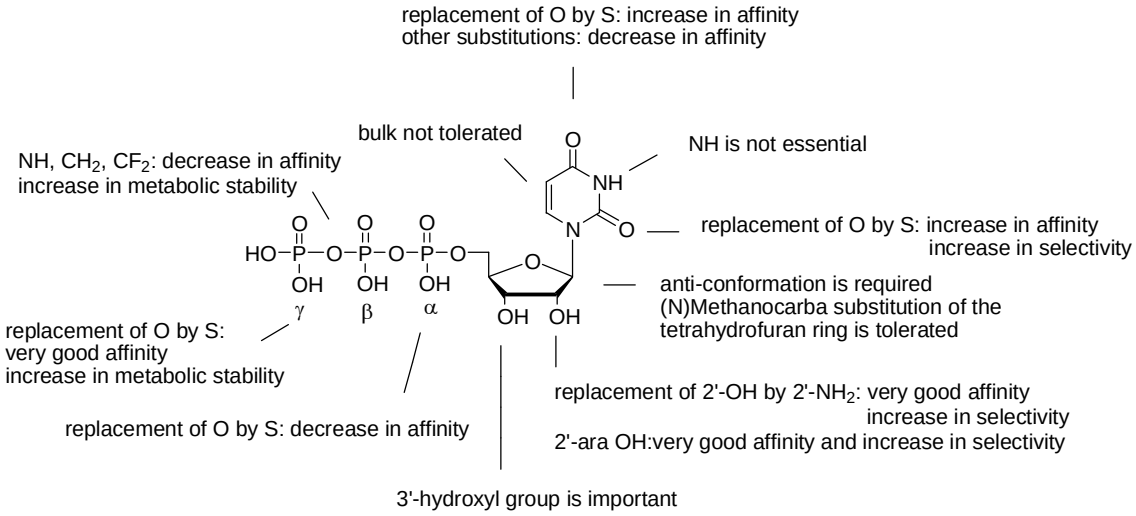
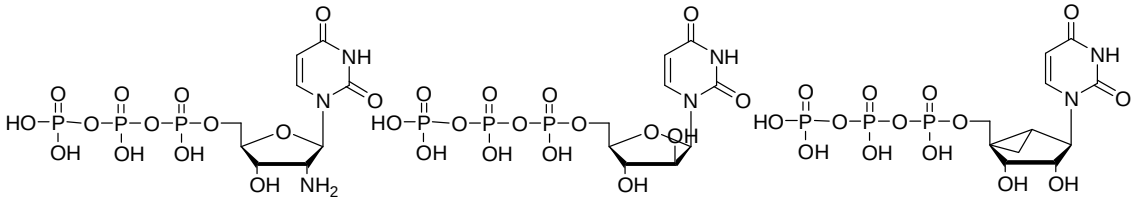



**Figure 1.21**

Structure-activity relationship of UTP derivatives as $P2Y_2$ agonists

### Ribose modifications

2'-Amino-2'-deoxy UTP (**1.42**) and 1-(β-D-arabinofuranosyl)uracil 5'-triphosphate (**1.43**) are equipotent with ATP at the $P2Y_2$ receptor and show a moderate selectivity over the $P2Y_4$ receptor. Other 2'-modifications decreased the $P2Y_2$ receptor potency. The only 3'-modification reported is 3'-*O*-methyluridine-5'-triphosphate, which proved inactive at both $P2Y_2$ and $P2Y_4$ receptors.[125] (N)-Methanocarba-UTP (**1.44**) is almost equipotent to UTP.[126] (Figure 1.22)

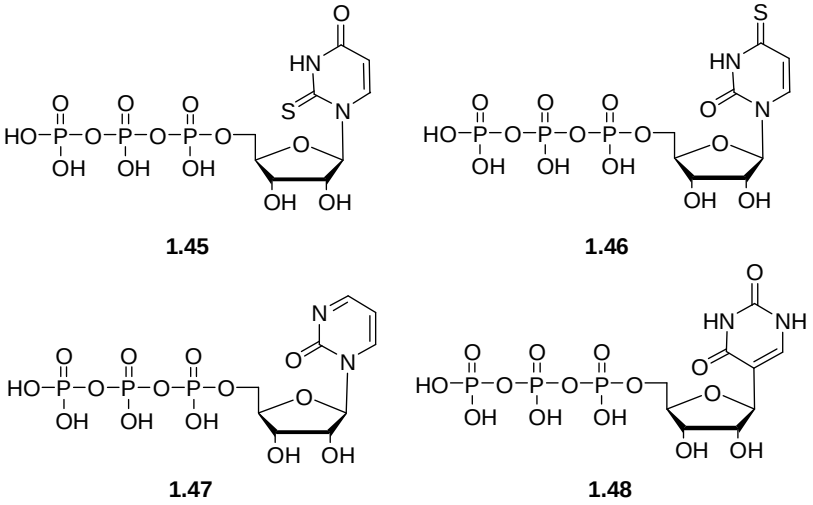


**1.42** **1.43** **1.44**

**Figure 1.22**

### Uracil modifications

The sulphur-containing nucleotides 2-thioUTP (**1.45**) and 4-thioUTP (**1.46**) (Figure 1.23) are very potent $P2Y_2$ receptor agonists, which are moderately selective (2-thioUTP) or nonselective (4-thioUTP) versus $P2Y_4$ receptors. All other variations in the 4-position (methoxy, hexyloxy, methylthio, hexylthio, amino, hexylamino, cyclopentylamino, morpholino) caused a significant loss in $P2Y_2$ receptor affinity.[125] Other uracil modifications such as 5-alkyl, 5-Br, 5-I, 5-Me, 6-aza and 3-methyl decreased the $P2Y_2$ receptor affinity.[125,127] Replacement (zebularine-5'-triphosphate, **1.47**) or reorientation (pseudouridine-5'-triphosphate, **1.48**) of the uracil ring also resulted in less potent analogues (Figure 1.23).[125]



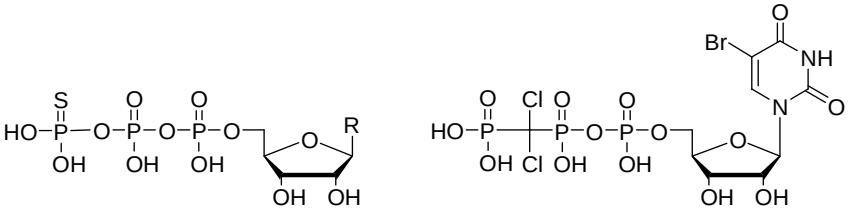
**1.45** **1.46**

**1.47** **1.48**

**Figure 1.23**

### Phosphate chain modifications

The phosphate chain modified nucleotides UTPγS (**1.49**), and ATPγS (**1.50**) (Figure 1.24) are full agonists at the $P2Y_2$ receptor. UTPγS is a relatively potent $P2Y_2$ agonist and is more stable against nucleotidases.[128] ATPγS is less potent than UTPγS.[129] α-phosphothioate modification, resulting in both stereoisomers of UTPαS, decreased the agonist potency.[123] The phosphate chain in UTP has been modified by replacement of the β,γ-oxygen by NH, $CH_2$ or $CF_2$. All these modifications resulted in a decrease in affinity.[130,131] β,γ-Dichloromethylene-5-bromo-UTP (**1.51**, Figure 1.24) also showed a decreased $P2Y_2$ receptor affinity, but showed an enhanced $P2Y_6$ receptor selectivity.[132]



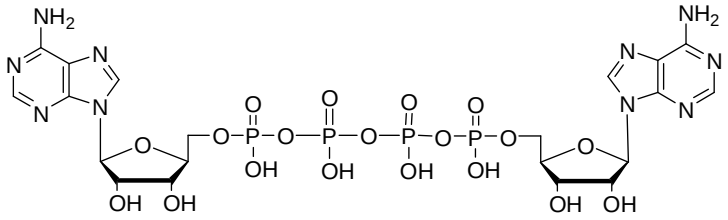
**1.49 R = uracil**
**1.50 R = adenine**

**1.51**

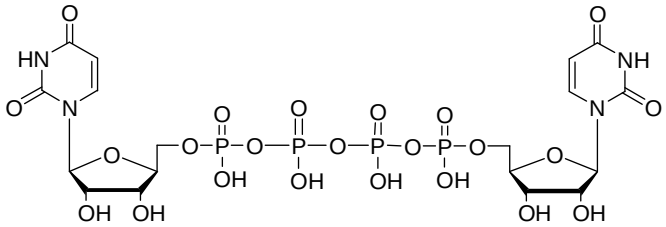
**Figure 1.24**

### Dinucleotides

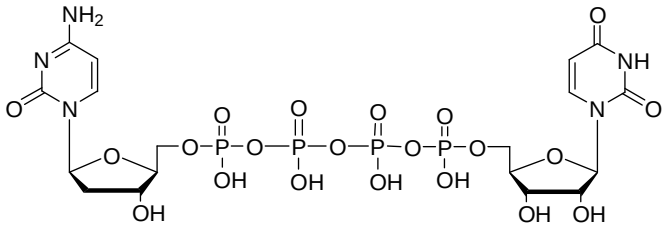
Mononucleotides are quickly dephosphorylated by cell surface ectonucleotidases. On the airway epithelial surface, UTP and UDP exhibit $t_{½}$ values (at 1 µM nucleotide) of 14 and 27 min, respectively.[133] The more metabolically stable dinucleotides such as diadenosinetetraphosphate (**1.52**, Ap4A),[134] Up4U (**1.53**, diquafosol, INS365)[135] and dCp4U (**1.54**, denufosol, INS37217) (Figure 1.25) are more slowly hydrolyzed to nucleoside mono- and triphosphosphates. Denufosol shows a 10-fold higher potency at the $P2Y_2$-receptor than at the $P2Y_4$-receptor. Moreover, this compound did not activate $P2Y_1$- or $P2Y_6$-receptors.[136]



**1.52**



**1.53**



**1.54**

**Figure 1.25**

### 1.3.3.2 $P2Y_2$ receptor Antagonists

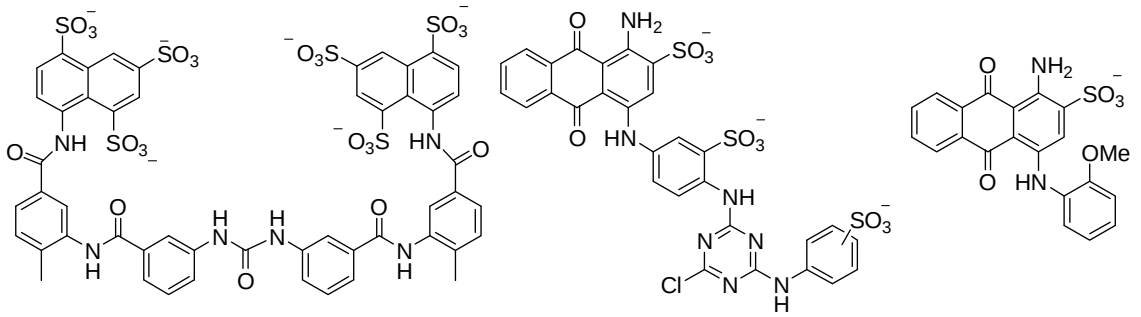
#### Standard Antagonists

The standard P2 receptor antagonists are suramin (**1.55**) and Reactive Blue 2 (**1.56**, RB-2) (Figure 1.26). Suramin is a naphtylsulfonate derivative that acts as a competitive antagonist at several P2 receptor subtypes.[137] Suramin does not block the $P2Y_4$ receptor. Consequently, the previously suggested receptor subpopulations defined by suramin sensitivity[2] may be the $P2Y_2$ receptor (suramin-sensitive P2U-purinoceptor) and the $P2Y_4$ receptor (suramin-insensitive P2U-purinoceptor). [138] Suramin exhibited an $IC_{50}$ value of ca. 50 µM. RB-2 is one of the most potent $P2Y_2$ antagonists known to date ($IC_{50}$ 1-5 µM). It is an anthraquinone derivative with a relatively high molecular weight (MW = 840 g/mol), containing three negatively charged sulfonate groups. RB-2 may be meta- or parasulfonated or a mixture of both isomers.

The usefulness of RB-2 and suramin as pharmacological tools is limited by their poor $P2Y_2$ receptor selectivity and their interaction with several other receptors and proteins, including P2X receptors,[139] ectonucleotidases,[140,141] kinases (RB-2),[142] and G proteins (suramin).[143]

#### RB-2 Derivatives

Müller and coworkers[144] recently discovered a simplified RB-2 derivative PSB-716 (**1.57**, Figure 1.26), which appeared to be selective versus other P2Y subtypes as well as nucleotide-metabolizing enzymes.



**1.55**
**$IC_{50}$ = 50 µM**

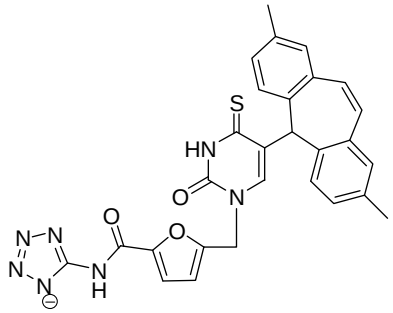
**1.56**
**$IC_{50}$ = 1-5 µM**

**1.57**
**$IC_{50}$ = 9 µM**

**Figure 1.26**

## Thiouracil Derivative

Structural UTP modifications to eliminate efficacy and improve the pharmacokinetic properties led to the development of a thiouracil derivative (**1.58**, Figure 1.27), bearing a tricyclic substituent in the 5-position and a ribose triphosphate- mimicking substituent at N-1. The compound appeared to be a $P2Y_2$-selective competitive antagonist and showed an $IC_{50}$ value of ca. 1 µM in a model of $P2Y_2$-agonist-induced mucin secretion in differentiated human bronchial epithelial cells.[145] The incorporation of a large heterocyclic substituent in the 5-position was earlier reported to preclude receptor activation.[146]

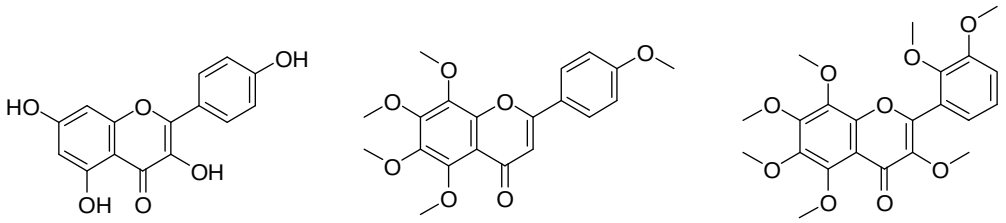


**1.58**

**Figure 1.27**

## Flavonoids

Several flavonoids were also identified as $P2Y_2$ receptor antagonists with $IC_{50}$ values in the low micromolar concentration range. The most potent $P2Y_2$ receptor antagonists of the present series, kaempferol (**1.59**), tangeretin (**1.60**), and heptamethoxyflavon (**1.61**), with $IC_{50}$ values between 6-19 µM, are depicted in Figure 1.28.[147]



**1.59** **1.60** **1.61**

**Figure 1.28**

### 1.3.3.3 Molecular Modeling of the $P2Y_2$ Receptor

An early model of the $P2Y_2$ receptor was constructed by Erb et al.[148] Recently, a molecular model was provided by a molecular dynamics (MD) simulation in a phospholipidic and aqueous environment.[149,150] This model of the $P2Y_2$ receptor is a rhodopsin-based homology model containing not only seven TMs, but also all extracellular hydrophilic loops, termini and phospholipids. Inclusion of the natural environment of the receptor was previously shown to significantly improve the results of a MD simulation, providing a more accurate structure of the receptor, especially in its loops and terminal regions.

#### Extracellular Regions

Recent MD simulation of the $P2Y_6$ receptor revealed that EL2 moved toward TM3 and further up into the extracellular space, opening the putative nucleotide binding cavity.[151] However, in the case of the $P2Y_2$ receptor, EL2 did not show a significant displacement during the MD simulation. Several electrostatic and disulfide bridges were proposed to fix this loop near the TM domain. In addition to the disulfide bridge conserved among all GPCRs of the rhodopsin family and formed between two cysteine residues located in TM3 and EL2 (C106 and C183 in the $P2Y_2$ receptor), several charged residues located in EL2 of the $P2Y_2$ receptor can interact with oppositely charged residues located around the loop (Figure 1.29A). Several other bridges in the extracellular region of the $P2Y_2$ receptor appeared in de MD simulation model.[150]

#### Intracellular Regions

Three possible pairs of charged residues were found in the intracellular region of the $P2Y_2$ receptor: D319-R340, R334-D342, and R335-D345 (Figure 1.29B). All these residues are located in the C-terminus domain.[150]

### Putative Interhelical Disulfide Bridges

Analysis of the model obtained after MD simulation suggested the formation of two interhelical disulfide bridges [C132(3.51) - C212(5.57) and C44(1.43) - C300 (7.47)].[*,150,152] (Figure 1.29B)

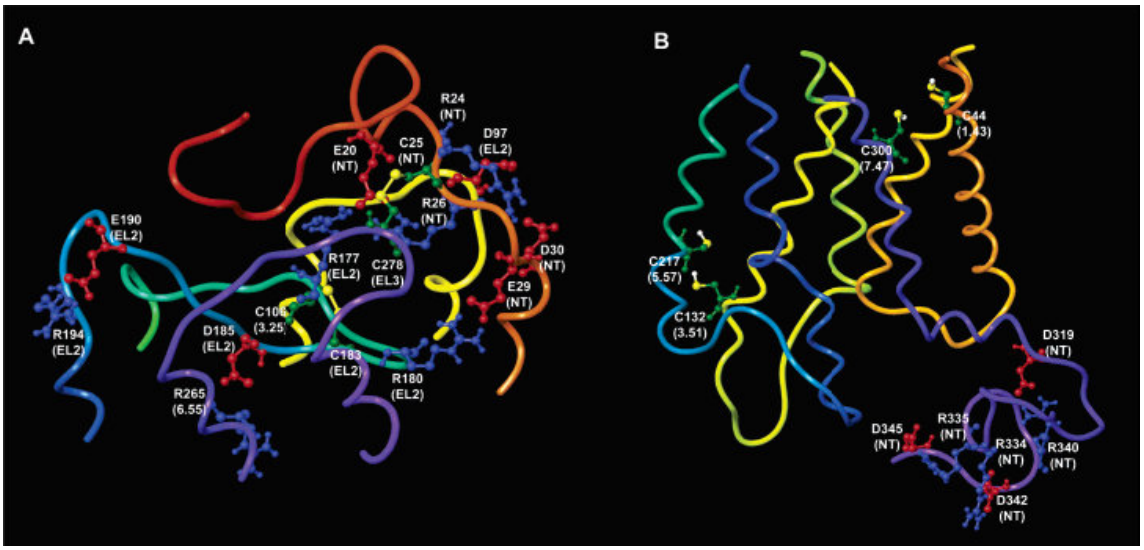



**Figure 1.29**

Putative electrostatic and disulfide bridges found in the extracellular (A) and intracellular (B) regions of the model of the $P2Y_2$ receptor

[*] Note on the residue indexing in this work
To facilitate comparison of aligned redidues in related GPCDs, residues are supplemented with an index, according to the van Rhee-convention.[149] The most conserved residue in a TM region X is given the index number (X.50). Residues within the given TM are then indexed relative to the "50" position. Note that the "50" position is not necessary the residue in the middle of the TM region.

### 1.3.3.4 Therapeutic Potential of $P2Y_2$ Receptor Agonists

*Cystic Fibrosis*

Cystic fibrosis (CF) is a recessive genetic disease caused by a mutation in the cystic fibrosis transmembrane regulator (CFTR) gene resulting in defective chloride secretion and excessive sodium absorption. This leads to a reduction of the airway surface liquid, causing defective ciliairy function and cough clearance. Subsequently, thickened mucus plaques adhere to the airway surface, generate hypoxic regions and promote bacterial infection which can finally cause respiratory failure. Cystic fibrosis has affected approximately 75 000 individuals worldwide. Their mean life expectancy is only about 32 years.[153]

Studies on $P2Y_2$-receptor-deficient mice verified the important role of the $P2Y_2$ receptor in regulating ion transport in epithelial cells.[154, 155] Receptor activation induces an increase in intracellular $Ca^{2+}$ concentration, which stimulates the $Cl^-$-secretion[156,157,158] via the $Ca^{2+}$-activated $Cl^-$ channels (CaCC) and inhibits the $Na^+$ absorption[158, 159, 160] via luminal epithelial $Na^+$ channels (ENaCs). The ENaCs are controlled by CFTR proteins, $Cl^-$-channels which are activated when the ENaCs are blocked. The net effect of activation of luminal $P2Y_2$ receptors in respiratory epithelium is inhibition of $Na^+$ absorption and stimulation of $Cl^-$-secretion.

As shown in the schematic cell model of a secretory respiratory epithelial cell (Figure 1.30), luminal $Cl^-$-extrusion by CFTR or CaCC requires basal $Cl^-$-influx mediated by the $Na^+/K^+/Cl^-$ cotransporter isoform 1 (NKCC-1). Nucleotide-mediated activation of secretion in the airways was also shown to encompass activation of basal $K^+$-secretion.[161]

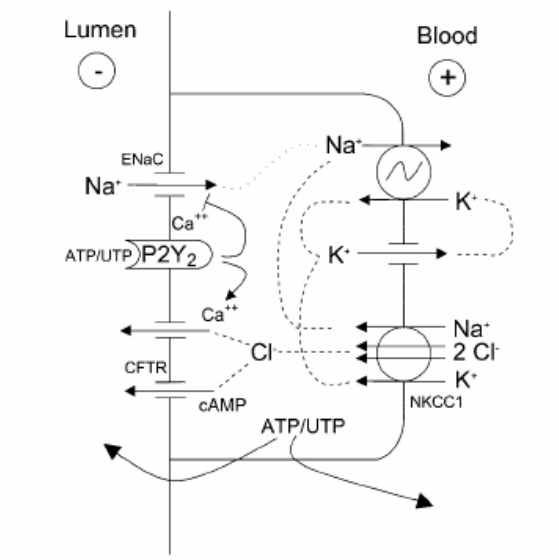



**Figure 1.30**

Schematic model of luminal $P2Y_2$ receptor-mediated ion transport regulation in respiratory epithelial cells. $Cl^-$ secretion requires: a) $Na^+/K^+/Cl^-$ cotransporter isoform 1 (NKCC1)-mediated; b) extrusion of $Cl^-$ via either luminal CFTR or $Ca^{2+}$-activated $Cl^-$ channels. [155]

CFTR proteins, $Cl^-$-channels that control the ENaC, are immature in CF patients. Consequently, the ENaC activity is up-regulated ($Na^+$-influx is increased) and the cell depends only on the CaCC for $Cl^-$-secretion. The impaired ion transport in the bronchi of CF patients can be bypassed by stimulation of $P2Y_2$ receptors to inhibit the ENaC and activate the CaCC.[162,163](Figure 1.31)

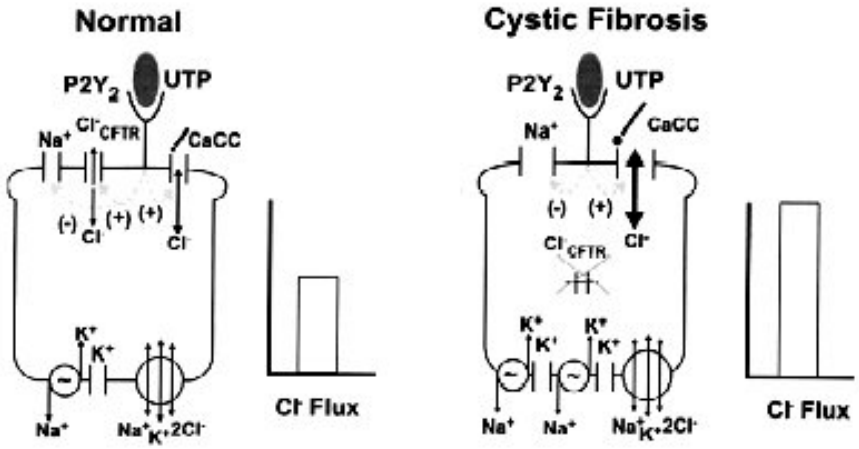



**Figure 1.31**

Normal – cystic fibrosis cell[163]

$P2Y_2$ receptor activation also stimulates the mucin secretion from goblet cells,[164] increases the ciliary beat frequency,[165] and promotes the surfactant release from type II alveolar cells.[166] A knockout study confirmed previous pharmacological data and identified the $P2Y_2$ receptor subtype as the crucial luminal P2 receptor in respiratory epithelium.[167] Therefore inhaled $P2Y_2$ receptor agonists are proposed for the treatment of cystic fibrosis.

UTP dose-dependently stimulates the mucociliary clearance and sputum expectoration in smokers and patients with a variety of airway disorders including CF. However, UTP has limited metabolic stability and, hence, a relatively short duration of action when administered by inhalation. Newer dinucleotide $P2Y_2$ agonists such as diquafosol (**1.53**) and denufosol (**1.54**) (Figure 1.25) have enhanced metabolic stability in CF sputum.[168] Denufosol as a treatment for CF received fast-track and orphan drug status in the U.S. and orphan drug status in Europe. The efficacy results of the first of two planned Phase III clinical trials with denufosol for CF are expected mid-year 2008. In February 2008, the second Phase III trial was initiated.[169]

*Dry Eye Syndrome*

A high expression level of $P2Y_2$ receptors is found in different cell types of the eye. Activation of $P2Y_2$ receptors appears to regulate ocular surface hydration by stimulation of conjunctival mucin and $Cl^-$-secretion. $P2Y_2$ agonists, such as diquafosol (**1.53**, Figure 1.25) stimulate chloride secretion and increase tear production.[170] To date, four Phase III clinical trials of diquafosol (Prolacria™) for the treatment of dry eye disease are completed.[169]

*Cancer*

ATP inhibits the growth of primary cell cultures of human oesophageal cancer as well as the growth of an oesophageal cancer cell line by inducing apoptosis and cell cycle arrest. The effect is thought to be mediated by stimulation of $P2Y_2$ receptors. Thus, $P2Y_2$ receptor agonists have been suggested as novel therapeutics for oesophageal cancer.[171] Therapeutically used anticancer and antiviral nucleoside antimetabolites are bioactivated by phosphorylation and might exert some of their effects (or side-effects) by interaction with P2 receptors.

### 1.3.3.5 Therapeutic Potential of $P2Y_2$ Receptor Antagonists

*Inflammation*

$P2Y_2$ receptors are expressed on epithelial cells in high density and are thought to be involved in defense mechanisms.[172] They are expressed on inflammatory cells, such as neutrophils and macrophages. $P2Y_2$ receptor antagonists may be potential antiinflammatory drugs, which is in agreement with the observations of Lackie et al. on the potential anti-inflammatory effects of chloride channel blockers.[173]

*Coronary Vasospastic Disorders*

Since stimulation of $P2Y_2$ besides P2X receptors mediates contraction of human coronary arteries, $P2Y_2$ receptor antagonists have been suggested as potential therapeutics for coronary vasospastic disorders.[174]

*Neuroprotective Agents*

P2Y receptor antagonists, including Reactive Blue-2 (**1.55**), have been reported to exhibit neuroprotective properties.[175] They may be useful for the treatment of epileptic seizures, stroke and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease.[176] The P2Y receptor subtype(s) responsible for these effects are not known. Since the $P2Y_2$ receptor is expressed in the brain and RB-2 is a relatively potent $P2Y_2$ receptor antagonist, $P2Y_2$ antagonism might be involved.

## 1.4 Objectives and Structure of this Thesis

The synthesis and biological evaluation of a series of $A_3$ adenosine receptor ligands (chapter 2 and 3) and $P2Y_2$ receptor ligands (chapter 4) will be discussed. The $A_3$AR and the $P2Y_2$ receptor are G-protein-coupled receptors, which belong to the purinergic receptors, which consist of 2 classes, termed P1-receptors or adenosine receptors and P2-receptors that recognize ATP, ADP, UTP and UDP as natural ligands.

In chapter 2 and 3 of this thesis, we will describe the design of novel ligands for the adenosine $A_3$ receptor by modification of the natural, non-selective ligand adenosine (Figure 1.32). Based on the known SAR, we aim to enhance $A_3$AR affinity and selectivity by introducing selected substitutions, possibly combined with 5'-*N*-(m)ethylcarbamoyl or 3'-amino modifications of the ribose moiety.

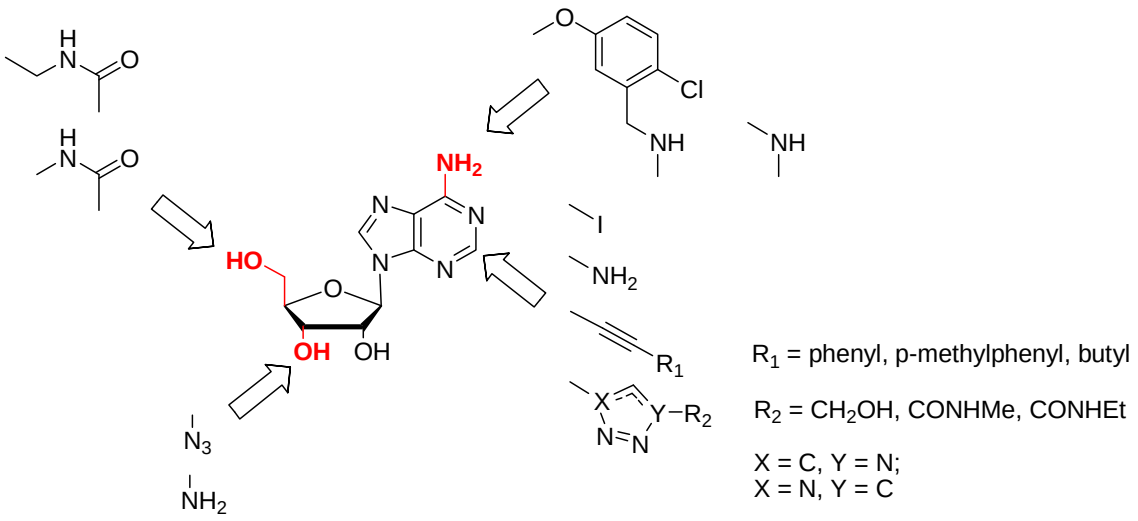



**Figure 1.32**

Summary of the performed adenosine modifications (Chapter 2-3)

In chapter 4, we will discuss the design of ligands for the $P2Y_2$ receptor. Toward this end, UTP will be used as a preferred template, because it is expected to yield a selective ligand and precludes the formation of active metabolites such as adenosine. Our work will focus on a 2-or 4-thio modification of the uracil base combined with modifications of the 2'-OH group of the ribose moiety. Furthermore, initial

modifications to enhance the metabolic stability of the 5'-triphosphate chain will be explored (Figure 1.33).

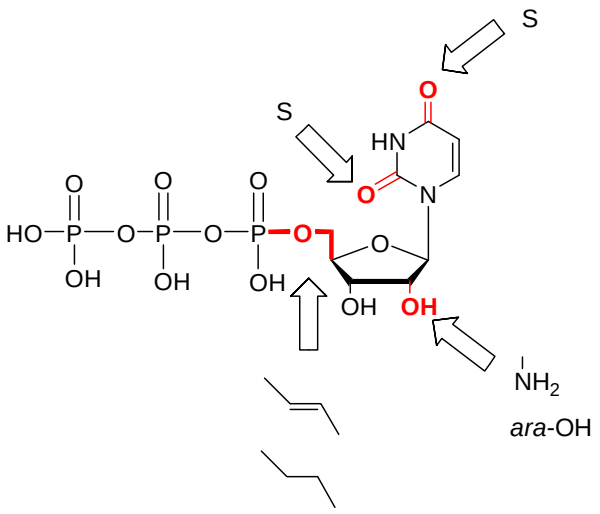



**Figure 1.33**

Summary of the planned UTP modifications (Chapter 4)

The biological evaluation of the synthesized $A_3AR$ receptor ligands, is performed by the group of prof. dr. Kenneth A. Jacobson, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA. The group of prof. dr. Kendall Harden, Department of Pharmacology, University of North Carolina, School of Medicine, North Carolina, USA performed the biological evaluation of the synthesized $P2Y_2$ receptor ligands. The molecular modeling of both $A_3A$ and $P2Y_2$ receptor ligands is performed under the guidance of prof. dr. Kenneth A. Jacobson. We intend to explain the observed biological effects of the synthesized compounds by means of the modeling results.

# 1.5 Note on the Nucleoside Nomenclature Used in this Work

In nucleosides, the ribofuranose ring numbering can be distinguished from purine heterocycle numbering by the use of the prime after the Arabic numerals. Moreover, this "generic" nucleoside notation, i.e. not according to IUPAC conventions, is used to indicate the positions of the nucleoside that are chemically modified (for example 2'-amino-2'-deoxyUTP, **1.42**, Figure 1.22) or to pinpoint particular regions to the nucleoside that interact with the biological (target) system (like the $N^6$-substituents as in **1.5**, Figure 1.7).

These conventions can lead to serious misinterpretations when modifications on the nucleoside significantly alter the parent nucleoside structure. Such a modification is the replacement of the 4'-$CH_2OH$ group of adenosine (**1.1**) by a *N*-ethylcarbamoyl functionality (NECA, **1.2**) (Figure 1.34), which is called a 5'-*N*-ethyluronamide or 5'-*N*-ethylcarbamoyl modification. Although the uronamide group can be seen as a 4'-substituent, the generic name of compound **1.2** is **5'-*N*-ethylcarbamoyladenosine** or **9-[5-*N*-ethylcarbamoyl)-β-D-ribofuranosyl] adenosine**. However, the latter can easily be mistaken for compound **1.62** (Figure 1.34).

Similar 5'-uronamide modifications are described throughout this work (chapter 2 and 3). In fact, to accurately describe the structures their IUPAC-name should be used. For example, the IUPAC-name of **1.2** is **(*2S,3S,4R,5R*)-5-(6-amino-9*H*-purin-9-yl)-*N*-ethyl-tetrahydro-3,4-dihydroxyfuran-2-carboxamide**. Since the IUPAC nomenclature does not allow rapid visualisation of the described structures, the "generic" nomenclature is generally accepted in the nucleoside (bio)chemistry and pharmacology field and will therefore be used throughout this work.

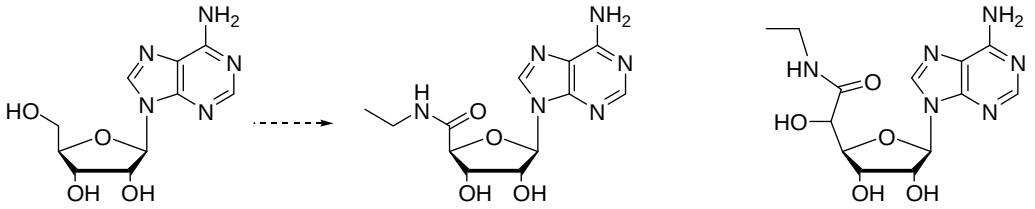



**Figure 1.34**

The nomenclature in nucleoside chemistry for modifications at the 5'-position

## CHAPTER 2:

# HYPERMODIFIED ADENOSINE ANALOGUES

# 2 HYPERMODIFIED ADENOSINE ANALOGUES

## 2.1 Introduction

Adenosine receptors are ubiquitously distributed throughout the body. As a consequence, ligands need to be highly selective in their action with respect to receptor subtype and tissue to be of therapeutic value.[177] Numerous structure-activity studies of adenosine derivatives as receptor agonists conclude that selectivity may be provided by specific substitutions of the adenine ring.[178,179] Substitution at the 8-position of the ring is not well tolerated by any AR subtype.[180,181] The nitrogen atoms at positions 3 and 7 are required for high affinity of adenosine at all subtypes.[179] 2-Alkynyl derivatives of NECA (**1.2**, Figure 1.7) possess high affinity at the $A_3$ receptor subtype. Moreover, the presence of 2-alkyne substituents enhanced the $A_3$AR selectivity.[182] (see 1.2.4.1 and Figure 1.8)

DeNinno et al.[48] discovered that introduction of an amino group at the 3'-position improves the selectivity for the human $A_3$AR, while enhancing the water solubility. The affinity drop caused by this 3'-substitution could be overcome by elaborating the $N^6$-substituents (**1.5**, Figure 1.7). The combination of a large $N^6$-subtitituent with a 2-alkynyl group has proven to be unsuccessful because of the steric hindrance caused by the two large substituents, reflected by a decrease in $A_3$AR affinity.[48] Therefore, the present study investigated the effect of a 2-alkynyl substituent in concert with a small $N^6$-substituent on the affinity and selectivity of a series of 3'-azido and 3'-amino adenosine derivatives. In addition, we evaluated the effect of the 5'-methylcarbamoyl modification on the overall affinity and efficacy of these compounds (Figure 2.1).

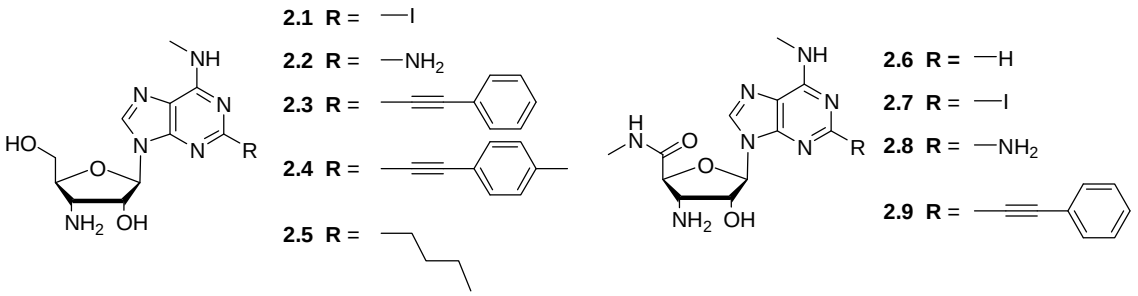



**Figure 2.1**

Overview of the synthesised hypermodified adenosine analogues

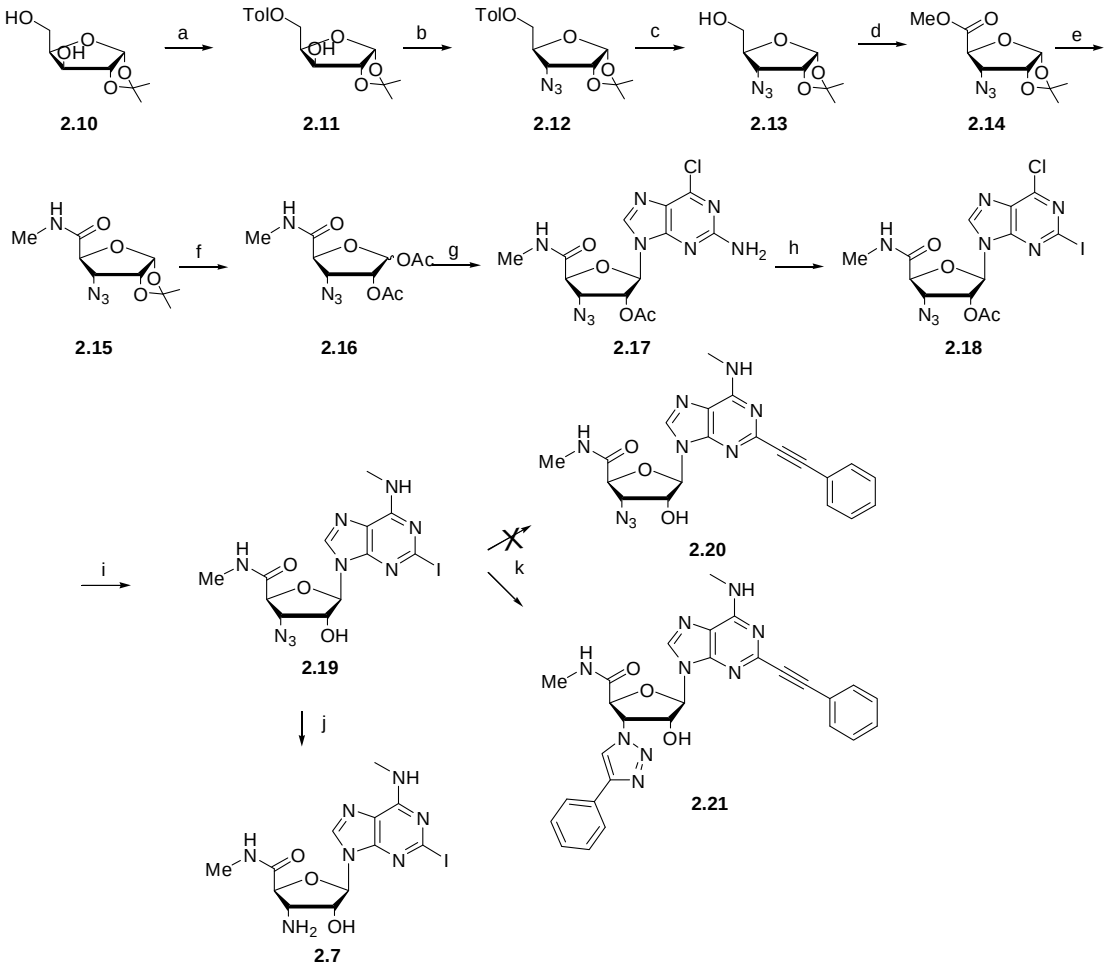
# 2.2 Chemistry

## 2.2.1 Synthesis of 5'-*N*-Methyluronamide 2-Phenylacetylene Adenosine Derivatives 2.7, 2.9 and 2.20

2-Iodo derivative **2.7** was prepared from the commercially available 1,2-*O*-isopropylidene-α-D-xylofuranose (**2.10**) as depicted in Scheme 2.1. Selective 5'-*p*-toluoyl protection allowed 3'-triflation. 3-Azido intermediate **2.12** was obtained by treatment of the resulting 3'-triflate with $NaN_3$ and subsequent 5'-deprotection, following a slightly altered literature procedure.[183] Periodate oxidation followed by esterification of the carboxylic acid and subsequent treatment with methylamine in a pressure tube afforded the ribofuranonamide **2.15**. One pot deprotection-acetylation gave the peracylated 3',5'-modified sugar **2.16**.[48] Vorbrüggen-glycosylation[184] (see also 2.2.4) with silylated 2-amino-6-chloropurine yielded nucleoside **2.17** in 79% yield. Classical procedures allowed a straightforward conversion of **2.17** to **2.19**. Triphenylphosphine reduction of the azido moiety yielded the corresponding amine **2.7**.

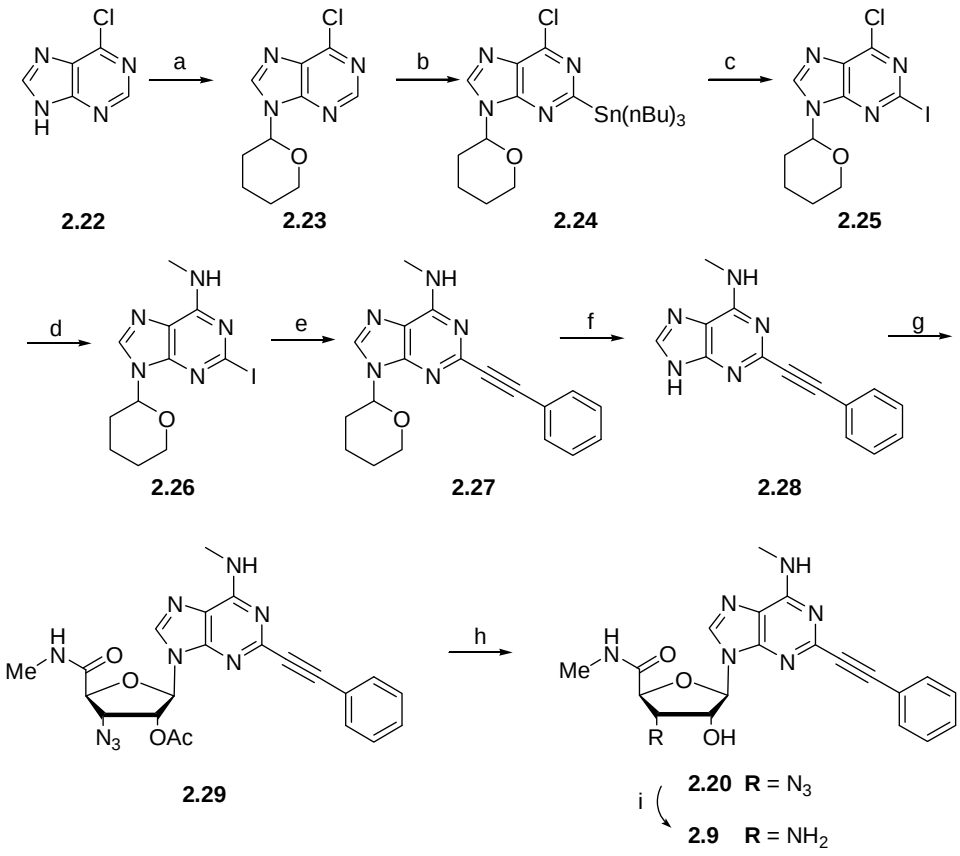
Based on the results of Cristalli et al.,[54] we have chosen phenylethynyl as the most promising C2-substituent. Reaction conditions used to perform a Sonogashira coupling[185] of **2.19** with phenylacetylene yielded the 3'-(4-phenyl-1,2,3-triazol-1-yl) derivative (**2.21**) of the 2-alkynylated compound (Scheme 2.1) This result was due to a $Cu^+$-catalysed Huisgen [3+2]cycloaddition[186] of the 3'-azide with phenylacetylene. Consequently, another strategy was used to gain acces to compound **2.20**, starting from 6-chloropurine (**2.22**) (Scheme 2.2). The purine N-9 was protected as the tetrahydropyran-2-yl (THP) derivative **2.23** by reacting **2.22** with the carbocation

formed *in situ* from 2,3-dihydro-*4H*-pyran and catalytic amounts of *p*-toluenesulfonic acid. The regioselectivity of this reaction had been described extensively.[187] Purine **2.23** was obtained as a mixture of enantiomers because of the stereogenicity of THP carbon C1'.[188] 6-chloro-2-iodo-(9-tetrahydropyran-2-yl)purine (**2.25**) was obtained via a lithiation-mediated stannyl transfer process followed by 2-tributylstannyl-iodine exchange.[189, 190, 191] Treatment of 6-Cl-purine derivative **2.25** with methylamine hydrochloride in the presence of DMAP provided $N^6$-methyladenine analogue **2.26**. Sonogashira coupling of **2.26**, followed by deprotection provided **2.28**. Unfortunately, classical Vorbrüggen coupling,[184] as described for the synthesis of **2.17** did not give satisfying results. By using *N,O*-bis(trimethylsilyl)acetamide (BSA) as silylating agent, [192] 9-(2-O-acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-phenylethynyl-adenine (**2.20**) was obtained in a poor yield.




**Scheme 2.1**

Synthesis of compound **2.7** and attempted synthesis of compound **2.20**. Reagents and conditions: (a) toluoyl chloride, pyridine, $CH_2Cl_2$, 0 ℃ (71%); (b) (i) $(CF_3SO_2)O$, pyridine, $CH_2Cl_2$, -10 ℃ (ii) $NaN_3$, DMF, rt, overnight (40%); (c) 0.1 N NaOMe in MeOH, rt (88%); (d) (i) $NaIO_4$, $RuCl_3$, $H_2O$, $CHCl_3$, $CH_3CN$, rt (ii) EDC, DMAP, dry MeOH, rt (40%); (e) 2M $CH_3NH_2$ in THF (89%); (f) $H_2SO_4$, $(CH_3CO)_2O$, $CH_3COOH$, 18h (55%); (g) (i) 2-amino-6-chloropurine, HMDS, $(NH_4)_2SO_4$,reflux, 20h, (ii) **2.16**, TMSOTf (79%); (h) Isoamyl nitrite, $I_2$, CuI, $CH_2I_2$ in THF, reflux (79%); (i) (i) $CH_3NH_3Cl$, $Et_3N$, EtOH, reflux; (ii) 7N $NH_3$ in MeOH (77%); (j) $Ph_3P$, $H_2O$, THF, 2 days (82%); (k) phenylacetylene, $(PPh_3)_2PdCl_2$,CuI, $Et_3N$, DMF (41%).



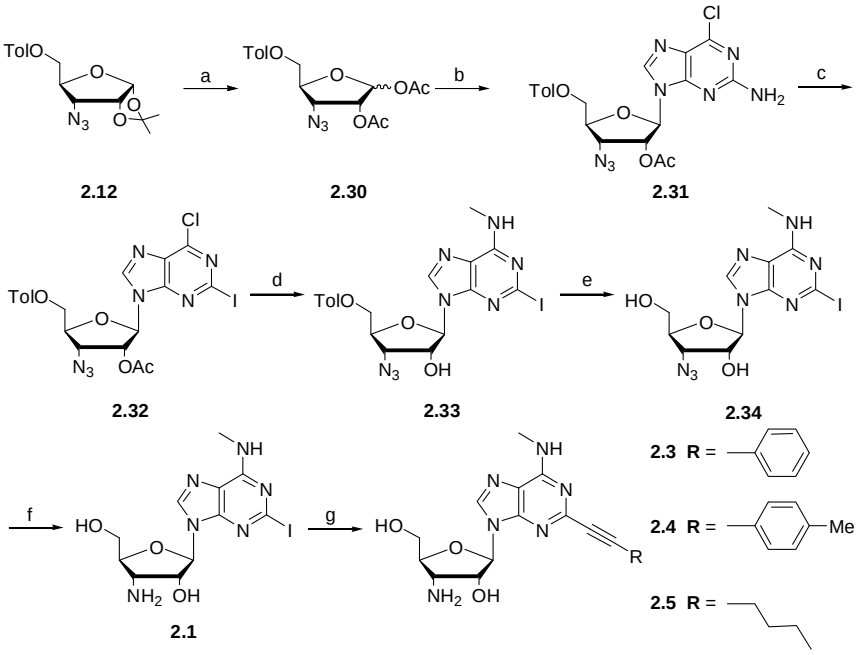

**Scheme 2.2**

Synthesis of compounds **2.20** and **2.9**. Reagents and conditions: (a) 2,3-dihydro-*4H*-pyran, *p*-toluene sulfonic acid, THF, 80 ℃, overnight (69%); (b) LiTMP, THF, -78 ℃ , then $(nBu)_3SnCl$ (95%); (c) $I_2$, THF, 24h (75%); (d) $CH_3NH_3Cl$, DMAP, EtOH, reflux (81%); (e) phenylacetylene, $(PPh_3)_2PdCl_2$,CuI, $Et_3N$, DMF (91%); (f) TFA, $CH_2Cl_2$ (72%); (g) **2.16**, BSA,TMSOTf, $CH_3CN$, reflux (24%); (h) 7N $NH_3$ in MeOH; (i) $Ph_3P$, $H_2O$, THF, 2 days (91%).

During the course of this work it became clear that the 3'-amino-analogues generally exhibit much better $A_3$AR affinities than their 3'-azide precursors. Consequently, we focussed on only the 3'-amino derivatives for further synthesis. This direction permitted us to reduce the 3'-azide to a 3'-amine before the Sonogashira coupling, avoiding the unwanted cycloaddition.

### 2.2.2 Synthesis of 2-Alkynylated 3'-Amino-adenosines 2.3-2.5

9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-$N^6$-methyl-2-iodopurine (**2.1**) served as a suitable synthon for the synthesis of the 2-alkynylated 3'-amino-adenosines **2.3-2.5** (Scheme 2.3). It was obtained by coupling of sugar **2.30** with 2-amino-6-chloropurine. Elaboration of the base moiety was essentially accomplished as for **2.19** to yield **2.34**. Staudinger reduction allowed the unmasking of the amine group. Finally, Sonogashira coupling on amine **2.1** provided the alkynylated analogues **2.3-2.5** in 80-82% yield. The choice of *p*-methyl phenylethynyl was based on a preliminary study performed in our lab, in which we compared different *para*-alkyl substituted acetylenes with the unsubstituted phenylacetylene at the 2-position of $N^6$-methyl adenosine. From this study, it was possible to conclude that a higher $A_3$AR affinity was obtained when a methyl group is introduced in para position of the phenyl ring ($K_i$ = 1.5 nM compared to $K_i$ = 3.4 nM for 2-phenylethynyl analogue **1.10**). Extending the size of the alkyl chain (*p*-propyl and *p*-pentyl) resulted in a decrease in affinity. 2-Hexynyl was chosen because it exhibited an excellent $A_3$AR affinity in Cristalli's study ($K_i$ = 1.1 nM).[54]



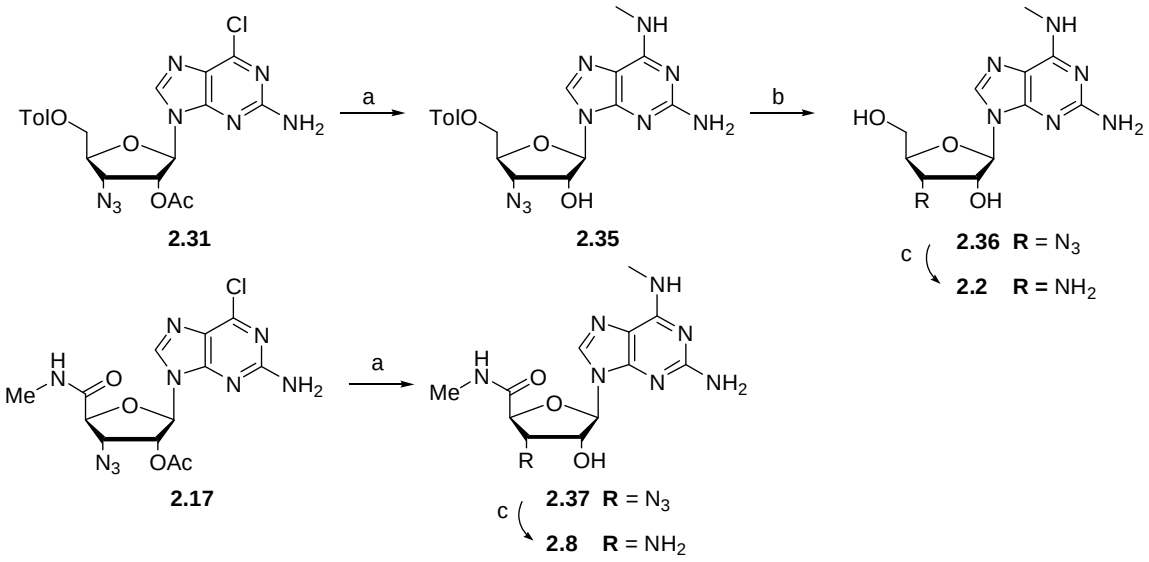

**Scheme 2.3**

Synthesis of compounds **2.3-2.5**. Reagents and conditions: (a) (i) 75% HCOOH, 50 ℃ (ii) $(CH_3CO)_2O$, pyridine, rt (90%); (b) 2-amino-6-chloropurine, BSA,TMSOTf, $CH_3CN$, reflux (65%); (c) Isoamylnitrite, $I_2$, CuI, $CH_2I_2$ in THF, reflux (81%); (d) (i) $CH_3NH_3Cl$, $Et_3N$, EtOH, reflux, (ii) 7N $NH_3$ in MeOH (69%); (e) Na° in MeOH (95%); (f) $Ph_3P$, $H_2O$, THF, 2 days (92%); (g) alkyne, $(PPh_3)_2PdCl_2$,CuI, $Et_3N$, DMF (80-82%).

### 2.2.3 Synthesis of Other 2-Substituted Adenosine Derivatives

To continue the exploration of the 2-position, we synthesised the 2-I and the 2-$NH_2$ derivatives of the 5'-OH and the 2-H and the 2-I derivatives of the 5'-methylcarbamoyl 3'-amino-$N^6$-aminomethyl adenosine analogues (Scheme 2.4).

3'-Amine **2.6** (Figure 2.1) was prepared by catalytic hydrogenation of the 3'-azide precursor which has been described.[193]




**Scheme 2.4**

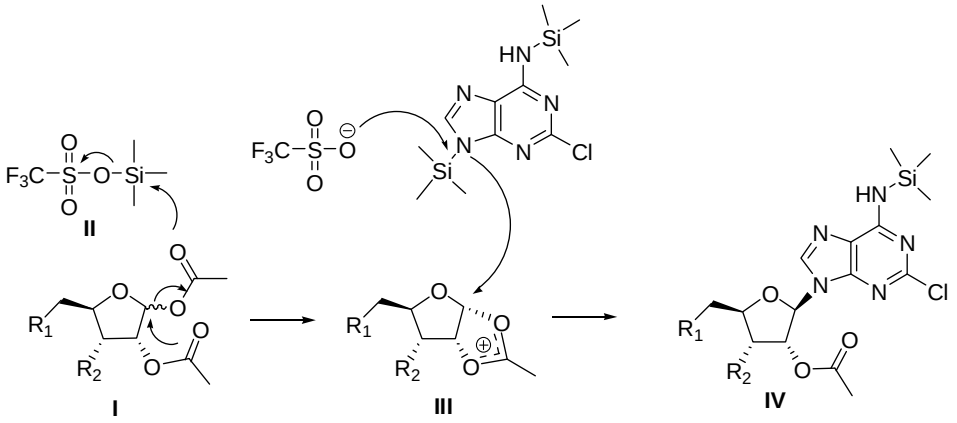
Synthesis of compounds **2.2** and **2.8**. Reagents and conditions: (a) (i) $CH_3NH_3Cl$, $Et_3N$, EtOH, reflux, (ii) 7N $NH_3$ in MeOH (68%); (b) Na° in MeOH (91%); (c) $Ph_3P$, $H_2O$, THF, 2 days (80%).

### 2.2.4 Mechanism of the Vorbrüggen Coupling Reaction

In the Vorbrüggen coupling method[184], the glycosidic bond formation is driven by the use of silylated nucleobases and strong Lewis acids. This highly reproducable method generally provides nucleoside formation in high yield with reliable and predictable stereochemistry. Generally, formation of a β-nucleoside, for which the nucleobase attacks from the β-side of the sugar, is desirable.

Silylation (e. g. with hexamethyldisilazane) converts the polar, often rather insoluble bases into lipophilic silyl compounds, which are readily soluble in organic solvents, permitting homogeneous reactions. Silylation also provides better nucleophiles because of the electron-releasing property of silicon.

The Vorbrüggen coupling mechanism is depicted in Scheme 2.5. Reaction of the 2'-acetate group of a 1,2-diacetylated ribofuranosyl sugar **I** and the Lewis acid catalyst (e.g. trimethylsilyl triflate **II**) results in the conversion of **I** into a stable 1,2-acetyloxonium salt **III**. Under these conditions the nucleophilic silylated base can only attack the sugar cation from the β-side, yielding the exclusive formation of β-nucleoside **IV**. Simultaneously, trimethylsilyl triflate is regenerated by reaction of the triflate anion with the persilylated nucleobase.




**Scheme 2.5**

The Vorbrüggen coupling reaction for the formation of D-ribonucleosides

## 2.3 Biological Evaluation

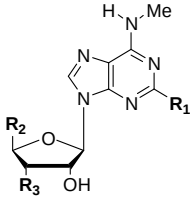
For the adenosine derivatives prepared in this study (**2.1- 2.9, 2.19-2.21**, **2.34**, **2.36**, **2.38**) we measured both the binding affinities at the $hA_1$, $hA_{2A}$ and $hA_3AR$ and their degree of activation of the $A_3AR$ subtype. The results are reported in Table 2.1. The ability of each of these adenosine derivatives to compete for radioligand binding at each of these hARs was evaluated at a fixed concentration of 10 µM, and full competition curves were determined at the $A_3AR$. Six different 2-substituents were included: H, I, $NH_2$, Ph-C≡C, *p*MePh-C≡C and nBu-C≡C. The choice of the methyl group as a small $N^6$ substituent was based on the results of Cristalli et al. who demonstrated that it increased the affinity for the human $A_3AR$ and significantly enhanced the $A_3AR$ selectivity.[48]

Results from the competition experiments showed that the $A_3AR$ affinities of the 3'-amines were much higher than those of the 3'-azides. For all derivatives studied, the 5'-methylcarbamoyl modification in general enhanced the affinity at the $A_3AR$ in comparison to 5'-$CH_2OH$. Except for **2.34**, all evaluated compounds showed a very high selectivity for the $A_3AR$ compared to the other ARs. The most potent compound (**2.9**) displayed a $K_i$ value of 16 nM at the $A_3AR$. The C-2 subsituent of this compound, a phenylethynyl moiety, was previously shown to enhance $A_3AR$ affinity and selectivity.[54] Although the excellent $A_3AR$ affinities of the 3'-unmodified *p*-methyl-phenylethynyl ($K_i$ = 1.5 nM) and 1-hexynyl ($K_i$ = 1.1 nM) $N^6$-methyladenosine analogues, the 2-phenylacetylene substituent of 3'-amino derivative **2.3** proved to have a superior contribution to $A_3AR$ affinity than a *p*-methyl-phenylethynyl (**2.4**) or a 1-hexynyl (**2.5**) moiety. Furthermore, the 2-phenylethynyl modification appeared to overcome the reduction of affinity caused by the 3'-azide (cf. $K_i$ = 78.9 nM for **2.20** vs. 1140 nM for **2.38**). Consequently, this C-2 substituent was selected to be combined with a 3'-amino and a 5'-methylcarbamoyl modification.

Previous studies[48] showed that 3'-amino derivatives exhibit a decreased affinity at the $A_3AR$ compared to their 3'-hydroxy analogues. The affinity reduction associated with this 3' modification could be overcome by elaborating the $N^6$-substituents, for example with a substituted benzyl group.[48] The high affinity of compound **2.9** ($K_i$ = 15.6 nM) demonstrated that a 2-phenylethynyl modification in concert with a small $N^6$-substituent was likewise capable to overcome this reduction in affinity. Note that in

our experiments the affinity of derivative **2.6** for the $A_3AR$ ($K_i$ = 32.3 nM) was 4-fold higher than reported by DeNinno et al.[48] The 2-I analogue **2.7** also showed appreciable $A_3AR$ affinity ($K_i$ = 71.4 nM). Conversely, the 2-$NH_2$ analogue **2.8** exhibited a weak $A_3AR$ affinity ($K_i$ = 536 nM).

The results of the cyclic cAMP-assay (Table 2.1) indicated that all 3'-azides were $A_3AR$ antagonists, except for compound **2.38** which showed partial agonist activity. Also the 3'-(4-phenyl-1,2,3-triazol-1-yl) derivative **2.21** appeared to be an $A_3AR$ antagonist. All other compounds were partial agonists, except for compound **2.8** that manifested full agonist activity.




| | $R_1$ | $R_2$ | $R_3$ | $K_i$ (nM) or %inh. at 10 µM | | | % Efficacy[b] |
|---|---|---|---|---|---|---|---|
| | | | | **$hA_1$** | **$hA_{2A}$** | **$hA_3$** | **$hA_3$** |
| **2.1** | I | $CH_2OH$ | $NH_2$ | 39% | 12% | 879±346 | 67±6% |
| **2.2** | $NH_2$ | $CH_2OH$ | $NH_2$ | 16% | 4% | 654±42 | 57±2% |
| **2.3** | Ph-C≡C | $CH_2OH$ | $NH_2$ | 50% | 14% | 126±4.3 | 36±6% |
| **2.4** | pMePh-C≡C | $CH_2OH$ | $NH_2$ | 33% | 0% | 145±35 | 23±3% |
| **2.5** | nBu-C≡C | $CH_2OH$ | $NH_2$ | 4% | 3% | 389±112 | 66±11% |
| **2.6** | H | CONHMe | $NH_2$ | 18% | 12% | 32.3±3.9 | 72±10% |
| **2.7** | I | CONHMe | $NH_2$ | 16% | 24% | 71.4±12.8 | 18±10% |
| **2.8** | $NH_2$ | CONHMe | $NH_2$ | -14% | 10% | 536±247 | 92±3% |
| **2.9** | **Ph-C≡C** | **CONHMe** | **$NH_2$** | **9%** | **9%** | **15.6±3.6** | **67±11%** |
| **2.19** | I | CONHMe | $N_3$ | 16% | 0% | 2530[c] | -8±5% |
| **2.20** | Ph-C≡C | CONHMe | $N_3$ | 31% | 16% | 78.9±12.4 | -11±8% |
| **2.21** | Ph-C≡C | CONHMe | 4-Ph-1,2,3-triazol-1-yl | 14% | 2% | 1820±770 | 0% |
| **2.34** | I | $CH_2OH$ | $N_3$ | 85%[c,d] | 27% | 6540±320 | -3±2% |
| **2.36** | $NH_2$ | $CH_2OH$ | $N_3$ | 49% | 16% | 28,800[b] | 0% |
| **2.38**[193] | H | CONHMe | $N_3$ | 12% | 10% | 1140±300 | 38±4% |

**Table 2.1**

Binding affinities of adenosine derivatives at human $A_1$, $A_{2A}$ and $A_3$ARs expressed in CHO cells[a]

(a) All $A_3AR$ experiments were performed using adherent CHO cells stably transfected with cDNA encoding one of the human adenosine receptors. Binding at human $A_1$, $A_{2A}$, and $A_3$ARs in this study was carried out as described in Methods using [$^3$H] PIA, [$^3$H]CGS 21680 or [$^{125}$I]AB-MECA as radioligand. Values from the present study are expressed as $K_i$ values (mean±s.e.m., n = 3, unless noted), or as percent displacement of radioligand.

(b) % Activation at 10 µM, relative to cyclic AMP inhibitory effect of 10 µM NECA = 100%. Cl-IB-MECA was also a full agonist (100%) in this assay.

(c) n=1

(d) $K_i$ ($A_1$ AR)= 1850 nM

## 2.4 Conclusions

The 2,3',5'-trisubstituted and 2,3'-disubstituted $N^6$-methyl adenosine derivatives described in the present study were synthesized in good overall yields. All the compounds had $A_3$AR affinities in the low micromolar or nanomolar range and showed very high $A_3$AR selectivity. The 3'-azides appeared to be $A_3$AR antagonists with a moderate $A_3$AR affinity. The 3'-amino modification significantly improved the $A_3$AR affinity and resulted in partial $A_3$AR agonists. For both the 3'-azido and the 3'-amino derivatives, the 5'-methylcarbamoyl modification improved the overall affinity. Curiously, the presence of a 5'-uronamide did not restore full $A_3$AR efficacy in 2-substituted derivatives, as was demonstrated in the case of $N^6$-substituents that reduced efficacy. The 2-phenylethynyl derivative **2.9** demonstrated high $A_3$AR receptor affinity with a $K_i$ value of 15.6 nM and >1000-fold selectivity. Previous studies revealed that 3'-amines exhibit a decreased affinity compared to their 3'-hydroxy analogues. This study demonstrated that introduction of a 2-phenylethynyl substituent in concert with the $N^6$-methyl group is capable of overcoming this affinity drop.

## CHAPTER 3:

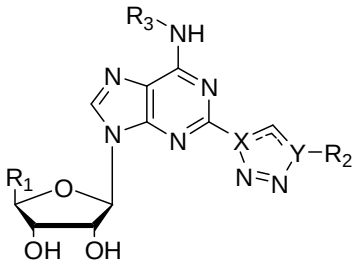
# 2-TRIAZOLE-SUBSTITUTED ADENOSINE ANALOGUES

# 3 2-TRIAZOLE-SUBSTITUTED ADENOSINE ANALOGUES

## 3.1 Introduction

As described in 1.2.4.2, adenosine receptor antagonists are diverse in structure. Most reported antagonists lack the ribose moiety, which seems to be essential for agonist activity.[194] However, the $A_3$AR, more than other AR subtypes, is amenable to the design of nucleoside-based antagonists. The efficacy of nucleoside derivatives in activation of the $A_3$AR is particularly sensitive to molecular substitution of the ligand.[195] A wide range of adenosine derivatives have been shown to antagonize this receptor, including the highly potent $A_1$AR agonist 2-chloro-$N^6$-cyclopentyladenosine (**1.33,** Figure 1.15). $N^6$-Benzyl groups are associated with reduced $A_3$AR efficacy, leading to partial agonists and antagonists. However, many of the nucleosides so far demonstrated to be antagonists of the $A_3$AR are not highly subtype-selective.[196] Nucleoside-based $A_3$AR antagonists maintaining an intact ribose moiety were reported by Volpini et al.,[197] with a series of 8-alkynyladenosine derivatives (**1.36**, Figure 1.15) that exhibited $A_3$AR selectivity, but suffered from weak $A_3$AR affinity. A spirolactam derivative, in which the 5'-*N*-alkyluronamide group was cyclized onto the 4'-carbon (**1.35**, Figure 1.15), was found to potently and selectively antagonize the $A_3$AR.[198,199] An important advantage of nucleoside-based $A_3$AR antagonists over other heterocyclic antagonists is the ability to achieve high affinity at murine species, which permits in vivo evaluation using wild-type $A_3$AR mice.

Recently, researchers from CV Therapeutics described a series of 2-pyrazolyladenosine analogues (**1.11**, Figure 1.8).[200] Several representative compounds containing a $N^6$-methyl substituent proved to display high affinity and selectivity for the $A_3$AR. This study confirms the former finding[54] that introduction of a methyl group into the $N^6$-position increases the affinity for the human $A_3$AR and enhances the selectivity versus $A_1$ and $A_{2A}$ARs. On the basis of these results, we explored the versatile "click chemistry" approach[201] to synthesize two series of $N^6$-methyl-2-(1,2,3-triazolyl)adenosine derivatives and evaluated their affinity, selectivity, and efficacy at the $A_3$AR. Figure 3.1 presents an overview of the performed modifications.




$R_1$ = $CH_2OH$, CONHMe, CONHEt
$R_2$ = alkyl, aryl, arylalkyl, cycloalkylmethyl
$R_3$ = Me, substituted Bn

X = C, Y = N;
X = N, Y = C

**Figure 3.1**

Overview of the performed modifications

Although a number of 1,2,3-triazole nucleoside derivatives have been described,[202] most involve the replacement of the nucleobase moiety with with a 1,2,3-triazole or introduction of 1,2,3-triazole at C8 or at the sugar moiety. A few examples are depicted in Figure 3.2.

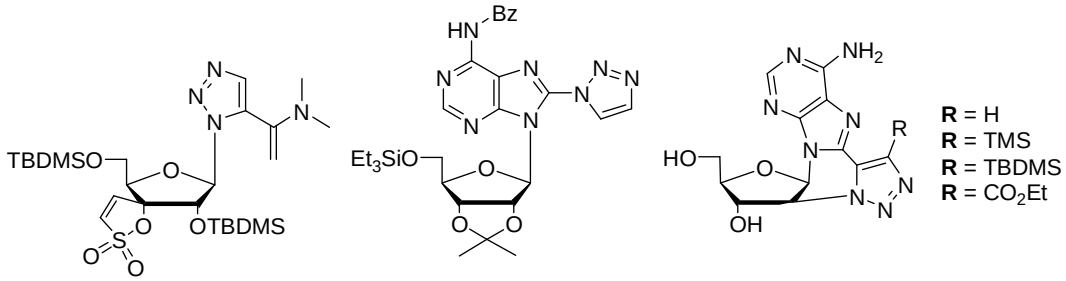



**Figure 3.2**

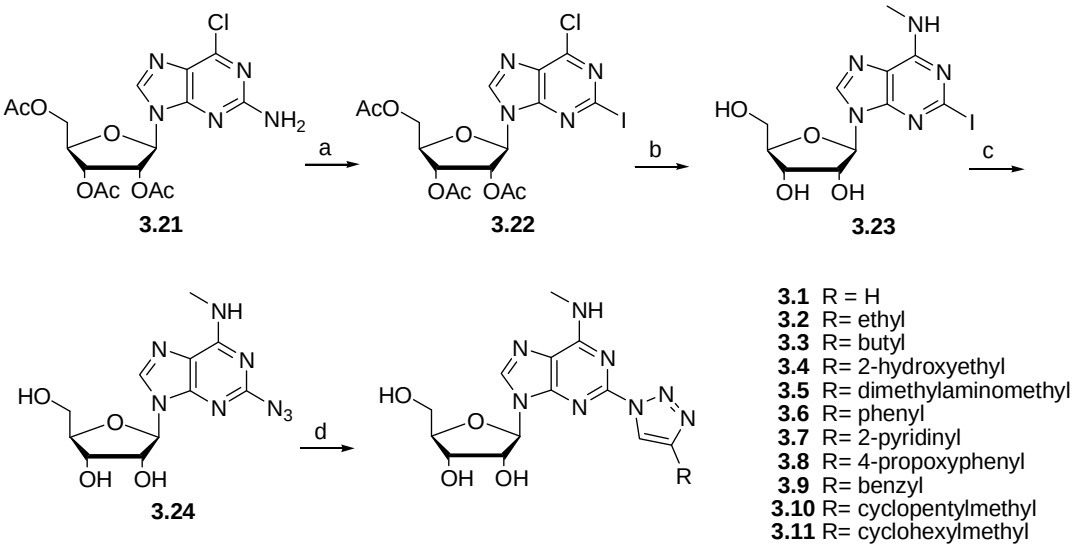
Examples of 1,2,3-triazole nucleoside derivatives described previously

# 3.2 Chemistry

### 3.2.1 Synthesis of 2-[(1,2,3)-Triazol-1-yl]adenosine Derivatives 3.1-3.11

The synthesis of the 1,2,3-triazol-1-yladenosine derivatives is depicted in Scheme 3.1. Classical Vorbrüggen coupling[184] of silylated 2-amino-6-chloropurine and β-D-ribofuranose 1,2,3,5-tetraacetate yielded nucleoside **3.21**. 2-Iodine substitution was followed by treatment of the resulting 2-iodo derivative **3.22**[203] with 2.0 M $CH_3NH_2$ in THF, allowing simultaneous introduction of the $N^6$-methylamine and deprotection of the acetyl groups to obtain 2-Iodo-$N^6$-methyladenosine **3.23**. This one step procedure was more convenient and resulted in a higher yield (99%) than our earlier described method (e.g. 77% yield for **2.19** from **2.18**), in which $CH_3NH_2$ was generated in situ from its hydrochloride salt by treatment with $Et_3N$. Moreover, further treatment with 7N $NH_3$ in methanol to complete deacetylation was not necessary. Since the reaction conditions[204] for a Cu(I)-catalyzed nucleophilic substitution are very similar to those used in the "click" variant of Huisgen's [3+2]cycloaddition, we initially attempted to perform a one-pot conversion of **3.23** to the desired 1,4-disubstituted 1,2,3-triazoles. The 2-azido derivative **3.24** was isolated as the main reaction product, and only a minor amount of the appropriate triazole was formed. This event forced us to perform the reaction in two steps. First the azido intermediate **3.24** was prepared in 66% yield from **3.23**. $^1H$ and $^{13}C$ NMR in DMSO-$d_6$ proved the presence of a tautomeric fused tetrazole form (17%) of the 2-azidoadenosine derivative **3.24**, due to a spontaneous cyclization (Figure 3.3). Such azido/tetrazole tautomerism has been previously reported for 2-azidoadenine and for 2-azidoadenosine.[205,206]

Next we applied a Cu(I)-catalyzed [3+2]cycloaddition reaction of azide **3.24** with the appropriate alkyne to generate the triazole analogues **3.1-3.11** (Scheme 3.1).[207] Generally, the use of a water/butanol mixture as a solvent for the [3+2]cycloaddition allowed simple isolation of the desired compounds, which precipitated from the reaction medium.




**Scheme 3.1**

Synthesis of 1,2,3-triazol-1-yl analogues of $N^6$-methyl-adenosine **3.1 -3.11**. Reagents and conditions: (a) isoamyl nitrite, $I_2$, CuI, $CH_2I_2$ in THF, reflux (79%); (b) $CH_3NH_2$ in THF, 2 days (99%); (c) $CuSO_4.5H_2O$, sodium ascorbate, L-proline, $Na_2CO_3$, $NaN_3$, $H_2O$:*t*-BuOH 1:1, 60 ℃ (66%); (d) $CuSO_4.5H_2O$, sodium ascorbate, appropriate alkyne, $H_2O$:*t*-BuOH 3:1, rt (32-82%).

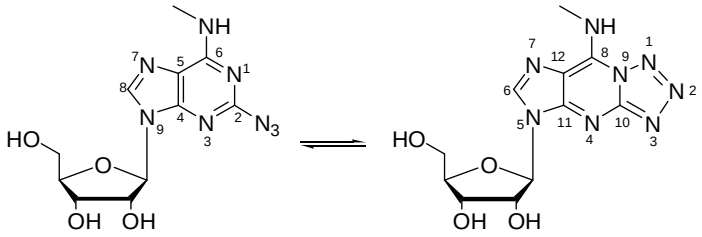
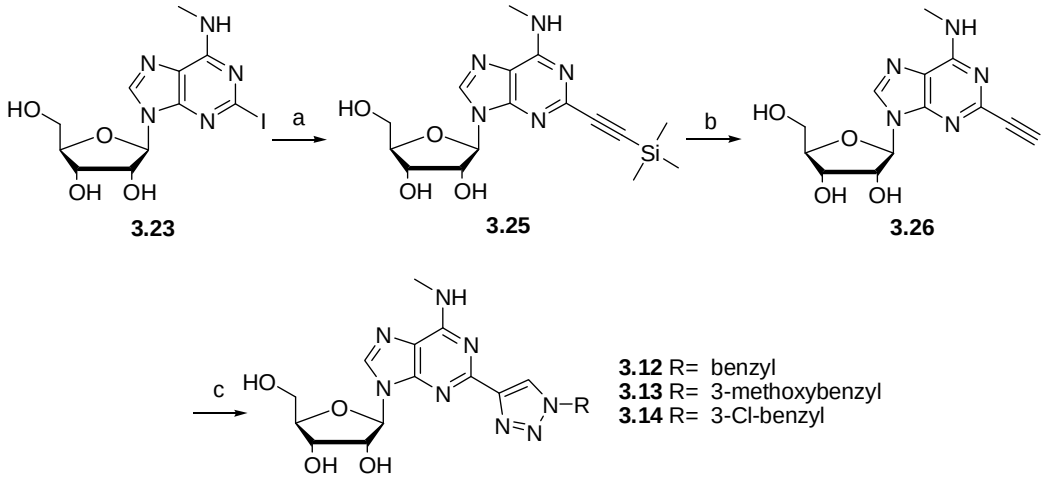


**Figure 3.3**

Azido/tetrazol tautomerism of a 2-substituted adenosine derivative **3.24**.

### 3.2.2 Synthesis of 2-[(1,2,3)-Triazol-4-yl]adenosine Derivatives 3.12-3.14

Similarly, the 1,2,3-triazol-4-yl analogues **3.12-3.14** (Scheme 3.2) were prepared by a Cu+-catalyzed Huisgen [3+2]cycloaddition reaction of 2-ethynyl-$N^6$-methyladenosine (**3.26**) with the appropriate azide.

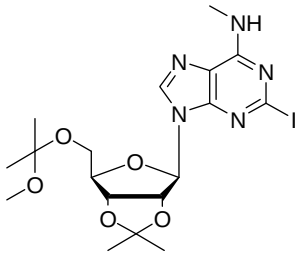



**Scheme 3.2**

Synthesis of 1,2,3-triazol-4-yl analogues of $N^6$-methyl-adenosine **3.12-3.14**. Reagents and conditions: (a) trimethylsilylacetylene, CuI, $(Ph_3P)_3PdCl_2$, DMF (66%); (b) 7N $NH_3$ in MeOH, 0 ℃ (65%); (c) $CuSO_4.5H_2O$, sodium ascorbate, appropriate azide, $H_2O$:*t*-BuOH 3:1, rt (73-80%).

### 3.2.3 Synthesis of 5'-Uronamide-2-[(1,2,3)-triazol-1-yl]adenosine Analogues 3.15a,b-3.19a,b

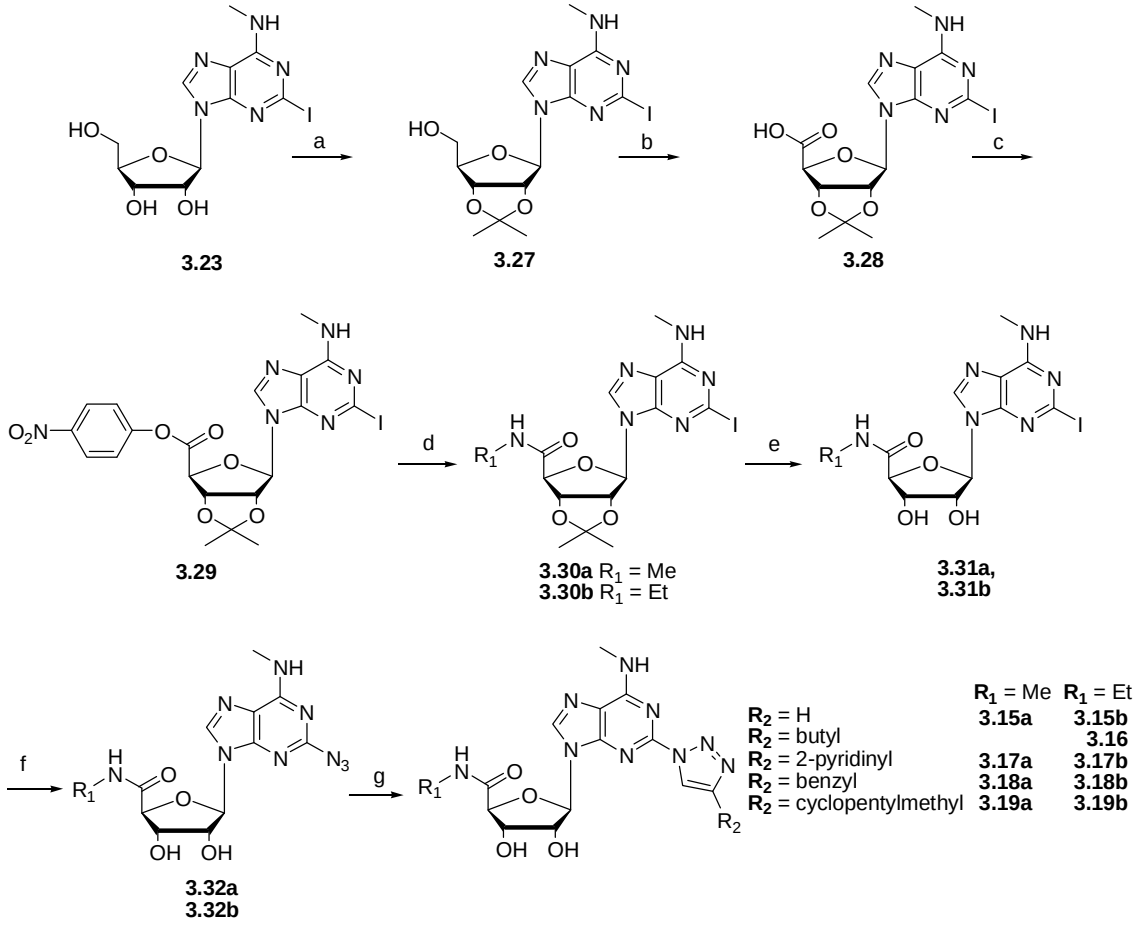
The synthesis of the 5'-*N*-methylcarbamoyl and 5'-*N*-ethylcarbamoyl 2-(1,2,3)-triazol-1-yladenosine analogues **3.15a,b-3.19a,b** (Scheme 3.3) started with the 2',3'-*O*-isopropylidene protection of **3.23**. Therefore, **3.23** was treated with 2,2-dimethoxypropane and *p*-toluene sulfonic acid. The low yield of this reaction (45%) was caused by the formation of the important side product **3.27bis** (Figure 3.4), by reaction of 2,2-dimethoxypropane with the 5'-hydroxyl group. Treatment of **3.27bis** with 80% trifluoroacetic acid allowed us to recuperate the starting material. 2',3'-Protection of **3.23** with $H_2SO_4$ and anhydrous $CuSO_4$ in dry acetone allowed to obtain **3.27** in 68% yield.




**3.27bis**

**Figure 3.4**

To obtain carboxylic acid **3.28**, both permanganate oxidation and 2,2,6,6-tetramethylpiperidinoxy/[bis(acetoxy)iodo]benzene (TEMPO/BAIB) oxidation of **3.27** were evaluated. However the yields were equally, the TEMPO/BAIB oxidation was much better reproducable. Carboxylic acid **3.28** was converted into its *p*-nitrophenyl ester **3.29**. Treatment with methylamine or ethylamine gave methyluronamide **3.30a** and ethyluronamide **3.30b**. Deprotection with 80% trifluoroacetic acid yielded 2-iodo-adenosine analogues **3.31a** and **3.31b**,[208] which were converted into the 2-azido intermediates **3.32a** and **3.32b** in 79% yield. Also here, the presence of a tautomeric fused tetrazole form (20%) of the 2-azidoadenosine derivatives, due to a spontaneous cyclization, was observed in the NMR spectrum. Finally we applied the Cu(I)-catalyzed [3+2]cycloaddition reaction of azides **3.32a** and **3.32b** with the appropriate alkyne to generate the triazole analogues **3.15a,b-3.19a,b.** (Scheme 3.3)

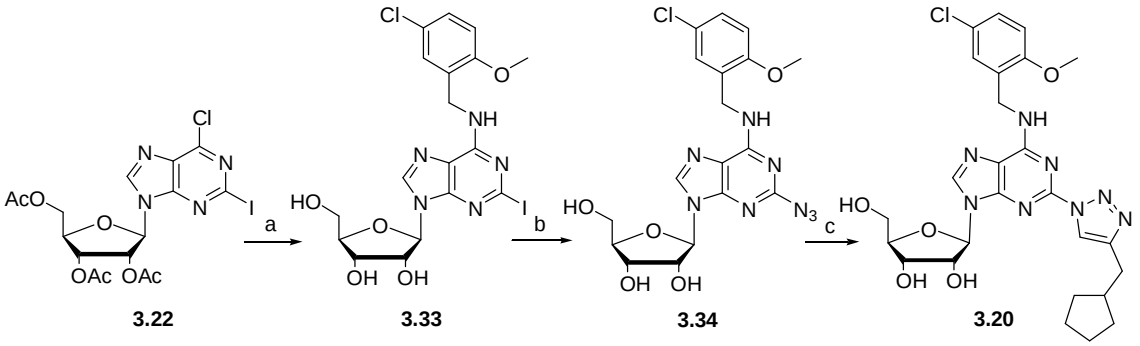



**Scheme 3.3**

Synthesis of 1,2,3-triazol-1-yl analogues **3.15a,b-3.19a,b**. Reagents and conditions: (a) anh. $CuSO_4$, $H_2SO_4$, dry acetone; (b) TEMPO, BAIB, $CH_3CN$:$H_2O$ 1:1 (75%); (c) *p*-nitrophenol, EDCI, DMF, rt; (d) methylamine or ethylamine; (e) 80% TFA/$H_2O$ (63% for **3.31a**; 78% for **3.31b**, overall yield from **3.28**); (f) $CuSO_4.5H_2O$, sodium ascorbate, L-proline, $Na_2CO_3$, $NaN_3$, $H_2O$:*t*-BuOH 1:1, 60 ℃ (**3.32a**: 68% yield; **3.32b**: 79% yield); (g) $CuSO_4.5H_2O$, sodium ascorbate, alkyne, $H_2O$:*t*-BuOH 1:1, rt (32-54%).

### 3.2.4 Synthesis of Compound 3.20

$N^6$-(5-chloro-2-methoxybenzyl)-2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl)-9-(β-D-ribofuranosyl)adenine (**3.20**) was prepared in three steps starting from intermediate **3.22**, as depicted in Scheme 3.4.




**Scheme 3.4**

Synthesis of compound **3.20**, 2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl)-$N^6$-(2-chloro-5-methoxybenzyl)-adenosine. Reagents and conditions: (a) (i) 2-chloro-5-methoxy-benzylammonium chloride, $Et_3N$, EtOH, reflux, (ii) 7N $NH_3$ in MeOH (80%); (b) $CuSO_4.5H_2O$, sodium ascorbate, L-proline, $Na_2CO_3$, $NaN_3$, $H_2O$:*t*-BuOH 1:1, 60 ℃ (82%); (c) $CuSO_4.5H_2O$, sodium ascorbate, alkyne, $H_2O$:*t*-BuOH 1:1, rt (59%).

### 3.2.5 Mechanism of the Cu(I) Catalyzed [3+2]Cycloaddition of Azides and Alkynes

Via a concerted mechanism, the Huisgen [3+2]cycloaddition[186] of an organic azide and a terminal alkyne usually afforded a mixture of 1,4- and 1,5-disubstituted regioisomers (Figure 3.5), unless the acetylene component is attached to an electron-withdrawing group such as a carbonyl or perfluoroalkyl.[209]

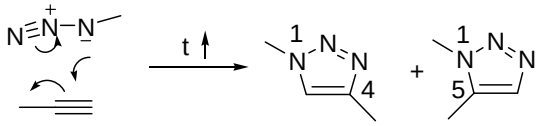



**Figure 3.5**

By using Cu(I) to catalyse the reaction, a complete regioselectivity for forming only 1,4- over 1,5-disubstituted triazoles has been achieved with a very efficient procedure.[210,211] The non-concerted mechanism of this Cu(I) catalyzed variant is depicted in Figure 3.6. The sequence begins with the conversion to the alkyne to the acetylide **I**, by coordination of the alkyne to the Cu(I) species, displacing one of the ligands. With acetonitrile as a ligand, this step was slightly endothermic (0.6 kcal/mol), while with water as a ligand, the displacement process becomes exothermic (-11.7 kcal/mol). This is in good agreement with the experimental observation that the reaction proceeds much faster in aqueous solutions and does not require an amine base.

In the next step, the azide replaces one of the ligands and binds to the copper atom via the nitrogen proximal to carbon, forming intermediate **II**. After that, the distal nitrogen of the azide in **II** attacks the C-2 carbon of the acetylide, forming the unusual six-membered copper metallocycle **III**. This reactive intermediate **III** undergoes ring contraction with formation of the triazolyl-copper derivative **IV**. Proteolysis of releases the triazole product, thereby completing the catalytic cycle.

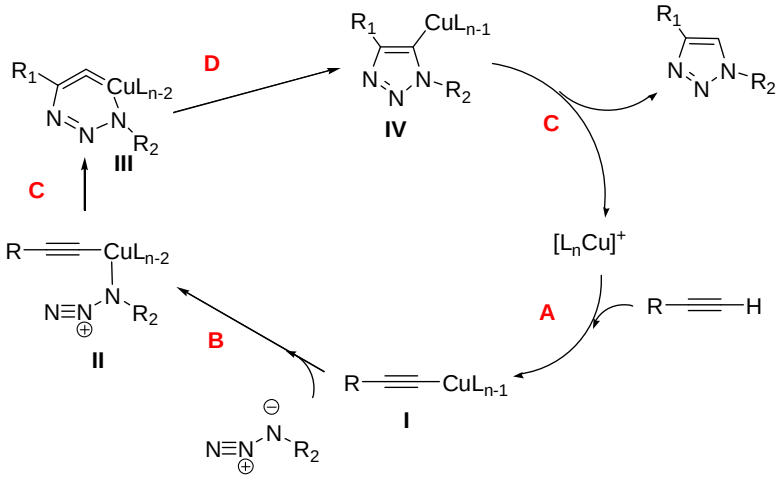



**Figure 3.6**

A mechanistic proposal for the catalytic cycle of the Cu(I) Catalyzed [3+2]Cycloaddition of Azides and Alkynes

Cu(I) salts (e.g. CuI) can be used directly or be generated in situ by reduction of Cu(II) salts (e.g. $CuSO_4$. $5H_2O$). The in situ Cu(I) formation is preferred, because formation of undesired byproducts, primarily diacetylenes, bis-triazoles, and 5-hydroxytriazoles are observed using Cu(I) salts, and Cu(II) salts are less costly and often purer than Cu(I) salts. Ascorbic acid or sodium ascorbate proved to be excellent reductants. Reactions with Cu(I) salts require acetonitrile as co-solvent and one equivalent of a nitrogen base. Acetonitrile can stabilize the Cu(I) oxidation state and can keep the catalytic species in the solution.

This Cu(I)-catalyzed variant is described as one of the most reliable processes of “click chemistry”, and therefore often referred to simply as the "click reaction". The concept of click chemistry, launched by Sharpless in 2001, [212] is an approach to generate substances by joining small units together with heteroatom links. The required click chemistry process characteristics include simply reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation. Since 2001, the click chemistry concept has grown in impact within drug discovery.[213,214] Especially the Huisgen [3+2]cycloaddition reacting terminal alkynes with azides forming 1,2,3-triazoles has been a convenient reaction. The reaction is chemoselective, it accepts almost all other functional groups, it can be performed in aqueous mixtures, and all atoms are used without the production of side products.

## 3.3 Biological Evaluation

The binding affinities of the newly synthesized adenosine derivatives were measured at the $hA_1$, $hA_{2A}$, and $hA_3$ARs expressed in CHO (Chinese hamster ovary) cells as previously described,[215] and their relative efficacy to activate the $A_3$AR was determined (Table 3.1). The binding affinity of more potent compounds at the ARs was evaluated with full competition curves, while the weaker compounds at the $hA_1$ and $hA_{2A}$ ARs were measured at a fixed concentration of 10 µM. Several compounds showed affinity for the $A_3$AR in the low nanomolar range, a very high ratio of $A_3/A_{2A}$ selectivity, and a moderate-to-high $A_3/A_1$ selectivity ratio. The functional $A_3$AR activity was measured relative to the full agonist NECA (10 µM) = 100% by a cAMP accumulation assay by the method of Nordstet and Fredholm[216] or a [$^{35}$S]GTP$\gamma$S binding assay measured by a variation of the method described by Jacobson et al.[217] The range of efficacies observed depended on the nature of the groups at the 2 and $N^6$-positions.

The 2-azido precursor **3.24** showed high binding affinity at the $A_3$AR ($K_i$ = 10.8 nM) and modest selectivity in comparison to the $A_1$AR. The 1,2,3-triazol-1-yl derivatives obtained by 1,3-dipolar cycloaddition of azide **3.24** with acetylene (**3.1**), butyne (**3.2**), and hexyne (**3.3**) maintained high affinity for the $A_3$AR and increased selectivity. They displayed $K_i$ values of 10.4 nM, 13.8 nM, and 11.7 nM, respectively. Also, aromatic triazole substituents (**3.6**, **3.7**, **3.9**) resulted in similar $K_i$ values of about 10 nM and even greater selectivity. Introducing nitrogen or oxygen including substituents at position 4 of the 1,2,3-triazole ring (**3.4**, **3.5**, and **3.8**) reduced the $A_3$AR affinity. Among the investigated analogues, the 4-cyclopentylmethyl derivative **3.10** exhibited the highest affinity for the $A_3$AR ($K_i$ = 1.3 nM) and 260-fold selectivity in comparison to the $A_1$AR. Replacement of the cyclopentyl ring with a phenyl (**3.9**) or cyclohexyl (**3.11**) moiety adversely affected $A_3$AR affinity. Remarkably, the 1,2,3-triazol-4-yl regiomers (**3.12–3.14**) showed decreased affinity for the $A_3$AR in comparison to similar 1,2,3-triazol-1-yl regiomers. In particular, a comparison of homologous compounds **3.12** and **3.9** indicated a 6-fold loss of affinity at the $A_3$AR for the 4-yl isomer, approximately the same affinity at the $A_1$AR, and no significant measurable gain in affinity at the $A_{2A}$AR.

Replacement of the ribose 4'-hydroxymethyl moiety of the 2-azido derivative **3.24** by a 5'-*N*-ethyluronamide did not appreciably affect affinity at any of the AR subtypes (**3.32b**). However, a similar substitution in the 2-(1,2,3-triazol-1-yl)-substituted series provided a modest (2- to 5-fold) increase in $A_3$AR affinity and small or no changes in $A_1$AR affinity, as demonstrated for the unsubstituted, 4-butyl, 4-pyridin-2-yl, and 4-benzyl substituted 1,2,3-triazol-1-yl combinations (**3.15b**, **3.16b**, **3.17b**, and **3.18b**, in comparison to **3.1**, **3.3**, **3.7**, and **3.9**, respectively). Curiously, one 5'-*N*-ethyluronamide, compound **3.19b**, exhibited decreased $A_3$AR affinity ($K_i$ = 11.5 nM) compared to its potent 5'-OH analogue **3.10** ($K_i$ = 1.3 nM), while introduction of a similar 5'-*N*-methyluronamide modification was able to partially restore the affinity drop (**3.19a**, $K_i$ = 4.5 nM). This 5'-*N*-methyluronamide modification also showed affinity enhancing effects when applied to the 2-azido derivative **3.24**. 2-Azido 5'-*N*-methyluronamide derivative **3.32a** exhibited enhanced $A_3$AR affinity ($K_i$ = 5.1 nM), compared to the corresponding 5'-hydroxymethyl (**3.24**, $K_i$ = 10.8 nM) and 5'-*N*-ethyluronamide (**3.32b**, $K_i$ = 11.4 nM) analogues.

For the unsubstituted (**3.15a**), the 4-benzyl (**3.18a**) and 4-pyridin-2-yl (**3.17a**) substituted 1,2,3-triazol-1-yl derivatives, the 5'-*N*-methyluronamide modification displayed varying results. The unsubstituted 2-(1,2,3-triazol-1-yl) derivative **3.15a** exhibited a greatly reduced $A_3$AR affinity ($K_i$ = 76 nM), in comparison to the corresponding 5'-hydroxymethyl (**3.1**, $K_i$ = 10.4 nM) and 5'-*N*-ethyluronamide (**3.15b**, $K_i$ = 2.1 nM) analogues. For the 4-benzyl substituted 1,2,3-triazol-1-yl derivatives, the 5'-*N*-methyluronamide modification (**3.18a**, $K_i$ = 12.2 nM) did not affect the $A_3$AR affinity compared to the corresponding 5'-hydroxymethyl analogue **3.9** ($K_i$ = 9.5 nM), while for the 4-pyridin-2-yl substituted analogues, the 5'-*N*-methyluronamide modification yielded better $A_3$AR affinity (**3.17a**, $K_i$ = 3.6 nM, in comparison to **3.7**, $K_i$ = 10.3 nM). However, for both the 4-benzyl and 4-pyridin-2-yl substituted analogues, the 5'-*N*-ethyluronamide modification demonstrated the best $A_3$AR affinity.

Replacement of the $N^6$-methyl substituent of the 2-azido precursor **3.24** by a sterically demanding 2-chloro-5-methoxybenzyl group yielded **3.34**, which manifested very high $A_3$AR affinity ($K_i$ = 1.4 nM). A similar replacement of the $N^6$-methyl group of an analogue **3.10**, also having a bulky 2-position substituent, to yield **3.20** reduced $A_3$AR affinity but not appreciably. This was in accordance with previous observations that a simultaneous substitution at the 6- and 2-positions did not improve $A_3$AR

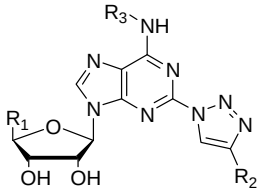
affinity.[200,40] Thus, the effects of substitution at the 2- and $N^6$-positions were not independent; however, it was possible to retain considerable $A_3$AR selectivity (46-fold in compound **3.20**). This was not representative of findings in a previous study in which double substitution greatly diminished the affinity and selectivity at the human $A_3$AR.[54,200]

Whereas some previously synthesized 2-substituted adenosine derivatives[200] displayed selective $A_3$AR agonist activity, all 2-triazol-1-yl-*N*$^6$-methyladenosine analogues synthesized with an unmodified ribose moiety (**3.1-3.11** and **3.20**) behaved as antagonists or weak partial agonists. Similar findings were reported for 2-ester derivatives of adenosine, in which a combination of 2 and $N^6$ substitution reduced efficacy.[218] Direct ring substitution at the 4-position of the 1,2,3-triazole with alkyl or aryl groups resulted in weak partial agonists (**3.1**, **3.2**, **3.4-3.8**), but subtle changes of structure resulted in a loss of efficacy, e.g., the 4-butyl derivative **3.3**. 2-Triazol-1-yl-*N*$^6$-methyladenosine analogues with a methylene spacer between the 1,2,3-triazole moiety and a ring system yielded full $A_3$AR antagonists (**3.9-3.11, 3.20**), since they bound to the receptor but did not activate it. The 2-triazol-4-yl-*N*$^6$-methyladenosine derivatives (**3.12-3.14**) also behaved as full $A_3$AR antagonists. Thus, the 5'-OH derivatives **3.3**, **3.9**-**3.14**, and **3.20** appeared to be $A_3$AR antagonists with the following order of decreasing selectivity for the $A_3$AR in comparison to the $A_1$AR: 4-cyclopentylmethyl-*N*$^6$-methyl **3.10** (260-fold) >4-butyl-*N*$^6$-methyl **3.3** (72-fold), 4-cyclohexylmethyl-*N*$^6$-methyl **3.11** (67-fold) >4-cyclopentylmethyl-*N*$^6$-(5-chloro-2-methoxybenzyl) **3.20**. Interestingly, the 5'-*N*-ethyluronamide modification was able to reestablish the $A_3$AR agonist activity in analogues with sterically bulky substitution at the 2-position. This is consistent with previous findings that similar 5'-uronamides overcome the efficacy-reducing activity of substitution at the adenine 2- and $N^6$-positions but not at the ribose 3'-position. [199,219,220] Indeed, all 5'-*N*-ethyluronamide analogues studied here (**3.15b-3.19b**) proved to be full agonists at the $A_3$AR. Among them are highly selective $A_3$AR agonists, *N*$^6$-methyladenosine-5'-*N*-ethyluronamide 2-(1,2,3-triazol-1-yl) derivatives: pyridin-2-yl **3.17b** (910-fold) > unsubstituted **3.15b** (280-fold) > benzyl **3.18b** (180-fold). Remarkebly, in the 5'-*N*-methyluronamide series only 4-pyridin-2-yl substituted 1,2,3-triazol-1-yl derivative **3.17a** was able to completely restore the agonist activity, while the other derivatives (**3.15a**, **3.18a**, **3.19a**) exhibited only partial agonist activity.

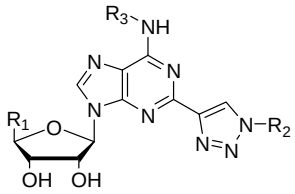
The 2-azido-$N^6$-methyl precursors **3.24, 3.32a** and **3.32b** showed full agonist activity, whereas azide **3.34** having a bulky $N^6$ group showed partial agonist activity. The only 2-pyrazolyl derivative of which the relative efficacy to activate the $A_3$AR was investigated exhibited full agonist activity at the $A_3$AR,[55] which highlights the remarkably antagonist or weak partial agonist activity of the structurally related 2-triazolyl adenosine analogues. Although previously reported 2-alkynyl compounds were claimed to be adenosine $A_3$AR agonists,[54] there are no supporting data reported on their $A_3$AR efficacy. In order to be able to compare our observations with the efficacy results of the 2-alkynyl adenosine derivatives, we evaluated the $A_3$AR efficacy of a few 2-alkynyl substituted adenosine analogues (see 2.2.2). In contrast to what the authors presumed, these 2-alkynyl derivatives exhibited full antagonist activity (results not shown). Consequently, the introduction of an acetylene or 1,2,3-triazolyl spacer resulted in $A_3$AR antagonists or partial agonists, whereas a similar pyrazolyl spacer probably yielded full $A_3$AR agonists. These observations showed that subtle changes or small modifications could influence the $A_3$AR efficacy dramatically, which illustrates the difficultty to predict $A_3$AR receptor affinity and efficacy. However, the fast and easy preparation of the 1,2,3-triazole series and the commercial availability of a large amount of alkynes, permitted us to explore the 2-position profoundly, including the effects of the different substituents on the $A_3$AR efficacy of all the compounds, which was hardly studied in the previously reported studies exploring the 2-position.[54,55]

Selected potent agonists in this series were measured in a functional assay of the human $A_{2B}$AR. At a concentration of 10 µM, compounds **3.3**-**3.7**, **3.15**, and **3.16** did not significantly stimulate adenyate cyclase in human $A_{2B}$AR-expressing CHO cells (<10% of the effect of 10 µM NECA, as a full agonist). Compounds **3.2**, **3.8**-**3.14**, **3.17b**, **3**.**19b**, and **3.24** at 10 µM, stimulated adenylate cyclase by <50%. Compounds **3.18b** and **3.32b** produced approximately 50% stimulation at 10 µM. Thus, selectivity for the $A_3$AR was demonstrated; these nucleosides that activate the $A_3$AR at low nanomolar concentrations activated the $A_{2B}$AR only at substantial micromolar concentrations. On the basis of previous findings, it is predicted that only the analogues containing the substituted $N^6$-benzyl group (5-chloro-2-methoxy), i.e., full agonist **3.20** and partial agonist **3.34**, would be expected to bind in the nanomolar range to the rat $A_3$AR. Small alkyl groups at the $N^6$-position, such as methyl and ethyl,

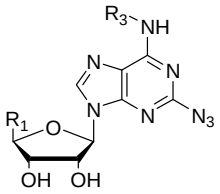
although conducive to high affinity at the human $A_3$AR, led to negligible affinity at the rat homologue of the receptor. Selected compounds were measured in binding to the rat $A_3$AR expressed in CHO cell membranes using [$^{125}$I]I-AB-MECA. The $K_i$ values determined were as follows: compounds **3.5**, **3.7**, and **3.8**, $K_i$ > 10 µM; compound **3.9**, $K_i$ = 1.79 µM; compound **3.10**, $K_i$ = 0.312 µM.



**3.1-3.11, 3.15-3.20**



**3.12-3.14**



**3.23**, **3.31**, **3.33**

| | $R_1$ | $R_2$ | $R_3$ | $K_i$ (nM) or (% inhibition) at 10µM | | | %Efficacy[b] |
|---|---|---|---|---|---|---|---|
| | | | | **$hA_1$** | **$hA_{2A}$** | **$hA_3$** | **$hA_3$** |
| **3.1** | $CH_2OH$ | H | $CH_3$ | 1000±30 | (13±3%) | 10.4±0.2 | 41±6 |
| **3.2** | $CH_2OH$ | ethyl | $CH_3$ | 2920±910 | (18%) | 13.8±3.3 | 23[a] |
| **3.3** | $CH_2OH$ | butyl | $CH_3$ | 848±76 | (23%) | 11.7±3.1 | 3±8 |
| **3.4** | $CH_2OH$ | 2-hydroxyethyl | $CH_3$ | 1270±260 | (14%) | 45.0±4.4 | 25[a] |
| **3.5** | $CH_2OH$ | dimethylaminomethyl | $CH_3$ | 3800±600 | (6%) | 117±25 | 8[a] |
| **3.6** | $CH_2OH$ | phenyl | $CH_3$ | (36%) | (5%) | 14.9±1.7 | 14±8 |
| **3.7** | $CH_2OH$ | pyridin-2-yl | $CH_3$ | 1970±210 | (40%) | 10.3±1.5 | 11±4 |
| **3.8** | $CH_2OH$ | 4-propoxyphenyl | $CH_3$ | (49%) | (14%) | 25.2±2.6 | 31[a] |
| **3.9** | $CH_2OH$ | benzyl | $CH_3$ | 589±55 | (20%) | 9.5±0.7 | -1±5 |
| **3.10** | **$CH_2OH$** | **cyclopentylmethyl** | **$CH_3$** | **335±13** | **(39%)** | **1.3±0.4** | **-5±7** |
| **3.11** | $CH_2OH$ | cyclohexylmethyl | $CH_3$ | 1430±60 | (16±1%) | 21.3±8.1 | 2±5 |
| **3.12** | $CH_2OH$ | benzyl | $CH_3$ | 770±210 | (21±5%) | 53.9±6.6 | 2±3 |
| **3.13** | $CH_2OH$ | 3-methoxybenzyl | $CH_3$ | 957±65 | (43±10%) | 86.1±3.8 | -1±3 |
| **3.14** | $CH_2OH$ | 3-Cl-benzyl | $CH_3$ | 956±6 | (39±10%) | 81.1±5.0 | 0±5 |
| **3.15a** | MeNHCO | H | $CH_3$ | 1000±250 | (9±7%) | 76±10 | 64.5±0.5[c] |
| **3.15b** | EtNHCO | H | $CH_3$ | 590±70 | (18±3%) | 2.1±0.1 | 102±5 |
| **3.16** | EtNHCO | butyl | $CH_3$ | 750±110 | (43±1%) | 5.6±0.2 | 89±3 |
| **3.17a** | MeNHCO | pyridin-2yl | $CH_3$ | (38±4%) | (26±6%) | 3.6±1.4 | 95.5±2.6[c] |
| **3.17b** | **EtNHCO** | **pyridin-2-yl** | **$CH_3$** | **1640±90** | **(45±12%)** | **1.8±0.6** | **90±7** |
| **3.18a** | MeNHCO | benzyl | CH3 | 590±80 | (30±16%) | 12.2±2.8 | 53.3±1.2[c] |
| **3.18b** | EtNHCO | benzyl | $CH_3$ | 510±50 | (33±2%) | 2.8±1.3 | 86±5 |
| **3.19a** | MeNHCO | cyclopentylmethyl | $CH_3$ | 1680±620 | (26±2%) | 4.5±1.2 | 39.0±1.4[c] |
| **3.19b** | EtNHCO | cyclopentylmethyl | $CH_3$ | 1250±150 | (36±7%) | 11.5±1.4 | 83[a] |
| **3.20** | $CH_2OH$ | cyclopentylmethyl | 2-Cl-5-MeO-Bn | 830±40 | 6000 | 18±11 | -6±3 |
| **3.24** | $CH_2OH$ | | $CH_3$ | 230±10 | (23%) | 10.8±3.1 | 84±9 |
| **3.32a** | MeHCO | | $CH_3$ | 1180±630 | (32±1%) | 5.1 ±1.7 | 91.5±6.0[c] |
| **3.32b** | EtNHCO | | $CH_3$ | 429±55 | (18±3%) | 11.4±4.2 | 112[a] |
| **3.34** | $CH_2OH$ | | 2-Cl-5-MeO-Bn | 60±10 | 1800±500 | 1.4±0.1 | 44±5 |

**Table 3.1**

Binding affinities of adenosine derivatives at human $A_1$, $A_{2A}$ and $A_3$ARs expressed in CHO cells and relative efficacy at the $A_3$AR.[a]

(a) All $A_3$AR experiments were performed using cells stably transfected with cDNA encoding one of the human ARs. Binding at human $A_1$, $A_{2A}$, and $A_3$ARs in this study was carried out as described in Methods using [$^3$H]CCPA, [$^3$H]CGS 21680 or [$^{125}$I]AB-MECA as radioligand. Values from the present study are expressed as $K_i$ values (mean±s.e.m., n = 3, unless noted), or as percent displacement of radioligand.

(b) % Activation at 10 µM, relative to cyclic AMP inhibitory effect of 10 µM NECA = 100%. Cl-IB-MECA was also a full agonist (100%) in this assay.

(c) Activity of nucleoside derivatives tested in a functional assay consisting of guanine nucleotide binding ([$^{35}$S]GTP$\gamma$S) in membranes of CHO cells expressing the human $A_3$AR. The effect of 10 µM NECA is considered 100%.

(d) n = 2.

## 3.4 Molecular Modeling

To explain the structural basis for the high binding affinity of the nucleoside 2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl)-*N*$^6$-methyl-9-(β-D-ribofuranosyl)adenine **3.10** at h$A_3$AR, we performed a computational study of ligand docking in a previously derived $A_3$AR model based on the high-resolution structure of bovine rhodopsin. [221, 222] Various bound conformations of the C2-substituent and c1 angles for the adenine ring were generated for an energetically favorable binding location and orientation, and the resulting conformations were compared energetically in the putative binding site. The result of docking **3.10** in the putative binding site of the $A_3$AR is shown in Figure 3.7. The purine ring was surrounded by a hydrophobic pocket, defined by L91 (3.33) and L246 (6.51). In addition, the H-bonds formed between the exocyclic amine and the hydroxyl group of S247 (6.52) and between the purine N1 atom and the side chain of N250 (6.55). The 2'-OH group of the ribose moiety formed a H-bond with the side chain of Q167 (EL2), and the 3'-OH group formed an intramolecular H-bond with the 5'-OH group. Unlike the previously reported docking models of N$^6$-substituted adenosines,[221] here the 5'-OH group H-bonded with the side chain of H272 (7.43) and the backbone carbonyl group of S271 (7.42). The cyclopentyl moiety interacted with aliphatic hydrophobic residues, M177 (5.38) and V178 (5.39), through a hydrophobic interaction and were situated in proximity to F168 (EL2) F182 (5.43), consistent with the optimized binding affinity of compound **3.10**. A comparison of the docking modes of Cl-IB-MECA (**1.9**, Figure 1.8) and compound **3.10** in the putative binding domain showed considerable overlap of the ribose rings and of the adenine moieties, although in compound **3.10** both were situated a little closer to extracellular loop 2 (Figure 3.8). Previously, it was noted that 5'-uronamide analogues, typically of derivatives having bulky N$^6$-subsitituents, generally gain affinity in comparison to the corresponding 5'-hydroxyl analogue. Here, the 5'-uronamide analogue **3.19** (agonist) of the most potent 5'-hydroxyl analogue **3.10** (antagonist) displayed a lower binding affinity, which could be explained by the shift of the ribose position in adenosine analogue having a bulky 2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl) substituent in comparison to those having N$^6$ bulky substituents. The binding of the cyclopentylmethyl group in **3.10** was directed more toward the upper part of TM5, partially overlapping with the binding site of the 3-iodophenyl ring in Cl-IB-MECA.

Curiously, other closely related triazoloderivatives displayed a higher potency of the 5'-uronamide analogues; thus, the compound **3.10** must bind to the receptor in a very distinct manner. There was a subtle difference in orientation between the 2-cyclopentyl group and bulkier groups like benzyl (**3.9**) or cyclohexylmethyl (**3.11**), which were associated with unfavorable van derWaals interactions and resulted in a decrease of binding affinity of 7- and 16-fold, respectively. In addition, the same preferred c1 angles of the energetically favorable bound conformation, common to Cl-IB-MECA and compound **3.10**, were consistent with the empirical finding that the combination of bulky $N^6$ and C2 substituents was unfavorable for $A_3$AR selectivity because of competitive interaction of these bulky substituents. Thus, the modeling has demonstrated that the human $A_3$AR preference of 2-(4-cyclopentylmethyl-1,2,3-triazol-1-yl) derivatives in the 5'-OH series might be explained by optimal van der Waals interactions.

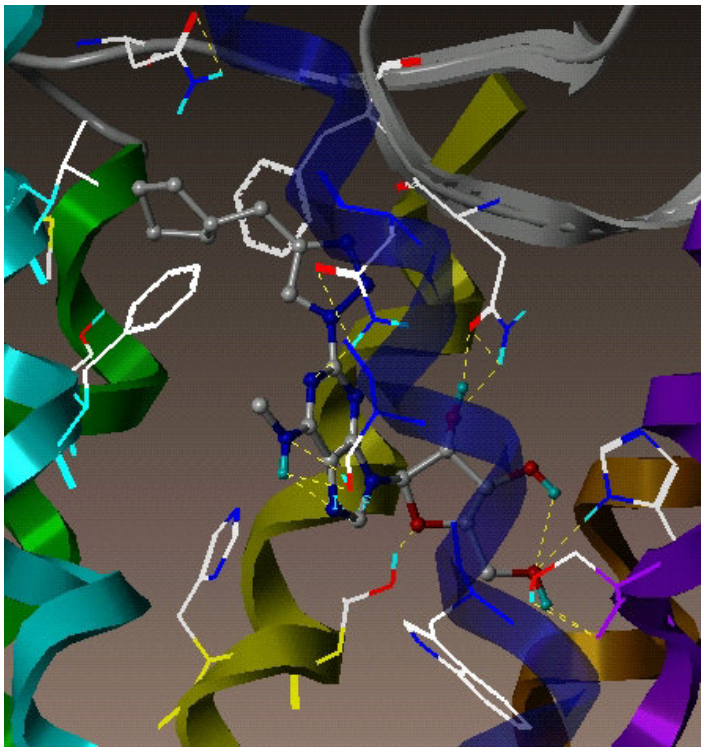


**Figure 3.7**

Docking complexes of analogue **3.10**

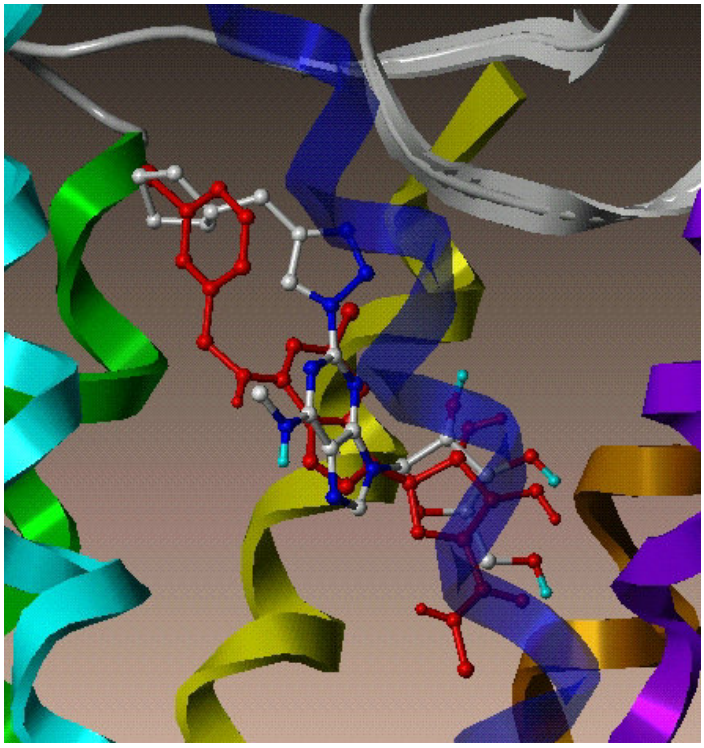


**Figure 3.8**

Superimposition of Cl-IB-MECA in red and compound **3.10** in color by atom type.

Figure 3.7 and Figure 3.8. Molecular docking of compound **3.10** performed under the guidance of prof. dr. Kenneth A. Jacobson (see Experimental Section). Residues that were within 5 Å proximity to the ligand in this putative binding site were: L91 (3.33), T94 (3.36), H95 (3.37), Q167 (EL2), F168 (EL2), M172 (EL2), S181 (5.42), M177 (5.38), V178 (5.39), F182 (5.43), W243 (6.48), L246 (6.51), S247 (6.52), N250 (6.55), C251 (6.56), I268 (7.39), S271 (7.42), and H272 (7.43). All ligands are represented by ball-and-stick models. The H-bonds are indicated with yellow dots. Using MOLCAD ribbon surface program, the $A_3$AR is shown in a ribbon model with different colors for each TM (TM1: red, TM2: orange, TM3: yellow, TM4: green, TM5: cyan, TM6: blue, TM7: purple, H8: violet).

# 3.5 Conclusions

Several 2-(1,2,3-triazol-1-yl)-$N^6$-methyl-substituted adenosine derivatives described in the present study displayed $A_3$AR affinities in the low nanomolar range, showed very high $A_3/A_{2A}$, and a moderate to high $A_3/A_1$ selectivity. Contrary to what we expected, the 2-triazole analogues with an unmodified ribose moiety (**3.1**-**3.14**) showed antagonist or weak partial agonist activity at the $A_3$AR. A 2-[4-cyclopentylmethyl-(1,2,3-triazol-1-yl)]-$N^6$-methyl derivative **3.10** was 260-fold selective in binding in comparison to the $A_1$AR. The binding of the 4-cyclopentylmethyl group in **3.10**, in distinction to the binding of closely related bulky groups pendent on the triazole ring, was directed more toward the upper part of TM5 partially overlapping with the binding site of the 3-iodophenyl ring in Cl-IB-MECA. The 5'-*N*-ethyluronamide modification was dominant over the efficacy reducing effects at the 2-position and was capable of fully re-establishing the $A_3$AR agonist activity, resulting in highly potent and selective $A_3$AR agonists **3.15b-3.19b**. The most selective agonist derivative was compound **3.17b**, 9-(5-ethylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-(4-pyridin-2-yl-1,2,3-triazol-1-yl) adenine, which was 910-fold selective in binding to the $A_3$AR in comparison to the $A_1$AR. Its 5'-*N*-methylcarbamoyl analogue (**3.17a**) also demonstrated highly selective full agonist activity at the $A_3$AR, while other 5'-*N*-methylcarbamoyl derivatives (**3.15a**, **3.18a**, **3.19a**) showed partial agonist activity. The retention of high human $A_3$AR affinity in compound **3.20** was not typical of previous findings that double bulky substitution at the 2- and $N^6$-positions tended to reduce $A_3$AR affinity markedly. Our observations and the comparison with previous findings showed that subtle changes or small modifications could influence the $A_3$AR efficacy dramatically, which illustrates the difficultty to predict $A_3$AR receptor affinity and efficacy.

The 2-triazol-1-yl-$N^6$-methyladenosine analogues **3.1-3.11** and **3.15-3.20** constitute a novel class of highly potent and selective nucleoside-based $A_3$AR partial agonists and antagonists (all of which maintain an intact ribose in the molecular structure) and agonists. Since the reported analogues show excellent affinity for the $A_3$AR and span the full intrinsic activity range, they might be useful as pharmacological tools or as leads for further optimization.

## CHAPTER 4 :

# PYRIMIDINE NUCLEOTIDE ANALOGUES

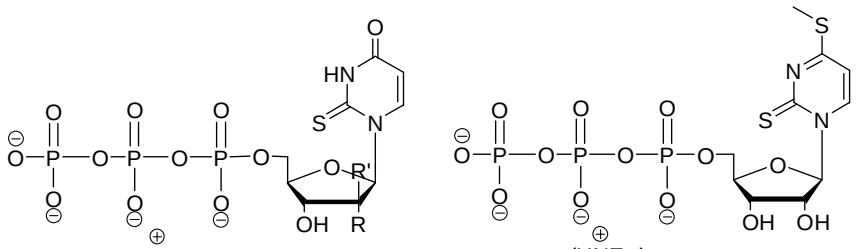
# 4 PYRIMIDINE NUCLEOTIDE ANALOGUES

## 4.1 Synthesis And Evaluation of 2-Thio UTP Derivatives 4.1-4.5

### 4.1.1 Introduction

A recent investigation by Jacobson et al. (results are shown in Table 1.1, Chapter 1) showed that distinct modifications of the natural, non-specific $P2Y_2$ receptor agonist UTP influenced the $P2Y_2$ receptor affinity and selectivity.[123,125] 2-Thiouracil modification, or replacement of the β-D-ribose part by 2-amino-2-deoxy ribose or by β-D-arabinofuranose enhanced both $P2Y_2$ receptor affinity and selectivity relative to UTP, while a 4-thio modification enhanced the $P2Y_2$ receptor affinity, but decreased $P2Y_2$ receptor selectivity.[125]

Based on these results, we combined the 2-thiouracil modification with, respectively, a β-D-2'-amino-2'-deoxy ribose and β-D-arabinofuranose to investigate the effect on $P2Y_2$ receptor affinity and selectivity. We also envisaged to combine the 2-thio and 4-thio modification to overcome the selectivity drop caused by 4-thionation. 5'-Phosphorylation of the synthesized 2-thio derivatives should gain access to new possible $P2Y_2$ receptor agonists. Figure 4.1 represents an overview of the synthesized compounds.




| | R | R' |
|---|---|---|
| **4.1** | H | OH |
| **4.2** | $NH_2$ | H |
| **4.3** | $NHCOCF_3$ | H |
| **4.4** | $NHCOCH_3$ | H |

**4.5**

**Figure 4.1**

Overview of the synthesized compounds **4.1**-**4.5**

## 4.1.2 Chemistry

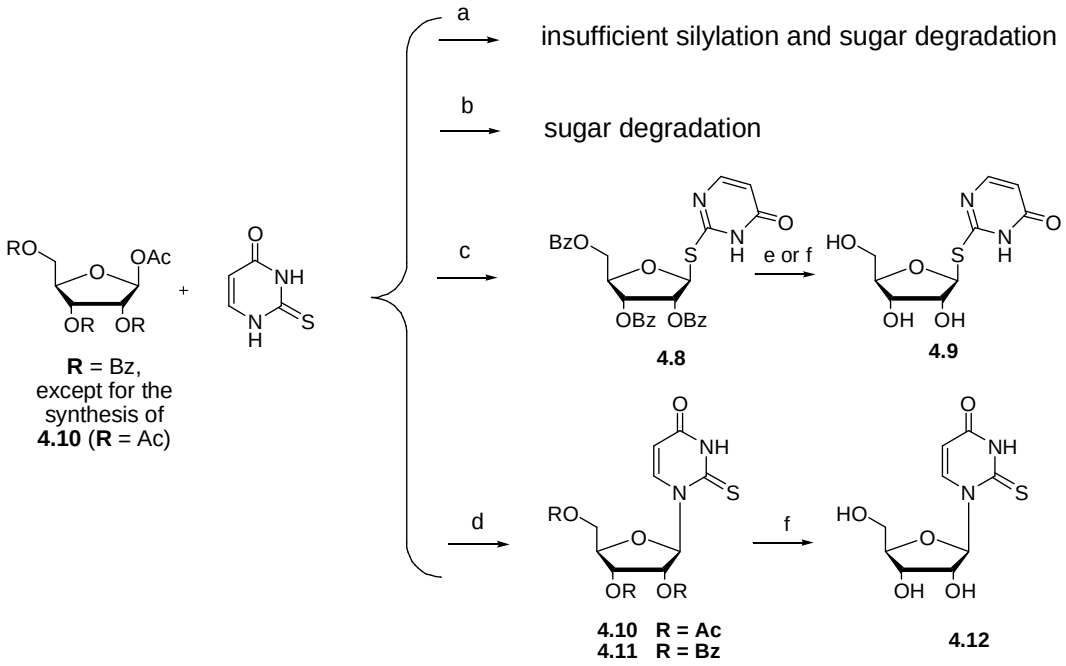
### 4.1.2.1 Synthesis of 2-Thiouridine 4.12

The different Vorbrüggen reaction conditions performed for the coupling of silylated 2-thiouracil with 1-*O*-acetyl 2,3,5-tri-*O*-benzoyl β-D-Ribofuranose are summarized in Scheme 4.1. We first explored conditions a-c to avoid the use of toxic stannic chloride and circumvent the difficulty in isolation of the desired product because of inseparable emulsion during extraction. Therefore, we used trimethylsilyltrifluoro-methanesulfonate (TMSOTf) as a catalyst. However, in the one-pot coupling reaction, using *N*,*O*-bis(trimethylsilyl)acetamide (BSA) as a silylating agent, the persistent turbidity of the mixture already indicated an unsuccessful silylation. After treatment with TMSOTf, sugar degradation occured.

Alternatively, distinct silylation with hexamethyldisilazane (HMDS) and $(NH_4)_2SO_4$ resulted in a clear solution after 6 h. Subsequent addition of the silylated base and TMSOTf to a solution of the ribose sugar should result in $N^1$-glycosylation of the base. However, when performing the latter step in dichloroethane sugar degradation was observed. Acetonitrile as coupling solvent yielded *S*-glycosylation, which was confirmed by the HMBC (Heteronuclear Multiple Bond Connectivity) NMR data: a $^3J(^1H\text{-}^{13}C)$ long range coupling between H-1' and C-2 was observed, while the expected H-1'-C-6 or H-1'-C-4 $^3J(^1H\text{-}^{13}C)$ long range couplings (typically for $N^1$- or $N^3$-glycosylation, respectively) were abscent. Earlier reported *S*-riboside formation[223,224] generally occurred because of sterical hindrance of the $N^1$-position by 6-substituents. The *S*-nucleoside was instable under ammonia debenzoylation conditions and degraded during silica gel purification. Therefore, sodium methoxide in methanol or potassium carbonate in methanol/THF was used for deprotection and the purification was executed by extraction of the resulting benzoic acid methyl esters with diethylether and lyofilisation of the water layer.

Despite our efforts to explore alternative coupling procedures, we were forced to apply the described coupling methods using stannic chloride as a catalyst.[225,226] The best yield (79%) was obtained using 1.1 equivalents sugar, 1 equivalent silylated base and 1.3 equivalents $SnCl_4$. We preferred to use the benzoylated sugar for the coupling, which allowed easy work-up after sodium methoxide deprotection. The

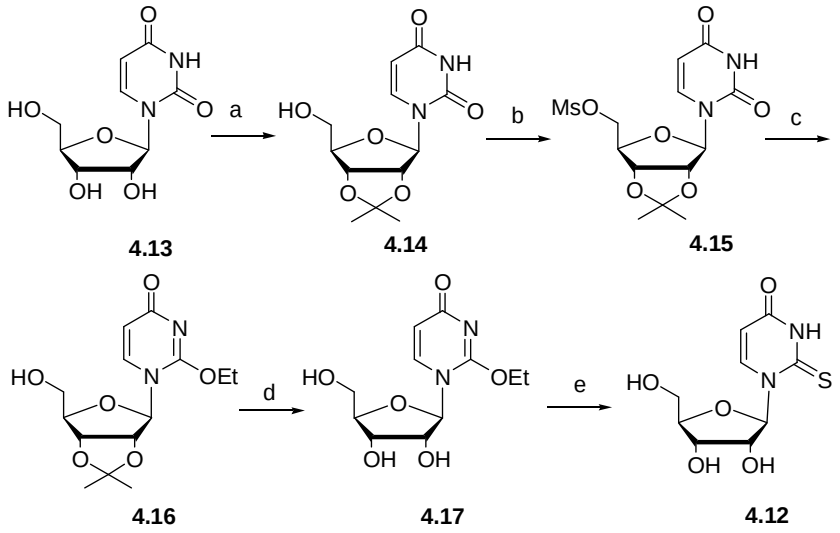
benzoic methyl esters were extracted with diethylether and subsequent lyophilisation of the water layer yielded 2-thiouridine without silica gel purification.




**Scheme 4.1**

Overview of the different Vörbruggen coupling attempts. Reagents and conditions: (a) BSA, TMSOTf, $CH_3CN$; (b) (i) HMDS, $(NH_4)_2SO_4$; (ii) TMSOTf, $C_2H_4Cl_2$; (c) (i) HMDS, $(NH_4)_2SO_4$; (ii) TMSOTf, $CH_3CN$ (51%); (d) (i) HMDS, $(NH_4)_2SO_4$; (ii) $SnCl_4$, $C_2H_4Cl_2$ (79%); (e) $K_2CO_3$, MeOH:THF (97%); (f) NaOMe, MeOH (96%).

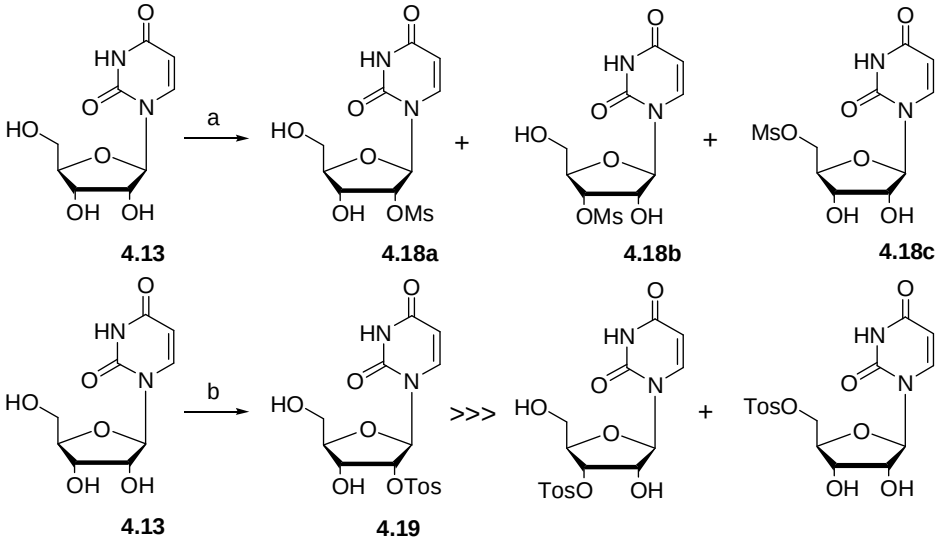
Alternatively, we started from 2',3'-*O*-isopropylidene-uridine (**4.14**) and adapted the method of Manoharan[227] or the synthesis of 2-thiouridine (Scheme 4.2). In this procedure, 5'-mesylation of 2',3'-*O*-isopropylidene-uridine (**4.14**) was followed by in situ generation of 2,5'-*O*-anhydrouridine and subsequently opening of the anhydro bridge with ethoxide. Isopropylidene deprotection of compound **4.16** with 80% TFA in water yielded 2-*O*-ethyluridine (**4.17**), that was treated with $H_2S$ in anhydrous pyridine to allow 2-thionation, resulting in 2-thiouridine (**4.12**).




**Scheme 4.2**

Alternative synthetic route for 2-thiouridine (**4.12**). Reagents and conditions: (a) 2,2-dimethoxypropane, p-toluene sulfonic acid, acetone, 4h (77%); (b) methanesulfonyl chloride, pyridine, 0 ℃, 2h (91%); (c) DBU, dry EtOH, reflux, 10h (91%); (d) 80% trifluoroacetic acid aq., 2h (86%); (e) $H_2S$, pyridine, 250 psi, 48h (86%).

2',3'-*O*-Isopropylidene protection of uridine was performed because the selective 5'-mesylation of uridine resulted in an unseparable mixture (**4.18 a,b,c**, 61%, 23% and 16%, resp.). The attempt to perform selective 5'-tosylation resulted in 2'-tosyluridine (**4.19**) as the major product. (Scheme 4.3)

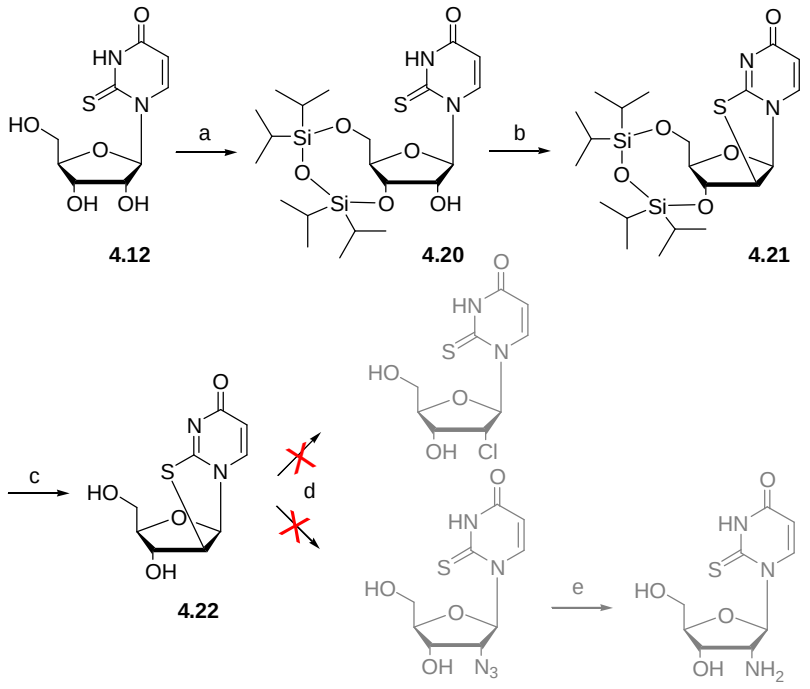



**Scheme 4.3**

Unsuccessful selective 5'-mesylation and 5'-tosylation. Reagents and conditions: (a) methane sulfonyl chloride, pyridine; (b) *p*-toluene sulfonyl chloride, pyridine (51%).

### 4.1.2.2 Synthesis of 2'-Amino-2'-deoxy 2-thiouridine 4.33

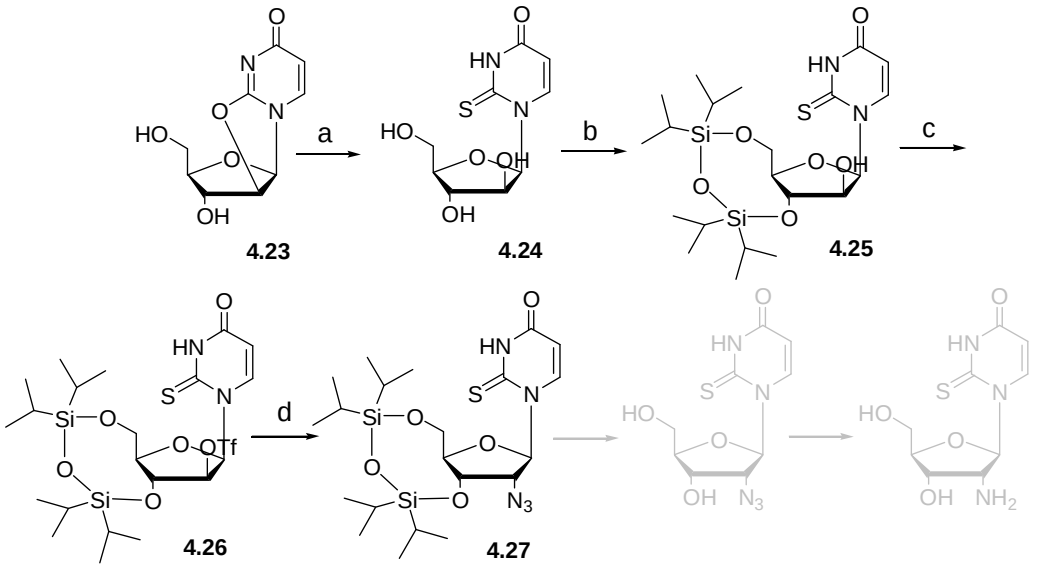
Our first attempt for the synthesis of **4.33** (Scheme 4.6) started from 2-thiouridine (**4.12**) (Scheme 4.4). After the synthesis of 2,2'-*S*-anhydro-1-β-D-arabinofuranosyl-2-thiouracil (**4.22**) from 2-thiouridine (**4.12**), we intended to open the anhydro ring with $NaN_3$ and to subsequently reduce the resulting 2'-azido-2'-deoxy-2-thiouridine to the corresponding 2'-amine. We planned to follow a similar route to gain access to 2'-chloro-2'-deoxy-2-thiouridine. Compound **4.22** was synthesized starting from 2-thiouridine. Selective 2'-mesylation was performed after tetraisopropyl disiloxanylidene (TIPDS) protection of the 3'- and 5'-hydroxyl groups. Under basic conditions, intramolecular nucleophilic attack of the 2-thio group resulted in the 2,2'-*S*-anhydro bridge formation (**4.21**). After TIPDS deprotection, we obtained 2,2'-*S*-anhydro-1-β-D-arabinofuranosyl-2-thiouracil (**4.22**), which was treated with, respectively, $NaN_3$ and HCl to afford the desired 2'-substituted 2-thiouridine derivatives. Unfortunately, both attempts to open the 2,2'-*S*-anhydro bridge resulted in degradation of the starting material (see experimental section).




**Scheme 4.4**

First attempt for the synthesis of 2'-modified 2-thiouridine. Reagents and conditions: (a) $TIPDSCl_2$, imidazole, DMF, 0 ℃ → 30 ℃, overnight (77%); (b) (i) MsCl, pyridine, 4h (ii) DBU, $CH_3CN$ (62%); (c) TBAF, THF, 2h (74%); (d) R = $N_3$: $NaN_3$, benzoic acid, DMF; R = Cl: HCl in dioxane, 75 ℃, overnight or R = Cl: (i) $(Ac)_2O$, pyridine, overnight (ii) AcCl, benzoic acid, $CH_3CN$.

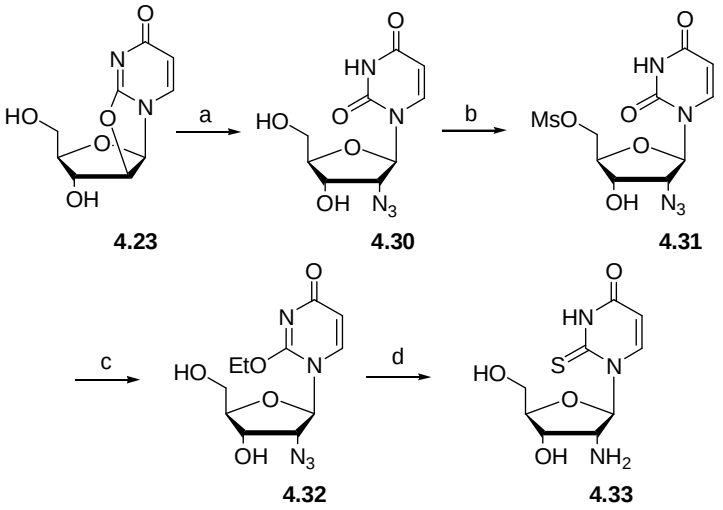
Consequently, we started to investigate an alternative synthetic procedure (Scheme 4.5), starting from 2,2'-*O*-anhydrouridine (**4.23**), which is commercially available. 2,2'-*O*-Anhydrouridine (**4.23**) was treated with $H_2S$ and triethylamine in anhydrous DMF to yield arabino analogue **4.24**. TIPDS protection of the 3'- and 5'- hydroxyl groups was followed by 2'-triflation with triflic chloride and DMAP. Nucleophilic substitution of the resulting triflate **4.26** with *N*,*N*,*N'*,*N'*-tetramethylguanidiniumazide gave in a very low yield (10%) 2'-azide **4.27**, which again forced us to investigate an alternative synthetic route to obtain 2'-amino-2'-deoxy-2-thiouridine (**4.33**).




**Scheme 4.5**

Second attempt for the synthesis of 2'-amino-2'-deoxy-2-thiouridine. Reagents and conditions: (a) $H_2S$, triethylamine, DMF, 240 psi, 48 h (70%); (b) $TIPDSCl_2$, imidazole, DMF (81%); (c) TfCl, DMAP, $CH_2Cl_2$; (d) *N*,*N*,*N'*,*N'*-tetramethylguanidiniumazide, DMF (10% from **4.25**).

To get round these difficulties, we adapted Manoharan's synthesis procedure[227] for the synthesis of 2'-amino-2'-deoxy-2-thiouridine (**4.33**) (Scheme 4.6). 2'-Azido-2'-deoxyuridine (**4.30**) was obtained via opening of 2,2'-anhydrouridine (**4.23**) with $NaN_3$ in DMF.[228] After selective mesylation of the 5'-hydroxyl group of **4.30**, the resulting 5'-methanesulfonate ester **4.31** was converted to 2'-azido-2'-deoxy-2-*O*-ethyluridine (**4.32**)**.** Treatment of **4.32** with $H_2S$ in anhydrous pyridine allowed simultaneous 2-thionation and reduction of the 2'-azido group, resulting in 2'-amino-2'-deoxy-2-thiouridine (**4.33**). The 2-thionation was confirmed by the $^{13}C$ NMR resonance signal of C-2, which shifts to low magnetic field (177.66 ppm) compared to uridine (150.66 ppm).




**Scheme 4.6**

Synthesis of 2'-amino-2'-deoxy 2-thiouridine **4.33**. Reagents and conditions (a) $NaN_3$, DMF, 150 ℃, 15h (52%); (b) MsCl, pyridine, 0 ℃ (61%); (c) $NaHCO_3$/EtOH, reflux, 36h (57%); (d) $H_2S$, pyridine, 50 ℃, 250 psi, 24h (77%).

### 4.1.2.3 Attempts to Synthesize Other 2'-Substituted 2-Thiouridine Derivatives

Introduction of a 2'-fluorine is described to lock the sugar pucker in the North conformation. The anomeric effect of the 2'-fluorine favours the pseudoaxial position of the electronegative ligand at the C-1'-position, which forces the sugar to adopt the North conformation. [229] In search for a general synthetic method for 2'-substituted 2-thiouridine to investigate the influence of electronegative ligands such as 2'-F and 2'-Cl on the 2-thioUTP conformation and receptor binding and to obtain an alternative route to synthesize 2'-amino derivative **4.33**, we decided to explore the use of Lawesson's reagent[227] (Scheme 4.8 and Scheme 4.9). Lawesson's reagent has been generally used for the conversion of the carbonyl group of nucleobases to a thiocarbonyl group. For uracil and thymine, the reactivity of the 4-carbonyl group is much higher than that of the 2-carbonyl group. Therefore, 4-*O*-protection is necessary for selective 2-thionation.

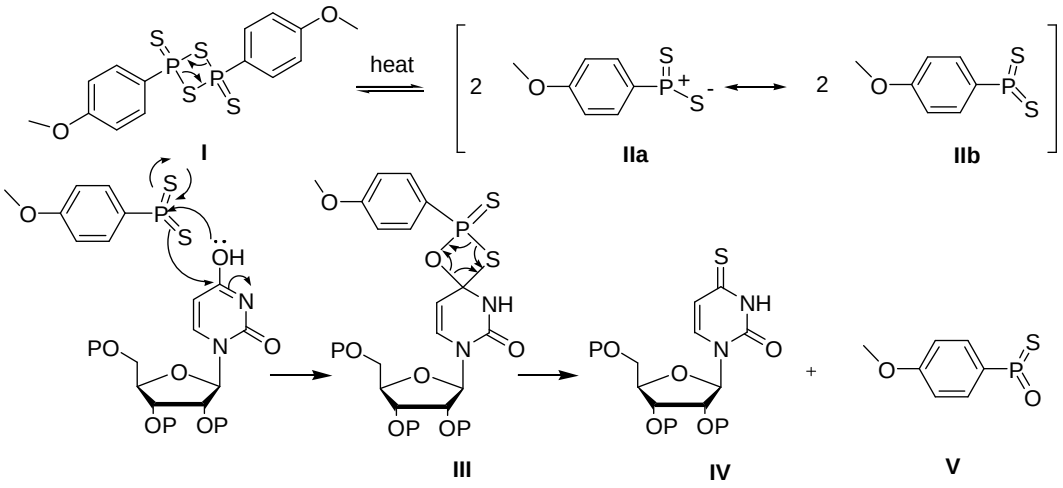
.

#### 4.1.2.3.1 Lawesson's Reagent[230]

Lawesson's reagent (LR) or 2,4-bis(*p*-methoxyphenyl)-1,3-dithiaphosphetane-2,4-disulfide can be prepared easily and safely by the reaction of phosphorous pentasulfide with refluxing anisole, but is also commercially available. The usual

method of thionation is performed in refluxing benzene, toluene, or xylene, in which the suggested mechanism involves dissociation equilibriums, which yield **II** (Scheme 4.7). These decomposition products can then react with a carbonyl functional group to form a four-membered ring (**III**), which decomposes to the corresponding thioketones in a Wittig-like reaction. The formation of thionated products is promoted by the thermodynamic stability of the resulting strong P=O bond.

Thionation of nucleosides, purines, and pyrimidines with LR is widely applied to obtain sulfur analogues. To avoid side reactions, the hydroxyl groups are properly protected as ethers or esters. Unprotected hydroxyl groups are transformed to thiols with 0.5 equivalents of LR, while higher equivalents lead to alkenes as elimination products and in some cases rearrangement products are observed. When two carbonyl groups are present at the 2- and 4-positions of the pyrimidine, LR is used for selective thionation of the 4-carbonyl group. An excess of LR helps for thionation of both carbonyl groups.



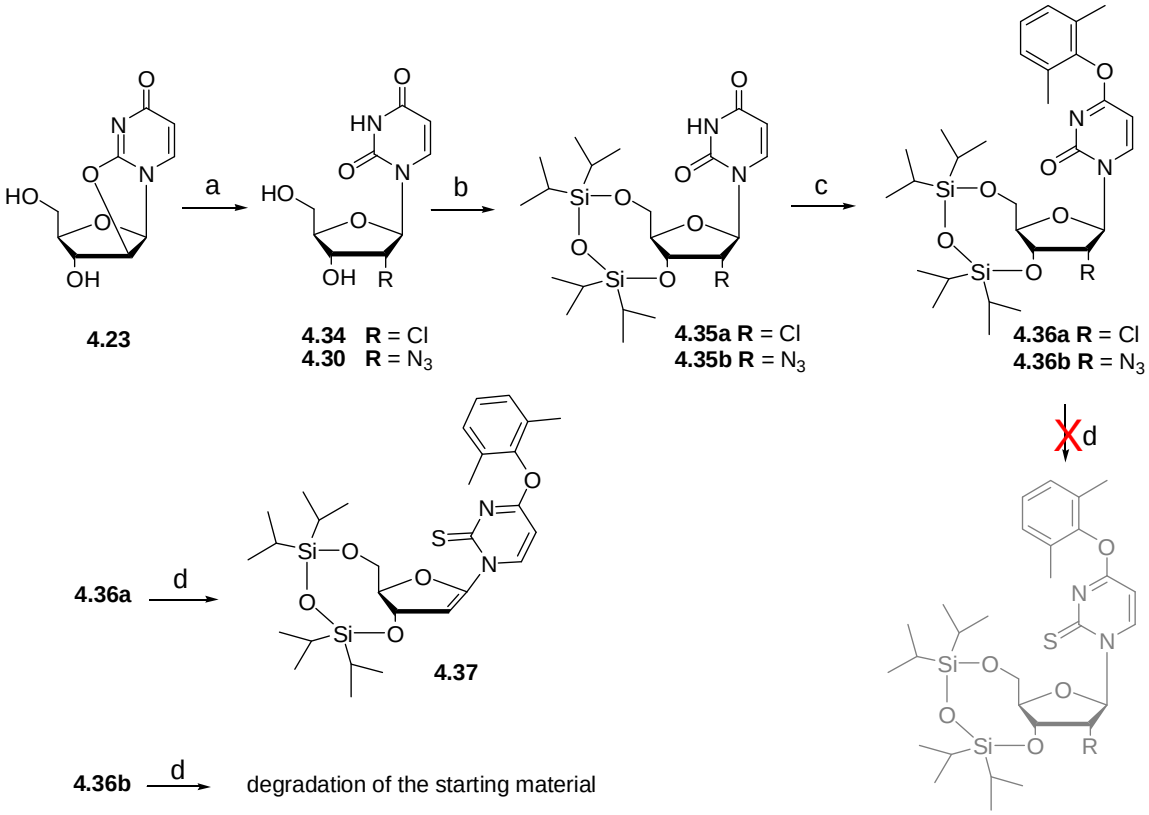

**Scheme 4.7**

Suggested mechanism of thionation using Lawesson's reagent (P = protective group)

#### 4.1.2.3.2 Attempted Synthesis of 2'-Chloro-2'-deoxythiouridine and 2'-Azido-2'-deoxythiouridine

2'-Chloro-2'-deoxyuridine (**4.34**) was obtained by treatment of 2,2'-*O*-anhydrouridine (**4.23**) with saturated HCl in dioxane.[231] After protection of the 3' and 5' hydroxyl groups as a cyclic TIPDS ether (**4.35a**), 4-*O*-protection is necessary given the much higher reactivity of the 4-carbonyl group in comparison to the 2-carbonyl group.

Based on Sekine's comparison of different possible 4-*O*-protective groups in thionation reactions with Lawesson's reagent, we have chosen 2,6-dimethylphenyl ether protection. [232] Treatment of 4-*O*-protected **4.36a** with Lawesson's reagent resulted in 2-thionation in concert with unexpected 1',2'-elimination (**4.37**, 20% yield). The low yield was due to degradation of the starting material during the reaction with Lawesson's reagent. Similarly, the attempted synthesis of 2'-azido-2'-deoxy-2-thiouridine started with the 3',5'-*O*-TIPDS-protection and 4-*O*-protection of compound **4.30**. Subsequent reaction with Lawesson's reagent only resulted in degradation of the starting material.

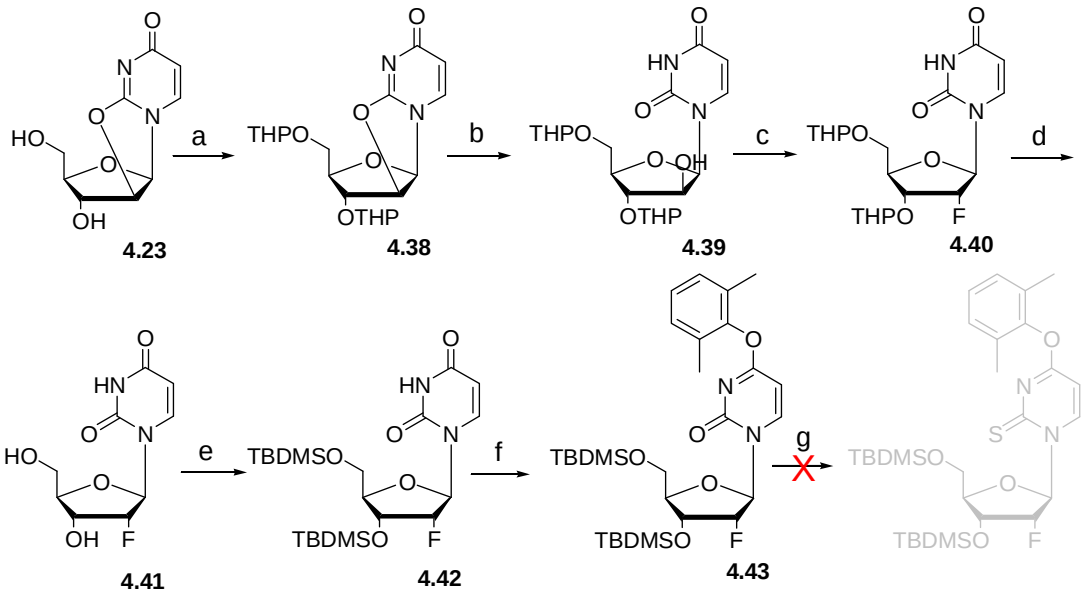



**Scheme 4.8**

Attempted synthesis of 2'-chloro-2'-deoxy-2-thiouridine and 2'-azido-2'-deoxy-2-thiouridine. Reagents and conditions. (a) **4.30**: $NaN_3$, DMF, 150 ℃, 15h (52%, Scheme 4.6); **4.34**: HCl in dioxane (69%); (b) $TIPDSCl_2$, imidazole, DMF (78%); (c) (i) $Na_2CO_3$, tetrabutylammonium bromide, 2,4,6-triisopropylbenzene sulfonylchloride, $CH_2Cl_2$; (ii) 2,6-dimethylphenol, DABCO, $Et_3N$, $CH_3CN$ (84-88%); (d) Lawesson's reagent, toluene.

#### 4.1.2.3.3 Attempted Synthesis of 2'-Deoxy-2'-fluoro-2-thiouridine

For the synthesis of 2'-deoxy-2'-fluorouridine (**4.41**), the 3'- and 5'-hydroxyl groups of 2,2'-*O*-anhydrouridine (**4.23**) were protected with tetrahydropyran groups. Subsequently opening of the anhydro bridge with sodium hydroxide yielded arabino nucleoside **4.39**. After treatment with diethylamino sulphur trifluoride (DAST) to introduce the 2'-fluoro group, deprotection of the tetrahydropyran groups with *p*-toluene sulfonic acid was performed to obtain 2'-fluoro analogue **4.41**.




**Scheme 4.9**

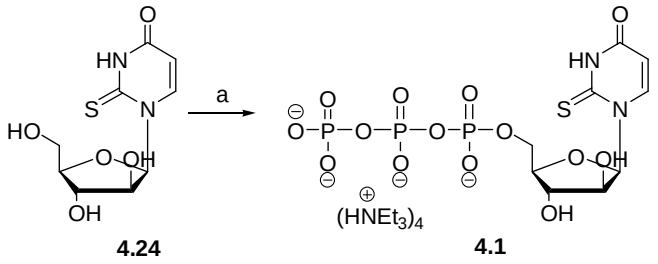
Attempted synthesis of 2'-deoxy-2'-fluoro-2-thiouridine. Reagents and conditions: (a) 3,4-dihydro-2*H*-pyrane, *p*-toluene sulfonic acid, DMF; (b) NaOH, EtOH:$H_2O$ (89% from **4.23**); (c) DAST, pyridine, $CH_2Cl_2$; (d) *p*-toluene sulfonic acid, MeOH (71% from **4.39**); (e) TBDMSCl, imidazole, DMF (61%); (f) (i) $Na_2CO_3$, tetrabutylammonium bromide, 2,4,6-triisopropylbenzene sulfonylchloride, $CH_2Cl_2$; (ii) 2,6-dimethylphenol, DABCO, $Et_3N$, $CH_3CN$ (82%); (g) Lawesson's reagent, toluene.

After protection of the 3' and 5'-OH as TBDMS ether, followed by 4-O-protection, attempted 2-thionation with Lawesson's reagent also resulted in degradation of the starting material.

Alternatively, we attempted to apply the procedure we used for the synthesis of 2'-amino-2'-deoxyuridine (Scheme 4.6), starting from 2'-deoxy-2'-fluorouridine (**4.41**). Therefore, selective 5'-mesylation or 5'-tosylation was necessary to allow the 2,5'-*O*-anhydro bridge formation. Unfortunately, 3',5'-*O*-dimesylation was obtained, while no reaction was observed under 5'-tosylation conditions.

### 4.1.2.4 5'-Phosphorylation of 2-Thio Uridine Derivatives 4.1-4.4

Triphosphate **4.1** was prepared by phosphorylation of the corresponding nucleoside in two steps (**4.24**) (Scheme 4.10). The unprotected nucleoside **4.24** was first treated with phosphorous oxychloride to yield the 5'-monophosphate, which was isolated, activated with 1,1'-carbodiimidazole and treated with bis(tri-n-butylammonium)pyro-phosphate.

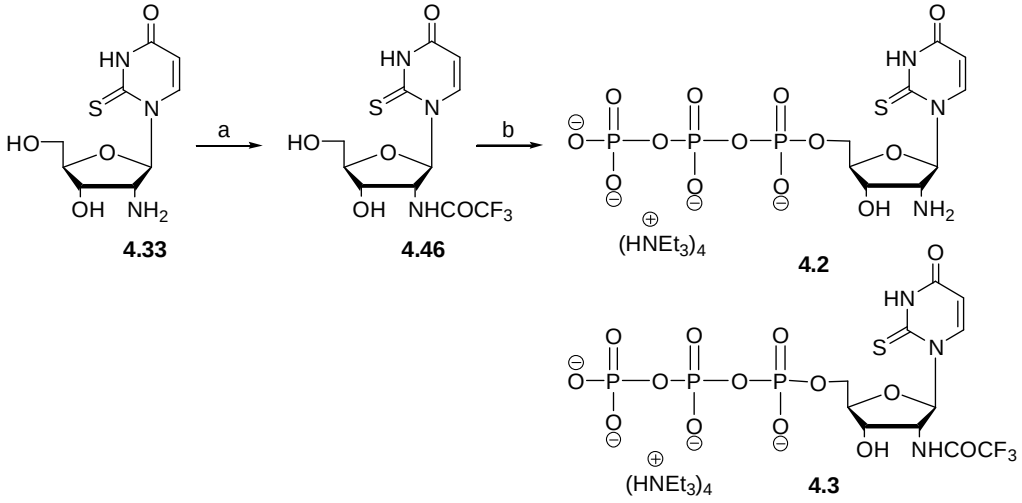



**4.24** **4.1**

**Scheme 4.10**

5'-Phosphorylation of **4.24**. Reagents and conditions: (a) (i) $POCl_3$, $PO(OCH_3)_3$, 4h, 0 °C.; (ii) $Bu_3N$, CDI, DMF; $(Bu_3NH)_2H_2P_2O_7$; TEAB 1M (6.6%).

After protection of the 2'-amino group of nucleoside **4.33** by trifluoroacetylation, the phosphorylation was performed as shown in Scheme 4.11. During purification by ion exchange, partial hydrolysis yielded a mixture of **4.2** and **4.3**, which was readily separable by HPLC.

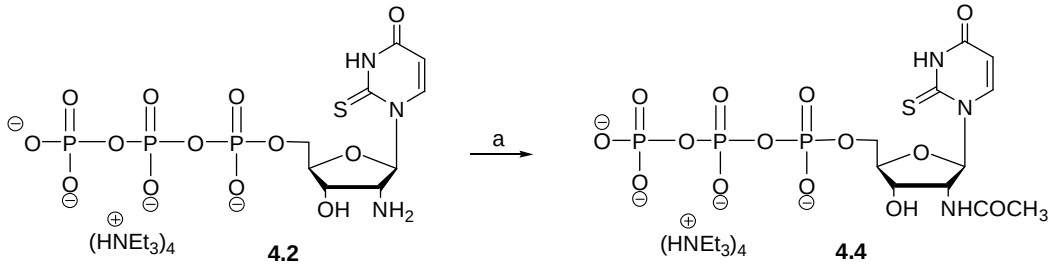



**4.33** **4.46** **4.2** **4.3**

**Scheme 4.11**

Synthesis of 5'-triphosphates **4.2** and **4.3**. Reagents and conditions: (a) DIEA, ethyl trifluoroacetate, DMF, room temperature, 13h (88%); (b) (i) $POCl_3$, $PO(OCH_3)_3$, Proton Sponge, 0 °C, 2h; (ii) $(Bu_3NH)_2P_2O_7H_2$, $Bu_3N$, DMF, 10min; (iii) 0.2 M triethylammonium bicarbonate solution, room temperature, 1 h (12% of **4.2**; 7% of **4.3**).

A 2'-acetamido derivative was obtained by direct acetylation of **4.2** (Scheme 4.12).

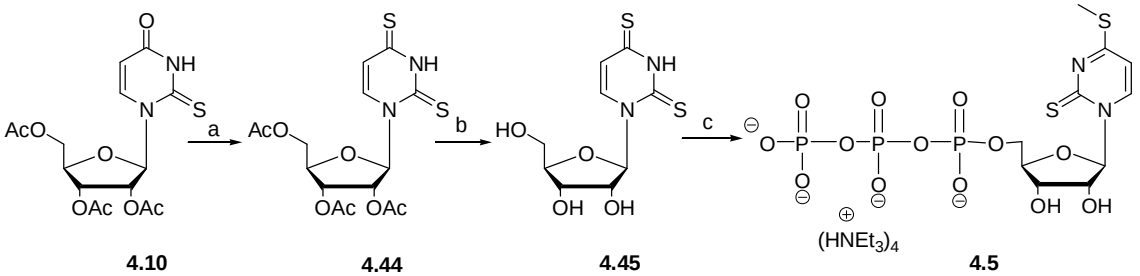



**Scheme 4.12**

2'-Acetylation of compound **4.2**. Reagents and conditions: (a) acetic anhydride, $H_2O$, room temperature, 6 h (57%).

### 4.1.2.5 Synthesis of 2,4-Dithiouridine and Phosphorylation Attempt: Synthesis of 4-Methylthio Analogue 4.5

The synthesis of 4-methylthio analogue **4.5** is depicted in Scheme 4.13. 4-Thionation of 2',3',5'-*O*-triacetyl-2-thiouridine (**4.10**) using Lawesson's reagent, [230] followed by sugar deprotection afforded 2,4-dithiouridine (**4.45**) in 75% yield from **4.10**. An attempt to synthesize the corresponding triphosphate led to isolation only of the 4-methylthio analogue (**4.5**). A possible explanation for the observed S-methylation is a nucleophilic attack of the 4-S to the trimethylphosphate reagent. Attempts to perform the 5'-phosphorylation in the absence of trimethylphosphate or in the absence of proton sponge, probably responsible for the nucleophilic character of 4-S, by NH-deprotonation, were not successful.

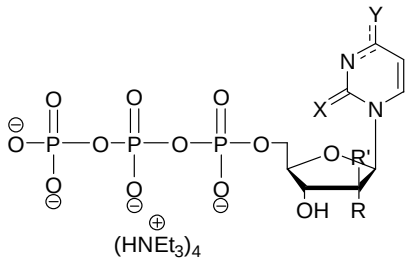



**Scheme 4.13**

Attempted synthesis of 2,4-dithioUTP. Reagents and conditions: (a) Lawesson's reagent, toluene, 80 ℃, overnight (83%); (b) NaOMe, MeOH, reflux, 4 h (91%); (c) (i) $POCl_3$, proton sponge, $PO(OMe)_3$, 0 ˚C; (ii) $(Bu_3NH)_2H_2P_2O_7$, $Bu_3N$, DMF, 0 ˚C (6.4%).

## 4.1.3 Biological Evaluation

Activation of phospholipase C by a range of concentrations of each nucleotide derivative was studied in 1321N1 astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors (Table 4.1), by reported methods.[123,125, 233, 234] Tritiated inositol phosphates produced from a radiolabeled myo-inositol precursor were measured using a standard ion exchange method. 5'-Triphospate **4.1** demonstrated a moderately potent and a highly selective agonist activity at the $P2Y_2$ receptor, while 5'-triphosphate **4.2** was 6 times more potent than the natural ligand UTP and 300-fold selective in activation of the $P2Y_2$ receptor in comparison with the $P2Y_4$ receptor (Table 4.1, Figure 4.2). The potency of **4.2** was greatly reduced upon acetylation (**4.4**) and reduced to a lesser degree upon trifluoroacetylation (**4.3**). 4-Methylthio modification (**4.5**) dramatically affected the potency of 2-thioUTP (**1.45**). The synthesized compounds **4.1**-**4.5** were nearly inactive at the $P2Y_6$ receptor.




| | R' | R | X | Y | $EC_{50}$ at $hP2Y_2$ (nM)[a] | $EC_{50}$ at $hP2Y_4$ (nM)[a] | $EC_{50}$ At $hP2Y_6$ (nM)[a] |
|---|---|---|---|---|---|---|---|
| **UTP** | H | OH | O | O | 49 ± 12 | 73 ± 2 | >10,000 |
| **1.45** | H | OH | S | O | 35 ± 4 | 350 ± 10 | 1,500 |
| **1.42** | H | $NH_2$ | O | O | 62 ± 8 | 1200 ± 300 | >100,000 |
| **1.43** | OH | H | O | O | 87 ± 10 | 710 ± 80 | No data |
| **4.1** | OH | H | S | O | 140 ± 10 | 7930 ± 800 | NE[b] |
| **4.2** | **H** | **$NH_2$** | **S** | **O** | **8 ± 2** | **2400 ± 800** | **>10,000** |
| **4.3** | H | $NHCOCF_3$ | S | O | 470 ± 60 | 8300 ± 1200 | NE[b] |
| **4.4** | H | $NHCOCH_3$ | S | O | 6500 ± 1400 | NE[b] | NE[b] |
| **4.5** | H | OH | S | SMe | 2740 ± 520 | ~10,000 | >10,000[c] |

**Table 4.1**

*In vitro* pharmacological data for UTP and its analogues in the stimulation of PLC at recombinant $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors expressed in 1321N1 astrocytoma cells.

(a) Agonist potencies reflect stimulation of PLC determined as reported[123,125,126] and were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). $EC_{50}$ values (mean standard error) represent the concentration at which 50% of the maximal effect is achieved.

(b) NE - no effect at 10 µM

(c) ≤50% effect at 10 µM

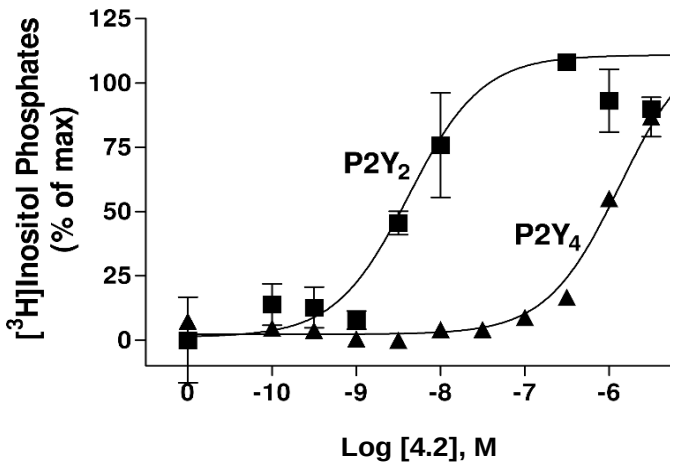



**Figure 4.2**

Activation by compound **4.2** of PLC in 1321N1 astrocytoma cells expressing the human $P2Y_2$ receptor or $P2Y_4$ receptor.

## 4.1.4 Molecular Modeling

The putative binding modes of the synthesized $P2Y_2$ receptor agonists were studied by molecular docking with a Monte Carlo Multiple Minimum (MCMM) conformational search analysis on several known nucleotide ligands of this receptor subtype. As described in the Experimental Section (5.3.2.3), UTP (**1.41**) initially was manually docked inside the putative binding site of the $P2Y_2$ receptor. The published data of site-directed mutagenesis combined with computational studies of P2Y receptors were taken into account.[123,149,151] Because the Northern (N) conformation of the ribose ring of the ligand was proposed to be important for recognition,[234] UTP and all other studied ligands were sketched and initially docked in their (N)-conformation. In addition, the anti-conformation of the ligand base ring was used during the modeling studies. Interestingly, in the original rhodopsin-based model of the $P2Y_2$ receptor, the side chain of one of the key cationic residues, namely R3.29, was oriented in the opposite direction from the putative binding pocket, but during the simulation it shifted toward the binding cavity. After MCMM refinement of the initially obtained docking complex, the $\alpha$-phosphate group of the UTP triphosphate chain was bonded to R7.39 whereas R6.55 could interact with both $\alpha$- and $\gamma$-phosphate groups and R3.29 interacted with the $\gamma$-phosphate group of UTP (Figure 4.3A). In addition, the $\gamma$-phosphate group of the ligand formed H-bonds with H184, located in EL2 directly after the conserved cysteine residue, with the backbone nitrogen atom of D185 and with the hydroxyl group of Y6.59. Another tyrosine residue, Y3.33, was H-bonded to the $\beta$-phosphate group.

In the model obtained after MCMM calculations, the 2'-hydroxyl group of UTP appeared near the conserved F6.51 (in the $P2Y_{11}$ and $P2Y_{14}$ receptors, this position is Y6.51). This observation suggests the possibility of OH/$\pi$ H-bonding between the 2'-hydroxyl group and the aromatic ring of F6.51. Several examples of similar interactions seen in protein crystallographic structures were reviewed by Meyer et al.[235] The hypothesis of H-bonding of the 2'-hydroxyl group to the receptor is consistent with the experimental SAR.[149] For example, 2'-deoxy-UTP ($EC_{50}$ = 1.08 µM) is 22-fold less potent than UTP ($EC_{50}$ = 0.049 µM), whereas the replacement of the 2'-hydroxyl group by a 2'-methoxy group ($EC_{50}$ = 14.3 µM) reduced the potency further (290-fold weaker than UTP). In the latter case, this model showed that not

only was the suggested OH/π H-bond lost but the 2'-*O*-methyl group also interfered sterically with the aromatic ring of F6.51. Agonist activities of the ligands, taken together with the binding mode obtained, strongly suggest that the hydroxyl group at the 2'-position is important as a donor but not as an acceptor of a H-bond.

The binding mode of 2'-deoxy-2'-amino-UTP (**1.42**), which was found to maintain potency in activation of the $P2Y_2$ receptor,[149] was studied with MCMM calculations. The 2'-amino group (p*K*a = 6.2 in a related derivative)[128,236] was examined in both unprotonated and protonated positively charged forms. Similar to the 2'-hydroxyl group of UTP, the 2'-amino group of **1.42** was found near and oriented toward the aromatic ring of F6.51. Both protonated and unprotonated forms of the ligands interacted with this residue. However, the protonated form of this amino group was involved in a stronger cation-π interaction with F6.51. In addition, the protonated 2'-$NH_3^+$ group acted as a H-bond donor to the hydroxyl of Y3.33 in this model (Figure 4.3B).

In the receptor-ligand complexes obtained for all studied agonists, the 3'-hydroxyl group of the ligand appeared to be H-bonded to the α-phosphate group of the triphosphate chain. This could be an important intramolecular interaction to stabilize the ribose ring in its (N)-conformation, to facilitate the interaction of the 2'-hydroxyl group with F6.51.

In this model, the oxygen atom at position 4 of the UTP ring was H-bonded to the hydroxyl group of U1.39, and S7.43 accepted a H-bond from the 3-NH group of the ligand. In contrast, the oxygen atom at position 2 was not involved in H-bonding with the receptor. In addition, F3.22, conserved among all subtypes of $P2Y_2$ receptors, was found to be involved in π-π interactions with the uracil ring of UTP.

The binding mode of the potent and selective compound **4.2** was also studied with MCMM calculations, and the results were similar to those for **1.42**, in which the 2'-amino group in its protonated form was involved in cation-π interaction with F6.51 and as H-bond donor to Y3.33 (Figure 4.3B). The potency of **4.2** was greatly reduced upon acetylation (**4.4**) and reduced to a lesser degree upon trifluoroacetylation (**4.3**). These observations are consistent with the binding modes obtained for compound **4.4**. In the model of the $P2Y_2$ receptor complex with compound **4.4**, the H-bond between F6.51 and the NH group of the acetamide moiety was not observed.

Moreover, the methyl group of this moiety undesirably apearded near the OH-group of Y3.33 and the $NH_2$ group of R6.55. The oxygen atom of the acetyl group was not involved in interactions with the receptor. We speculate that in the case of compound **4.3**, the $CF_3$ group, which is more electronegative than $CH_3$, provides some favourable interactions with positively charged R6.55, improving the potency of compound **4.4**.

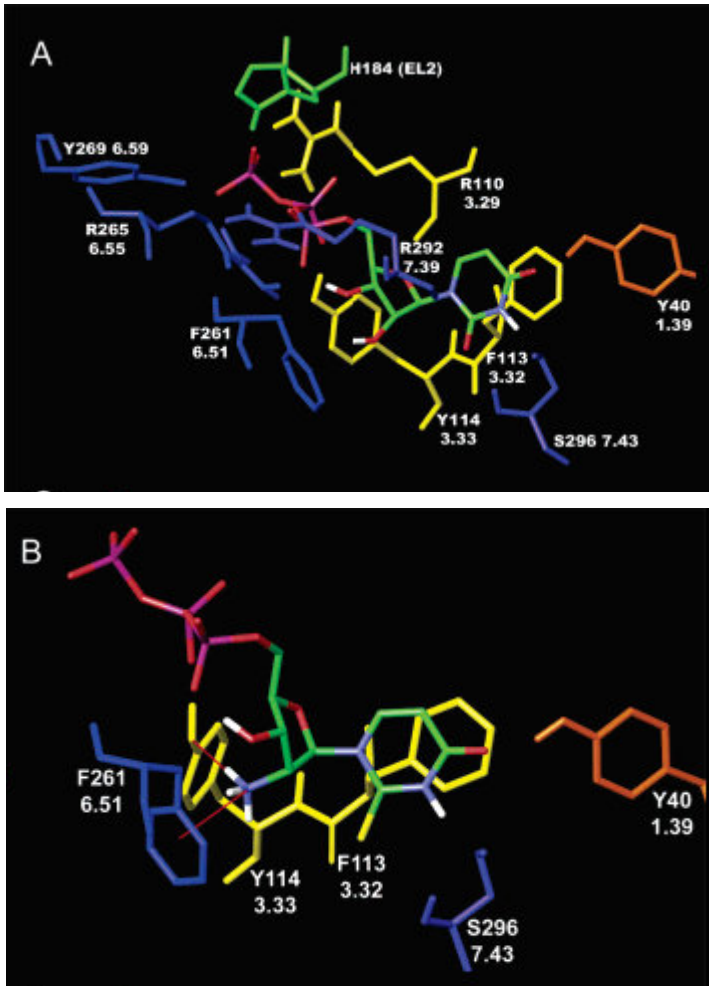



**Figure 4.3**

Molecular modeling of the synthesized $P2Y_2$ receptor agonists performed under the guidance of prof. dr. Kenneth A. Jacobson (see experimental section). Binding modes of UTP (A) and 2'-amino-2'-deoxy-2-thioUTP (A) to the human $P2Y_2$ receptor following MCMM calculations. The 2'-amino group is shown in its protonated form, which can be involved in cation-π interactions with F6.51 and can form an additional H-bond with Y3.33

## 4.1.5 Conclusions

The combination of two favourable UTP modifications resulted in the highly potent $P2Y_2$ receptor agonist **4.2**, which was 300-fold selective in activation of the $P2Y_2$ receptor in comparison to the $P2Y_4$ receptor. 5'-Triphosphate **4.2** should prove to be very useful as a pharmacological probe for studying $P2Y_2$ receptor action. Modeling provided an explanation for the general stabilizing effect of the 2'-amino modification of UTP in $P2Y_2$ receptor recognition.

# 4.2 Synthesis and Evaluation of Uridine 5'-Phosphonodiphosphates 4.6 and 4.7

## 4.2.1 Introduction

Even when applied for topical use (cystic fibrosis) the natural $P2Y_2$ receptor ligand UTP and its many reported analogues of this messenger are sensitive to degradation by ecto-nucleotidase(s) at the airway surface, which reduces the duration of action when administered by inhalation. Replacement of the $\alpha$-phoshate group by an isosteric phosphonate group should enhance the metabolic stability, since a phosphorous-carbon bond is not susceptible to phosphatase hydrolysis.

To assess the influence of this isosteric modification on $P2Y_2$ receptor affinity and efficacy, we synthesized a 5'-phosphonodiphosphate analogues of UTP (**4.6** and **4.7**), starting from 2',3'-*O*-isopropylidene-uridine (**4.14**) (Scheme 4.14).

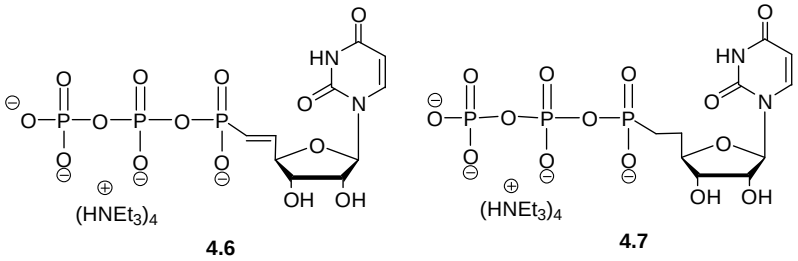


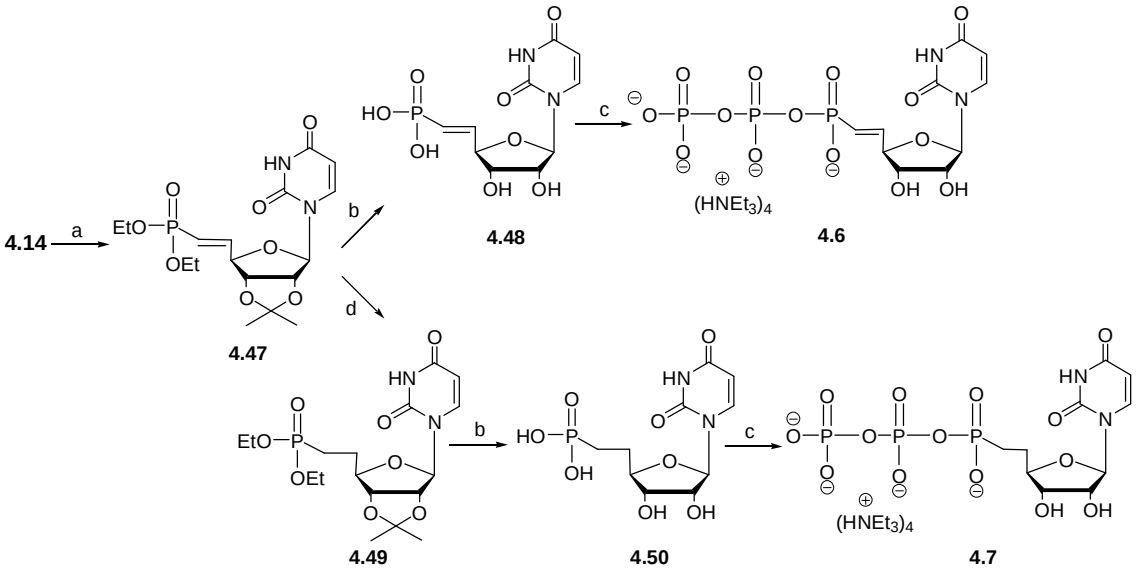

**Figure 4.4**

Synthesized 5'-phosphonodiphosphate analogues of UTP

## 4.2.2 Synthesis

### 4.2.2.1 Synthesis of Uridine 5'-Phosphonodiphosphates 4.6 and 4.7

The synthesis of the target phosphonodiphosphate **4.7** is depicted in scheme 4.14. 5',6'-Vinyl phosphonate **4.47** was synthesized by oxidation of 2,3-*O*-isopropylidene-uridine (**4.14**) to the 5'-aldehyde intermediate, which was immediately reacted with freshly prepared [(diethoxyphosphinyl)methylidene] triphenylphosphorane **4.57**.[237] Catalytic hydrogenation of the resulting vinyl phosphonate ester **4.47** in the presence of palladium on carbon gave the corresponding saturated phoshonate ester **4.49**. Simultanous deprotection of the phosphonate diester and the acetonide was achieved upon treatment with TMSBr, yielding the corresponding 5'-phosphonate **4.50**. To prepare the diphosphate derivative, **4.50** was activated with CDI in DMF, followed by the addition of bis(tri-*n*-butylammonium)pyrophosphate. The resulting diphosphate contained a 2',3'-cyclic carbonate moiety resulting from a reaction of the 2'- and 3'-hydroxyl groups with CDI. Hydrolysis of the cyclic carbonate was achieved upon treatment with triethylammonium bicarbonate (TEAB) buffer to obtain the desired 5'-phosphonodiphosphate **4.7**. To assess the influence of a double bond, intermediate **4.47** was also converted to the corresponding diphosphate **4.6**.

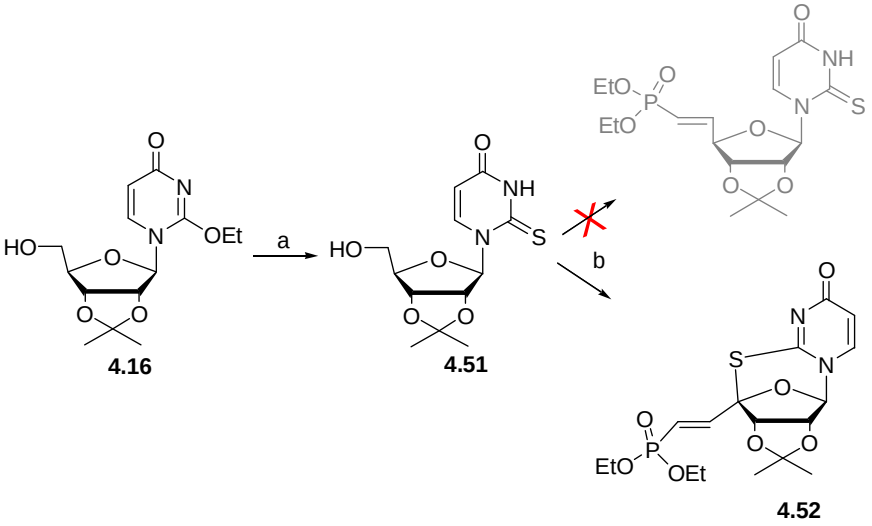



**Scheme 4.14**

Synthesis of uridine 5'-phosphonodiphosphates **4.6** and **4.7**. Reagents and conditions: (a) (i) TFA, DCC, pyridine, DMSO or Dess-Martin periodinane, pyridine, $CH_3CN$ or IBX, $CH_3CN$; (ii) $(EtO)_2P(O)C=PPh_3$ (**4.57**), DMSO (34-44%); (b) TMSBr, $CH_2Cl_2$ (62%); (c) $Bu_3N$, CDI, DMF; $(Bu_3NH)_2H_2P_2O_7$; TEAB 1M (5.4%-22%); (d) $H_2$, Pd/C (84%).

### 4.2.2.2 Attempts to Synthesize 2-Thiouridine 5'-Phosphonodiphosphate

Based on the $P2Y_2$ receptor affinity and selectivity enhancing effects of 2-thioUTP[125] and 2'-amino-2'-deoxy-2-thioUTP (**4.2**) compared to the corresponding uracil analogues UTP and 2'-amino-2'-deoxy-UTP (**1.42**) (results are shown in Table 4.1), we have chosen to synthesize the 2-thio analogue of **4.7** starting from intermediate **4.16** (Scheme 4.15). 2',3'-*O*-Isopropylidene-2-thiouridine (**4.51**) was prepared by treatment of **4.16** with $H_2S$ in pyridine. In an attempt to convert this nucleoside to the vinylic phosphonate, we followed the same two-step procedure as described for **4.47**. Remarkably, the $^1$H NMR spectrum of the major reaction product indicated the absence of a hydrogen at C-4'. Apparently, expected 5'-oxidation followed by condensation of the 5'-aldehyde intermediate with phosphor ylide **4.57** occurred in concert with an intramolecular $S^2$,4'-anhydro ring formation, resulting in compound **4.52**. The structural assignement of **4.52** was made from the following observations: in the $^1$H NMR spectrum, H-3' appeared as a sharp doublet, C-4' and N-3 were devoid of a proton. The absence of the two protons was confirmed by mass spectrometry ($[M+H]^+$ calculated for the desired compound: 433.1, $[M+H]^+$ found: 431.1). The $^{13}$C NMR signals for C-4' and C-2 at 113.68 and 156.33 ppm, respectively, were in agreement with the reported signals of a similar earlier reported $O^2$,4'-anhydro uridine derivative.[238]




**Scheme 4.15**

Unexpected synthesis of $S^2$,4'-anhydro derivative **4.52**. Reagents and conditions: (a) $H_2S$, pyridine, 250 psi, 48h (88%); (b) (i) DCC, TFA, pyridine, DMSO; (ii) $(EtO)_2P(O)C=PPh_3$ (**4.57**)(56%)

The mechanism of the intramolecular ring closing is currently unclear, because we were not capable to isolate any reaction intermediate. We suppose nucleophilic sulphur attack is involved, as in the hypothetical mechanism in Figure 4.5. A participating role of the ylid in the formation of **4.52** is likely, since several attempts to produce aldehyde **V** as such were unsuccessful. In an effort to prevent nucleophilic attack of the 2-sulfur atom, we protected N-3 as a benzyloxymethyl ether (BOM).[239] Unfortunately, this BOM-protected analogue failed to undergo the DMSO-mediated oxidation reaction, probably through sterical hindrance. Alternative formation of the aldehyde with iodoxybenzoic acid (IBX) resulted in complete degradation of the starting material.

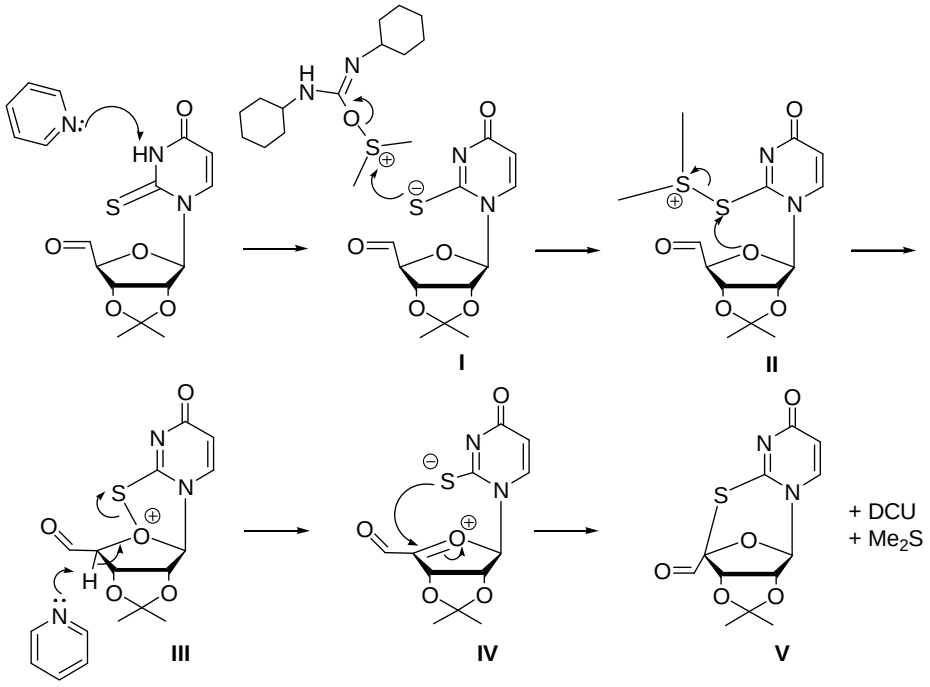
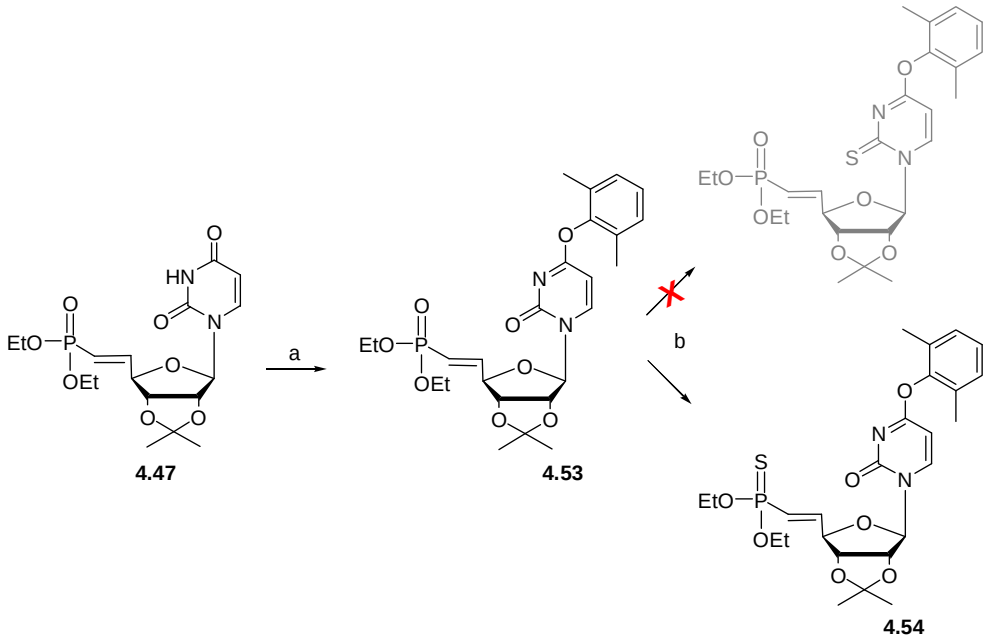



**Figure 4.5**

Supposed mechanism of $S^2$,4'-anhydroformation

Consequently, we attempted to convert compound **4.47** to its 2-thio analogue using Lawesson's reagent (Scheme 4.16). For that purpose, previous 4-*O*-protection as a 2,6-dimethylphenyl ether was performed (see also 4.1.2.3). Treatment of the 4-*O*-protected **4.53** with Lawesson's reagent resulted in thionation of the phosphonate ester as the major reaction product. The structure was confirmed by the $^1$H NMR spectrum, which was devoid of the 2-thionation related shift of the resonance signal of H-1' to lower magnetic field.[232] The conversion of phosphonates to thiophosphates was earlier reported.[240,241]

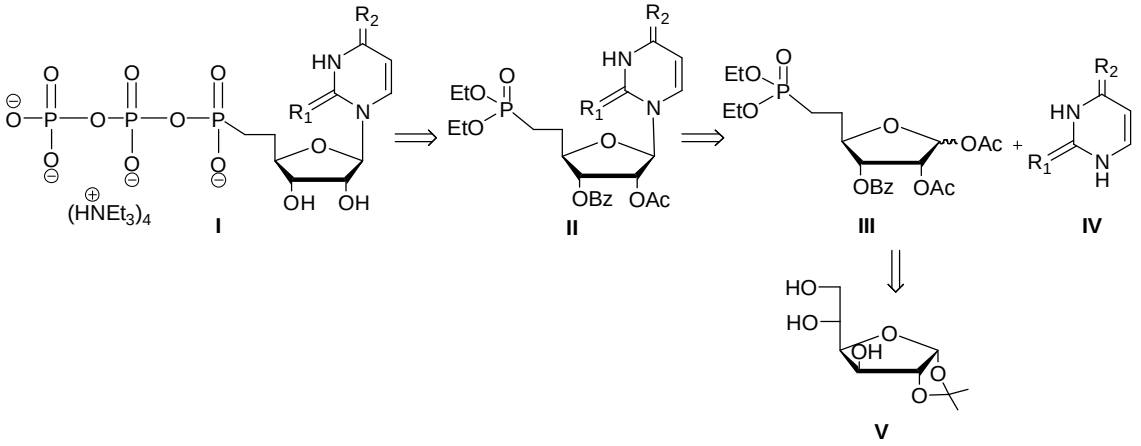



**Scheme 4.16**

Unexpected synthesis of 5'-thiophosphonate ester **4.54**. Reagents and conditions: (a) (i) $Na_2CO_3$, tetrabutylammonium bromide, 2,4,6-triisopropylbenzenesulfonyl chloride, $CH_2Cl_2$; (ii) 2,6-dimethylphenol, DABCO, $CH_3CN$; (b) Lawesson's reagent, toluene.

The introduction of a phosphonate ester group was not possible in the presence of the 2-thiocarbonyl moiety, neither was it possible to perform 2-thionation of the uridine 5'-phosphonate ester (**4.53**). Hence, in Scheme 4.17, we propose an alternative retrosynthetic route to obtain the desired 5'-phosphonodiphosphates ($R_1$= O or S; $R_2$= O or S). Unfortunately, a shortage of time prevented us to perform this reaction pathway, so we suggest this route as a future perspective.

A key step will be the Vörbruggen coupling of the appropriate silylated pyrimidine with a suitably protected 5-deoxy-5-methylphosphonate ribofuranose (**III**), as reported by Mikhailov and coworkers.[242] This sugar intermediate is accessible from the commercially available 1,2-*O*-isopropylidene glucofuranose (**V**). In case $R_1$= O, this method further allows to modify the 2'-OH group by other functionalities.




**Scheme 4.17**

Proposed retrosynthetic route for the desired 2-thiouridine 5'-phosphonodiphosphates ($R_1$= O or S; $R_2$= O or S)

## 4.2.3 Biological Evaluation

Activation of phospholipase C by 5'-phosphonodiphosphate **4.7** was studied in 1321N1 astrocytoma cells stably expressing the human $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors, by reported methods.[123,125,243,244] Tritiated inositol phosphates produced from a radiolabeled myo-inositol precursor were measured using a standard ion exchange method. The investigated compound exhibited weak partial $P2Y_2$ agonist activity, which was surprising, given the $P2Y_2$ receptor full agonist activities of the previously synthesized 5'-triphosphates and 5'-triphosphate isosteres (see 1.3.3.1 and 4.1.3). However, HPLC and mass screening of the 200 mM Hepes (N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid) storage solution containing **4.7**, showed nearly quantitative hydrolysis of 5'-phosphonodiphosphate **4.7** to the corresponding 5'-phosphonate **4.50**. This was in contrast to an earlier reported higher chemical stability of a similar 5'-γ-phosphonate group in comparison to the corresponding 5'-γ-phosphate.[245] The presumption that the partial agonist activity was caused by the hydrolysis product **4.50** was confirmed by the repetition of the PLC activation assay with pure 5'-phosphonate **4.50**, yielding the same PLC activation curves for both **4.50** and **4.7** (hydrolysed) (Figure 4.6A).

5'-Phosphonate **4.50** caused a $P2Y_2$ receptor-dependent increase in phospholipase C activity that does not occur in wild type 1321N1 cells or in 1321N1 cells expressing the $P2Y_4$ or $P2Y_6$ receptors, which is presented as fold stimulation of [$^3$H]inositol phosphate accumulation over background [$^3$H]inositol phosphate accumulation (Figure 4.6B). So far, we assumed **4.50** to be a $P2Y_2$ receptor- selective partial agonist. However, experiments to determine the antagonist potency with up to 10 µM of the 5'-phosphonate did not illustrate an inhibition of the effect of UTP. Therefore, a higher concentration of **4.50** may be needed or **4.50** may exhibit some other action, for example through an allosteric interaction, to cause an enhancement of the effect of UTP, that is always in the medium in low concentrations because of basal release of cellular UTP. The latter presumption is supported by the $P2Y_2$ receptor-dependent increase in inositol phosphate accumulation in the absence of added UTP (Figure 4.6B) and the reduction of the basal activity by adding a nucleotide-hydrolyzing enzyme (apyrase) to the medium. Additional tests would be necessary to determine the role of **4.50** unambiguously. The additional amount of the 5'-phosponate needed

for these tests was not possible to provide during the period of this work due to a lack of time and is suggested as a future perspective.

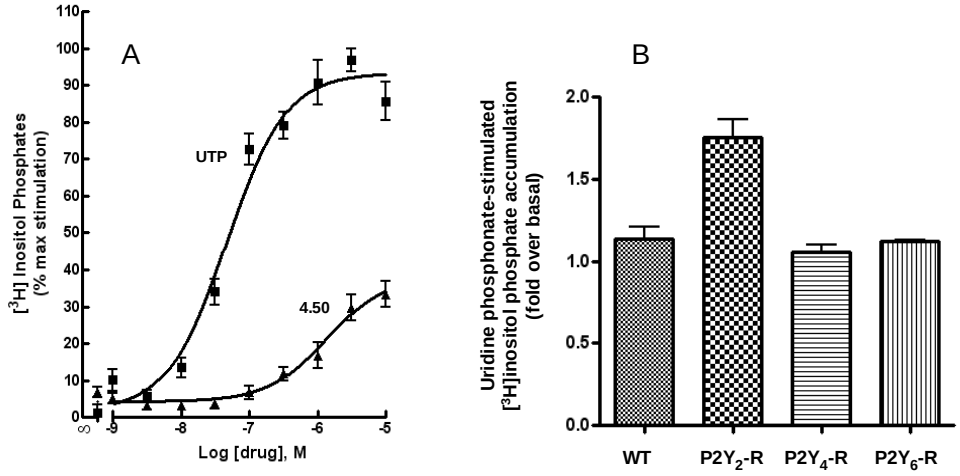



**Figure 4.6**

Partial agonist activity of 5'-phosphonate **4.50** at the $P2Y_2$ Receptor. A) Concentration effect curves were generated for UTP or uridine 5'-phosponate (**4.50)** in the presence of 10 mM LiCl in $P2Y_2$-R-1321N1 cells prelabeled with [$^3$H]inositol. B) The capacity of 10 µM uridine phosphonate to stimulate [$^3$H]inositol phosphate accumulation was measured in the presence of 10 mM LiCl in wild-type 1321N1 cells or in 1321N1 cells stably expressing the human $P2Y_2$-R, $P2Y_4$-R, or $P2Y_6$-R. The data are presented as fold stimulation of [$^3$H]inositol phosphate accumulation over background [$^3$H]inositol phosphate accumulation. The data are the mean ± SEM of six (A) or three (B) independent experiments.

The chemical stability and the biological data of vinyl analogue **4.6** are currently under investigation by prof. K. Harden and its group, Department of Pharmacology, University of North Carolina, USA.

## 4.2.4 Conclusions

Uridine 5'-phophonodiphosphate **4.7** exhibited nearly quantitative hydrolysis to the corresponding 5'-phosphonate **4.50** in the 200 mM Hepes buffer storage solution. Uridine 5'-phosphonate **4.50** exhibited weak partial $P2Y_2$ agonist activity and caused a $P2Y_2$ receptor-selective increase in phospholipase C activity. Therefore, uridine 5'-phosphonate may be a selective partial agonist at the $P2Y_2$ receptor but we have not proven this unambiguously. Uridine 5'-phosphonate may exhibit some other action, for example through an allosteric interaction, to cause $P2Y_2$ receptor-dependent increases in PLC activity. Additional tests would be necessary to further determine the role of **4.50**.

## CHAPTER 5 :

# EXPERIMENTAL SECTION

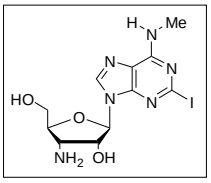
# 5 EXPERIMENTAL SECTION

## 5.1 Synthesis

### 5.1.1 General

All reagents were from standard commercial sources and of analytic grade, except for the benzylic azides, which were prepared by treating the corresponding benzylic bromides with $NaN_3$ in DMF. Precoated Merck silica gel F254 plates were used for TLC, and spots were examined under UV light at 254 nm and further visualized by sulfuric acid–anisaldehyde spray. Column chromatography was performed on ICN silica gel (63–200 µm, 60 Å, ICN Biochemicals, Eschwege, Germany). NMR spectra were obtained with a Varian Mercury 300 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to the residual solvent signals, which in the case of DMSO-$d_6$ were 2.54 ppm for $^1H$ and 40.5 ppm for $^{13}C$, in the case of $CDCl_3$ 7.26 ppm for $^1H$ and 77.23 ppm for $^{13}C$ and in the case of $D_2O$ 4.79 ppm for $^1H$. Structural assignment was confirmed with COSY and HSQC. All signals assigned to amino, amide hydrogen and hydroxyl groups were exchangeable with $D_2O$. Exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qToF 2, Micromass, Manchester, U.K.) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in a 2-propanol/water (1:1) mixture at 3 µL/min.

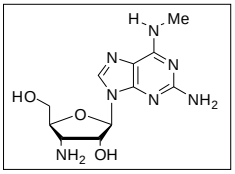
## 5.1.2 Hypermodified Adenosine Analogues



**9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-2-iodo-$N^6$-methyladenine (2.1)**

This compound was synthesized from 30 mg (0.069 mmol) of **2.34** by the procedure described for the synthesis of **2.7**; yield: 26 mg (92%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$) : δ 1.66 (br s, 2H, $NH_2$), 2.87 (d, 3H, *J* = 4 Hz, $N^6$-$CH_3$), 3.41 (app t, 1H, *J* = 6.0 Hz, H-3'), 3.54-3.71 (m, 3H, H-4' and H-5'A and H-5'B), 4.10 (br s, 1H, 2'-OH), 4.19 (dd, 1H, *J* = 2.6 and 4.4 Hz , H-2'), 4.98 (br s, 1H, 5'-OH), 5.82 (d, 1H, *J* = 2.6 Hz, H-1'), 8.11 (d, 1H, *J* = 4 Hz, $N^6$-H), 8.30 (s, 1H, H-8).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{11}H_{16}N_6O_3I$ $[M+H]^+$: 407.0330, found 407.0332.



**9-(3-Amino-3-deoxy-β-D-ribofuranosyl)- 2-amino-$N^6$-methyladenine (2.2)**

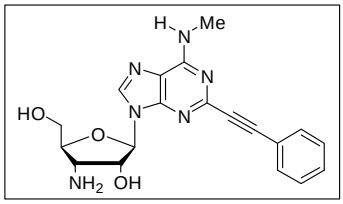
This compound was synthesized from **2.36** (35 mg, 0.11 mmol) by the procedure described for the synthesis of **2.7**; yield: 30 mg (93%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$) : δ 1.70 (br s, 2H, 3'-$NH_2$), 2.86 (s, 3H, $N^6$-$CH_3$), 3.34 (app t, 1H, *J* = 6.0 Hz, H-3'), 3.52-3.70 (m, 3H, H-4' and H-5'A and H-5'B), 4.16 (br s, 2H, 2'-OH and H-2'), 5.16 (br s, 1H, 5'-OH), 5.74 (d, 1H, *J* = 2.8 Hz, H-1'), 5.83 (s, 2H, 2-$NH_2$), 7.24 (br s, 1H, $N^6$-H), 7.91 (s, 1H, H-8);
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{12}H_{18}N_7O_3$ $[M+H]^+$: 296.1471, found 296.1470.

**General procedure for the synthesis of alkynes 2.3, 2.4 and 2.5 from 2.1**

Compound **2.1** (50 mg, 0.12 mmol) was dissolved in $Et_3N$ (1.5 mL) and DMF (1mL). After purging the solution with $N_2$, $(PPh_3)_2PdCl_2$ (8.6 mg, 0.012 mmol) and CuI (2.3 mg, 0.012 mmol) were added. The appropriate alkyne (2 equiv.) was subsequently

added dropwise and the mixture was stirred at room temperature for 3h. The solvents were removed under reduced pressure, the residue was taken up in ethyl acetate, and the solution filtered over a celite pad. The residue remaining after solvent evaporation was purified on silica gel column ($CH_2Cl_2$:MeOH, 90:10).

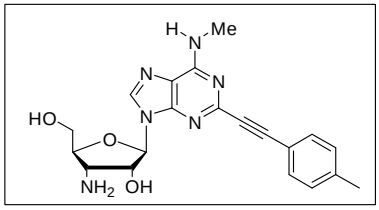


**9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-$N^6$-methyl-2-phenylethynyladenine (2.3)**

The reaction of **2.1** (50 mg, 0.12 mmol) with phenylacetylene (27 µL, 0.24 mmol) gave compound **2.3** in 81% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$) δ 2.07 (br s, 2H, 3'-$NH_2$), 2.94 (s, 3H, $N^6$-$CH_3$), 3.44 (app t, 1H, *J* = 5.3 Hz, H-3'), 3.56-3.75 (m, 3H, H-4' and H-5'A and H-5'B), 4.09 (br s, 1H, 2'-OH), 4.24 (dd, 1H, *J* = 2.6 and 4.4 Hz, H-2'), 5.11 (br s, 1H, 5'-OH), 5.94 (d, 1H, *J* = 2.3 Hz, H-1'), 7.46 (m, 3H, Ph), 7.61 (m, 2H, Ph), 7.95 (br s, 1H, $N^6$-H), 8.47 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{19}H_{21}N_6O_3$ $[M+H]^+$: 381.1675, found 381.1675.

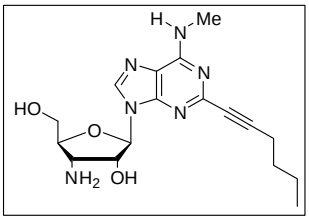


**9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-$N^6$-methyl-2-(4-methyl-phenyl) ethynyladenine (2.4)**

The reaction of **2.1** (50 mg, 0.12 mmol) with 4-methyl-phenylacetylene (31 µL, 0.24 mmol) gave compound **2.4** in 82% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$) δ 2.33 (s, 3H, $CH_3$Ph), 2.94 (s, 3H, $N^6$-$CH_3$), 3.50-3.79 (m, 4H, H-3', H-4', H-5'A and H-5'B), 4.31 (dd, 1H, *J* = 2.4 and 4.5 Hz, H-2'), 5.15 (br s, 1H, 5'-OH), 5.74 (s, 1H, 2'-OH), 5.96 (d, 1H, *J* = 2.4 Hz, H-1'), 7.25 (m, 2H, Ph), 7.50 (m, 2H, Ph), 7.95 (br s, 1H, $N^6$-H), 8.46 (s, 1H, H-8).

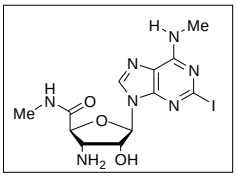
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{20}H_{23}N_6O_3$ $[M+H]^+$: 395.1831, found 395.1823.



**9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-*N*$^6$-methyl-2-(1-hexyn-1-yl)adenine (2.5)**

The reaction of **2.1** (50 mg, 0.12 mmol) with 1-hexyn (28 µL, 0.24 mmol) gave compound **2.5** in 80% yield.

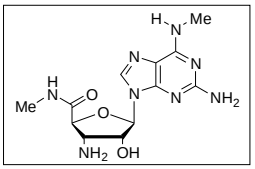
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.90 (t, 3H, *J* = 7.03 Hz, $CH_2CH_3$), 1.37-1.54 (m, 4H, $CH_2CH_2CH_3$), 2.41 (t, 2H, *J* = 7.04 Hz, C≡C$CH_2$), 2.88 (s, 3H, *N*$^6$-$CH_3$), 3.48-3.73 (m, 4H, H-3', H-4', H-5'A and H-5'B), 4.10-4.23 (m, 2H, H-2' and 2'-OH), 5.10 (br s, 1H, 5'-OH), 5.90 (d, 1H, *J* = 2.5 Hz, H-1'), 7.83 (br s, 1H, *N*$^6$-H), 8.41 (s, 1H, H-8).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{17}H_{25}N_6O_3$ $[M+H]^+$: 361.1988, found 361.1982.



**9-(3-Amino-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyl-2-iodoadenine (2.7)**

Compound **2.19** (50 mg, 0.11 mmol) and $PPh_3$ (57 mg, 0.21 mmol) were dissolved in THF (2mL). After stirring for 10 minutes, $H_2O$ was added (270 µL, 15 µmol) and the reaction was stirred for 2 days. The residue obtained after solvent evaporation was purified by column chromatography ($CH_2Cl_2$:MeOH 95:5) to yield 82% of compound **2.7**.

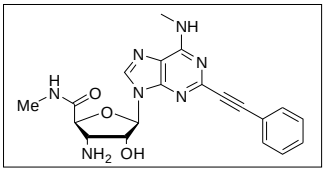
**$^1$H NMR** (300 MHz, DMSO-$d_6$) : δ 1.79 (s, 2H, 3'-$NH_2$), 2.69 (d, 3H, *J* = 4.7 Hz, $CH_3$NHCO), 2.88 (d, 3H, *J* = 4.1 Hz, *N*$^6$-$CH_3$), 3.54 (t, 1H, *J* = 11.1 Hz, H-3'), 4.10 (d, 1H, *J* = 6.2 Hz, H-4'), 4.30 (app t, 1H, *J* = 8.8 Hz, H-2'), 5.91 (br s, 1H, 2'-OH), 5.93 (d, 1H, *J* = 3.8 Hz, H-1'), 8,03 (d, 1H, *J* = 4,7 Hz, N*H*CO), 8.13 (d, 1H, *J* = 4.1 Hz, *N*$^6$-H), 8.48 (s, 1H, H-8).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{12}H_{17}ClIN_7O_3$ $[M+H]^+$: 434.0439, found 434.0445.



**9-(3-Amino-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl]-2-amino-*N*$^6$-methylpurine (2.8)**

Compound **2.37** (40 mg, 0.12 mmol) and $PPh_3$ (66 mg, 0.25 mmol) were dissolved in THF (2 mL). After stirring for 10 minutes, $H_2O$ was added (310 µL, 17 mmol) and the mixture was stirred for 2 days. The residue obtained after solvent evaporation was purified by column chromatography ($CH_2Cl_2$:MeOH 80:20) to give compound **2.8** in 80% yield.

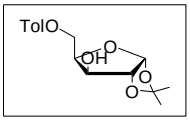
**$^1$H NMR** (300 MHz, DMSO-$d_6$) : δ 2.95 (br s, 2H, 3'-$NH_2$), 2.66 (d, 3H, *J* = 4.7 Hz, *CH*$_3$NHCO), 2.86 (s, 3H, *N*$^6$-$CH_3$), 3.54 (app t, 1H, *J* = 5.4 Hz, H3'), 4.05 (d, 1H, *J* = 5.6 Hz, H-4'), 4.37 (app t, 1H, *J* = 4.7 Hz, H-2'), 5.82 (d, 1H, *J* = 4.1 Hz, H-1'), 5.88 (s, 2H, 2-$NH_2$), 7.31 (s, 1H, *N*$^6$-H), 8.04 (s, 1H, H-8), 8.27 (d, 1H, *J* = 4.4 Hz, N*H*CO).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{12}H_{19}N_8O_3$ $[M+H]^+$: 323.1580, found 323.1579.



**9-(3-Amino-3-Deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyl-2-phenylethynyladenine (2.9)**

This compound was synthesized by the procedure described for the synthesis of **2.7** from 20 mg (0.046 mmol) of **2.20** in 96% yield (18 mg).

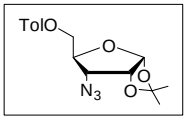
**$^1$H NMR** (300 MHz, DMSO-$d_6$) : δ 1.78 (s, 2H, 3'-$NH_2$), 2.71 (d, 3H, *J* = 4.4 Hz, *CH*$_3$NHCO), 2.94 (s, 3H, *N*$^6$-$CH_3$), 4.33 (dd, 1H, *J* = 5.0 and 5.3 Hz, H-3'), 4.13 (d, 1H, *J* = 5.3 Hz, H-4'), 4.36 (br s, 1H, H-2'), 5.94 (s, 1H, 2'-OH), 6.02 (d, 1H, *J* = 4.40, H-1'), 7.46 (m, 3H, Ph), 7.61 (m, 2H, Ph), 8.04 (d, 1H, *J* = 4.4 Hz, *N*$^6$-H), 8.41 (d, 1H, *J* = 4.7 Hz, N*H*CO), 8.62 (s, 1H, H-8).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{20}H_{22}N_7O_3$ $[M+H]^+$: 408.1784, found 408.1787.




**1,2-*O*-isopropylidene-5-*O*-(toluoyl)-α-D-xylofuranose (2.11)**

A solution of toluoyl chloride (13.2 mL, 100 mmol) in dry $CH_2Cl_2$ (40 mL) was added dropwise to a solution of 1,2-*O*-isopropylidene-α-D-xylofuranose **2.10** (20 g, 105.15 mmol) in dry pyridine (50 mL) and dry $CH_2Cl_2$ (150 mL). After stirring at 0 ℃ for 2h, the reaction mi xture was quenched with water (250 mL) and extracted with $CH_2Cl_2$. The combined organic phases were evaporated to dryness. The residue was dissolved in a minimal amount of EtOAc and crystallized upon treatment with hexane to obtain 23g (71%) of compound **2.11**.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.32 (s, 3H, $CH_3$), 1.51 (s, 3H, $CH_3$), 2.42 (s, 3H, Ph-$CH_3$), 2.78 (d, 1H, *J* = 4.1 Hz, 3-OH), 4.18 (dd, 1H, *J* = 2.5 and 5.2 Hz, H-3), 4.24-4.90 (m, 4H, H-2, H-4, H-5A and H-5B), 5.96 (d, *J* = 3.6 Hz, H-1), 7.26 (d, 2H, Ph), 8.00 (d, 2H, Ph). (The $^1$H NMR spectrum is accordance with the literature report.[183])
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{16}H_{21}O_6$ $[M+H]^+$ 309.1338, found 309.1343.

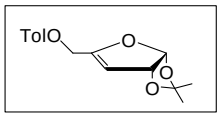



**3-Azido-3-deoxy-1,2-*O*-isopropylidene-5-*O*-(toluoyl)-α-D-ribofuranose (2.12)**

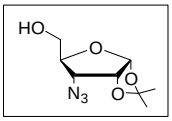
To a solution of **2.11** (23 g, 74.6 mmol) in $CH_2Cl_2$ (250 mL) and pyridine (20 mL) trifluoromethanesulfonic anhydride (15 mL, 89.5 mmol) was added dropwise and the reaction mixture was stirred at -10 ℃ for 2h. sat. aq. $NaHCO_3$ was added and stirring continued for 20 min. The organic phase was washed with $H_2O$, dried over $MgSO_4$, filtered and evaporated to dryness. The residue was dissolved in DMF (250 mL) and $NaN_3$ (34 g, 522 mmol) was added. The reaction mixture was stirred overnight at room temperature. DMF was evaporated *in vacuo*, and sat. aq. $NaHCO_3$ (250 mL) and EtOAc (250 mL) were added. The organic phase was dried over $MgSO_4$, filtered, evaporated to dryness and purified by column chromatography (pentane:EtOAc 95:5→84:15) to yield 10 g (40%) of compound **2.12**.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.36 (s, 3H, $CH_3$), 1.58 (s, 3H, $CH_3$), 2.38 (s, 3H, Ph-$CH_3$), 3.33 (dd t, 1H, *J* = 4.2 and 8.6 Hz, H-3), 4.21-4.61 (m, 3H, H-4, H-5A and H-

5B), 4.73 (dd, 1H, *J* = 4.5 and 8.5 Hz, H-2), 5.79 (d, *J* = 3.6 Hz, H-1), 7.16 (d, 2H, Ph), 7.86 (d, 2H, Ph). (The $^1$H NMR spectrum is accordance with the literature report.[183])
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{16}H_{19}N_3O_5Na$ $[M+Na]^+$ 356.1222, found 356.1235.

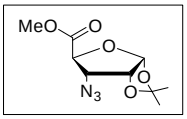



Next to the desired compound, 40% of the elimination product was obtained.
**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.47 (s, 3H, $CH_3$), 1.50 (s, 3H, $CH_3$), 2.40 (s, 3H, Ph-$CH_3$), 4.83 (s, 2H, H-5'A and H-5'B), 5.31 (m, 2H, H-2 and H-3), 4.51 (s, 2H, H-2 and H-3), 6.13 (1H, d, *J* = 4.1 Hz, H-1), 7.16 (d, 2H, Ph), 7.86 (d, 2H, Ph). (The $^1$H NMR spectrum is accordance with the literature report.[183])




**3-azido-3-deoxy-1,2-*O*-isopropylidene-α-D-ribofuranose (2.13)**
Compound **2.12** (6g, 15 mmol) was dissolved in 0.1 N $NaOCH_3$ in $CH_3OH$ (150 mL), stirred for 2h, neutralized with a $H_2O$:$CH_3COOH$ (9:1) solution, evaporated and purified by column chromatography ($CH_2Cl_2$:MeOH 99:1) to yield 3.4 g (88%) of **2.13**.

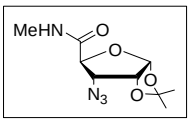
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.28 (s, 3H, $CH_3$), 1.44 (s, 3H, $CH_3$), 3.45-3.52 (m, 2H, H-3 and H-5A), 3.64 (ddd, 1H, *J* = 2.9, 5.3 and 12.3 Hz, H-5B), 3.94 (ddd, 1H, *J* = 2.9, 4.3 and 9.8 Hz, H-4), 4.77 (app t, 1H, *J* = 4.3 Hz, H-2), 4.90 (dd, 1H, *J* = 5.4 and 6.0 Hz, 5-OH), 5.77 (d, 1H, *J* = 3.5 Hz, H-1). (The $^1$H NMR spectrum is accordance with the literature report.[246])
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_8H_{13}N_3O_4Na$ $[M+Na]^+$ 238.0803, found 238.0801.




**3-Azido-3-deoxy-1,2-*O*-isopropylidene-α-D-ribofanuronic acid methyl ester (2.14)**
A biphasic mixture of water (30 mL), $CHCl_3$ (20 mL) and $CH_3CN$ (20 mL) containing compound **2.13** (2.03 g, 9.43 mmol), $RuCl_3$ (97 mg, 0.47 mmol) and $NaIO_4$ (8.2 g, 38.7 mmol) was vigorously stirred for 4.5 h at room temperature. The

reaction mixture was then diluted with 0.05 M HCl (75 mL) and extracted with $CH_2Cl_2$ (2 x 150 mL). The combined organic phase was dried over $MgSO_4$, filtered and evaporated. A dark green oily residue was triturated with diethyl ether to precipitate the ruthenium salts, which were removed by filtration through celite. Concentration of the filtrate *in vacuo*, resulted in a lightly coloured oil that was used without further purification. A mixture of this crude 3-azido-3-deoxy-1,2-*O*-isopropylidene-α-D-ribofuronic acid, EDC (4.4 mL, 23.58 mmol) and DMAP (113 mg, 0.92 mmol) in dry methanol (40 mL) was stirred at room temperature for 24 h. The reaction mixture was partionned between $CH_2Cl_2$ (150 mL) and water (75 mL). The aqeous phase was extracted with $CH_2Cl_2$ (3 x 150 mL) and the combined organic phase was dried over $MgSO_4$, filtered, evaporated and purified on a silica gel column ($CH_2Cl_2$: MeOH 98:2) to yield 2.23 g of compound **2.14** (40%).

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.36 (s, 3H, $CH_3$), 1.58 (s, 3H, $CH_3$), 3.69 (dd, 1H, *J* = 4.4 and 9.7 Hz, H-3), 3.84 (s, 3H, $OCH_3$), 4.56 (d, 1H, *J* = 9.7 Hz, H-4), 4.74 (app t, 1H, *J* = 4.1 Hz, H-2), 5.91 (d, 1H, *J* = 3.5 Hz, H-1).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_9H_{14}N_3O_5$ $[M+ H]^+$ 244.0934 , found 244.0941.

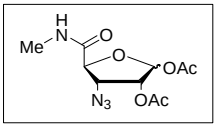



**3-Azido-3-deoxy-1,2-*O*-isopropylidene-α-D-ribofuranuronic acid methyl amide (2.15)**

Compound **2.14** (2.2 g, 8.77 mmol) was dissolved in 2 M methylamine in THF (20 mL) and was heated for 24h at 55 ℃ in a sealed tube. After cooling, the reaction mixture was concentrated to dryness and purified by silica gel chromatography (pentane: EtOAc 90:10) to obtain uronamide **2.15** in 89% yield (1.89 g).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.29 (s, 3H, $CH_3$), 1.46 (s, 3H, $CH_3$), 2.62 (d, 3H, *J* = 4.7 Hz, $CH_3$-N), 3.75 (dd, 1H, *J* = 4.5 and 9.5 Hz, H-3), 4.27 (d, 1H, *J* = 9.7 Hz, H-4), 4.77 (app t, 1H, *J* = 4.0 Hz, H-2), 5.85 (d, 1H, *J* = 3.5 Hz, H-1), 8.12 (d, 1H, *J* = 4.4 Hz, N-H). (The $^1$H NMR spectrum is accordance with the literature report.[48])

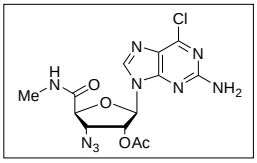
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_9H_{14}N_4O_4Na$ $[M+ Na]^+$ 265.0912 , found 265.0917.



**N-Methyl 3-azido-3-deoxy-1,2-di-*O*-acetyl-D-ribofuronamide (2.16)**

A mixture of **2.15** (1.8 g, 7.4 mmol), concentrated sulphuric acid (2.2 mL, 41.3 mmol) and acetic anhydride (7.4 mL, 78.6 mmol) in glacial acid (25 mL) was stirred for 18h at room temperature. After cooling in an ice bath, a saturated $NaHCO_3$ solution (75 mL) and $CH_2Cl_2$ (75 mL) were slowly added and the mixture was stirred for another 10 min. After separation, the organic phase was extracted with saturated $NaHCO_3$ solution and with brine, dried over $MgSO_4$, filtered and evaporated to dryness to yield 1.18 g (55%) of crude **2.16** as a yellowish foam.

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{10}H_{15}N_4O_6$ $[M+ H]^+$ 287.0991, found 287.0987.



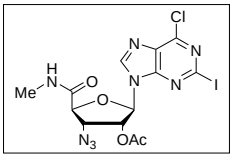
**9-(2-*O*-Acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-2-amino-6-chloropurine (2.17)**

Silylation of the base: 2-Amino-6-chloropurine (462 mg, 2.7 mmol) was treated with 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 40 mL) and $(NH_4)_2SO_4$ (0.27 mmol, 36 mg) and refluxed for 20h. The silylated compound was evaporated to dryness and used without further purification.

Vorbrüggen coupling: 3-Azido-3-deoxy-1,2-di-*O*-acetyl-α-D-ribofuronamide (**2.16**, 600 mg, 2.09 mmol) in dry 1,2-dichloroethane (25 mL) was added to the crude silylated 2-amino-6-chloropurine. The solution was gently refluxed, and after 5 minutes TMSOTf (417µL, 2.3 mmol) was added dropwise. After 4 h the mixture was cooled to room temperature, quenched with a cold saturated $NaHCO_3$ solution (80 mL) and extracted with $CH_2Cl_2$ (40 mL). The organic layer was dried with $MgSO_4$, filtered and evaporated to dryness. The residue was purified by column chromatography ($CH_2Cl_2$:MeOH 98:2) to give 650 mg (79%) of compound **2.17**.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 2.15 (s, 3H, $CH_3CO$), 2.90 (d, 3H, *J* = 5.0 Hz, *$CH_3$*N), 4.53 (d, 1H, *J* = 3.2 Hz, H-4'), 4.90 (dd, 1H, *J* = 3.5 and 5.0 Hz, H-3'), 5.14 (s, 2H, $NH_2$), 5.94 (t, 1H, *J* = 5.3 Hz, H-2'), 5.97 (d, 1H, *J* = 6.2 Hz, H-1'), 7.09 (br s, 1H, N*H*CO), 7.82 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{13}H_{15}ClN_9O_4$ $[M+H]^+$: 396.0935, found 396.0932.

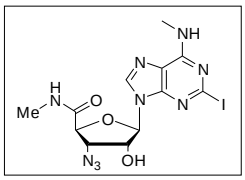



**9-(2-*O*-Acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-6-chloro-2-iodopurine (2.18)**

Isoamylnitrite (681 µL, 4.98 mmol) was added to a mixture of **2.17** (650 mg, 1.65 mmol), $I_2$ (418 mg, 1.65 mmol), $CH_2I_2$ (1.37 mL, 16.5 mmol) and CuI (330 mg, 1.72 mmol) in 15 mL THF. The mixture was refluxed for 45 minutes and then cooled to room temperature. Insoluble materials were removed by filtration, and the filtrate was concentrated to dryness. The residue was purified by means of a silica gel column, which was washed with $CH_2Cl_2$ until the iodine colour disappeared and then eluted with $CH_2Cl_2$:MeOH (98:2). Compound **2.18** was obtainded in 79% yield.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 2.13 (s, 3H, $CH_3CO$), 3.06 (d, 3H, *J* = 4.98 Hz, *$CH_3$*N), 4.56 (d, 1H, *J* = 2.9 Hz, H-4'), 4.80 (dd, 1H, *J* = 2.9 and 5.6 Hz, H-3'), 5.75 (dd, 1H, *J* = 5.9 and 7.0 Hz, H-2'), 6.06 (d, 1H, *J* = 7.3 Hz, H-1'), 7.09 (br s, 1H, N*H*CO), 8.11 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{13}H_{13}ClIN_8O_4$ $[M+H]^+$: 506.9794, found 506.9822.



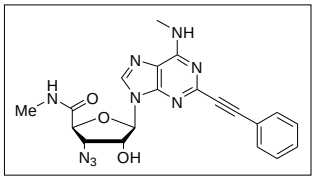

**9-(3-Azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyl-2-iodoadenine (2.19)**

Compound **2.18** (460 mg, 0.91 mmol) was dissolved in EtOH (10 mL). Methylammonium chloride (92 mg, 1.36 mmol) and $Et_3N$ (158 µL,1.13 mmol) were added, and the solution was refluxed overnight. The mixture was concentrated to dryness, dissolved in 7N $NH_3$ in methanol and stirred at

room temperature for 2 h to deprotect the 2'-hydroxyl group. The volatiles were removed under reduced pressure, and the residue was purified by silica gel column ($CH_2Cl_2$:MeOH 98:2). The product, **2.19,** was realized, in 77% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$ ): $\delta$ 2.71 (d, 3H, *J* = 4.7 Hz, *CH*$_3$NHCO), 2.89 (d, 3H, *J* = 4.4 Hz, $N^6$-$CH_3$), 4.33 (d, 1H, *J* = 3.5 Hz, H-4'), 4.47 (dd, 1H, *J* = 3.5 and 5.0 Hz, H-3'), 4.92 (dd, 1H, *J* = 5.6 and 11.4 Hz, H-2'), 5.89 (d, 1H, *J* = 6.5 Hz, H-1'), 6.33 ( d, 1H, *J* = 5.6 Hz, 2'-OH), 8.10 (d, 1H, *J* = 4,7 Hz, N*H*CO), 8.17 (d, 1H, *J* = 4.7 Hz, $N^6$-H), 8.36 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{12}H_{15}IN_9O_3$ $[M+H]^+$: 460.0344, found 460.0350.

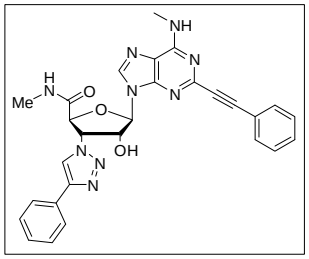



**9-(3-Azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-phenylethynyladenine (2.20)**

A solution of compound **2.29** (30 mg, 0.06 mmol) and 10 mL 7N $NH_3$ in methanol was kept at room temperature for 2h to allow deprotection of the 2'-hydroxyl group. The mixture was concentrated to dryness and purified on a silica gel column ($CH_2Cl_2$:MeOH 97:3). A 91% yield of compound **2.20** was obtained.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): $\delta$ 2.73 (d, 3H, *J* = 3.5 Hz, *CH*$_3$NHCO), 2.96 (s, 3H, $N^6$-$CH_3$), 4.33 (d, 1H, *J* = 2.6 Hz, H-4'), 4.51 (dd, 1H, *J* = 3.0 and 5.27 Hz, H-3'), 4.96 (app t, 1H, *J* = 5.3 Hz, H-2'), 5.98 (d, 1H, *J* = 6.7 Hz, H-1'), 7.46 (m, 3H, Ph), 7.64 (m, 2H, Ph), 8.12 (s, 1H, $N^6$-H), 8.51 (s, 1H, H-8), 8.53 (d, 1H, *J* = 4.4 Hz, N*H*CO).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{20}H_{20}N_9O_3$ $[M+H]^+$: 434.1688, found 434.1686.

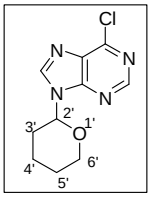


**Attempted synthesis of compound 2.20: Synthesis of 2.21**

Attempted conversion of **2.19** to **2.20** using the procedure as described for **2.3**, **2.4** and **2.5** failed to give **2.20**, but resulted in the formation of triazole **2.21** as sole reaction product (41% yield).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.78 (d, 3H, J= 4.6 Hz, $N^6$-$CH_3$), 3.0 (br s, 3H, $CH_3$NHCO), 5.15 (app q, 1H, *J* = 6.3 Hz, H-2'), 5.17 (d, 1H, *J* = 3.4 Hz, H-4'), 5.61 (dd, 1H, *J* = 3.6 and 6.3 Hz, H-3'), 6.26 (d, 1H, *J* = 5.4 Hz, 2'-OH), 6.27 (d, 1H, *J*= 6.3 Hz, H-1'), 7.36 (t, 1H, *J* = 7.3 Hz, 4''-Ph), 7.46-7.51 (m, 2H, 4''-Ph and 3H, C≡CPh), 7.65 (br d, 2H, *J* = 6.8 Hz, C≡CPh), 7.90 (d, 2H, *J* = 7.6 Hz , 4''-Ph), 8.59 (s, 1H, H-8), 8.71 (m, 2H, H-5'' and N*H*CO).

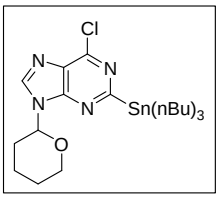
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{28}H_{25}N_9O_3$ $[M+H]^+$: 536.2158, found 536.2166.



**6-chloro-9-(tetrahydropyran-2-yl)-9*H*-purine (2.23)**

To a solution of 6-chloropurine (**2.22**, 6 g, 38.8 mmol) and *p*-toluene sulfonic acid (100 mg, 0.53 mmol) in dry THF (60 mL), 2,3-dihydro-4*H*-pyran (4 mL, 43.74 mmol) was added and the reaction mixture was heated at 80 ℃ for 15h. After dropwise addition of concentrated ammonia (3.5 mL), the reaction was cooled to room temperature, and evaporated to dryness. The yellowish oil was dissolved in ethyl acetate (150 mL), washedwith brine (75 mL), water (2x 75 mL), and then dried over $MgSO_4$. The organic phase was evaporated to dryness and boiling petroleum ether (60-80 ℃) was added. The ether extract was cooled in a fridge overnight to precipitate a colourless powder that was recrystallised from petroleum ether 60-80 ℃ to afford **2.23** (6.3 g, 69%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.58 (m, 3H, THP), 2.00 (m, 2H, THP), 2.30 (m, 1H, THP), 3.70 (m, 1H, THP H-3'), 4.00 (d, *J* = 10.8 Hz, 1H, THP H-2'), 5.76 (dd, *J* = 2.3 and 11.0 Hz, 1H, THP H-1'), 8.79 (s, 1H, H-8), 8.90 (s, 1H, H-2). (The $^1$H NMR spectrum is accordance with the literature report.[188] )

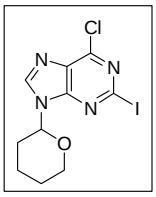


**6-Chloro-9-(tetrahydro-pyran-2-yl)-2-(tributylstannyl)-9*H*-purine (2.24)**

To a stirred solution of 2,2,6,6-tetramethylpiperidine (19.5 mL, 115.5 mmol) in THF (15 mL) and dry THF (30 mL) was added dropwise at -78 ℃ *n*-butyllithium (48.5 mL, 2.6 M solution in hexane, 121.3 mmol) over 30 minutes. Following stirring at the same temperature for 1 h, a solution of compound **2.23** (5.5 g, 23.1 mmol) in dry THF (30 mL) was added dropwise. After 30 minutes of stirring at -78 ℃ tributylstannyl chlor ide (31.3 mL, 115.5 mmol) was added dropwise to the dark mixture and stirring at the same temperature was continued for 1 hour. The resulting dark solution was quenched by dropwise addition of a saturated aqueous ammonium chloride solution (50 mL). Following overnight warming to room temperature with stirring, saturated aqueous $NaHCO_3$ (50 mL) was added. Extraction with ethyl acetate (2 × 50 mL) and drying of the combined organic phase over $MgSO_4$ afforded after evaporation to dryness a yellowish oil. Purification by silica gel column chromatography (hexane:EtOAc 80:20) afforded the 2-stannylated purine **2.24** (11.6 g, 95%) as a colourless oil.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 0.81 (t, 9H, *J*= 7.4 Hz, 3 x $CH_3$), 1.13 (t, 6H *J*= 7.9 Hz, 3 x $CH_2$), 1.27 (m, 6H, *J* = 7.7 Hz, 3 x $CH_2$), 1.55 (m, 9H, 3 x $CH_2$ and THP), 1.75 (m, 2H, THP) 2.10 (m, 2H, THP), 3.72 (td, 1H, *J* = 3.3 and 11.8 Hz, THP H-3'), 4.13 (m, 1H, THP H3'), 5.71 (dd, 1H, *J* = 2.8 and 7.2 Hz, THP H-1'), 8.15 (s, 1H, H-8). (The $^1$H NMR spectrum is accordance with the literature report.[188])

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{22}H_{38}N_4OClSn$ $[M+H]^+$: 539.1751, found 529.1746.

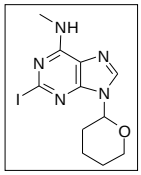


**6-Chloro-2-iodo-9-(tetrahydro-pyran-2-yl)-9*H*-purine (2.25)**

To a stirred solution of 2-stannylated purine **2.24** (11.5 g, 21.9 mmol) in dry THF (190 mL) iodine (8.55 g, 33.5 mmol) was added portionwise. Stirring was continued for 24 hours under $N_2$ atmosphere. Following treatment of the remaining iodine with saturated sodium metabisulfite, and subsequent stirring for one hour, the solution was extracted with dichloromethane (3 × 100 mL). The combined organic layers were washed with brine (100 mL), water (100 mL), dried over magnesium sulfate, and evaporated to full dryness. Trituration

with *n*-hexane (75 mL) precipitated the 2-iodopurine **2.25**, which was isolated as a pale yellow solid (6 g, 75%).

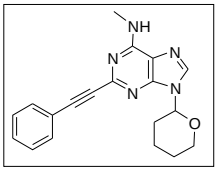
**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.46 (m, 3H, THP), 1.78 (m, 2H, THP), 2.01 (m, 1H, THP), 3.72 (td, *J* = 3.6 and 11.5 Hz, 1H, THP H3'), 4.10 (m, 1H, THP H-2'), 5.70 (dd, 1H, *J* = 2.3 and 10.5 Hz, THP H-1'), 8.22 (s, 1H, H-8). (The $^1$H NMR spectrum is accordance with the literature report.[188])
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{10}H_{11}ClN_4OI$ $[M+H]^+$: 364.9661, found: 364.9665.



**2-Iodo-*N*$^6$-methyl-9-(tetrahydropyran-2-yl)adenine (2.26)**

Methylammonium chloride (28 mg, 0.41 mmol) and DMAP (67 mg, 0.54 mmol) were added to 100 mg (0.27 mmol) of compound **2.25** in EtOH (6 mL), and the solution was refluxed overnight. The mixture was concentrated to dryness and the residue purified on a silica gel column (pentane: EtOAc 50:50). The title compound was obtained in 81% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.52 (m, 6H, H-2A' and H-2B', H-3A' and H-3B', H-4A' and H-4B'), 2.87 (s, 3H, NH*CH*$_3$), 3.68 (t, 1H, *J*= 11.4 Hz, H-5'A), 3.96 (app d, 1H, *J* = 11.4 Hz, H-5'B), 5.43 (dd, 1H, *J* = 2.3 and 10.9 Hz, H-1'), 7.26 (br s, 1H, N*H*$CH_3$), 7.87 (s, 1H, H-8).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{11}H_{15}N_5O_3I$ $[M+H]^+$: 360.0323, found 360.0333.

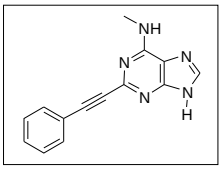


***N*$^6$-Methyl-2-phenylethynyl-9-(tetrahydropyran-2-yl)adenine (2.27)**

Compound **2.26** (200 mg, 0.557 mmol) was dissolved in a mixture of $Et_3N$ (3 mL) and DMF (1mL) and the solution was purged with $N_2$. $(PPh_3)_2PdCl_2$ (39 mg, 0.056 mmol) and CuI (10.6 mg, 0.056 mmol) were added. Phenyl acetylene (112 µL, 1.11 mmol) was subsequently added dropwise, and the mixture was stirred at room temperature for 3h. The solvents were removed under reduced pressure, the residue was taken up in ethyl acetate and the resulting

solution filtered over a pad of celite. After solvent evaporation, the residue was purified on a silica gel column (pentane:EtOAc 50:50) to give compound **2.27** in 91% yield.

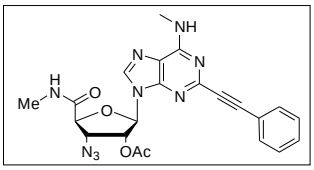
**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.18-2.25 (m, 6H, H-2A' and H-2B', H-3A' and H-3B', H-4A' and H-4B'), 3.21 (s, 3H, NH*CH*$_3$), 3.72 (app t, 1H, *J* = 11.4 Hz, H-5'A), 3.96 (app d, 1H, *J* = 10.3 Hz, H-5'B), 5.79 (d, 1H, *J* = 9.1 Hz, H-1'), 7.20 (s, 1H, H-8), 7.30 (d, 3H, *J* = 5.3 Hz, H-Ph), 7.61 (m, 2H, H-Ph).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{19}H_{20}N_5O$ $[M+H]^+$: 334.1667, found 334.1671.



**$N^6$-Methyl-2-phenylethynylpurine (2.28)**

To a solution of **2.27** (170 mg, 0.510 mmol) in 10 mL of $CH_2Cl_2$ was added slowly a solution of 0.78 mL TFA (10.2 mmol) and 0.78 mL $CH_2Cl_2$. After stirring at room temperature for 1h, the solvent was evaporated, and the residue was taken up in ethyl acetate, and the solution washed with 7% $NaHCO_3$. After silica gel chromatography ($CH_2Cl_2$:MeOH 97:3) pure **2.28** was obtained in a 72% yield.

**$^1$H NMR** (300 MHz, DMSO): δ 2.97 (s, 3H, NH*CH*$_3$), 7.30 (m, 3H, H-Ph), 7.61 (m, 2H, H-Ph), 7.88 (br s, 1H, N*H*$CH_3$), 8.24 (s, 1H, H-8).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{14}H_{12}N_5$ $[M+H]^+$: 250.1092, found 250.1073.

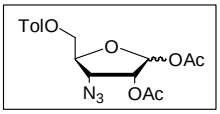


**9-(2-Acetyl-3-azido-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-phenylethynyladenine (2.29)**

To a mixture of **2.28** (150 mg, 0.602 mmol) and methyl 3-azido-3-deoxy-1,2-di-*O*-acetyl-α-D-ribofuronamide (**2.16**) (207 mg, 0.722 mmol) in 3 mL $CH_3CN$ was successively added 223 µL (0.903 mmol) of *N,O*-bis(trimethylsilyl)-acetamide (BSA) and 131 µL (0.722 mmol) of TMSOTf. The suspension was refluxed for 10 h. After being cooled to room temperature, the

reaction was quenched with 7% $NaHCO_3$ and extracted with $CH_2Cl_2$. The organic layer was washed with brine, filtered through a short pad of celite and evaporated to dryness. The crude material was purified by column chromatography ($CH_2Cl_2$:MeOH, 99:1), and compound **2.29** was obtained in 24.5% yield (70 mg).

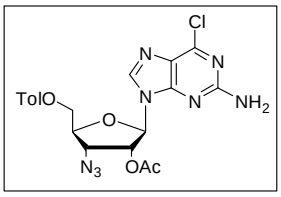
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.12 (s, 3H, $CH_3CO$), 3.02 (d, 3H, *J*= 4.69 Hz, *$CH_3$*NHCO), 3.25 (s, 3H, *N*$^6$-$CH_3$ ), 4.58 (d, 1H, *J* = 2.6 Hz, H-4'), 4.78 (dd, 1H, *J* = 2.1 and 5.3 Hz, H-3'), 5.78 (dd, 1H, *J* = 7.3 and 12.9 Hz, H-2'), 5.92 (s, 1H, *N*$^6$H) 6.02 (d, 1H, *J* = 7.6 Hz, H-1'), 7.40 (app d, 3H, *J* = 7.0 Hz H-Ph), 7.63 (app d, 2H, *J* = 9.1 Hz, H-Ph), 7.81 (s, 1H, H-8), 8.83 (s, 1H, N*H*CO).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{22}H_{22}N_9O_4$ $[M+H]^+$: 476.1794, found 476.1800.




**3-Azido-3-deoxy-1,2-di-*O*-acetyl-5-O-Toluoyl)-D-ribofuranose (2.30)**

The azido derivative **2.12** (5g, 15 mmol) was added to a 75% HCOOH solution (150 mL). The mixture was heated to 50 ℃ for 2h and evaporated to dryness. The residue was dissolved in a mixture of acetic anhydride:pyridine (125 mL, 2:3, v:v) at room temperature. Stirring was continued for 3h and $CH_2Cl_2$ (100 mL) and sat. aq. $NaHCO_3$ (100 mL) were added. The aqeous phase was washed with $CH_2Cl_2$ (100 mL) and the combined organic phases were dried over $MgSO_4$, filtered and evaporated *in vacuo*. The residue was precipitated from methanol to give **2.30** as a white solid (5.1 g, 90%).

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.90 (s, 3H, $CH_3CO$), 2.15(s, 3H, $CH_3CO$), 2.48 (s, 1H, $CH_3$-Ph), 4.10-4.68 (m, 4H, H-3, H-4, H-5A and H-5B), 5.31 (d, 1H, *J* = 4.6 Hz, H-2), 6.09 (s, 1H, H-1) 7.16 (d, 2H, *J* = 8.1 Hz, Ph), 7.88 (d, 1H, *J* = 8.1 Hz, Ph).
**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{17}H_{19}N_3O_7Na$ $[M+Na]^+$: 400.1120, found 400.1198.

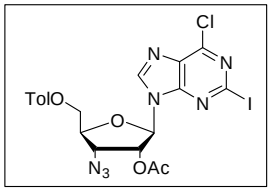



**9-(2-Acetyl-3-azido-3-deoxy-5-*O*-toluoyl-β-D-ribofuranosyl)-2-amino-6-chloropurine (2.31)**

To a mixture of 2-amino-6-chloropurine (90 mg, 0.53 mmol) and compound **2.30** (240 mg, 0.64 mmol) in 3 mL $CH_3CN$ was successively added 196 µL (0.79 mmol) BSA and 115 µL (0.64 mmol) TMSOTf. The suspension was heated at 80 ℃ for 3 h. After being cooled to room temperature, the reaction was quenched with 7% $NaHCO_3$ and extracted with $CH_2Cl_2$. The organic layer was washed with brine, filtered through a short celite pad and evaporated to dryness. The residue was purified by column chromatography ($CH_2Cl_2$:MeOH 99:1) to yield 200 mg (65%) of compound **2.31**.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 2.19 (s, 3H, $CH_3CO$), 2.41(s, 3H, $CH_3$-Ph), 4.40 (m, 1H, H-4'), 4.52-4.58 (m, 1H, H-5'A'), 4.76-4.82 (m, 2H, H3' and H-5'B), 5.13 (br s, 2H, $NH_2$), 5.94 (d, 1H, *J* = 3.8 Hz, H-1'), 5.80 (dd, 1H, *J* = 3.8 and 5.6 Hz, H-2'), 7.23 (d, 2H, *J* = 7.63 Hz, Ph ), 7.79 (s, 1H, H-8), 7.86 (d, 2H, *J* = 8.2 Hz, Ph).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{20}H_{19}N_8O_5Cl$ $[M+H]^+$: 487.1245, found 487.1246.

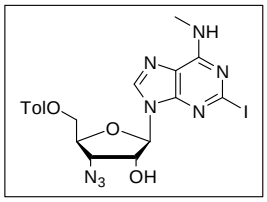



**9-(2-Acetyl-3-azido-3-deoxy-5-*O*-toluoyl-β-D-ribofuranosyl)-6-chloro-2-iodopurine (2.32)**

This compound was prepared by the procedure described for the synthesis of **2.18** from **2.31** (200 mg, 0.41 mmol); yield: 200 mg (81%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.15 (s, 3H, $CH_3CO$), 2.35 (s, 3H, $CH_3$-Ph), 4.37 (dd, 1H, *J* = 4.1 and 7.6 Hz, H-4'), 4.52 (dd, 1H, *J* = 4.4 and 12.3 Hz, H-5'A'), 4.65 (dd, 1H, *J* = 3.2 and 12.3 Hz, H-5'B), 4.96 (dd, 1H, *J* = 5.8 and 7.6 Hz, H-3'), 6.03 (dd, 1H, *J* = 2.6 and 5.28 Hz, H-2'), 6.29 (d, 1H, *J* = 2.9 Hz, H-1'), 7.24 (d, 2H, *J* = 7.9 Hz, Ph ), 7.68 (d, 2H, *J* = 8.2 Hz, Ph), 8.74 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{20}H_{17}N_7O_5ClINa$ $[M+Na]^+$: 619.9924, found 619.9920.

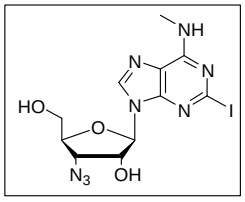



**9-(3-Azido-3-deoxy-2-hydroxyl-5-*O*-toluoyl-β-D-ribofuranosyl)-2-iodo-$N^6$-methyladenine (2.33)**

The title compound was prepared as described for the synthesis of **2.19** from **2.32** (200 mg, 0.335 mmol); yield: 127 mg (69%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.36 (s, 3H, *CH*$_3$-Ph), 2.87 (d, 3H, *J* = 3.5 Hz, $N^6$-$CH_3$), 4.29 (dd, *J* = 5.3 and 9.7 Hz, H-4'), 4.46-4.60 (m, 3H, H-3', H5'A and H-5'B), 5.01 (t, 1H, *J* = 4.7 Hz, H-2'), 5.87 (d, 1H, *J* = 4.7 Hz, H-1'), 6.43 (d, 1H, *J* = 5.3 Hz, 2'-OH), 7.29 (d, 2H, *J* = 8.2 Hz, Ph ), 7.78 (d, 2H, *J* = 8.2 Hz, Ph), 8.17 (d, 1H, *J* = 4.4 Hz, $N^6$-H), 8.21 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{19}H_{20}N_8O_4I$ $[M+H]^+$: 551.0653, found 551.0649.

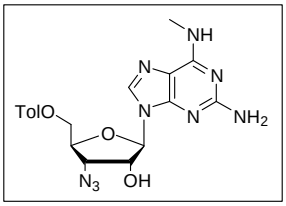



**9-(3-Azido-3-deoxy-β-D-ribofuranosyl)- 2-iodo-$N^6$-methyl-adenine (2.34)**

Ester **2.33** (127 mg, 0.23 mmol) was dissolved in 2.5 mL of MeOH. Na° (11.28 mg, 0.32 mmol) was added and the mixture was stirred at room temperature for 1h. The reaction was quenched by adding a mixture of $CH_3COOH$:$H_2O$ (9:1, v:v) to pH 7. The solution was concentrated to dryness, and the residue purified by column chromatography ($CH_2Cl_2$:MeOH 98:2) to yield 100 mg (95 %) of compound **2.34**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.88 (d, 3H, *J* = 4 Hz, $N^6$-$CH_3$), 3.52-3.67 (m, 2H, H-5'A and H-5'B), 3.94 (dd, 1H, *J* = 7.26 and 3.81 Hz, H-4'), 4.28 (app t, 1H, *J* = 4.5 Hz, H-3'), 4.89 (app t, 1H, *J* = 5.4 Hz, H-2'), 5.20 (br s, 1H, 5'-OH), 5.80 (d, 1H, *J* = 6.16 Hz, H-1'), 6.25 (br s, 1H, 2'-OH), 8.18 (d, 1H, *J* = 4 Hz, $N^6$-H), 8.28 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{11}H_{14}N_8O_3I$ $[M+H]^+$: 433.0235, found 433.0237.

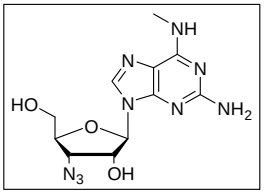


**9-(3-Azido-3-deoxy-5-*O*-toluoyl-β-D-ribofuranosyl)-2-amino-$N^6$-methyl-adenine (2.35)**

Derivative **2.31** (100 mg, 0.21 mmol) was solubilized in EtOH (5 mL). Methylammonium chloride (35 mg, 0.515 mmol) and $Et_3N$ (72 µL, 0.515 mmol) were added, and the solution was refluxed overnight. The mixture was concentrated to dryness, the residue redissolved in methanolic $NH_3$, and the solution stirred at room temperature for 2h to allow deprotection of the 2'-hydroxyl group. The mixture was concentrated to dryness and purified on a silica gel column ($CH_2Cl_2$:MeOH, 97:3). Compound **2.35** was obtained in 68% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ2.36 (s, 3H, *$CH_3$*-Ph), 2.84 (br s, 3H, *$N^6$*-$CH_3$), 4.23 (dd, *J* = 5.27 and 9.38 Hz, H-4'), 4.43 (dd, 1H, *J* = 5.6 and 11.6, H-5'A), 4.55 (m, 2H, H-3 and H-5'B), 4.48 (app t, 1 H, 4.7 Hz, H-2'), 5.78 (d, 1H, *J* = 4.7 Hz, H1'), 5.95 (br s, 2H, 2-$NH_2$), 6.34 (d, 1H, *J* = 7.9 Hz, 2'-OH), 7.28 (br s, 1H, *$N^6$*-H), 7.29 (d, 2H, *J* = 7.9 Hz, Ph ), 7.79 (s, 1H, H-8), 7.82 (d, 2H, *J* = 8.2 Hz, Ph).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{19}H_{22}N_9O_4$ $[M+H]^+$: 440.1794, found 440.1789.

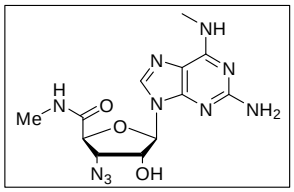


**9-(3-Azido-3-deoxy-β-D-ribofuranosyl)-2-amino-$N^6$-methyladenine (2.36)**

The title compound was synthesized from **2.35** (60 mg, 0.013 mmol) by the procedure described for the synthesis of **2.34**; yield: 40 mg (91%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.85 (br s, 3H, *$N^6$*-$CH_3$), 3.48-3.64 (m, 2H, H-5'A and H-5'B), 3.89 (dd, 1H, *J* = 3.2 and 7.0 Hz, H-4'), 4.25 (dd, 1H, *J* = 3.2 and 5.6 Hz, H-3'), 4.88 (app t, 1H, *J* = 5.3 Hz, H-2'), 5.58 (t, *J* = 6.8 Hz, 1H, 5'-OH), 5.71 (d, 1H, *J* = 6.2 Hz, H-1'), 5.85 (s, 2H, $NH_2$), 6.16 (s, 1H, 2'-OH), 7.31 (br s, 1H, *$N^6$*-H), 7.89 (s, 1H, H-8).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{11}H_{16}N_9O_3$ $[M+H]^+$: 322.1376, found 322.1365.



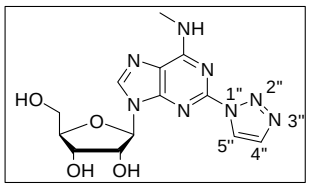
**9-[3-azido-3-deoxy-5-(methylcarbamoyl)-β-D-ribofuranosyl]-2-amino-6-chloropurine (2.37)**

Derivative **2.17** (120 mg, 0.30 mmol) was dissolved in EtOH (6 mL). Methylammonium chloride (30 mg, 0.46 mmol) and $Et_3N$ (53 µL, 0.38mmol) were added, and the solution was refluxed overnight. The mixture was concentrated to dryness, the remaining solid dissolved in 7N $NH_3$ in methanol and, the solution stirred at room temperature for 2 h to deprotect the 2'-hydroxyl group. The mixture was concentrated to dryness and the residue purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5). Compound **2.37** was obtained in 79% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.68 (d, 3H, *J* = 4.7 Hz, *CH*$_3$NHCO), 2.87 (s, 3H, *N*$^6$-$CH_3$), 4.24 (d, 1H, *J* = 2.9 Hz, H-4'), 4.42 (dd, 1H, *J* = 2.9 and 5.3 Hz, H-3'), 4.97 (dd, 1H, *J* = 5.6 and 11.7 Hz, H-2'), 5.81 (d, 1H, *J* = 6.8 Hz, H-1'), 5.91 (s, 2H, $NH_2$), 6.26 (d, 1H, *J* = 5.3 Hz, 2'-OH ), 7.31 (s, 1H, *N*$^6$-H), 7.96 (s, 1H, H-8), 8.35 (d, 1H, *J* = 4.7 Hz, N*H*CO).

**HRMS** (ESI-MS, *i*PrOH:$H_2O$): calculated for $C_{12}H_{17}N_{10}O_3$ $[M+H]^+$: 349.1484, found 349.1491.

## 5.1.3 2-Triazol-Substituted Adenosine Analogues



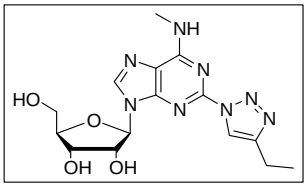
**$N^6$-Methyl-9-(β-D-ribofuranosyl)-2-(1,2,3-triazol-1-yl)adenine (3.1).**

In a pressure tube was added **3.24** (165 mg, 0.51 mmol) trimethylsilylacetylene (292 µL, 2.05 mmol) and 4 mL DMF. The mixture was stirred at 105 ℃ for 15h. After so lvent evaporation, the yellowish residue was dissolved in 2 mL of a 1.0 solution of tetrabutylammonium fluoride in THF and stirred for 5h. The reaction was monitored by NMR After evaporation of the solvent, the residue was dissolved in ethyl acetate. Water was added and the triazole product precipitated in the water layer. After overnight cooling and filtration, the precipitate was further purified on a silica gel column ($CH_2Cl_2$:MeOH 92:8) and yielded compound **3.1** as a white solid (82 mg, 46 %).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.06 (d, 3H, *J* = 4.5 Hz, $N^6$-$CH_3$), 3.54-3.61 (m, 1H, H-5'A), 3.65-3.72 (m, 1H, H-5'B), 3.96 (dd, 1H, *J* = 3.8 and 7.9 Hz, H-4'), 4.20 (dd, 1H, *J* = 4.7 and 8.2 Hz, H-3'), 4.65 (app q, *J* = 5.9 Hz, H-2'), 4.98 (t, 1H, *J* = 5.6 Hz, 5'-OH), 5.23 (d, 1H, *J* = 5.0 Hz, 3'-OH), 5.48 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.95 (d, 1H, *J* = 5.9 Hz, H-1'), 7.92 (d, 1H, *J* = 1.2 Hz, H 4"), 8.37 (d, 1H, *J* = 4.6 Hz, $N^6$-H), 8.46 (s, 1H, H-8), 8.82 (br s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.84 ($N^6$-$CH_3$), 62.19 (C-5'), 71.13 (C-3'), 74.30 (C-2'), 86.42 (C-4'), 88.01 (C-1'), 119.75 (C-5), 124.67 (C-5"), 134.25 (C-4"), 141.13 (C-8), 149.56 and 149.85 (C-2 and C-4), 156.082 (C-6).

**HRMS** (ESI-MS) $C_{13}H_{17}N_8O_4$: $[M+H]^+$: 349.1367 found; 349.1372 calculated.



**2-(4-Ethyl-1,2,3-triazol-1-yl)-$N^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.2).**

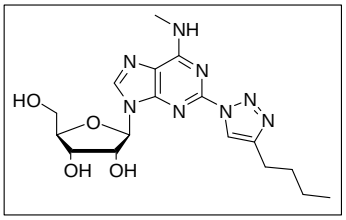
Compound **3.24** (70 mg, 0.217 mmol), sodium ascorbate (8.6 mg, 0.043 mmol) and $CuSO_4.5H_2O$ (2.2 mg, 0.009 mmol) were suspended in 20 mL $H_2O$:*t*-BuOH (3:1). The mixture was saturated with 1-butyne and stirred for 4 d at room temperature in a parr apparatus. Purification on a preparative silica gel TLC ($CH_2Cl_2$:MeOH 90:10) resulted in compound **3.2** as a white solid in 40% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.27 (t, 3H, *J* = 7.62 Hz, $CH_3$), 2.75 (q, 2H, *J* = 7.6 Hz, $CH_2$), 3.05 (d, 3H, *J* = 4.4 Hz, $N^6$-$CH_3$), 3.51-3.59 (m, 1H, H-5'A), 3.61-3.70 (m, 1H, H-5'B), 3.94 (dd, 1H, *J* = 4.0 en 7.6 Hz, H-4'), 4.15 (dd, 1H, *J* = 4.4 and 7.9 Hz, H-3'), 4.60 (app q, *J* = 5.6 Hz, H-2'), 4.97 (t, 1H, *J* = 5.6 Hz, 5'-OH), 5.22 (d, 1H, *J* = 4.7 Hz, 3'-OH), 5.47 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.93 (d, 1H, *J* = 6.2 Hz, H-1'), 8.34 (d, 1H, *J* = 4.4 Hz, $N^6$-H), 8.45 (s, 1H, H-8), 8.55 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$) ): δ 14.32 ($CH_3$), 19.08 ($CH_2$), 27.84 ($N^6$-$CH_3$), 62.13 (C-5'), 71.15 (C-3'), 74.34 (C-2'), 86.43 (C-4'), 87.84 (C-1'), 119.57 (C-5), 120.962 (C-5''), 140.92 (C-8), 149.31, 149.63, 149.88 (C-2, C4 and C-4"), 156.05 (C-6).

**HRMS** (ESI-MS) $C_{15}H_{21}N_8O_4$ $[M+H]^+$: 377.1682 found; 377.1685 calculated.

**General procedure for the synthesis of 4"-substituted 2-(1,2,3-triazol-1-yl)adenosine derivatives 3.3-3.11.** Compound **3.24** (70 mg, 0.217 mmol), sodium ascorbate (8.6 mg, 0.043 mmol) and $CuSO_4.5H_2O$ (2.2 mg, 0.009 mmol) were suspended in 2 mL $H_2O$:*t*-BuOH (3:1). The appropriate alkyne (2 equiv.) was subsequently added and the mixture was stirred overnight at room temperature. The 2-triazol-1-yl compounds (generally) precipitated from this reaction medium and were isolated by filtration with water.




**2-(4-Butyl-1,2,3-triazol-1-yl)-$N^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.3).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 1-hexyne (50 µL, 0.435 mmol) gave compound **3.3** in 59% yield.[†]

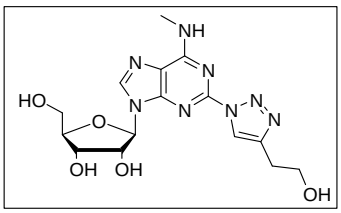
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.93 (t, 3H, *J* = 7.3 Hz, $CH_3$), 1.38 (m, 2H, $CH_2$), 1.66 (m, 2H, $CH_2$), 2.71 (t, 2H, *J* = 7.2 Hz, C4''-$CH_2$), 3.05 (d, 3H, *J* = 4.0 Hz, $N^6$-$CH_3$), 3.52-3.62 (m, 1H, H-5'A), 3.62-3.72 (m, 1H, H-5'B), 3.95 (dd, 1H, *J* = 3.6 and 7.2 Hz,

[†] For the compounds that precipitated from the reaction medium, the yields were calculated from the amount obtained after filtration and are lower than the actual yields, since in most cases a considerable amount remained in solution.

H-4'), 4.18 (dd, 1H, *J* = 4.8 and 8.1 Hz, H-3'), 4.62 (app q, 1H, *J* = 5.7 Hz, H-2'), 5.01 (t, 1H, *J* = 5.2 Hz, 5'-OH), 5.29 (d, 1H, *J* = 4.0 Hz, 3'-OH), 5.54 (d, 1H, *J* = 5.6 Hz, 2'-OH), 5.94 (d, 1H, *J* = 5.9 Hz, H-1'), 8.36 (d, 1H, *J* = 4.1 Hz, $N^6$-H), 8.45 (s, 1H, H-8), 8.56 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 14.37 ($CH_3$), 22.37 ($CH_2$), 25.21 ($CH_2$), 27.83 ($N^6$-$CH_3$), 31.68 ($CH_2$), 62.14 (C-5'), 71.15 (C-3'), 74.36 (C-2'), 86.43 (C-4'), 87.88 (C-1'), 119.56 (C-5), 121.33 (C-5"), 140.96 (C-8), 147.88 and 149.90 (C-2, C-4 and C4"), 156.07 (C-6).

**HRMS** (ESI-MS) $C_{17}H_{25}N_8O_4$ $[M+H]^+$: 405.1992 found, 405.1998 calculated.



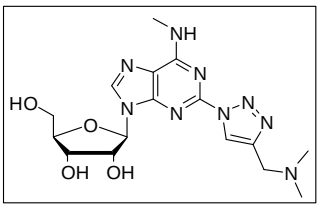

**2-[4-(2-Hydroxyethyl)-1,2,3-triazol-1-yl]-$N^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.4).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 3-butyn-1-ol (33 µL, 0.435 mmol) afforded compound **3.4** without precipitation. After solvent evaporation, the mixture was purified on a silica gel column ($CH_2Cl_2$:MeOH 90:10 + 1% 7N $NH_3$ in MeOH) yielding compound **3.11** as a white solid in 68% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.85 (t, 2H, *J* = 6.9 Hz, $CH_2$), 3.03 (d, 3H, *J* = 4.7 Hz, $N^6$-$CH_3$), 3.52-3.59 (m, 1H, H-5'A), 3.63-3.72 (m, 3H, H-5'B and $CH_2$), 3.94 (dd, 1H, *J* = 3.5 and 7.3 Hz, H-4'), 4.16 (dd, 1H, *J* = 4.8 and 8.1 Hz, H-3'), 4.59 (app q, 1H, *J* = 5.4 Hz, H-2'), 4.74 (t, 1H, *J* = 5.8 Hz, $CH_2$-OH), 4.97 (app t, 1H, *J* = 5.6 Hz, 5'-OH), 5.24 (d, 1H, *J* = 5.0 Hz, 3'-OH), 5.50 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.93 (d, 1H, *J* = 6.2 Hz, H-1'), 8.31 (d, 1H, *J* = 4.7 Hz, $N^6$-H), 8.43 (s, 1H, H-8), 8.54 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.83 ($N^6$-$CH_3$), 29.70 ($CH_2$), 60.85 ($CH_2$-OH), 62.13 (C-5'), 71.15 (C-3'), 74.35 (C-2'), 86.43 (C-4') 87.81 (C-1'), 119.56 (C-5), 122.02 (C-5"), 140.49 (C-8), 145.49, 149.88 and 149.65 (C-2, C-4 and C4"), 156.05 (C-6).

**HRMS** (ESI-MS) $C_{15}H_{21}N_8O_5$ $[M+H]^+$: 393.1630 found, 393.1634 calculated.



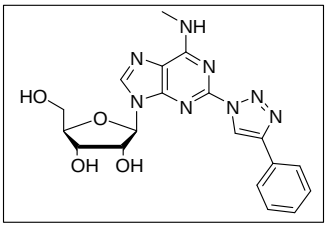
**2-(4-Dimethylaminomethyl-1,2,3-triazol-1-yl)-*N*$^6$-methyl-9-(β-D-ribofuranosyl) adenine (3.5).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 1-dimethylamino-2-propyne (47 µL, 0.435 mmol) gave compound **3.5** without precipitation. The volatiles were removed under reduced pressure, and the residue purified on a silica gel column ($CH_2Cl_2$:MeOH 80:10 + 1% 7N $NH_3$ in MeOH). Compound **3.5** was obtained as a white solid in 67% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.20 (s, 6H, 2 x $CH_3$), 3.05 (d, 3H, *J* = 4.4 Hz, *N*$^6$-$CH_3$), 3.54-3.62 (m, 3H, H-5'A and $CH_2$), 3.65-3.72 (m, 1H, H-5'B), 3.95 (dd, 1H, *J* = 4.1 and 7.6 Hz, H-4'), 4.18 (dd, 1H, *J* = 5.0 and 8.2 Hz, H-3'), 4.62 (app q, 1H, *J* = 5.4 Hz, H-2'), 4.98 (t, 1H, *J* = 5.6 Hz, 5'-OH), 5.23 (d, 1H, *J* = 5.0 Hz, 3'-OH), 5.49 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.95 (d, 1H, *J* = 6.2 Hz, H-1'), 8.38 (d, 1H, *J* = 4.4 Hz, *N*$^6$-H), 8.45 (s, 1H, H-8), 8.64 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$) ): δ 27.85 (*N*$^6$-$CH_3$), 45.18 (*N*$(CH_3)_2$), 53.85 ($CH_2$), 62.10 (C-5'), 71.12 (C-3'), 74.38 (C-2'), 86.41 (C-4'), 87.84 (C-1'), 119.64 (C-5), 123.328 (C-5''), 140.97 (C-8), 144.41, 149.59, 149.79 (C-2, C4 and C-4"), 156.05 (C-6).

**HRMS** (ESI-MS) $C_{16}H_{24}N_9O_4$ $[M+H]^+$: 406.1944 found, 406.1951 calculated.



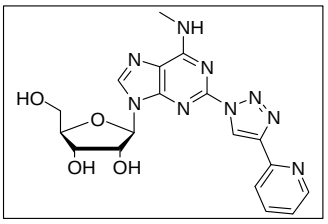
***N*$^6$-Methyl-2-(4-phenyl-1,2,3-triazol-1-yl)-9-(β-D-ribofuranosyl)adenine (3.6).**

The reaction of **3.24** (70 mg, 0.217 mmol) with phenylacetylene (48 µL, 0.435 mmol) yielded compound **3.6** (54%) as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.11 (d, 3H, *J* = 4.5 Hz, *N*$^6$-$CH_3$), 3.55- 3.63 (m, 1H, H-5'A), 3.67-3.74 (m, 1H, H-5'B), 3.97 (dd, *J* = 3.6 and 7.2 Hz, H-4'), 4.21 (dd, *J* = 4.8 and 8.1 Hz, H-3'), 4.66 (app q, *J* = 5.4 Hz, H-2'), 5.03 (app t, 1H, *J* = 5.6 Hz, 5'-OH), 5.29 (d, 1H, *J* = 5.0 Hz, 3'-OH), 5.54 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.99 (d, 1H, *J* = 5.9 Hz, H-1'), 7.4 (t, 1H, *J* = 7.3 Hz, Ph), 7.50 (t, 2H, *J* = 7.5 Hz, Ph), 8.06 (d, 2H, *J* = 7.3 Hz, Ph), 8.44 (d, 1H, *J* = 4.5 Hz, *N*$^6$-H), 8.49 (s, 1H, H-8), 9.31 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.92 ($N^6$-$CH_3$), 62.16 (C-5'), 71.17 (C-3'), 74,39 (C-2'), 86.48 (C-4'), 87.94 (C-1'), 119.85 (C-5), 120.68 (C-5"), 126.27, 128.95, 129.61 and 130.85 (Ph), 141.082 (C-8), 147.02 and 149.77 (C-2, C-4 and C-4"), 156.10 (C-6).

**HRMS** (ESI-MS) $C_{19}H_{21}N_8O_4$ $[M+H]^+$: 425.1689 found, 425.1685 calculated.



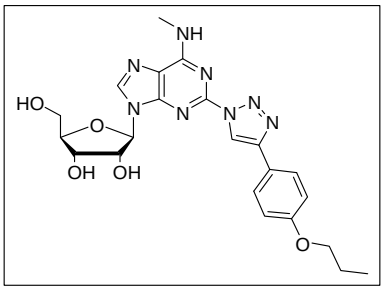
**$N^6$-Methyl-2-[4-pyridin-2-yl-1,2,3-triazol-1-yl]-9-(β-D-ribofuranosyl)adenine (3.7).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 2-ethynylpyridine (44 µL, 0.435 mmol) afforded compound **3.7** as a white solid in 55% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.09 (d, 3H, *J* = 4.1 Hz, $N^6$-$CH_3$), 3.57-3.66 (m, 1H, H-5'A), 3.67-3.76 (m, 1H, H-5'B), 3.97 (dd, 1H, *J* = 3.9 and 7.5 Hz, H-4'), 4.19 (dd, 1H, *J* = 4.8 and 8.1 Hz, H-3'), 4.64 (app q, 1H, *J* = 6.0 Hz, H-2'), 4.99 (t, 1H, *J* = 5.6 Hz, 5'-OH), 5.26 (d, 1H, *J* = 5.0 Hz, 3'-OH), 5.54 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.99 (d, 1H, *J* = 5.9 Hz, H-1'), 7.42 (m, 1H, pyridin-2-yl) , 7.96 (m, 1H, pyridin-2-yl), 8.16 (d, 1H, *J* = 7.3 Hz, pyridin-2-yl), 8.42 (d, 1H, *J* = 4.1 Hz, 1H, $N^6$-H), 8.49 (s, 1H, H-8), 8.68 (d, 1H, *J* = 4.1 Hz, pyridin-2-yl), 9.16 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.92 ($N^6$-$CH_3$), 62.13 (C-5'), 71.14 (C-3'), 74.49 (C-2'), 86.47 (C-4'), 88.16 (C-1'), 119.92 (C-5), 120.53 (C-5''), 121.07 (pyridin-2-yl), 122.25 (pyridin-2-yl), 137.79 (pyridin-2-yl), 141.28 (C-8), 148.47, 150.45 and 150.56 (C-2, C-4, C-4"and pyridin-2-yl), 156.28 (C-6).

**HRMS** (ESI-MS) $C_{18}H_{19}N_9O_4Na$ $[M+Na]^+$: 448.1458 found, 448.1457 calculated.



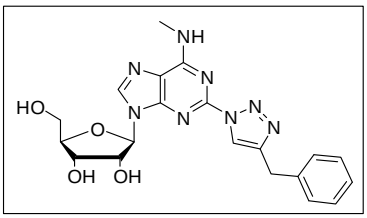
**$N^6$-Methyl-2-[4-(4-propoxyphenyl)-1,2,3-triazol-1-yl]-9-(β-D-ribofuranosyl)adenine (3.8).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 1-ethynyl-4-propoxybenzene (57 µL, 0.435 mmol) afforded compound **3.8** as a white solid in 36% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.99 (t, 3H, *J* = 7.3 Hz, $CH_3$), 1.71-1.78 (m, 2H, $CH_2$), 3.10 (d, 3H, *J* = 4.1 Hz, $N^6$-$CH_3$), 3.54-3.62 (m, 1H, H-5'A), 3.64-3.72 (m, 1H, H-5'B), 3.98 (m, 3H, H-4'and $CH_2$), 4.18 (dd, 1H, *J* = 4.8 and 8.1 Hz, H-3'), 4.64 (app q, 1H, *J* = 5.4 Hz, H-2'), 4.99 (t, 1H, *J* = 5, 7 Hz, 5'-OH), 5.25 (d, 1H, *J* = 4.1 Hz, 3'-OH), 5.50 (d, 1H, *J* = 5.9 Hz, 2'-OH), 5.97 (d, 1H, *J* = 6.2 Hz, H-1'), 7.03 (d, 2H, *J* = 8.8 Hz, Ph), 7.94 (d, 2H, *J* = 8.8 Hz, Ph), 8.36 (d, 1H, *J* = 4.1 Hz, 1H, $N^6$-H), 8.45 (s, 1H, H-8), 9.15 (s, 1H, H-5").

**$^{13}$C NMR** (300 MHz, DMSO-$d_6$): δ 11.08 ($CH_3$), 22.73 ($CH_2$), 27.90 ($N^6$-$CH_3$), 62.16 (C-5'), 69.71 ($OCH_2$), 71.18 (C-3'), 74.38 (C-2'), 86.46 (C-4'), 87.91 (C-1'), 115.50 (Ph), 119.60 (C-5), 123.28 (C-5''), 127.66 (Ph), 140.01 (C-8), 147.01, 149.82, 150.45 (C-4, C-2 and C-4"), 156.10 (C-6).

**HRMS** (ESI-MS) $C_{22}H_{27}N_8O_4$ $[M+H]^+$: 483.2109 found, 483.2104 calculated.



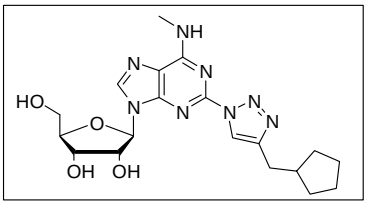

***N*$^6$-Methyl-2-(4-benzyl-1,2,3-triazol-1-yl)-9-(β-D-ribofuranosyl)adenine (3.9).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 3-phenyl-1-propyne (54 µL, 0.435 mmol) gave compound **3.9** as a white solid in 43% yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.03 (d, 3H, *J* = 3.8 Hz, $N^6$-$CH_3$), 3.53-3.62 (m, 1H, H-5'A), 3.64-3.71 (m, 1H, H-5'B), 3.95 (dd, 1H, *J* = 3.7 and 7.2 Hz, H-4'), 4.11 (s, 2H, $CH_2$), 4.17 (dd, 1H, *J* = 4.8 and 8.1 Hz, H-3'), 4.61 (app q, 1H, *J* = 5.7 Hz, H-2'), 4.97 (app t, 1H, *J* = 5.3 Hz, 5'-OH), 5.22 (d, 1H, *J* = 4.7 Hz, 3'-OH), 5.47 (d, 1H, *J* = 5.9 Hz, 2'-OH), 5.94 (d, 1H, *J* = 5.9 Hz, H-1'), 7.23 (m, 1H, Ph), 7.32 (d, 4H, *J* = 4.4 Hz, Ph), 8.34 (d, 1H, *J* = 3.8 Hz, $N^6$-H), 8.45 (s, 1H, H-8), 8.59 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 25.32 ($N^6$-$CH_3$), 32.51 ($CH_2$), 62.11 (C-5'), 71.13 (C-3'), 74.43 (C-2'), 86.41 (C-4'), 87.96 (C-1'), 121.42 (C-5), 121.97 (C-5"), 126.91, 129.12, 129.21 and 140.02 (Ph), 140.88 (C-8), 147.42, 149.58 and 149.81 (C-4, C-2 and C-4"), 156.09 (C-6).

**HRMS** (ESI-MS) $C_{20}H_{23}N_8O_4$ $[M+H]^+$: 439.1846 found, 439.1842 calculated.



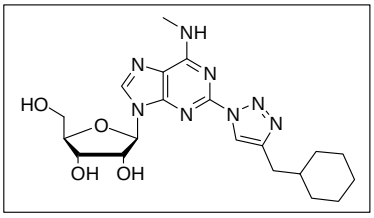
**2-(4-Cyclopentylmethyl-1,2,3-triazol-1-yl)-*N*$^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.10).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 3-cyclopentyl-1-propyne (57 µL, 0.435 mmol) yielded compound **3.10** (32%) as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.23-1.28 (m, 2H, cyclopentyl), 1.48-1.62 (m, 4H, cyclopentyl), 1.71-1.75 (m, 2H, cyclopentyl), 2.19-2.25 (m, 1H, cyclopentyl), 2.72 (d, 2H, *J* = 7.3 Hz, $CH_2$-cyclopentyl), 3.05 (d, 3H, *J* = 3.9 Hz, *N*$^6$-$CH_3$), 3.53-3.61 (m, 1H, H-5'A), 3.63-3.72 (m, 1H, H-5'B), 3.96 (dd, 1H, *J* = 3.6 and 7.2 Hz, H-4'), 4.19 (dd, 1H, *J* = 4.8 and 8.1 Hz, H-3'), 4.62 (app q, 1H, *J* = 5.9 Hz, H-2'), 4.98 (t, 1H, *J* = 5.3 Hz, 5'-OH), 5.23 (d, 1H, *J* = 4.7 Hz, 3'-OH), 5.49 (d, 1H, *J* = 6.5 Hz, 2'-OH), 5.95 (d, 1H, *J* = 5.9 Hz, H-1'), 8.34 (d, 1H, *J* = 3.9 Hz, *N*$^6$-H), 8.45 (s, 1H, H-8), 8.55 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 25.37 (cyclopentyl), 27.83 (*N*$^6$-$CH_3$), 31.56 ($CH_2$), 32.55 (cyclopentyl), 62.15 (C-5'), 71.15 (C-3'), 74.34 (C-2'), 86.43 (C-4'), 87.87 (C-1'), 119.60 (C-5), 121.58 (C-5"), 140.92 (C-8), 147.33, 149.89 (C-4, C-2 and C4"), 156.07 (C-6).

**HRMS** (ESI-MS) $C_{19}H_{27}N_8O_4$ $[M+H]^+$: 431.2153 found, 431.2155 calculated.



**2-(4-Cyclohexylmethyl-1,2,3-triazol-1-yl)-*N*$^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.11).**

The reaction of **3.24** (70 mg, 0.217 mmol) with 3-cyclohexyl-1-propyne (63 µL, 0.435 mmol) gave compound **3.11** in 82 % yield.

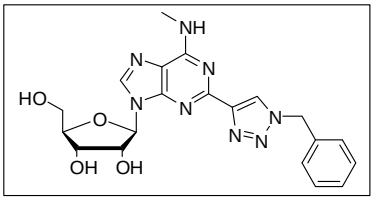
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.86-1.28 (br m, 6H, cyclohexyl), 1.54-1.72 ( br m, 5H, cylcohexyl), 2.58 (d, 2H, *J* = 6.9 Hz, $CH_2$), 3.03 (d, 3H, *J* = 3.9 Hz, *N*$^6$-$CH_3$), 3.51-3.59 (m, 1H, H-5'A), 3.63-3.70 (m, 1H, H-5'B), 3.94 (dd, 1H, *J* = 4.2 and 7.5 Hz, H-4'), 4.16 (dd, 1H, *J* = 4.8 and 8.1 Hz, H-3'), 4.59 (app q, 1H, *J* = 6.3 Hz, H-2'), 4.97 (t, 1H, *J* = 5.5 Hz, 5'-OH), 5.22 (d, 1H, *J* = 4.8 Hz, 3'-OH), 5.47 (d, 1H, *J* = 6.3 Hz, 2'-

OH), 5.93 (d, 1H, *J* = 6.0 Hz, H-1'), 8.30 (d, 1H, *J* = 3.9 Hz, $N^6$-H), 8.43 (s, 1H, H-8), 8.51 (s, 1H, H-5").

**$^{13}$C NMR** (300 MHz, DMSO-$d_6$): δ 26.31 (cyclohexyl), 26.66 (cyclohexyl), 27.84 ($N^6$-$CH_3$), 33.16 (cyclohexyl), 38.22 ($CH_2$), 62.14 (C-5'), 71.15 (C-3'), 74.35 (C-2'), 86.44 (C-4'), 87.86 (C-1'), 119.60 (C-5), 121.93 (C-5"), 140.92 (C-8), 146.45, 149.92 (C-4, C-2 and C-4"), 153.51 (C-6).

**HRMS** (ESI-MS) $C_{20}H_{29}N_8O_4$ $[M+H]^+$: 445.2305 found, 445.2311 calculated.

**General procedure for the synthesis of 4"-substituted 2-(1,2,3-triazol-4-yl)adenosine derivatives 3.12-3.14.** Compound **3.26** (100 mg, 0.32 mmol), sodium ascorbate (13 mg, 0.06 mmol mmol) and $CuSO_4.5H_2O$ (3 mg, 0. 013 mmol) were suspended in 30 mL $H_2O$:*t*-BuOH (3:1). The appropriate azide[‡] (2 equiv.) was subsequently added and the mixture was stirred overnight at room temperature. The 2-triazol-4-yl compounds (generally) precipitated from this reaction medium and were isolated by filtration with water.




**2-(1-Benzyl-1,2,3-triazol-4-yl)-$N^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.12).**

The reaction of **3.26** (100 mg, 0.32 mmol) with 85 mg (0.64 mmol) benzylazide gave compound **3.12** in 78 % yield (110 mg).

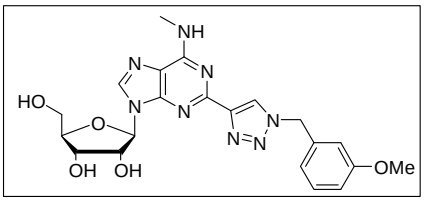
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.03 (br s, 3H, $N^6$-$CH_3$), 3.52-3.58 (m, 1H, H-5'A), 3.60-3.66 (m, 1H, H-5'B), 3.92 (app d, H-4', *J* = 2.9 Hz, H-4'), 4.15 (dd, 1H, *J* = 4.7 and 7.6 Hz, H-3'), 4.60 (app q, 1H, *J* = 5.9 Hz, H-2'), 5.08 (t, 1H, *J* = 5.4 Hz, 5'-OH), 5.19 (d, 1H, *J* = 3.5 Hz, 3'-OH), 5.44 (d, 1H, *J* = 5.6 Hz, 2'-OH), 5.68 (s, 2H, $CH_2$), 5.97 (d, 6.2 Hz, H-1'), 7.38 (br s, 5H, Ph), 7.82 (br s, 1H, $N^6$-H), 8.36 (s, 1H, H-8), 8.66 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.54 ($N^6$-$CH_3$), 53.62 ($CH_2$), 62.29 (C-5'), 71.30 (C-3'), 74.30 (C-2'), 86.41 (C-4'), 87.77 (C-1'), 119.52 (C-5), 126.41 (C-5"), 128.63,

[‡] The benzylic azides were prepared by treating the corresponding benzylic bromides with $NaN_3$ in DMF.

128.86, 129.48 and 136.71 (Ph), 140.27 (C-8), 148.22, 153.56, 153.72 (C-2, C-4 and C-4'), 155.69 (C-6).

**HRMS** (ESI-MS) $C_{20}H_{23}N_8O_4$ $[M+H]^+$: 439.1834 found, 439.1842 calculated.



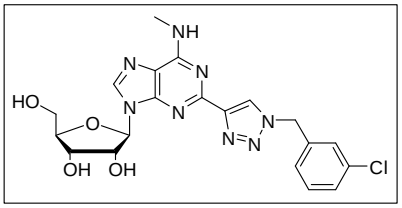

**2-[1-(3-Methoxybenzyl)-1,2,3-triazol-4-yl)-*N*$^6$-methyl-9-(β-D-ribofuranosyl) adenine (3.13).**

The reaction of **3.26** (100 mg, 0.32 mmol) with 104 mg (0.64 mmol) 3-methoxybenzylazide gave compound **3.13** in 80 % yield (120 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.03 (br s, 3H, $N^6$-$CH_3$), 3.52-3.60 (m, 1H, H-5'A), 3.64-3.72 (m, 1H, H-5'B), 3.95 (app d, H-4', *J* = 2.9 Hz, H-4'), 4.15 (dd, 1H, *J* = 4.7 and 7.6 Hz, H-3'), 4.60 (app q, 1H, *J* = 6.4 Hz, H-2'), 5.07 (t, 1H, *J* = 5.1 Hz, 5'-OH), 5.21 (d, 1H, *J* = 4.2 Hz, 3'-OH), 5.46 (d, 1H, *J* = 6.3 Hz, 2'-OH), 5.62 (s, 2H, $CH_2$), 5.95 (d, 1H, *J* = 6.6 Hz, H-1'), 6.91 (m, 3H, Ph), 7.29 (t, 1H, *J* = 7.9 Hz, Ph), 7.79 (br s, 1H, $N^6$-H), 8.34 (s, 1H, H-8), 8.63 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.59 ($N^6$-$CH_3$), 53.45 ($CH_2$), 55.82 ($OCH_3$), 62.30 (C-5'), 71.33 (C-3'), 74.25 (C-2'), 86.42 (C-4'), 87.70 (C-1'), 114.44 and 114.20 (Ph), 119.51 (C-5), 120.72 (Ph), 130.65 (C-5"), 138.21 (C-8), 148.22, 153.56, 153.64 (C-2, C-4 and C-4'), 155.69 (C-6), 160.14 (Ph).

**HRMS** (ESI-MS) $C_{21}H_{25}N_8O_5$ $[M+H]^+$: 469.1938 found, 469.1947 calculated.




**2-[1-(3-Chlorobenzyl)-1,2,3-triazol-4-yl)-*N*$^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.14).**

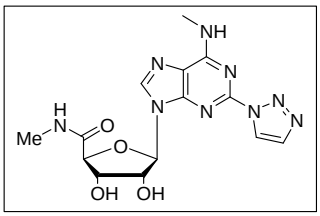
The reaction of **3.26** (100 mg, 0.32 mmol) with 107 mg (0.64 mmol) 3-chlorobenzylazide gave compound **3.14** in 73 % yield (110 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.04 (br s, 3H, $N^6$-$CH_3$), 3.53-3.60 (m, 1H, H-5'A), 3.64-3.71 (m, 1H, H-5'B), 3.96 (app d, H-4', *J* = 2.9 Hz, H-4'), 4.17 (dd, 1H, *J* = 4.7 and 7.6 Hz, H-3'), 4.64 (app q, 1H, *J* = 5.8 Hz, H-2'), 5.09 (t, 1H, *J* = 5.3 Hz, 5'-OH), 5.20 (d, 1H, *J* = 4.4 Hz, 3'-OH), 5.45 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.97 (d, 1H, *J* = 6.2

Hz, H-1'), 7.31-7.36 (m, 1H, Ph), 4.41-7.47 (m, 3H, Ph), 7.83 (br s, 1H, $N^6$-H), 8.37 (s, 1H, H-8), 8.72 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.54 ($N^6$-$CH_3$), 52.80 ($CH_2$), 62.31 (C-5'), 71.33 (C-3'), 74.27 (C-2'), 86.43 (C-4'), 87.69 (C-1'), 119.56 (C-5), 126.63 (C-5"), 127.53, 128.53, 128.84, 131.42, 133.98 and 139.15 (Ph), 140.36 (C-8), 148.22, 150.03 and 153.43 (C-2, C-4 and C-4'), 155.78 (C-6).

**HRMS** (ESI-MS) $C_{20}H_{22}N_8O_4Cl$ $[M+H]^+$: 473.1452 found, 473.1452 calculated.



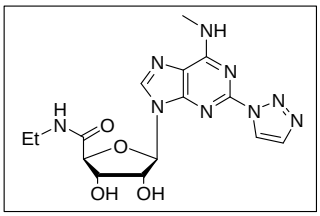

**9-(5-Methylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-(1,2,3-triazol-1-yl)adenine (3.15a).**

In a pressure tube was added **3.32a** (50 mg, 0.14 mmol) trimethylsilylacetylene (102 µL, 0.72 mmol) and 2.5 mL DMF. The mixture was stirred at 110 °C for 15h. Solvent evaporation yielded a yellowish solid that was dissolved 750 µL THF and 172 µL (0.17 mmol) of a 1.0 M solution of tetrabutylammonium fluoride in THF and stirred for 5 h. After solvent evaporation, the residue was dissolved in ethyl acetate. Water was added and the triazole precipitated in the water layer. After overnight cooling and centrifugation, the precipitate was further purified on a silica gel column ($CH_2Cl_2$:MeOH 93:7) and yielded compound **3.15a** as a white solid (23 mg, 42 %).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.60 (d, 3H, *J* = 4.7 Hz, CONHC*H*$_3$), 3.06 (d, 3H, *J* = 3.9 Hz, $N^6$-$CH_3$), 4.26 (m, 1H, H-3'), 4.34 (d, 1H, *J* = 1.8 Hz, H-4'), 4.75 (app q, 1H, *J* = 6.3 Hz, H-2'), 5.60 (d, 1H, *J* = 6.3 Hz, 2'-OH), 5.72 (d, 1H, *J* = 4.2 Hz, 3'-OH), 5.03 (d, 1H, *J* = 7.2 Hz, H-1'), 7.94 (s, 1H, H-4''), 8.04 (d, 1H, *J* = 4.7 Hz, CON*H*), 8.45 (d, 1H, *J* = 4.2 Hz, $N^6$-H), 8.55 (s, 1H, H-8), 8.83 (s, 1H, H-5'').

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 26.34 (CONH*CH*$_3$), 27.86 ($N^6$-$CH_3$), 73.32 (C-3'), 73.58 (C-2'), 85.03 (C-4'), 88.02 (C-1'), 119.99 (C-5), 124.75 (C-5"), 134.34 (C-4"), 141.78 (C-8), 149.60 and 149.86 (C-2 and C-4), 156.10 (C-6), 170,42 (*C*ONH).

**HRMS** (ESI-MS) $C_{14}H_{17}N_9O_4Na$ $[M+H]^+$: 398.12941 found, 398.13013 calculated.



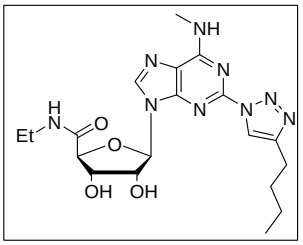
**9-(5-Ethylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-(1,2,3-triazol-1-yl) adenine (3.15b).**

The reaction of compound **3.32b** (110 mg, 0.30 mmol) with trimethylsilylacetylene (259 µL, 1.81 mmol) and subsequently treatment with 360 µL (0.36 mmol) 1.0 M tetrabutylammoniumfluoride was performed as described for the synthesis of compound **3.15a**. Compound **3.16b** was obtained as a white solid in 42% yield (65 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.90 (t, 3H, $CH_3$), 3.05-3.21 (m, 2H, *N*-$CH_2$), 3.05 (d, 3H, *J* = 4.2 Hz, $N^6$-$CH_3$), 4.26 (m, 1H, H-3'), 4.33 (d, 1H, *J* = 2.1 Hz, H-4'), 4.74 (app q, 1H, *J* = 6.5 Hz, H-2'), 5.61 (d, 1H, *J* = 6.2 Hz, 3'-OH), 5.71 (d, 1H, *J* = 4.7 Hz, 2'-OH), 6.04 ( d, 1H, *J* = 7.2 Hz, H-1'), 7.91 (d, 1H, *J* = 1.2 Hz, H-4"), 8.07 (t, 1H, *J* = 5.3 Hz, N*H*CO), 8.41 (d, 1H, *J* = 4.2 Hz, $N^6$-H), 8.54 (s, 1H, H-8), 8.82 (br s, 1H, H-5").
**$^{13}$CMR** (75 MHz, DMSO-$d_6$): δ 15.16 ($CH_3$), 27.85 ($N^6$-$CH_3$), 34.07 ($CH_2$), 73.64 (C-2' and C-3'), 84.98 (C-4'), 88.02 (C-1'), 119.91 (C-5), 124.70 (C-5"), 134.29 (C-4"), 141.59 (C-8), 149.69 and 149.90 (C-2 and C-4), 156.14 (C-6), 169.73 (C=O).
**HRMS** (ESI-MS) $C_{15}H_{20}N_9O_4$ $[M+H]^+$: 390.1676 found, 390.1683 calculated.

**General procedure for the synthesis of 4"-substituted 2-(1,2,3-triazol-1-yl) adenosine derivatives 3.16, 3.17a,b-3.19a,b.**

To a mixture of **3.32a (31b)** (100 mg, 0.28 mmol), CuI (5 mg, 0.03 mmol) and triethylamine (40 µL, 0.28 mmol) in water:acetonitrile (1:1), the appropriate alkyne (2 equiv.) was added. The mixture was stirred for 5 d at room temperature. The reaction was monitored by $^1$H-NMR. The product was precipitated with water and cooled overnight. After filtration, the yellowish solid was purified on a silica gel column ($CH_2Cl_2$: MeOH 90:10) to obtain the 1,2,3-triazol-1-yl adenosine derivative as a white solid.



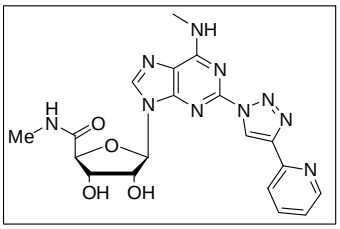
**2-(4-Butyl-1,2,3-triazol-1-yl)-9-(5-ethylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyladenine (3.16).**

The reaction of compound **3.32b** (100 mg, 0.28 mmol) with 1-hexyne (64 µL, 0.56 mmol) gave 65 mg (53%) of compound **3.16** as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$) δ 0.88-0.95 (m, 6H, 2 x $CH_3$), 1.31-1.43 (m, 2H, $CH_2$), 1.61-1.71 (m, 2H, $CH_2$), 2.72 (t, 2H, *J* = 7.8 Hz, C4''-$CH_2$), 3.05-3.22 (m, 2H, *N*-$CH_2$), 3.05 (d, 3H, *J* = 4.1 Hz, $N^6$-$CH_3$), 4.25 (m, 1H, H-3'), 4.33 (d, 1H, *J* = 2.1 Hz, H-4'), 4.73 (m, 1H, H-2'), 5.59 (d, 1H, *J* = 6.3 Hz, 3'-OH), 5.69 (d, 1H, *J* = 4.5 Hz, 2'-OH), 6.04 (d, 1H, *J* = 6.9 Hz, H-1'), 8.09 (t, 1H, *J* = 5.4 Hz), 8.37 (d, 1H, *J* = 4.1 Hz, $N^6$-H), 8.53 (s, 1H, H-8), 8.56 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): 14.36 ($CH_3$), 15.17 ($CH_3$), 22.35 ($CH_2$), 25.21 ($CH_2$), 27.84 ($N^6$-$CH_3$), 31.70 ($CH_2$), 34.07 ($CH_2$), 73.60 and 73.64 (C-2' and C-3'), 84.99 (C-4"), 87.91 ( C-1'), 119.79 (C-5), 121.45 (C-5"), 141.49 (C-8), 147.97, 149, 72 and 149.93 (C-2, C-4 and C-4"), 156.11 (C-6), 169.73 (C=O).

**HRMS** (ESI-MS) $C_{19}H_{28}N_9O_4$ $[M+H]^+$: 446.2256 found, 446.2264 calculated.



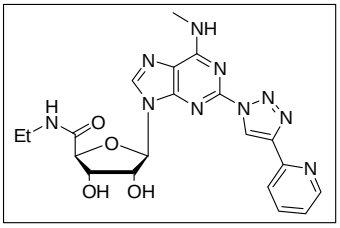
**9-(5-Methylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyl-2-(4-pyridin-2-yl-1,2,3-triazol-1-yl)adenine (3.17a).**

The reaction of compound **3.32a** (70 mg, 0.20 mmol) with 2-ethynyl-pyridine (41 µL, 0.40 mmol) gave 48 mg (53 %) of compound **3.17a** as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.64 (d, 3H, *J* = 4.4 Hz, CONHC*H*$_3$), 3.10 (d, 3H, *J* = 4.4 Hz, $N^6$-$CH_3$), 4.26 (m, 1H, H-3'), 4.36 (d, 1H, *J* = 1.8 Hz, H-4'), 4.77 (app q, 1H, *J* = 6.6 Hz, H-2'), 5.63 (d, 1H, *J* = 6.5 Hz, 2'-OH), 5.74 (d, 1H, *J* = 4.7 Hz, 3'-OH), 6.07 (d, 1H, *J* = 7.0 Hz, H-1'), 7.42 (t, 1H, *J* = 5.3 Hz, pyridin-2-yl), 7.96 (t, 1H, *J* = 7.6 Hz, pyridin-2-yl), 8.06 (d, 1H, *J* = 4.4 Hz, CON*H*), 8.16 (d, 1H, *J*= 7.6 Hz , pyridin-2-yl), 8.49 (d, 1H, *J* = 4.4 Hz, $N^6$-H), 8.58 (s, 1H, H-8), 8.67 (d, 1H, *J*= 4.4 Hz, pyridin-2-yl), 9.17 (s, 1H, H-5'').

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 26.38 ($CH_3$), 27.95 ($N^6$-$CH_3$), 73.36 and 73.63 (C-2' en C-3'), 85.04 (C-4'), 88.04 (C-1'), 120.68 and 120.18 (C-5 en C-5''), 122.22 and 124.16 (pyridin-2-yl), 138.07 (pyridin-2-yl), 141.92 (C-8), 147.96, 149.69 and 150.53 (C-2, C-4, C-4" and pyridyn-2-yl), 156.13 (C-6), 170.45 (C=O).

**HRMS** (ESI-MS) $C_{19}H_{20}N_{10}O_4Na$ $[M+Na]^+$: 475.15592 found, 475.15668 calculated.



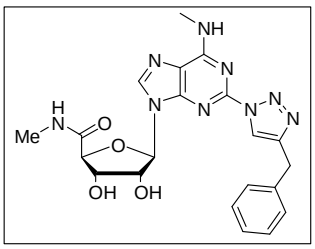
**9-(5-Ethylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyl-2-(4-pyridin-2-yl-1,2,3-triazol-1-yl)adenine (3.17b).**

The reaction of compound **3.32b** (50 mg, 0.14 mmol) with 2-ethynyl-pyridine (56 µL, 0.56 mmol) gave 35 mg (54 %) of compound **3.17b** as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.93 (t, 3H, *J* = 7.2 Hz, $CH_3$), 3.09-3.21 (m, 2H, *N*-$CH_2$), 3.10 (d, 3H, *J* = 4.2 Hz, $N^6$-$CH_3$), 4.25 (m, 1H, H-3'), 4.35 (d, 1H, *J* = 2.1 Hz, H-4'), 4.74 (m, 1H, H-2'), 5.68 (d, 1H, *J* = 6.6 Hz, 2'-OH), 5.75 (d, 1H, J = 4.5 Hz, 3'-OH), 6.08 (d, 1H, *J* = 6.6 Hz, H-1'), 7.41 (m, 1H, pyridin-2-yl), 7.96 (m, 1H, pyridin-2-yl), 8.10 (t, 1H, *J* = 6.0 Hz, N*H*CO), 8.16 (d, 1H, *J* = 8.1 Hz, pyridin-2-yl), 8.48 (d, 1H, *J* = 4.1 Hz, 1H, $N^6$-H), 8.60 (s, 1H, H-8), 8.67 (d, H, *J* = 5.1 Hz, pyridin-2-yl), 9.17 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 15.18 ($CH_3$), 27.94 ($N^6$-$CH_3$), 34.11 (*N*-$CH_2$), 73.69 and 73.78 (C-2' and C-3'), 84.95 (C-4'), 87.98 (C-1'), 120.04 (C-5), 120.65 (C-5"), 122.20 and 124.11 (pyridin-2-yl), 138.027 (pyridin-2-yl), 141.64 (C-8), 147.95, 149,64, 149.67 and 149.98 (C-2, C-4, C-4" and pyridin-2-yl), 150.49 (pyridin-2-yl), 156.13 (C-6), 169.76 (C=O).

**HRMS** (ESI-MS) $C_{20}H_{23}N_{10}O_4$ $[M+H]^+$: 467.1899 found, 467.1903 calculated.



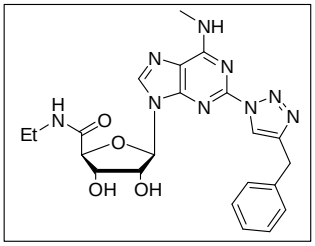
**2-(4-Benzyl-1,2,3-triazol-1-yl)-9-(5-methylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyladenine (3.18a).**

The reaction of compound **3.32a** (70 mg, 0.20 mmol) with 3-phenyl-1-propyne (51 µL, 0.40 mmol) gave 37 mg (40 %) of compound **3.18a** as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.60 (d, 3H, *J* = 4.7 Hz, CONHC*H*$_3$), 3.04 (d, 3H, *J* = 4.3 Hz, *N*$^6$-CH$_3$), 4.11 (s, 2 H, CH-Ph), 4.22 (m, 1H, H-3'), 4.33 (d, 1H, *J* = 1.8 Hz, H-4'), 4.70 (m, 1H, H-2'), 5.59 (d, 1H, *J* = 6.5 Hz, 2'-OH), 5.72 (d, *J* = 4.5 Hz, 1H, 3'-OH) , 6.01 (d, 1H, *J* = 7.0 Hz, H-1'), 7.22 (m, 1H, Ph), 7.32 (d, 4H, *J* = 4.4 Hz, Ph), 8.03 (d, 1H, *J* = 4.4 Hz, CON*H*), 8.41 (d, 1H, *J* = 4.8 Hz, *N*$^6$-H), 8.53 (s, 1H, H-8), 8.60 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-*d*$_6$): δ 26.37 (CH$_3$), 27.85 (*N*$^6$-CH$_3$), 31.71 (CH$_2$), 73.32 and 73.58 (C-2' and C-3'), 85.041 (C-4'), 87.86 (C-1'), 119.88 (C-5), 122.17 (C-5"), 126.95, 129.16, 129.23 and 140.071 (Ph), 141.66 (C-8), 147.09 (C-4), 149.62 and 149.84 (C-2 and C-4"), 156.07 (C-6), 170.40 (C=O).

**HRMS** (ESI-MS) $C_{21}H_{23}N_9O_4Na$ $[M+Na]^+$: 488.17631 found, 488.17708 calculated.



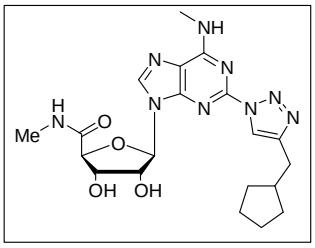

**2-(4-Benzyl-1,2,3-triazol-1-yl)-9-(5-ethylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyladenine (3.18b).**

The reaction of compound **3.32b** (70 mg, 0.19 mmol) with 3-phenyl-1-propyne (49 µL, 0.56 mmol) gave 35 mg (38 %) of compound **3.18b** as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.87 (t, 3H, *J* = 7.5 Hz, CH$_3$), 3.03-3.20 (m, 2H, *N*-CH$_2$), 3.03 (d, 3H, 4.5 Hz, *N*$^6$-CH$_3$), 4.11 (s, 2H, CH$_2$-Ph), 4.24 (m, 1H, H-3'), 4.32 (d, 1H, *J* = 2.1 Hz, H4'), 4.72 (m, 1H, H-2'), 5.59 (d, 1H, *J* = 6.6 Hz, 2'-OH), 5.69 (d, 1H, *J* = 4.5 Hz, 3'-OH), 6.03 (d, 1H, *J* = 6.9 Hz, H1'), 7.22 (m, 1H, Ph), 7.32 (d, 4H, *J* = 4.2 Hz, Ph), 8.06 (t, 1H, *J* = 5.7 Hz, N*H*CO), 8.38 (d, 1H, *J* = 4.1 Hz, *N*$^6$-H), 8.54 (s, 1H, H-8), 8.60 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 15.16 (CH3), 27.84 (*N*$^6$-CH$_3$), 31.72 (CH$_2$), 34.07 (N-CH$_2$), 73.64 (C-2' and C-3'), 84.98 (C-4'), 87.87 (C-1'), 119.91 (C-5), 122.11 (C-5"), 126.93, 129.13, 129.22 and 140.07 (Ph), 140.48 (C-8), 147.05, 149.87 (C-4, C-2 and C-4"), 156.12 (C-6), 169.71 (C=O).

**HRMS** (ESI-MS) $C_{22}H_{26}N_9O_4$ $[M+H]^+$: 480.2098 found, 480.2107 calculated.



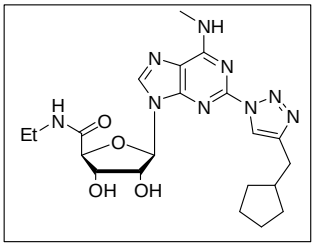
**2-(4-Cyclopentylmethyl-1,2,3-triazol-1-yl)-9-(5-methylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyladenine (3.19a).**

The reaction of compound **3.32a** (60 mg, 0.17 mmol) with 3-cyclopentyl-1-propyn (45 µL, 0.34 mmol) gave 25 mg (32%) of compound **3.16a** as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.23-1.27 (m, 2H, cyclopentyl), 1.49-1.75 (m, 6H, cyclopentyl), 2.20-2.27 (m, 1H, CH, cyclopentyl), 2.62 (d, 3 H, $J$ = 4.7 Hz, $N^6$-$CH_3$), 2.72 (d, 2H, $J$ = 7.3 Hz, $CH_2$), 3.06 (3H, $J$ = 4.4 Hz, CONHC$H_3$), 4.24 (m, 1H, H-3'), 4.34 (d, 1H, $J$ = 2.1 Hz, H-4'), 4.72-4.76 (app q, 1H, $J$ = 6.4 Hz, H-2'), 5.59 (d, 1H, $J$ = 6.4 Hz, 2'-OH), 5.72 (d, 1H, $J$ = 4.4 Hz, 3'-OH), 6.02 (d, 1H, $J$ = 7.0 Hz, H-1'), 8.04 (d, 1H, $J$ = 4.7 Hz, CONH), 8.40 (d, 1H, $J$ = 4.7 Hz, $N^6$-H), 8.53 (s, 1H, H-8), 8.56 (s, 1H, H-5'').

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 25.37 (cyclopentyl), 26.36 ($CH_3$), 27.86 ($N^6$-$CH_3$), 31.55 (cyclopentyl), 32.53 ($CH_2$), 73.27 and 73.58 (C-2' and C-3'), 85.06 (C-4'), 87.93 (C-1'), 119.86 (C-5), 121.71 (C-5''), 141.68 (C-8), 147.49, 149.64 and 149.90 (C-2, C-4 and C-4''), 156.09 (C-6), 170.42 (C=O).

**HRMS** (ESI-MS) $C_{20}H_{27}N_9O_4Na$ $[M+Na]^+$: 480.20778 found, 480.20838 calculated.



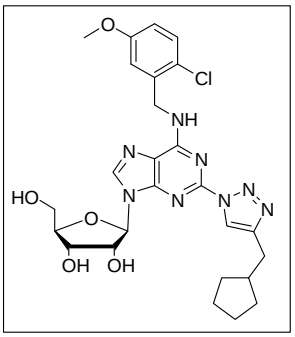
**2-(4-Cyclopentylmethyl-1,2,3-triazol-1-yl)-9-(5-ethylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyladenine (3.19b).**

Compound **3.32b** (60 mg, 0.17 mmol), sodium ascorbate (13 mg, 0.066 mmol) and $CuSO_4.5H_2O$ (3.5 mg, 0.013 mmol) were suspended in 4 mL *t*-BuOH:$H_2O$ (1:1). 3-cyclopentyl-1-propyne (58 µL, 0.44 mmol) was subsequently added and the mixture was stirred two d at room temperature. The 2-triazol-1-yl compound precipitated from the reaction medium. Water was added and the mixture was cooled overnight. The precipitate was filtered off and washed with water and hexane to obtain **3.19b** as a white solid in 33 % yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.89 (t, 3H, $CH_3$), 1.19-1.28 (m, 2H, cyclopentyl), 1.45-1.75 (m, 6H, cyclopentyl), 2.15-2.25 (m, 1H, CH, cyclopentyl), 2.72 (d, 2H, *J* = 7.2 Hz, $CH_2$), 3.06 (d, 3H, *J* = 4.5 Hz, $N^6$-$CH_3$), 3.09-3.22 (m, 2H, *N*-$CH_2$), 4.24 (dt, 1H, *J* = 1.5 and 4.8 Hz, H-3'), 4.33 (d, 1H, *J* = 2.1 Hz, H-4'), 4.71-4.76 (app q, 1H, *J* = 6.6 Hz, H-2'), 5.61 (d, 1H, *J* = 6.3 Hz, 2'-OH), 5.71 (d, 1H, *J* = 4.8 Hz, 3'-OH), 6.03 (d, 1H, *J* = 6.9 Hz, H-1'), 8.10 (t, 1H, *J* = 5.7 Hz, N*H*CO), 8.40 (d, 1H, *J* = 5.1 Hz, $N^6$-H), 8.54 (s, 1H, H-8), 8.56 (s, 1H, H-5").

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 15.17 ($CH_3$), 25.36 (cyclopentyl), 27.85 ($N^6$-$CH_3$), 31.53 (cyclopentyl), 31.51 ($CH_2$), 34.07 ($CH_2$), 73.63 and 73.56 (C-2' and C-3'), 84.99 (C-4'), 87.93 (C-1'), 119.77 (C-5), 121.69 (C-5"), 141.51 (C-8), 147.48 and 149.92 (C-2, C-4 and C-4"), 156.10 (C-6), 169.74 (C=O).

**HRMS** (ESI-MS) $C_{21}H_{30}N_9O_4$ $[M+H]^+$: 472.2415 found, 472.2420 calculated.



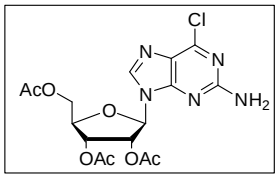

**$N^6$-(5-Chloro-2-methoxybenzyl)-2-(4-cyclopentyl methyl-1,2,3-triazol-1-yl)-9-(β-D-ribofuranosyl)adenine (3.20).**

Compound **3.34** (100 mg, 0.22 mmol), sodium ascorbate (17 mg, 0.086 mmol) and $CuSO_4.5H_2O$ (3.5 mg, 0.017 mmol) were suspended in 4 mL $H_2O$:*t*-BuOH (1:1). 3-Cyclopentyl-1-propyne (29 µL, 0.44 mmol) was subsequently added and the mixture was stirred two d at room temperature. The 2-triazol-1-yl compound precipitated from the reaction medium. Water was added and the mixture was cooled overnight. The precipitate was filtered off and washed with water and hexane to obtain **3.20** as a white solid in 59 % yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.23-1.28 (m, 2H, cyclopentyl), 1.48-1.61 (m, 4H, cyclopentyl), 1.66-1.73 (m, 2H, cyclopentyl), 2.15-2.25 (m, 1H, cyclopentyl), 2.72 (d, 2H, *J* = 7.2 Hz, $CH_2$-cyclopentyl), 3.56-3.61 (m, 1H, H-5'A), 3.66-3.71 (m, 1H, H-5'B), 3.97 (m, 1H, H-4'), 4.20 (m, 1H, H-3'), 4.64 (m, 1H, H-2'), 4.73 (br s, 2H, $N^6$-$CH_2$), 4.96 (t, 1H, *J* = 6.0 Hz, 5'-OH), 5.22 (d, 1H, *J* = 4.8 Hz, 3'-OH), 5.48 (d, 1H, *J* = 5.7 Hz, 2'-OH), 5.95 (d, 1H, *J* = 6.3 Hz, H-1'), 7.04 (d, 1H, *J* = 9.0 Hz, Ph), 7.25-7.29 (m, 2H, Ph), 8.40 (s, 1H, H-8), 8.81 (s, 1H, H-5"), 8.81 (br s, 1H, $N^6$-H);

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 25.36 (cyclopentyl), 31.54 ($CH_2$), 32.54 (cyclopentyl), 38.86 ($CH_2$), 56.57 ($OCH_3$), 62.13 (C-5'), 71.15 (C-3'), 74.37 (C-2'), 86.46 (C-4'), 88.07 (C-1'), 113.15 (Ph), 119.58 (C-5), 121.37 (C-5"), 124.66, 128.23 and 129.93 (Ph), 141.37 (C-8), 147.40, 149.66 and 150.12 (C-2,C-4 and C-4"), 155.51 (Ph), 156.33 (C-6).

**HRMS** (ESI-MS) $C_{26}H_{32}N_8O_5Cl$ $[M+H]^+$: 571.2184 found, 571.2184 calculated.

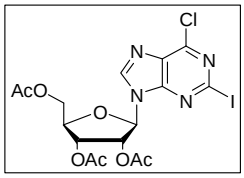



**2-Amino-6-chloro-9-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)purine (3.21)**

Silylation of the base: 2-Amino-6-chloropurine (500 mg, 2.94 mmol) was treated with 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 40 mL) and $(NH_4)_2SO_4$ (0.29 mmol, 39 mg) and refluxed for 20h. The silylated compound was concentrated and used without further purification.

Vorbrüggen coupling: β-D-ribofuranose 1,2,3,5-*O*-tetraacetate (720 mg, 2.26 mmol) in dry 1,2-dichloroethane (25 mL) was added to the silylated 2-amino-6-chloropurine. The solution was gently refluxed, and after 5 minutes TMSOTf (584 µL, 3.0 mmol) was added dropwise. After 4 h the mixture was cooled to room temperature, quenched with a cold saturated $NaHCO_3$ solution (80 mL) and extracted with $CH_2Cl_2$ (40 mL). The organic layer was dried with $MgSO_4$, filtered and evaporated to dryness. The residue was purified by column chromatography ($CH_2Cl_2$:MeOH 99:1) to give 705 mg (56%) of compound **2.17**.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 2.08, 2.10, 2.14 (s, 3H, $CH_3$), 4.3-4.47 (m, 3H, H-4', H-5'A and H-5'B), 5.20 (br s, 2H, 2-$NH_2$), 5.74 (app t, 1H, *J* = 5.0 Hz, H-3'), 5.95 (app t, 1H, *J* = 5.0 Hz, H-2'), 6.01 (d, 1H, *J* = 5.0 Hz, H-1'), 7.87 (s, 1H, H-8). (The $^1$H-NMR is in accordance with the literature report.[247])

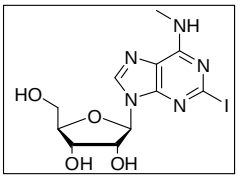



**6-chloro-2-iodo-9-(2',3',5'-*O*-triacetyl-β-D-ribofuranosyl)purine (3.22)**

Isoamylnitrite (1 mL, 7.55 mmol) was added to a mixture of **3.21** (1.05 g, 2.45 mmol), $I_2$ (620 mg, 2.45 mmol), $CH_2I_2$ (2.04 mL,

25.2 mmol) and CuI (490 mg, 2.57 mmol) in 20 mL THF. The mixture was refluxed for 45 minutes and then cooled to room temperature. Insoluble materials were removed by filtration, and the filtrate was concentrated to dryness. The residue was purified by means of a silica gel column, which was washed with $CH_2Cl_2$ until the iodine colour disappeared and then eluted with $CH_2Cl_2$:MeOH (99:1). Compound **3.22** was obtainded in 79% yield.

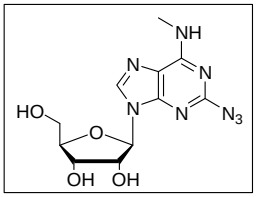
**$^1$H NMR** (300 MHz, $CDCl_3$) : δ 2.10, 2.13, 2.17 (s, 3H, $CH_3$), 4.41 (m, 2H, H-5'A and H-5'B), 4.48 (dd, 1H, *J* = 3.2 and 4.4 Hz, H-4'), 5.58 (dd, 1H, *J* = 5.6 and 4.4 Hz, H-3'), 5.78 (app t, 1H, *J* = 5.6 Hz, H-2'), 6.21 (d, 1H, *J* = 5.3 Hz, H-1'), 8.19 (s, 1H, H-8). (The $^1$H-NMR is in accordance with the literature report.[203])
**HRMS** (ESI-MS) $C_{16}H_{16}N_4O_7I_1Cl_1$ $[M+Na]^+$: 560.9654 found, 560.9652 calculated.



**2-iodo-N$^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.23)**

2.22 (280 mg, 0.52 mmol) was dissolved in 3 mL 2.0 M $CH_3NH_2$ in THF and stirred for 2 days. After solvent evaporation and purification on a silica gel column ($CH_2Cl_2$:MeOH 93:7) compound **3.23** was obtained as a white solid (210 mg, 99% yield).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.88 (d, 3H, *J* = 3.9 Hz, $N^6$-$CH_3$), 3.48-3.55 (m, 1H, H-5'A), 3.59-3.66 (m, 1H, H-5'B), 3.91 (dd, 1H, *J* = 3.8 and 7.3 Hz, H-4'), 4.09 (m, 1H, H-3'), 4.51 (app q, 1H, *J* = 5.7 Hz, H-2'), 5.04 ( t, 1H, *J* = 5.3 Hz, 5'-OH), 5.23 (d, 1H, *J* = 4.7 Hz, 3'OH), 5.47 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.79 (d, 1H, *J* = 6.2 Hz, H-1'), 8.14 (d, 1H, *J* = 3.9 Hz, $N^6$-H), 8.28 (s, 1H, H-8).
**HRMS** (ESI-MS) $C_{11}H_{15}N_5O_4I$: $[M+H]^+$: 408.0173 found; 408.0170 calculated.

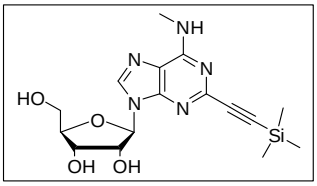


**2-Azido-*N*$^6$-methyl-9-(β-D-ribofuranosyl)adenine (3.24).**

Sodium ascorbate (19.4 mg; 0.098 mmol) and $CuSO_4.5H_2O$ (12.2 mg; 0.049 mmol) were added to a mixture of **3.23** (200 mg; 0.491 mmol), sodium azide (38,3 mg; 0.589 mmol), L-proline (11,3 mg; 0.098 mmol) and sodium carbonate (10.4 mg; 0.098 mmol) in 10 mL $H_2O$:*t*-

BuOH (1:1). The reaction was stirred overnight at 65 ℃ and was monitored by $^1$H-NMR. 50 mL dilute $NH_4OH$ was added and the crude mixture extracted with ethyl acetate (3 x 60 mL). The organic layer was washed with brine (60 mL), dried over $MgSO_4$, and purified on a silica gel column ($CH_2Cl_2$:MeOH, 95:5) to afford compound **3.24** as a slightly yellow solid in 66% yield.

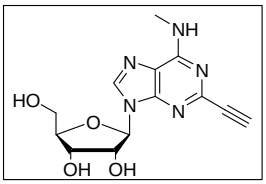
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.91 (d, 3H, *J* = 4.4 Hz, $N^6$-$CH_3$), 3.48-3.55 (m, 1H, H-5'A), 3.58-3.66 (m, 1H, H-5'B), 3.90 (dd, 1H, *J* = 3.8 and 7.3 Hz, H-4'), 4.10 (dd, 1H, *J* = 4.7 and 9.7 Hz, H3'), 4.53 (app q, 1H, *J* = 5.9 Hz, H-2'), 5.04 (dd, 1H, *J* = 5.2 and 6.2 Hz, 5'-OH), 5.19 (d, 1H, *J* = 5.0 Hz, 3'-OH), 5.43 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.78 (d, 1H, *J* = 6.2 Hz, H-1'), 8.12 (d, *J* = 4.4 Hz, $N^6$-H), 8.27 (s, 1H, H-8), small peaks from 1/6 tetrazole tautomeric form: δ 3.15 (d, 3H, *J* = 5.0 Hz, $N^6$-$CH_3$), 3.95 (d, H-4'), 4.15 (d, H-3'), 5.51 (d, 2'-OH), 5.94 (d, H-1'), 8.51(s, H-8).
**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 27.54 ($N^6$-$CH_3$), 62.21 (C-5'), 71.17 (C-3'), 74.12 (C-2'), 86.35 (C-4'), 88.01 (C-1'), 118.07 (C-5), 139.99 (C-8), 156.06 and 156.20 (C-2 and C-6), small peaks from 1/6 tetrazole tautomeric form: δ 31.89 ($N^6$-$CH_3$), 61.79 (C-5'), 70.77 (C-3'), 74.37 (C-4'), 112.30 (C-12), 142.91 (C-11), 147.60 (C-6).
**HRMS** (ESI-MS) $C_{11}H_{21}N_8O_4$ $[M+H]^+$: 323.1208 found, 323.1216 calculated.




**$N^6$-Methyl-9-(β-D-ribofuranosyl)-2-[2-trimethylsilylethyn-1-yl]adenine (3.25).**

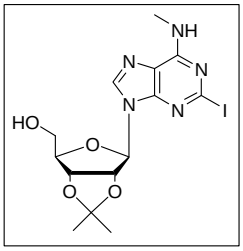
Compound **3.23** (500 mg, 1.23 mmol), CuI (12 mg, 0.062 mmol) and $(Ph_3P)_3PdCl_2$ were dissolved in 9 mL DMF. Triethylamine (205 µL, 1.47 mmol) and trimethylsilylacetylene (210 mg, 1.47 mmol) were added and the reaction mixture was stirred overnight. After solvent evaporation, the residue was dissolved in $CH_2Cl_2$ and filtered through a pad of Celite. Purification on a silica gel column ($CH_2Cl_2$:MeOH 95:5) yielded 305 mg (66%) of compound **3.25**.
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.00 (9H, s, $(CH_3)_3Si$), 2.7, 3H, $N^6$-$CH_3$), 3.26-3.34 (m, 1H, H-5'A), 3.37-3.44 (m, 1H, H-5'B), 3.68 (dd, 1H, *J* = 3.5 Hz, H-4'), 3.86 (dd, 1H, *J* = 3.4 and 8.2 Hz, H-3'), 4.23 (app q, 1H, *J* = 5.9 Hz , H-2'), 4.87 (t, 1H, *J* = 5.0, 5'-OH), 4.94 (d, 1H, *J* = 4.99 Hz, 3'-OH), 5.21 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.63 (d, 1H, *J* = 6.2 Hz, H-1'), 7.69 (br s, 1H, $N^6$-H), 8.19 (s, 1H, H-8).

**HRMS** (ESI-MS) $C_{16}H_{24}N_5O_4Si$: $[M+H]^+$: 378.1586 found; 378.1597 calculated.



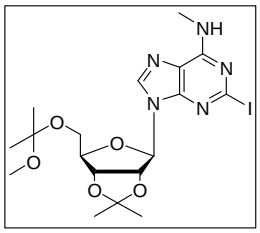
**2-Ethynyl-*N*$^6$-methyl-9-(β-D-ribofuranosyl)purine (3.26).**
300 mg (0.8 mmol) of compound **3.25** was dissolved in 7N ammonia in methanol and stirred for 2 h at 0 ℃. After solvent evaporation, the residue was purified by silica gel chromatography ($CH_2Cl_2$:MeOH 95:5) to obtain 160 mg (65%) of derivative **3.26**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.91(s, 3H, *N*$^6$-$CH_3$), 3.48-3.56 (m, 1H, H-5'A), 3.61-3.68 (m, 1H, H-5'B), 3.92 (m, 1H, H-4'), 4.02 (s, 1H, C≡CH), 4.11 (dd, 1H, *J*= 5.0 and 8.2 Hz, H-3'), 4.53 (app q, 1H, *J*= 5.9 Hz, H-2'), 5.15 (m, 2H, 3'-OH and 5'-OH), 5.44 (d, 1H, *J*= 6.15 Hz, 2'-OH), 5.84 (d, 1H, *J*= 6.16 Hz, H-1'), 7.95 (br s, 1H, *N*$^6$-H), 8.40 (s, 1H, H-8).
**HRMS** (ESI-MS) $C_{13}H_{16}N_5O_4$: [ $M+H]^+$: 306.1197 found; 306.1202 calculated.

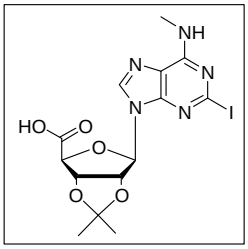


**2',3'-*O*-isopropylidene-2-iodo-*N*$^6$-methyladenosine (3.27)**
**First method**: *p*-Toluene sulfonic acid (213 mg, 1.12 mmol) was added to a solution of compound **3.23** (4.55 g, 11.2 mmol) in dry acetone (100 mL) and 2,2-dimethoxypropane (6.9 mL, 56 mmol) and the reaction was stirred at room temperature for 4h. After neutralization with sat. aq. $NaHCO_3$, the mixture was evaporated to dryness and purified on a silica gel column ($CH_2Cl_2$:MeOH 99:1) to yield 2.2 g (44 %) of compound **3.27**. The low yield was caused by the formation of an important side product (**3.27bis**), that was treated with 80% trifluoroacetic acid to recuperate the starting material **3.23**.
**Second method**: A mixture of compound **3.23**, anhydrous $CuSO_4$ (1.18 g, 7.36 mmol), and 98% sulfuric acid (49 µL, 0.92 mmol) in dry acetone (45mmL), was stirred for 24h, and neutralized with triethylamine. After solvent evaporation, the residue was purified by silica gel chromatography to obtain compound **3.27** in 68% yield (1.12 g).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.33 (s, 3H, $CH_3$), 1.55 (s, 3H, $CH_3$), 2.90 (d, 3H, *J* = 4.2 Hz, $N^6$-$CH_3$), 3.53 (m, 2H, H-5'A and H-5'B), 4.20 (m, 1H, H-4'), 4.90 (dd, 1H, *J* = 3.0 and 5.0 Hz, H-3'), 5.04 (t, 1H, *J* = 5.4 Hz, 5'-OH), 5.25 (m, 1H, H-2'), 6.06 (d, 1H, *J* = 2.7 Hz, H-1'), 8.14 (d, 1H, *J* = 4.5 Hz, $N^6$-H), 8.27 (s, 1H, H-8).
**MS** (m/z) $C_{14}H_{19}IN_5O_5$ $[M+H]^+$: 448.1 found; 448,0 calculated.



**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.30, 1.33, 1.40, 1.61 (s, 3H, $CH_3$), 3.10 (s, 3H, $OCH_3$), 3.16 (d, 3H, *J* = 4.2 Hz, $N^6$-$CH_3$), 3.63 (m, 2H, H-5'A and H-5'B), 4.41 (m, 1H, H-4'), 5.09 (dd, 1H, *J* = 3.0 and 6.0 Hz, H-3'), 5.25 (dd, 1H, J = 2.2 and 6.0 Hz H-2'), 6.09 (d, 1H, *J* = 2.2 Hz, H-1'), 7.80 (s, 1H, H-8), 8.12 (d, 1H, *J* = 4.5 Hz, $N^6$-H). **MS** (m/z) $C_{18}H_{27}IN_5O_5$ $[M+H]^+$: 520.1 found; 520,1 calculated.



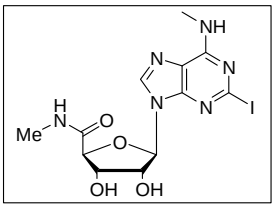
**1-Deoxy-1-(6-methylamino-2-iodo-9*H*-purin-9-yl)-2,3-*O*-isopropylidene-β-D-ribufuranuronic acid (3.28).**

**First method**: To a stirred solution of 3.8 g (8.5 mmol) of **3.27** in 560 mL $H_2O$ were added 1.4 g KOH and, dropwise, a solution of 4.03 g (25.5 mmol) of $KMnO_4$ in 110 mL of $H_2O$. The mixture was stirred in the dark for 20 h, cooled to 0 °C and quenched with 30 mL 7% $H_2O_2$. The mixture was filtered through Celite, the filtrate was concentrated *in vacuo* and then acidified to pH 4 with 3N HCl. The resulting precipitate was filtered off and successively washed with water and ether to give 2.98 g (76%) of **3.27** as a white solid.

**Second method**: A mixture of compound **3.27** (660 mg, 1.5 mmol), 2,2,6,6-tetramethylpiperidinoxy (46 mg, 0.3 mmol) and iodosobenzenediacetate (1.046 g, 3.2 mmol) in 30 ml acetonitrile:water (1:1) was stirred in the dark for 24 h. After solvent evaporation, the residue was washed subsequently with acetone and diethylether. Isolation of the precipitate by centrifugation resulted in compound **3.28** as a white solid in 75% (510 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): $\delta$ 1.36 (s, 3H, $CH_3$), 1.51 (s, 3H, $CH_3$), 2.89 (d, 3H, *J* = 3.3 Hz, $N^6$-$CH_3$), 4.68 (d, 1H, *J* = 1.8 Hz, H-4'), 5.40 (d, 1H, *J* = 6.0 Hz, H-2'), 5.47 (dd, 1H, *J* = 6.0 and 1.8 Hz, H-3'), 6.28 (s, 1H, H-1'), 8.08 (d, 1H, *J* = 3.3 Hz, $N^6$-H), 8.16 (s, 1H, H-8).

**HRMS** (ESI-MS) $C_{14}H_{16}N_5O_5I$: $[M+H]^+$: 462.0273 found; 462.0276 calculated.

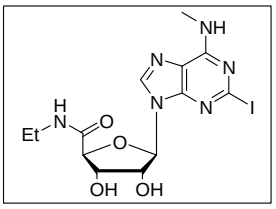



**2-Iodo-9-(5-methylcarbamoyl-β-D-ribofuranosyl)- $N^6$-methyladenine (3.31a).**

*p*-Nitrophenol (332 mg, 2.39 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (420 mg, 2.15 mmol) were added to a solution of **3.28** (920 mg, 1.99 mmol) in 10 mL dry DMF. The reaction mixture was stirred for 3 h at room temperature, cooled to 0 ℃ and 0.7 mL (19.9 mmol) of methylamine was added. The solution turned yellow immediately and was further stirred for 30 min at room temperature. After evaporating the volatiles, the residue was portioned between ethyl acetate (3 x 100 mL) and $H_2O$ (100 mL). The organic layer was washed with brine (100 mL), dried over $MgSO_4$ and concentrated to dryness. The residue was dissolved in 80% aquous TFA (20 mL) and stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*, coevaporated several times with EtOH and purified by silica gel chromatography ($CH_2Cl_2$:MeOH 95:5). Compound **3**.**31a** was obtained as a white solid in 63 % yield (547 mg).

**$^1$H NMR** (300MHz, DMSO- $d_6$): $\delta$ 2.77 (d, 3H, *J* = 4.8 Hz, CONHC$H_3$), 2.91 (d, 3H, *J* = 4.0 Hz, $N^6$-$CH_3$), 4.17 (m, 1H, H-3'), 4.31 (d, 1H, *J* = 1. 8 Hz, H-4'), 4.58 (app q, *J*= 6.7 Hz, 1H, H-2'), 5.57 (d, 1H, *J* = 6.2 Hz, 2'-OH), 5.71 (d, 1H, *J* = 4.4 Hz, 3'-OH), 5.90 (d, 1H, *J* = 7.3, H-1'), 8.05 (d, 3H, *J* = 4.8, CON*H*), 8.19 (d, 3H, *J* = 4.3, $N^6$-H), 8.37 (s, 1H, H-8).

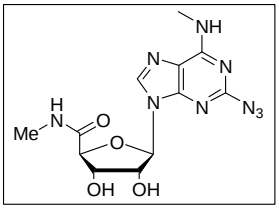
**HRMS** (ESI-MS) $C_{12}H_{16}N_6O_4I_1$ $[M+H]^+$: 435.02705 found, 435.02795 calculated.



**9-(5-Ethylcarbamoyl-β-D-ribofuranosyl)-2-iodo-*N*$^6$-methyladenine (3.31b).**

This compound was prepared by the procedure described for the synthesis of **3.31a** from **3.28** by using $EtNH_2$ instead of $MeNH_2$(1.11 g, 2.41 mmol); yield: 840 mg (78%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.05 (t, 3H, *J* = 7.2 Hz, $CH_3$), 2.91 (d, 3H, *J* = 4.4 Hz, *N*$^6$-$CH_3$), 3.19-3.29 (m, 2H, *N*-$CH_2$), 4.16 (dt, 1H, *J* = 2.1 and 4.4 Hz, H-3'), 4.31 (d, 1H, *J* = 2.1 Hz, H-4'), 4.58 (app q, 1H, *J* = 5.6 Hz, H-2'), 5.59 (d, 1H, *J* = 6.5 Hz, 2'-OH), 5.71 (d, 1H, *J* = 4.7 Hz, 3'-OH), 5.92 (d, 1H, *J* = 7.1 Hz, H-1'), 8.12 (t, 1H, *J* = 5.5 Hz, N*H*CO), 8.19 (d, 1H, *J* = 4.4 Hz, *N*$^6$-H), 8.38 (s, 1H, H-8).
**HRMS** (ESI-MS) $C_{13}H_{18}N_6O_4I$ $[M+H]^+$: 449.0429 found, 449.0436 calculated.



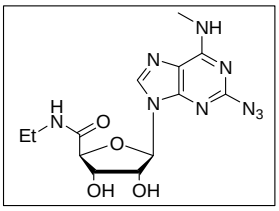
**2-Azido-9-(5-methylcarbamoyl-β-D-ribofuranosyl)-*N*$^6$-methyladenine (3.32a).**

Sodium ascorbate (61 mg, 0.31 mmol) and $CuSO_4.5H_2O$ (39 mg, 0.15 mmol) were added to a mixture of **3.31a** (670 mg, 1.54 mmol), sodium azide (200 mg, 3.08 mmol), L-proline (36 mg, 0.31 mmol) and sodium carbonate (33 mg, 0.31 mmol) in 20 mL $H_2O$:*t*-BuOH (1:1). The reaction was stirred for 2 d at 65 ℃ and monitored by $^1$H-NMR. 100 mL dilute $NH_4OH$ was added and the crude mixture extracted with ethyl acetate (5 x 150 mL) and washed with brine (150 mL). The organic layer was dried over $MgSO_4$ and purified on a silica gel column ($CH_2Cl_2$:MeOH 93:7) to afford compound **3.32a** as a white solid in 68 % yield (361 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.73 (d, 3H, *J*= 4.7 Hz, CONHC*H*$_3$), 2.96 (d, 3H, *J* = 4.6 Hz, *N*$^6$-$CH_3$), 4.14 (m, 1H, H-3'), 4.30 (d, 1H, *J* = 1.5 Hz, H-4'), 4.54 (dd, 1H, *J*= 6.5 Hz, H-2'), 5.54 (d, 1H, *J* = 6.45 Hz, 2'-OH), 5.72 (d, 1H, *J* = 3.52 Hz, 3'-OH), 5.89 (d, 1H, *J* = 7.33 Hz H-1'), 8.24 (d, 1H, *J* = 4.7 Hz, CON*H*), 8.34 (s, 1H, H-8), 8.55 (d, 1H, *J* = 4.8 Hz, *N*$^6$-$CH_3$) small peaks from 1/5 tetrazole tautomeric form: 2.77 (d, 3H, *J* = 4.7 Hz, CONHC*H*$_3$), 4.36 (d, 1H, H-4'), 6.02 (d, 1H, *J* = 7.3 Hz, H-1').

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 22.4 ($CH_3$), 26.74 ($N^6$-$CH_3$), 72.07 and 72.91 (C-2' and C-3'), 84.50 (C-4'), 87.41 (C-1'), 117.65 (C-5), 140.05 (C-8), 156.32 and 156.41 (C-2 and C-4) 169.67 (C-6).

**HRMS** (ESI-MS) $C_{12}H_{16}N_9O_4$ $[M+H]^+$: 350.13189 found, 350.13251 calculated.



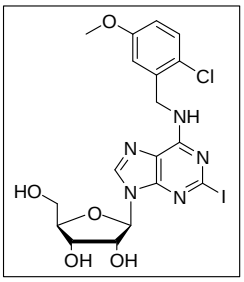
**2-Azido-9-(5-ethylcarbamoyl-β-D-ribofuranosyl)-$N^6$-methyladenine (3.32b).**

This compound was prepared by the procedure described for the synthesis of **3.32a.** From **3.31b** (780 mg, 1.74 mmol), 500 mg (79%) of **3.32b** was obtained.

**$^{1}$H NMR** (300 MHz, DMSO-$d_6$): δ 1.06 (t, 3H, *J* = 7.2 Hz, $CH_3$), 2.96 (d, 3H, *J* = 4.0 Hz, $N^6$-$CH_3$), 3.16-3.29 (m, 2H, *N*-$CH_2$), 4.14 (dt, 1H, *J* = 1.8 and 4.8 Hz, H-3'), 4.29 (d, 1H, *J* = 1.8, H-4'), 4.53-4.59 (app q, 1H, *J* = 6.3 Hz, H-2'), 5.52 (d, 1H, *J* = 6.3 Hz, 2'-OH), 5.68 (d, 1H, *J* = 4.8 Hz, 3'-OH), 5.90 (d, 1H, *J* = 7.2 Hz, H-1'), 8.20 (d, 1H, *J* = 4.0 Hz, $N^6$-H), 8.35 (s, 1H, H-8), 8.49 (t, 1H, *J* = 6.0 Hz, N*H*CO) small peaks from 1/5 tetrazole tautomeric form: 1.08-1.12 (t, 3H, *J* = 7.2 Hz, $CH_3$), 4.34 (d, 1H, *J* = 2.1 Hz, H-4'), 4.67-4.73 (app q, 1H, *J* = 7.2 Hz, H-2'), 5.54 (d, 1H, *J* = 4.5 Hz, 2'-OH) 6.00 (d, 1H, *J* = 7.2 Hz, H-1').

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 15.48 ($CH_3$), 27.52 ($N^6$-$CH_3$), 33.94 (*N*-$CH_2$), 73.06 and 73.75 (C-2' and C-3'), 85.15 (C-4'), 88.09 (C-1'), 118.36 (C-5), 140.69 (C-8), 156.03 and 156.14 (C-2 and C-4) 169.79 (C-6).

**HRMS** (ESI-MS) $C_{13}H_{18}N_9O_4$ $[M+H]^+$: 364.1473 found, 364.1481 calculated.



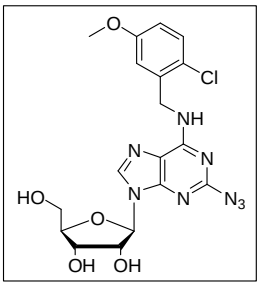
**$N^6$-(5-Chloro-2-methoxybenzyl)-2-iodo-9-(β-D-ribofuranosyl)adenine (3.33)**

Compound **3.22** (1 g, 1.86 mmol) was dissolved in EtOH (30 mL). 5-chloro-2-methoxybenzylammonium chloride (580 mg, 2.79 mmol) and $Et_3N$ (392 µL, 2.79 mmol) were added, and the solution was refluxed overnight. The mixture was concentrated to dryness, dissolved in 7N $NH_3$ in methanol and stirred at room temperature for 2 h to

deprotect the 2'-,3'- and 5'-hydroxyl groups. The volatiles were removed under reduced pressure, and the residue was purified by silica gel column ($CH_2Cl_2$:MeOH, 97:3). The product, compound **3.33,** was realized in 80 % yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.51-3.58 (m, 1H, H-5'A), 3.63-3.68 (m, 1H, H-5'B), 3.85 (s, 3H, *O*$CH_3$), 3.94 (m, 1H, H-4'), 4.13 (m, 1H, H-3'), 4.52-4.59 (m, 3H, $N^6$-$CH_2$ and H-2'), 5.03 (t, 1H, *J* = 5.6 Hz, 5'-OH), 5.21 (d, 1H, *J* = 5.0 Hz, 3'-OH), 5.48 (d, 1H, *J* = 5.8 Hz, 2'-OH), 5.83 (d, 1H, *J* = 6.2 Hz, H-1'), 7.03 (d, 1H, *J* = 8.8 Hz, Ph), 7.16 (d, 1H, *J* = 2.7 Hz, Ph), 7.29 (dd, 1H, *J* = 2.7 and 8.8 Hz, Ph), 8.35 (s, 1H, H-8), 8.62 (br s, 1H, $N^6$-H).

**HRMS** (ESI-MS) $C_{18}H_{20}N_5O_5ICl$ $[M+H]^+$: 548.0204 found, 548.0199 calculated.




**2-Azido-$N^6$-(5-chloro-2-methoxybenzyl)-9-(β-D-ribofuranosyl)adenine (3.34).**

Sodium ascorbate (14 mg, 0.073 mmol) and $CuSO_4.5H_2O$ (9 mg, 0.037 mmol) were added to a mixture of **3.33** (200mg, 0.365 mmol), sodium azide (47 mg, 0.73 mmol), L-proline (8 mg, 0.073 mmol) and sodium carbonate (8mg, 0.073 mmol) in 4 mL $H_2O$:*t*-BuOH (1:1). The reaction was stirred for 2 d at 65 ℃ and monitored by $^1$H-NMR. 10 mL dilute $NH_4OH$ was added and the crude mixture extracted with ethyl acetate (5 x 15 mL) and washed with brine (15 mL). The organic layer was dried over $MgSO_4$ and purified on a silica gel column ($CH_2Cl_2$:MeOH 97:3) to afford compound **3.34** as a white solid in 82 % yield.

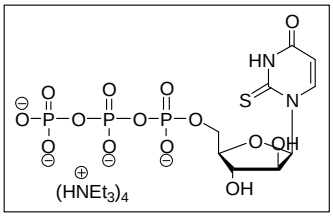
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.51-3.56 (m, 1H, H5'-A) , 3.60-3.66 (m, 1H, H5'-B), 3.93 ( m, 1H, H 4'), 4.13 ( m, 1H, H-3'), 4.56-4.62 (m, 3H, $N^6$-$CH_2$ and H-2'), 5.03 (t, 1H, *J* = 5.4 Hz, 5'-OH), 5.17 (d, 1H, *J* = 4.8 Hz, 3'-OH), 5.41 (d, 1H, *J* = 6.3 Hz, 2'-OH), 5.81 (d, 1H, *J* = 6.0 Hz, H-1'), 7.02 (d, 1H, *J* = 8.7 Hz, Ph), 7.15 (d, 1H, *J* = 3.0 Hz, Ph), 7.28 (dd, 1H, *J* = 2.7 and 8.7 Hz, Ph), 8.34 (s, 1H, H-8), 8.63 (br s, 1H, $N^6$-H).

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 38.59 ($CH_2$), 56.49 ($OCH_3$), 62.19 (C-5'), 71.15 (C-3'), 74.13 (C-2'), 86.36 (C-4'), 88.13 (C-1'), 113.02 (Ph), 119.58 (C-5), 124.29 (Ph),

128.10 (Ph), 129.93 (Ph), 140.43 (C-8), 150.69 (C-4), 155.51 (Ph and C-2), 156.33 (C-6).

**HRMS** (ESI-MS) $C_{18}H_{20}N_8O_5Cl$ $[M+H]^+$: 463.1248 found, 463.1245 calculated.

## 5.1.4 Pyrimidine Nucleotide Analogues




**1-(β-D-Arabinofuranosyl)-2-thio(1*H*)pyrimidin-4-one 5'-triphosphate triethyl ammonium salt (4.1)**

A solution of **4.24** (150 mg, 0.58 mmol) in trimethyl phosphate (5.8 mL) was cooled to 0 °C, $POCl_3$ (342 µL, 3.8 mmol) was added dropwise and the mixture was stirred for 4h at 0 °C and for 30 min at room tempe rature. The mixture was poured into ice-water (10 mL), neutralized with concentrated $NH_4OH$ and evaporated to dryness. The resulting residue was purified by column chromatography (*i*PrOH:$NH_4OH$:$H_2O$ 60:30:5). After lyophilisation of the collected pure fractions, the 5'-monophosphate analogue was obtained as a white solid (124 mg, 60%).

**5'-Monophosphate $^1$H NMR** (300 MHz, $D_2O$): δ 3.90-4.01 (m, 3H, H-4', H-5'A and H-5'B), 4.12 (app t, 1H, *J* = 4.8 Hz, H-3'), 4.48 (app t, 1H, *J* = 4.8 Hz, H-2'), 6.07 (d, 1H, *J* = 8.2 Hz, H-6), 6.78 (d, 1H, *J* = 5.0 Hz, H-1'), 7.97 (d, 1H, *J* = 8.2 Hz, H-5).
**$^{31}$P NMR** (121 MHz, $D_2O$-$d_6$): δ 3.42
**HRMS** (ESI-MS) $C_9H_{12}N_2O_8PSNa$: $[M+Na]^+$: 363.0271 found; 363.0281 calculated.

To a solution of the 5'-monophosphate (32 mg, 0.088 mmol) and tributylamine (21 µL, 0.088 mmol) in DMF (3.2 mL) was added 1,1'-carbonyldiimidazole (71 mg, 0.44 mmol). After stirring for 3h at room temperature, the reaction was quenched by addition of methanol (14 µL). Bis(*tri-n*-butylammonium)pyrophosphate (228 mg, 0.51 mmol) was added and the mixture was stirred for 3h and subsequently concentrated under reduced pressure. The resulting residue was stirred in 1M triethylammonium bicarbonate (TEAB) buffer (6mL, pH = 7.4) for 30 min, lyophilized and purified on a source 15 Q column (100% water → 100% 1 M TEAB/water in 45 min) to yield 10 µmol (11%) of compound **4.1**.

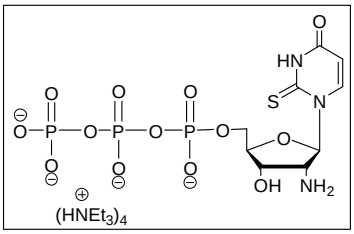
**Compound 4.1. $^1$H NMR** (300 MHz, $D_2O$-$d_6$): δ 1.14 (t, 36H, *J* = 7.2 Hz, 12 x $CH_3$), 3.07 (q, 24 H, *J* = 7.2 Hz, 12 x $CH_2$), 4.03 (m, 1H, H-4'), 4.16 (m, 3H, H-3', H-5'A and H-5'B), 4.48 (app t, 1H, *J* = 5.0 Hz, H-2'), 6.08 (d, 1H, *J* = 8.2 Hz, H-6), 6.80 (d, 1H, *J* = 5.2 Hz, H-1'), 7.90 (d, 1H, *J* = 8.1 Hz, H-5).

**$^{31}$P NMR** (121 MHz, $D_2O$-$d_6$): δ - 22.19 (t, *J* = 19.6 Hz), -10.35 (d, *J* = 19.6 Hz), -9.54 (d, *J* = 19.6 Hz).

**HRMS** (ESI-MS) $C_9H_{14}N_2O_{14}P_3S$ $[M-H]^-$: 498.9099 found; 498.9384 calculated.

**2'-Amino-2'-deoxy-2-thiouridine 5'-triphosphate triethylammonium salt (4.2) and 2'-trifluoroacetylamino-2'-deoxy-2-thiouridine 5'-triphosphate triethylammonium Salt (4.3).**

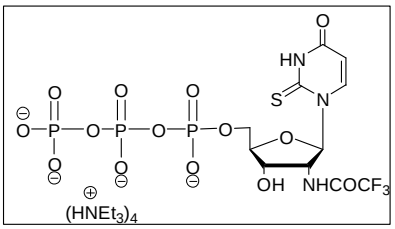
Phosphorus oxychloride (0.004 mL, 0.04 mmol) was added to a solution of **4.46** (7.2 mg, 0.020 mmol) and Proton Sponge (4 mg, 0.033 mmol) in trimethyl phosphate (1 mL) at 0 ℃. The reaction mixture was stirred for 2 h at 0 ℃. Then a mixture of tributylamine (0.02 mL, 0.08 mmol) and tributylammonium pyrophosphate (1.6 mol of $C_{12}H_{27}N$ per mol of $H_4PO_7$, 62 mg, 0.131 mmol) in DMF (0.3 mL) was added at once. After 10 min, 0.2 M triethylammonium bicarbonate solution (2 mL) was added, and the clear solution was stirred at room temperature for 1 h. The latter was lyophilized overnight, and the resulting residue was purified by ion-exchange column chromatography with a Sephadex DEAE A-25 resin with a linear gradient (0.01-0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase to get a mixture of **4.2** and **4.3** as the ammonium salts. The mixture was purified by HPLC (10mM TEAA:$CH_3CN$ from 100:0 to 90:10 in 20 min.) to obtain **4.2** (2.2 mg, 12%) and **4.3** (1.4 mg, 7%) as the triethylammonium salts.




**Compound 4.2.** **$^1$H NMR** ($D_2O$): δ 1.28 (t, *J* = 7.5 Hz, 36H, $N(CH_2CH_3)_3$), 3.20 (q, *J* = 7.5 Hz, 24H, $N(CH_2CH_3)_3$), 4.15 (dd, *J* = 6.9, 5.7 Hz, 1H, H-2'), 4.26 (m, 1H, H-5'A), 4.37 (m, 1H, H-5'B), 4.48 (m, 1H, H-4'), 4.86 (m, 1H, H-3'), 6.31 (d, *J* = 8.1 Hz, 1H, H-6), 7.22 (d, *J* = 7.2 Hz, 1H, H-1'), 8.12 (d, *J* = 8.1 Hz, 1H, H-5).

**$^{31}$P NMR** (121 MHz, $D_2O$): δ -22.64 (t, *J* = 20.2 Hz, -11.22 (d, *J* = 20.2 Hz), -9.63 (d, *J* = 19.6 Hz).

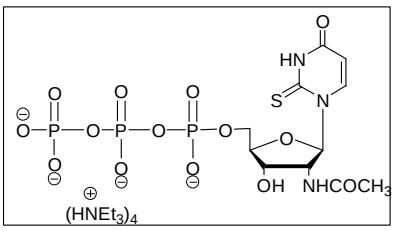
**HRMS** (*m/z)* $C_9H_{15}N_3O_{13}P_3S$ $[M - H]^-$: 497.9529 found; 497.9538 calculated.



**Compound 4.3.** **$^{1}$H NMR** ($D_2O$): δ 1.28 (t,36H, *J* = 7.5 Hz, $N(CH_2CH_3)_3$), 3.20 (q, 24H, *J* = 7.5 Hz, $N(CH_2CH_3)_3$), 4.32 (m, 2H, H-5'A and H-5'B), 4.58 (m, 1H, H-4'), 4.68 (m, 1H, H-2'), 4.80 (hidden by the water peak, 1H, H-3'), 6.26 (d, 1H, *J* = 8.1 Hz, H-6), 6.92 (d,1H, *J* = 5.4 Hz, H-1'), 8.13 (d, 1H, *J* = 8.4 Hz, H-5).

**$^{31}$P NMR** (121 MHz, $D_2O$): δ -22.28 (t, *J* = 19.6 Hz), -11.04 (d, *J* = 19.6 Hz), -8.54 (m).

**HRMS** (*m/z)* $C_{11}H_{14}N_3O_{14}P_3F_3S$ $[M-H]^-$: 593.9364 found; 593.9361 calculated.



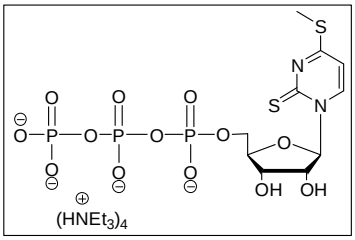
**2'-Acetylamino-2'-deoxy-2-thiouridine 5'-triphosphate triethylammonium salt (4.4)**

Acetic anhydride (0.07 mL, 0.74 mmol) was added to a solution of **4.2** (0.5 mg, 0.55 *μ*mol) in $H_2O$ (0.5 mL) at room temperature. After the reaction mixture was stirred for 6 h, the solvent was removed *in vacuo*. The residue was purified by HPLC (10mM TEAA:$CH_3CN$ from 100:0 to 90:10 in 20 min.) to obtain **4.4** (0.3 mg, 57%) as the triethylammonium salt.

**$^{1}$H NMR** ($D_2O$): δ 1.28 (t, 36H, *J* = 7.5 Hz, $N(CH_2CH_3)_3$), 3.20 (q, 24H, *J* = 7.5 Hz, $N(CH_2CH_3)_3$), 4.29 (m, 2H, H-5'A and H-5'B), 4.36 (m, 1H, H-4'), 4.53 (m, 2H, H-2' and H-3'), 6.27 (d, 1H, *J* = 8.1 Hz, H-6), 6.98 (d, 1H, *J* = 5.6 Hz, H-1'), 8.08 (d, 1H, *J* = 8.1 Hz, H-5).

**$^{31}$P NMR** (121 MHz, $D_2O$): δ -21.31 (t, *J* = 20.5 Hz), -10.84 (d, *J* = 20.8 Hz), -7.62 (m).

**HRMS** (*m/z*) $C_{11}H_{17}N_3O_{14}P_3S$ $[M-H]^-$: 539.9660 found; 539.9644 calculated.



**2-Thio-4-methylthiouridine 5'-triphosphate triethylammonium salt (4.5)**

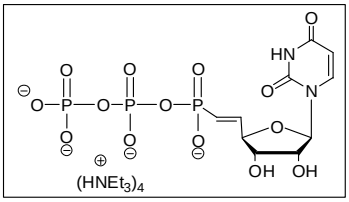
To a solution of **4.45** (15 mg, 0.054mmol) and Proton Sponge (23 mg, 0.11 mmol) in trimethyl phosphate (1 mL) was added phosphorous oxychloride (0.01 mL, 0.11 mmol) at 0 °C. The reaction

mixture was stirred for 2 hours at 0°C. Then a mixture of tributylamine (0.03 mL, 0.12 mmol) and tributylammonium pyrophosphate (1.6 moles $C_{12}H_{27}N$ per mole $H_4PO_7$, 110 mg, 0.23 mmol) in DMF (0.3 mL) was added at once. After 10 min, 0.2 M triethylammonium bicarbonate solution (2 mL) was added, and the clear solution was stirred at room temperature for 1 h. The latter was lyophilized overnight and the resulting residue was purified by ion-exchange column chromatography using a Sephadex-DEAE A-25 resin with a linear gradient (0.01-0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase to get **4.5** as the ammonium salts. The collected portions were purified by HPLC (5 mM TBAP:$CH_3CN$ 80:20 to 40 60 in 20 min) to obtain **4.5** (3.2 mg, 6.4 %) as the triethylammonium salts.

**$^1$H NMR** ($D_2O$) δ 1.28 (t, 36H, *J* = 7.5 Hz, $N(CH_2CH_3)_3$), 2.60 (s, 3H, $SCH_3$), 3.20 (q, 24H, *J* = 7.5 Hz, $N(CH_2CH_3)_3$), 4.38 (m, 3H, H4', H-5'A and H-5'B), 4.45 (m, 2H, H-2' and H-3'), 6.53 (br s, 1H, H-1'), 7.15 (d, 1H, *J* = 7.2 Hz, H-6), 8.51 (d, 1H, *J* = 7.5 Hz, H-5).

**$^{31}$P NMR** (121 MHz, $D_2O$): δ -21.02 (m), -11.00 (t, *J* = 19.2 Hz). -7.62 (m).

**HRMS** (*m/z)* $C_{10}H_{16}N_2O_{13}P_3S_2$ $[M-H]^-$: 528.9315 found; 528.9307 calculated.



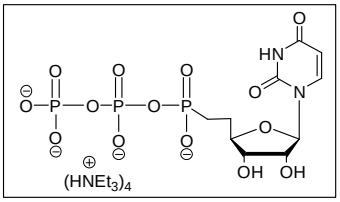

**1-[5,6-Dideoxy-6-[(hydroxypyrophosphoroxy) phosphonyl]-β-D-*ribo*-hex-5-enofuranosyl] uracil triethylammonium salt (4.6)**

To a solution of compound **4.48** (35 mg, 0.109 mmol) and tributylamine (27 µL, 0.109 mmol) in DMF (4mL) was added 1,1'-carbonyldiimidazole (118 mg, 1.1 mmol). After stirring for 3h at room temperature, the reaction was quenched by addition of methanol (40 µL). Bis(*tri-n*-butylammonium)pyrophosphate (282 mg, 0.63 mmol) was added, and the mixture was stirred and subsequently concentrated under reduced pressure. The resulting residue was stirred in 1M triethylammonium bicarbonate (TEAB) buffer (6mL, pH = 7.4) for 8h, lyofilized and purified on a source 15 Q column (1M TEAB:$H_2O$ from 0:100 to 100:0 in 45 min) to yield 24.27 µmol (22%) of compound **4.6**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$) : δ 1.20 (t, 36H, *J* = 7.3 Hz, 12 x $CH_3$), 3.16 (q, 24H, *J* = 7.3 Hz, 12 x $CH_2$), 4.15 (app t, 1H, *J* = 5.5 Hz, H-2'), 4.30 (app t, 1H, J = 4.9 Hz, H-3'), 4.53 (m, 1H, H-4'), 5.84 (d, 1H, *J* = 7.9 Hz, H-6), 5.88 (d, 1H, *J* = 3.5 Hz, H-1'), 6.10-6.23 (dt, 1H, *J* = 1.3 and 17.3 Hz, H-6'), 6.48-6.63 (ddd, 1H, $J_{H5'-H4'}$ = 5.6 Hz, $J_{H5'-H6'}$ 17.3 Hz, $J_{H5'-P}$ = 22.6 Hz, H-5'), 7.66 (d, 1H, J = 8.1 Hz, H-5).

**$^{31}$ P NMR** ($D_2O$) δ -23.3 (t, *J* = 21.5 Hz), -10.59 (d, *J* = 20.2 Hz), 3.68 (d, *J* = 23.0 Hz).

**MS** (m/z) $C_{10}H_{14}N_2O_{14}P_3$ $[M-H]^-$: 479.2 found; 479.0 calculated.



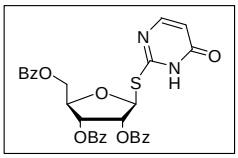

**1-[5,6-Dideoxy-6-[(hydroxypyrophosphoroxy) phosphonyl]-β-D-*ribo*-hexofuranosyl]uracil triethylammonium salt (4.7)**

This compound was synthesized by the procedure described for the synthesis of **4.6**. From compound **4.50** (36 mg, 0.11 mmol), 5'-triphosphate **4.7** was obtained in 5.4 % yield (5.99 µmol).

**$^1$H NMR** (300 MHz, DMSO-$d_6$) : δ 1.14 (t, 36H, *J* = 7.3 Hz, 12 x $CH_3$), 3.16 (q, 24 H, *J* = 7.3 Hz, 12 x $CH_2$), 1.78-1.97 (m, 4H, H-5' A-B and H-6' A-B), 3.98-4.05 (m, 2H, H-3' and H-4'), 4.27 (app t, 1H, *J* = 5.0 Hz, H-2'), 5.81 (d, 1H, *J* = 4.8, H-1'), 5.83 (d, 1H, *J* = 8.0 Hz, H-6), 7.64 (d, 1H, *J* = 8.1 Hz, H-5);

**$^{31}$P NMR** ($D_2O$): δ -23.32 (t, *J* = 20.5 Hz), -20.50 (d, *J* = 20.5 Hz), 18.22 (d, *J* = 26.0 Hz).

**MS** (m/z) $C_{10}H_{16}N_2O_{14}P_3$ $[M-H]^-$: 481.1 found; 481.0 calculated.



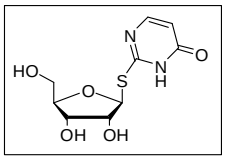

**2-*S*-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranos-1-yl)-2-thio-pyrimidin-4(*3H*)-one (4.8)**

Silylation of the base: 300 mg (2.34 mmol) 2-thiouracil was treated with 1,1,1,3,3,3-hexamethyldisilazane (20 mL) and some $(NH_4)_2SO_4$ crystals were added. After refluxing overnight, the silylated compound was concentrated to dryness and used without further purification.

Vorbrüggen coupling: 1-*O*-acetyl 2,3,5-tri-*O*-benzoyl β-D-Ribofuranose (1.047 g, 2.13 mmol) in 20 mL dry $CH_3CN$ was added to the silylated 2-thiouracil. TMSOTf (461 µL,

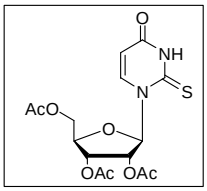
2.55 mmol) was added after 5 min and the solution was stirred overnight at room temperature. The reaction was quenched with cold aquous saturated $NaHCO_3$ and extracted with $CH_2Cl_2$. The organic layer was washed with brine, dried with $MgSO_4$ and purified by column chromatography ($CH_2Cl_2$:MeOH 99:1). Compound **4.8** was obtained in 51 % yield (680 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): $\delta$ 4.52-4.58 (dd, 1H, *J* = 4.1 and 12.3 Hz, H-5'A), 4.63-4.68 (dd, 1H, *J* = 3.8 and 12.3 Hz, H-5'B), 4.79-4.84 (ddd, 1H, *J* = 3.8, 4.1 and 5.3 Hz, H-4'), 5.89 (app t, 1H, *J* = 5.3 Hz, H-3'), 5.99 (app t, 1H, *J* = 4.7 Hz, H-2'), 6.24 (d, 1H, *J* = 5.28 Hz, H-6), 6.42 (d, 1H, *J* = 4.4 Hz, H-1'), 7.43-7.72 (m, 9H, Ph), 7.86-8.05 (m, 7H, Ph and H-5), 12.81 (br s, 1H, N-H).
**HRMS** (ESI-MS) $C_{30}H_{24}N_2O_8S_1$: $[M+H]^+$: 572.1249 found; 572.1253 calculated.




**2-*S*-(β-D-Ribofuranos-1-yl)-2-thio-pyrimidin-4(*3H*)-one (4.9)**
Compound **4.8** (229 mg, 0.4 mmol) was dissolved in 10 mL MeOH:THF (1:1) and 56 mg $K_2CO_3$ (0.4 mmol) was added. The mixture was stirred overnight at 35 ℃ and poured into water (10 mL). Chloroform (10mL) was added to extract the formed methylbenzoate esters. The water layer was treated with activated carbon and concentrated to dryness. The residue was extracted about 10 times with small portions of acetone and the residual salts were discarded. Evaporation to dryness yielded 101 mg (97 %) of compound **4.9**.

**$^1$H NMR** (500 MHz, DMSO-$d_6$): $\delta$ 3.38 (dd, 1H, *J* = 5.7 and 11.6 Hz, H-5'A), 3.48 (dd, 1H, *J* = 3.8 and 11.6 Hz, H-5'B), 3.78 (app q, 1H, *J* = 4.8 Hz, H-4'), 3.87 (app t , *J* = 5.0 Hz, H-3'), 3.97 (app t, *J* = 4.6 Hz, H-2'), 5.66 (d, 1H, *J* = 4.5 Hz, H-1'), 5.55 (d, 1H, *J* = 6.0 Hz, H-6), 7.46 (d, 1H, *J* = 6.0 Hz, H-5).
**$^{13}$C NMR** (125 MHz, DMSO-$d_6$): $\delta$ 62.56 (C-5'), 71.41 (C-3'), 75.82 (C-2'), 84.74 (C4'), 86.47 (C-1'), 108.22 (C-5), 153.20 (C-6), 167.87 (C-S), 172.55 (C=O).
**HRMS**(ESI-MS) $C_9H_{12}N_2O_5SK$: $[M+K]^+$: 299.0066 found; 299,0104 calculated.

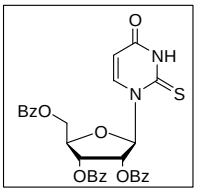



**2',3',5'- Tri-*O*-acetyl-2-thiouridine (4.10)**

A suspension of 2-thiouracil (2 g, 15.6 mmol) and trimethylsilyl chloride (1.8 mL) in hexamethyldisilazane (80 mL) was treated with a few crystals of ammonium sulphate and refluxed overnight. The clear greenish solution was evaporated and a solution of β-D-ribofuranose 1,2,3,5-tetraacetate (5.5 g, 17.3 mmol) in 20 mL dry dichloroethane was added. After a few minutes, stannic chloride (2.4 mL, 20.8 mmol) was added and after 1h, the mixture was poured into a sat. aq. $NaHCO_3$ solution under vigorous stirring and then allowed to stand for 1 h. The suspension was filtered over a silica gel pad, which was washed with $CH_2Cl_2$. The organic layer was separated, dried over $MgSO_4$ and evaporated to dryness. Silica gel chromatography ($CH_2Cl_2$:MeOH 99:1) yielded 4.75 g (79%) of compound **4.10**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.06 (s, 3H, $CH_3$), 2.09 (s, 6H, 2 x $CH_3$), 4.31-4.37 (dd, 1H, *J* = 3.2 and 13.5 Hz, H-5'A), 4.41-4.47 (m, 2H, H-5'B and H-4'), 5.20 (app t, 1H, *J* = 5.7 Hz, H-3'), 5.45 (dd, 1H, *J* = 3.8 and 5.6 Hz, H-2'), 6.62 (dd, 1H, *J* = 2.3 and 7.9 Hz, H-6), 6.68 (d, 1H, *J* = 3.8 Hz, H-1'), 7.49 (d, 1H, *J* = 7.9 Hz, H-5), 10.64 (br s, 1H, N-H).

**HRMS** (ESI-MS) $C_{15}H_{19}N_2O_8S$: $[M+H]^+$: 387.08604 found; 387.08620 calculated.

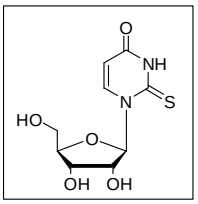



**2',3',5'-Tri-*O*-benzoyl-2-thiouridine (4.11)**

Vörbruggen coupling of silylated 2-thiouracil with 1-*O*-acetyl 2,3,5-tri-*O*-benzoyl β-D-Ribofuranose was performed as described for the synthesis of **4.10**. Nucleoside **4.11** was obtained in 79 % yield.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 4.77 (m, 1H, H-5'A and H-5'B), 4.86 (ddd, 1H, *J* = 4.1, 4.7 and 5.9 Hz, H-4'), 5.84 (dd, *J* = 5.9 and 6.2 Hz, H-3'), 5.90 (dd, 1H, *J* = 4.7 and 6.2 Hz, H-2'), 5.99 (d, 1H, *J* = 7.9 Hz, H-6), 7.22 (d, 1H, *J* = 4.7 Hz, H-1'), 7.43-7.72 (m, 9H, Ph), 7.86-8.04 (m, 7H, Ph and H-5), 12.80 (br s, 1H, N-H).

**HRMS** (ESI-MS) $C_{30}H_{25}N_2O_8S$: $[M+H]^+$: 572.1249 found; 572.1253 calculated.



**2-Thiouridine (4.12)**

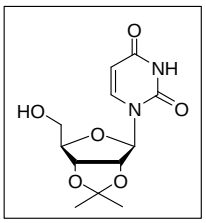
**1st Method**: Compound **4.11** (2.69 g, 4.7 mmol) was dissolved in a mixture of 60 mL dry methanol and 1.3 mL 30% methanolic $NaOCH_3$ solution. After stirring for 15h at room temp, the solution was neutralized by adding DOWEX-50 WX-8 (proton form), previously washed with methanol. The resin was filtered off, methanol was evaporated and 200 mL water was added. The benzoic methyl ester was extracted twice with 100 mL diethylether and the water fraction was lyophilized to obtain 1.1 g of 2-thiouridine (**4.12**, 96%).

**2nd Method**: In a parr apparatus compound **4.17** (430 mg, 1.58 mmol) was dissolved in pyridine (60mL). The solution was cooled to -40 ℃ and saturated with $H_2S$. The reaction mixture was heated at 50 ℃ for 48h resulting in a pressure of 250 psi. After cooling to room temperature, the remaining $H_2S$ was released, the solvent evaporated and the residue was purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5), yielding compound **4.12** (354 mg, 86 %) as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.56-3.62 (m, 1 H, H-5'A), 3.68-3.74 (m, 1H, H-5'B), 3.88-4.07 (m, 3H, H-2', H-3' and H-4'), 5.11 (d, 1H, *J* = 5.6 Hz, 3'-OH), 5.26 (t, 1H, *J* = 5.0 Hz, 5'-OH), 5.45 (d, 1H, *J* = 5.3 Hz, 2'-OH), 5.99 (d, 1H, *J* = 8.2 Hz, H-6), 6.54 (d, 1H, *J* = 3.8 Hz, H-1'), 8.18 (d, 1H, *J* = 8.2 Hz, H-5), 12.64 (s, 1H, N-H);

**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 60.51 (C-5'), 69.61 (C-3'), 75.23 (C-2'), 85.29 (C-4'), 93.26 (C-1'), 107.09 (C-5), 141.67 (C-6), 160.28 (C=O), 177.1 (C=S).

**HRMS** (ESI-MS) $C_9H_{13}N_2O_5S_1$: $[M+H]^+$: 261.05402 found; 261.05451 calculated.

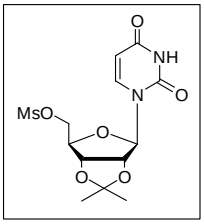


**2',3'-*O*-isopropylidene uridine (4.14)**

*p*-Toluenesulfonic acid (1.6 mmol) was added to a solution of uridine (4 g, 16 mmol) in dry aceton (160 mL) and 2,2-dimethoxypropane (10,6 mL, 80 mmol). The reaction was stirred at room temperature for 4h. After neutralization with sat. aq. $NaHCO_3$, the mixture was evaporated to dryness and purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5) to yield 3.5 g (77 %) of compound **4.14**.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.36 (s, 3H, $CH_3$), 1.57 (s, 3H, $CH_3$), 3.80 (dd, 1H, *J* = 4.5 and 11.9 Hz, H-5'A), 3.91 (dd, 1H, *J* = 3.6 and 11.9 Hz, H-5'B), 4.26-4.30 (m, 1H, H-4'), 4.95 (dd, 1H, *J* = 3.4 and 6.3 Hz, H-3'), 5.02 (dd, 1H, *J* = 2.8 and 6.3 Hz, H-2'), 5.56 (d, 1H, *J* = 8.0 Hz, H-5), 5.72 (d, 1H, *J* = 2.8 Hz, H-1'), 7.36 (d, 1H, *J* = 8.1 Hz, H-6). (The $^1$H NMR spectrum is accordance with the literature report.[248183])

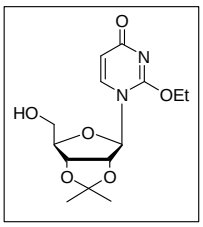
**HRMS** (ESI-MS) $C_{12}H_{17}N_2O_6$: $[M+H]^+$: 285.10881 found; 285,10866 calculated.




**1-(2,3-*O*-Isopropylidene-5-*O*-mesyl-β-D-ribofuranosyl)uracil (4.15)**

To a cooled solution of **4.14** (1 g, 3.52 mmol) in pyridine (12 mL) methanesulfonyl chloride (350 µL, 4.5 mmol) was added. The reaction was kept at 0 ℃ for 2h and then poured in to aquous saturated $NaHCO_3$, extracted with $CH_2Cl_2$ and dried over $MgSO_4$. The pyridine residue was coevaporated with toluene and after silica gel chromatography ($CH_2Cl_2$:MeOH 98:2) compound **4.15** was obtained in 91% yield (1.143 g).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.30 (s, 3H, $CH_3$), 1.50 (s, 3H, $CH_3$), 3.19 (s, 3H, $CH_3$), 4.23-4.28 (m, 1H, H-4'), 4.32-4.38 (dd, 1H, *J* = 6.9 and 10.8 Hz, H5'-A), 4.41-4.47 (dd, 1H, *J* = 4.4 and 10.8 Hz, H-5'B), 4.79-4.83 (dd, 1H, *J* = 4.1 and 6.5 Hz, H-3'), 5.08-5.11 (dd, 1H, *J* = 2.1 and 6.5 Hz, H-2'), 5.65 (d, 1H, *J* = 8.2 Hz, H-6), 5.82 (d, 1H, *J* = 2.1 Hz, H-1'), 7.71 (d, 1H, *J* = 8.2 Hz, H-5), 11.45 (s, 1H, N-H).
**HRMS** (ESI-MS) $C_{13}H_{19}N_2O_8$: $[M+H]^+$: 363.0877 found; 3630862 calculated.

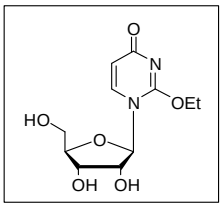



**2',3'-*O*-Isopropylidene-2-ethoxyuridine (4.16)**

Compound **4.15** (200 mg, 0.55 mmol) was suspended in ethanol (previously dried over 4 Å molecular sieves). After DBU (1.2 mmol, 181 µL) was added, the solution was boiled with reflux for 10h, cooled to room temperature and neutralized with saturated $NH_4Cl$ solution. The mixture was concentrated *in vacuo* and the residue was purfied on a silica gel column ($CH_2Cl_2$: MeOH 95:5) to yield 156 mg (91%) of compound **4.16**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.29-134 (6H, s and t, *J* = 7.0 Hz, 2 x $CH_3$), 1.50 (s, 3H, $CH_3$), 3.52-3.63 (m, 2H, H-5'A and H-5'B), 4.13-4.17 (dd, 1H, *J* = 4.1 and 7.6 Hz, H-4'), 4.31-4.38 (q, 2H, *J* = 7.0 Hz, $CH_2$), 4.73 (dd, 1H, *J* = 3.2 and 6.2 Hz, H-3'), 4.91 (dd, 1H, *J* = 2.6 and 6.2, H-2'), 5.15 (t, 5.1 Hz, 5'-OH), 5.81-5.84 (2 x d, *J* = 2.5 and 7.8 Hz, H1' and H-5).

**HRMS** (ESI-MS) $C_{14}H_{21}N_2O_6$: $[M+H]^+$: 313.1368 found; 313.1399 calculated.




**2-Ethoxyuridine (4.17)**

Compound **4.16** (150 mg, 0.48 mmol) was dissolved in 80% trifluoroacetic acid aquous solution. After stirring for 2h at room temperature, the volatiles were removed under reduced pressure. The residue was three times dissolved in ethanol and evaporated to dryness. Silica gel chromatography ($CH_2Cl_2$:MeOH 90:10) yielded 112 mg of compound **4.17** (86%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.31 (t, 3H, *J* = 7.0 Hz, $CH_3$), 3.54-3.59 (dd, 1H, *J* = 2.9 and 12.9 Hz, H-5'A), 3.63-3.68 (dd, 1H, *J* = 2.9 and 12.0 Hz, H-5'B), 3.88 (dd, 1H, *J* = 3.2 and 6.8 Hz, H-4'), 3.97 (app t, *J* = 4.5 Hz, H-3'), 4.06 (app t, 1H, *J* = 5.0 Hz, H-2'), 4.32-4.39 (q, 2H, *J* = 7.0 Hz, $CH_2$), 5.12 (br s, 2H, 3'-OH and 5'-OH), 5.46 (br s, 1H, 2'-OH), 5.72 (d, 1H, *J* = 4.8 Hz, H-1'), 5.84 (d, 1H, *J* = 7.7 Hz, H-6), 7.98 (d, 1H, *J* = 7.7 Hz, H-5).

**HRMS** (ESI-MS) $C_{11}H_{16}N_2O_6Na$: $[M+Na]^+$: 295.0901 found; 295.0906 calculated.

**Attempted synthesis of 2,5'-*O*-anhydrouridine via selective 5'-mesylation or 5'-tosylation of uridine**

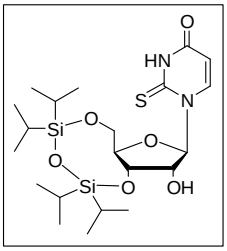
**a. Attempted selective 5'-mesylation of uridine: synthesis of 4.18a-c**

Methanesulfonyl chloride (350 µl, 4.5 mmol) was added dropwise to a cooled (- 30 ℃) solution of uridine (1g, 4.1 mmol) in pyridine (20 mL). After the reaction mixture was warmed to 0 ℃ over 1h, volatiles were removed under reduced pressure and the residue was purified on a silica gel column (95:5 $CH_2Cl_2$: MeOH), yielding 690 mg of an unseparable mixture of 5'- (61%), 3'- (16%) and 2'- (23%) mesylated uridine.

**b. Attempted selective 5'-tosylation of uridine: synthesis of 2'-*O*-*p*-toluene sulfonyl-uridine (4.19)**

*p*-Toluenesulfonyl chloride (476 mg, 2.25 mmol) was added to a cooled (-20 ℃) solution of uridine (500 mg, 2.05 mmol) in pyridine (20 mL) and allowed to warm to 0 ℃. After 4h, the reaction mixture was evaporated to dryness and the residue was dissolved in ethyl acetate. The organic phase was washed with water, dried over $NaSO_4$, filtered, evaporated to dryness and purified by column chromatography (95:5 $CH_2Cl_2$:MeOH). Surprisingly, 2'-*O*-*p*-toluene sulfonyl-uridine (**4.19**, 51% yield) was isolated as the major reaction product.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.38 (s, 3H, $CH_3$), 3.55 (app t, 2H, *J* = 4.5 Hz, H5'A and H-5'B), 3.93 (dd, 1H, *J* = 4.8 and 2.4 Hz, H-4'), 4.14 (dt, *J* = 5.1 and 2.1 Hz, H-3'), 4.84 (dd, 1H, *J* = 5.3 and 7.3 Hz, H-2'), 5.27 (t, 1H, *J* = 4.7 Hz, 5'-OH), 5.46 (d, 1H, *J* = 8.2 Hz, H-6), 5.90 (d, 1H, *J* = 5.6 Hz, 3'-OH), 6.00 (d, 1H, *J* = 7.0 Hz, H-1'), 7.36 (d, 2H, *J* = 7.8 Hz, 2x H-Ph), 7.55 (d, 1H, *J* = 8.4 Hz, H-5), 7.67 (d, 2H, *J* = 8.1 Hz, 2x H-Ph), 11.2 (br s, 1H, N-H).

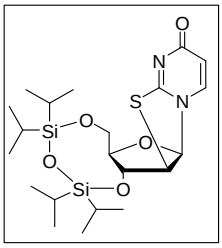



**3',5'-O-TIPDS-2-thiouridine (4.20)**

To a cooled (0 ℃) solution of 2-thiouridine (**4.12**) (500 mg, 1.92 mmol) and imidazole (653 mg, 9.6 mmol) were dissolved in 10 mL DMF, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (676 µL, 2.11 mmol) was added. The solution was stirred for 3h at room temperature and then overnight at 30 ℃. The mixture was poured into water and extracted twice with ethyl acetate. The organic layers were combined, dried with $MgSO_4$, filtered and evaporated to dryness. The residue was purified by column chromatography ($CH_2Cl_2$:MeOH 99:1) to give compound **4.20** in 77% yield (746 mg).

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 0.94-1.12 (m, 28H, 4 x isopropyl), 2.77 (d, 1H, *J* = 1.2 Hz, H-2'), 4.00 (d, 1H, *J* = 13.5 Hz, H-5'A), 4.22-4.29 (m, 4H, H-3', H-4', 2'-OH and H-5'B), 5.97 (d, 1H, *J* = 8.2 Hz, H-6), 6.32 (s, 1H, H-1'), 7.97 (d, 1H, *J* = 8.2 Hz, H-5), 10.25 (br s, 1H, N-H).

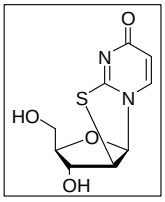
**HRMS** (ESI-MS) $C_{21}H_{38}N_2O_6SSi_2Na$: $[M+Na]^+$: 525.18864 found; 525.18869 calculated.



**1-(2,2'-*S*-anhydro-3,5-TIPDS-β-D-arabinofuranos-1-yl)-2-thiouracil (4.21)**

Compound **4.20** (746 mg, 1.48 mmol) was dissolved in 8 mL pyridine and methanesulfonyl chloride (138 µL, 1.78 mmol) was added. After stirring for 4h at room temperature, the solvent was evaporated. The residue was dissolved in ethyl acetate and the organic layer was washed with water, dried over $MgSO_4$, filtered and concentrated to dryness. The residue was dissolved in $CH_3CN$ (12 mL) and DBU (419 µL, 2.96 mmol) was added. After 3h, the mixture was cooled to room temperature, taken up in ethyl acetate and washed with water. The combined organic layers were dried over $MgSO_4$, filtered and concentrated to dryness. After purification on a silica gel column ($CH_2Cl_2$:MeOH 97:3), 447 mg (62 %) of compound **4.21** was obtained.

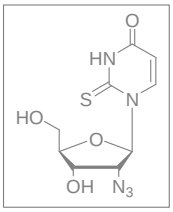
**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.96-1.03 (m, 28H, 4 x isopropyl), 3.90-4.02 (m, 3H, H-4', H-5'A and H-5'B), 4.40-4.50 (m, 2H, H-2' and H-3'), 5.92 (dd, 1H, *J* = 1.2 and 7.6 Hz, H-6), 6.25 (d, 1H, *J* = 6.5 Hz, H-1'), 7.76 (d, 1H, *J* = 7.6 Hz, H-5).
**HRMS** (ESI-MS) $C_{21}H_{36}N_2O_5SSi_2Na$: $[M+Na]^+$: 507.17833 found; 507.17813 calculated.



**1-(2,2'-*S*-anhydro-β-D-arabinofuranosyl)-2-thiouracil (4.22)**

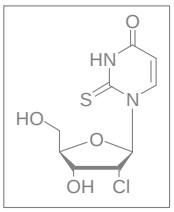
Compound **4.21** (440 mg, 0.91 mmol) was dissolved in 30 mL THF. After adding 2.7 mL of a 1.0 M solution of tetrabutylammonium fluoride in THF, the mixture was stirred for 2h at room temperature, evaporated to dryness and purified on a silica gel column ($CH_2Cl_2$:MeOH 90:10). Compound **4.22** was obtained in 74% yield (162 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.37-3.50 (m, 2H, H-5'A and H-5'B), 3.97 (m, 1H, H-4'), 4.30 (m, 2H, H-2' and H-3'), 4.97 (t, 1H, *J* = 5.3 Hz, 5'-OH), 5.86 (d, 1H, *J* = 7.6 Hz, H-6), 5.90 (br s, 1H, 3'-OH), 6.32 (d, 1H, *J* = 6.5 Hz, H-1'), 7.80 (d, 1H, *J* = 7.6 Hz, H-5).
**HRMS** (ESI-MS) $C_9H_{11}N_2O_4S$: $[M+H]^+$: 243.04322 found; 243.04394 calculated.



**Attempted synthesis of 2'-azido-2'-deoxy-2-thiouridine**

To a suspension of **4.22** (120 mg, 0.24 mmol) and 96 mg $NaN_3$ (1.49 mmol) in DMF (5 mL) was added benzoic acid (3 mg, 0.024 mmol). The mixture was heated overnight at 150 ℃, but no reaction occurred. After longer heating, the starting material degraded.

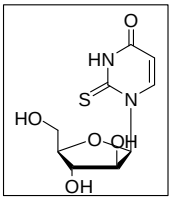


**First attempted synthesis of 2'-chloro-2'-deoxy-2-thiouridine**

A suspension of 200 mg **4.22** in 5 mL 4.0 M HCl in dioxane was heated in a glass cylinder at 75 ℃. After overnigh t reaction, the product appeared to be degraded.

**Second attempted synthesis 2'-chloro-2'-deoxy-2-thiouridine**

**4.22** (120 mg, 0.50 mmol) was dissolved in 2.5 mL dry pyridine. Acetic anhydride (2.62 mmol, 250 µL) was added and the solution was stired overnight. The mixture was poured into water (5 mL) and extracted with $CH_2Cl_2$. The combined organic layers were washed with 1N HCl (2 x 2.5 mL) and water (2 x 2.5 mL) and dried over $MgSO_4$. Evaporation of the solvent gave the diacetate quantitatively (163 mg), which was dissolved in dry acetonitrile. After adition of 0.8 mL acetyl chloride, the solution was refluxed for 5h. Because no reaction was observed, 0.1 equivalent benzoic acid was added, but no reaction occurred after overnight refluxing.



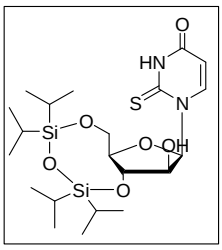
**1-β-D-Arabinofuranosyl)-2-thio(1*H*)pyrimidin-4-one (4.24)**

A solution of compound **4.23** (2 g, 8.8 mmol) in dry DMF (40mL) and triethylamine (6 mL) was transferred to a parr apparatus and saturated with $H_2S$ at -40 ℃ and allowed to warm to room temperature resulting in a pressure of 200 psi. After stirring for two days, the remaining $H_2S$ was released and the solvent evaporated to dryness. The brown residue was purified on a silica gel column ($CH_2Cl_2$:MeOH 96:4), yielding compound **4.24** (1.6 g, 70%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.61 (app t, 2H, *J* = 4.8 Hz, H-5' A en B), 3.83 (ddd, 1H, *J* = 3.0, 2.7 and 5.4 Hz, H-4'), 3.91 (m, 1H, H-3'), 4.18 (m, 1H, H-2'), 5.04 (t, 1H,

*J* = 5.2 Hz, 5'-OH), 5.47 (d, 1H, *J* = 3.9 Hz, 3'-OH), 5.59 (d, 1H, *J* = 5.4 Hz, 2'-OH), 5.93 (d, 1H, *J* = 8.4 Hz, H-6), 6.73 (d, 1H, *J* = 3.9 Hz, H-1'), 7.72 (d, 1H, *J* = 8.4 Hz, H-5), 12.59 (br s, 1H, N-H).

**HRMS** (ESI-MS) for $C_9H_{13}N_2O_5S$: $[M+H]^+$: 261.0540 found; 261.0545 calculated.

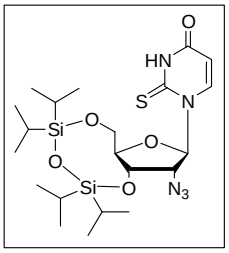


**1-(3,5-*O*-TIPDS-β-D-arabinofuranos-1-yl)-2-thiouracil (4.25)**

To a solution of compound **4.24** (840 mg, 3.0 mmol) and imidazole (1.1 g 16.0 mmol) in dry DMF (20 mL), $TIPDSCl_2$ (1.14 mL; 3.6 mmol) was added at 0 ℃. The mixture was allowed to warm to 30 ℃ over 3h. After overnight stirring, the solution was poured into water, extracted with ethyl acetate, dried over $MgSO_4$, and purified on a silica gel column ($CH_2Cl_2$:MeOH 98:2) to obtain 1.30 g of **4.25** (81 % yield).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.96-1.03 (m, 28 H, 4x isopropyl), 3.74-3.80 (m, 1H, H4'), 3.99 (m, 2H, H-5'A and H-5'B), 4.11 (app t, *J* = 6.1 Hz, H-3'), 4.34-4.40 (app q, 1H, *J* = 6.2 Hz, H-2'), 5.86 (d, 1H, *J* = 5.9 Hz, 2'-OH), 5.91 (d, 1H, *J* = 7.9 Hz, H-5), 6.82 (d, 1H, *J* = 5.9 Hz, H-1'), 7.57 (d, 1H, *J* = 8.1 Hz, H-6), 12.65 (br s, 1H, N-H).

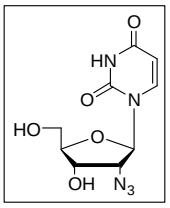
**HRMS** (ESI-MS) for $C_{21}H_{39}N_2O_6SSi_2$: $[M+H]^+$: 503.2064 found; 503.2067 calculated.



**1-(2-Azido-2-deoxy-3, 5-*O*-TIPDS-β-D-ribofuranos-1-yl)-2-thiouracil (4.27)**

To a solution of **4.25** (606 mg, 1.2 mmol) and DMAP (586 mg, 4.8 mmol) in dry dichloromethane (20 mL), trifluoromethanesulfonyl chloride (151.8 µl, 1.4 mmol) was added dropwise at 0 ℃. After 1 h, ice water was added. The mixture was subsequently extracted with $CH_2Cl_2$, dried over $MgSO_4$ and evaporated. The crude residue was dissolved in dry DMF (20 mL) and *N,N,N',N'*-tetramethylguanidinium azide (949 mg, 6 mmol) was added. After overnight reaction and extraction with $CH_2Cl_2$, the organic faction was washed with sat. aq. $NaHCO_3$ solution and purified by means of silica gel chromatography ($CH_2Cl_2$:MeOH 95:5) to obtain compound **4.27** in 10% yield (65 mg).

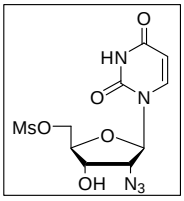
**$^1$H NMR** (300 MHz, $CDCl_3$): δ 0.88-1.05 (m, 28H, 4 x isopropyl), 3.73-3.77 (dd, 1H, *J* = 1.8 and 9.3 Hz, H-2'), 3.91-3.97 (dd, 1H, *J* = 2.7 and 13.5 Hz, H-5'A), 4.06-4.12 (m, 2H, H-4', H-5'B), 4.54-4.64 (m, 1H, H-3'), 5.94 (d, 1H, *J* = 8.1 Hz, H-6), 6.62 (d, 1H, *J* = 6.0 Hz, H-1'), 7.86 (d, 1H, *J* = 8.1 Hz, H-5), 9.35 (br s, 1H, N-H).
**HRMS** (ESI-MS) for $C_{21}H_{38}N_5O_5SSi_2$: $[M+H]^+$: 528.2140 found; 528.2132 calculated.



**2'-Azido-2'-deoxyuridine (4.30)**

To a suspension of 2,2'-anhydrouridine (**4.23**) (1g, 4.42 mmol) and 1.72 g $NaN_3$ (26.5 mmol) in DMF (25 mL) was added benzoic acid (540 mg, 4.42 mmol). The mixture was heated overnight at 150 ℃, cooled to room temperature, diluted with water and evaporated to dryness. Purification on a silica gel column ($CH_2Cl_2$:MeOH 95:5) yielded 617mg (52%) of compound **4.30** as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.53-3.60 (m, 1H, H-5'A), 3.62-3.69 (m, 1H, H-5'B), 3.89 (dd, 1H, *J* = 3.2 and 7.7 Hz, H-4'), 4.05 (app t, 1H, *J* = 5.4 Hz, H-2'), 4.30 (app q, 1H, *J* = 5.1 Hz, H-3'), 5.17 (t, 1H, *J* = 5.0 Hz, 5'-OH), 5.68 (dd, 1H, *J* = 7.9 and 2.1 Hz, H-6), 5.88 (d, 1H, *J* = 5.6 Hz, H-1'), 5.95 (d, 1H, *J* = 5.6 Hz, 3'-OH), 7.86 (d, 1H, *J* = 7.9 Hz, H-5), 11.45 (s, 1H, N-H). (The $^1$H NMR spectrum is accordance with the literature report.[249])
**HRMS** (ESI-MS) for $C_9H_{11}N_5O_5Na$ : $[M+Na]^+$: found, 292.0654; calculated 292.0658.

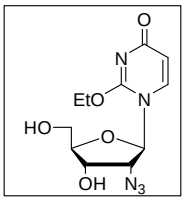


**2'-Azido-2'-deoxy-5'-*O*-methanesulfonyluridine (4.31)**

To a solution of **4.30** (610 mg, 2.3 mmol) in 10 mL pyridine at -78 ℃ was added methanesulfonyl chloride (210 µL, 2.7 mmol). The reaction was stirred for 1h at 0 ℃, quenched with aqueous saturated $NaHCO_3$, extracted with $CH_2Cl_2$, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by column chromatography ($CH_2Cl_2$:MeOH 96:4) to obtain compound **4.31** as a white solid (61%, 472 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.23 (s, 3H, $CH_3$), 4.06 (m, 1H, H4'), 4.25-4.48 (m, 4H, H-2', H-3', H-5'A and H-5'B), 5.68 (dd, 1H, *J* = 7.9 and 2.1 Hz, H-6), 5.82 (d, 1H, *J* = 5.3 Hz, 3'-OH), 6.2 (d, 1H, *J* = 5.6 Hz, H-1'), 7.62 (d, 1H, *J* = 8.2 Hz, H-5), 11.47 (s, 1H, N-H).

**HRMS** (ESI-MS) for $C_{10}H_{14}N_5O_7S$: $[M+H]^+$: 348.0618 found; 348.0613 calculated.

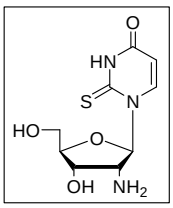



**2'-Azido-2'-deoxy-2-*O*-ethyluridine (4.32)**

Compound **4.31** (465 mg, 1.3 mmol) was refluxed in absolute ethanol (60 mL) in the presence of anhydrous sodium bicarbonate (281 mg, 3.3 mmol) under a nitrogen atmosphere for 36h. After cooling to room temperature, the reaction was diluted with ethyl acetate and the precipitated sodium salt was removed by filtration. The filtrate was concentrated *in vacuo* and purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5) to obtain compound **4.32** (222 mg, 57%) as a white solid.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 1.33 (t, 3H, *J* = 6.9 Hz, $CH_3$), 3.56-3.63 (m, 1H, H-5'A), 3.67-3.74 (m, 1H, H-5'B), 3.92 (m, 1H, H-4'), 4.12 (app t, 1H, *J* = 4.8 Hz, H-2'), 4.27-4.39 (m, 3H, $CH_2$ and H-3'), 5.23 (t, 1H, *J* = 4.8 Hz, 5'-OH), 5.85 (m, 2H, H-6 and H-1'), 6.01 (d, 1H, *J* = 5.4 Hz, 3'-OH), 7.98 (d, 1H, *J* = 7.8 Hz, H-5).

**HRMS** (ESI-MS) for $C_{11}H_{15}N_5O_5Na$ : $[M+Na]^+$: 320.0974 found; 320.0971 calculated.



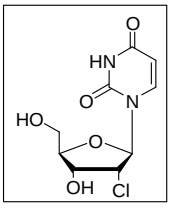

**2'-Amino-2'-deoxy-2-thiouridine (4.33)**

In a parr apparatus compound **4.32** (210 mg, 0.71 mmol) was dissolved in pyridine (40mL), cooled to -50 ℃ and saturated with $H_2S$. The mixture was heated at 50 ℃ for 24h resulting in a pressure of 250 psi. After cooling to room temperature, the remaining $H_2S$ was released, the solvent evaporated and the residue was purified on a silica gel column ($CH_2Cl_2$:MeOH 90:10), yielding compound **4.33** (141 mg, 77%), after subsequent crystallization from MeOH.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.32 (dd, 1H, *J* = 5.0 and 6.1, H-2'), 3.56-3.67 (m, 2H, H-5'A and H-5'B), 3.94 (m, 2H, H-3' and H-4'), 5.19 (t, *J* = 4.7 Hz, 5'-OH), 5.41 (br s, 1H, 3'-OH), 6.01 (d, 1H, *J* = 8.2 Hz, H-6), 6.53 (d, 1H, *J* = 6.0 Hz, H-1'), 8.09 (d, 1H, *J* = 8.2 Hz, H-5).

**$^{13}$C NMR** (300 MHz, DMSO-$d_6$): δ 59.74 (C-2'), 61.43 (C-5'), 70.91 (C-3'), 86.50 (C-4'), 93.49 (C-1'), 107.32 (C-5), 141.66 (C-6), 160.24 (C-4), 177.66 (C-2).

**HRMS** (ESI-MS) for $C_9H_{14}N_3O_4S$: $[M+H]^+$: 260.0692 found; 260.0704 calculated.

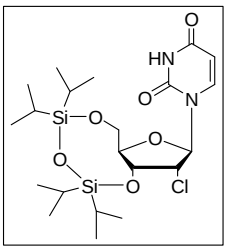



**2'-Chloro-2'-deoxyuridine (4.34)**

A suspension of 2,2'-anhydrouridine (2.0 g, 8.8 mmol) in 4.0 M HCl in dioxane (30 mL) was heated in a glass cylinder at 75 ℃. After 24 h, a white suspension was obtained. Solvent evaporation yielded 2'-chloro-2'-deoxyuridine **4.34** (1.6 g, 69%) as a white foam.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.55-3.71 (m, 2H, H-5'A and B), 3.94 (app q, 1H, *J* = 3.2 Hz, H-4'), 4.18 (app q,1H, *J* = 4.8 Hz, H-3'), 4.54 (app t, 1H, *J* = 5.3 Hz, H-2'), 5.22 (t, 1H, *J* = 5.0 Hz, 5-OH'), 5.66 (dd, 1H, *J* = 2.4 and 8.2 Hz, H-6), 5.84 (d, 1H, *J* = 5.6 Hz, H-1'), 5.99 (d, 1H, *J* = 8.1 Hz, H-5), 6.0 (d, 1H, *J* = 5.7 Hz, 3'-OH), 11.42 (br s, 1H, N-H). ). (The $^1$H NMR spectrum is accordance with the literature report.[250])

**HRMS** (ESI-MS) $C_9H_{12}N_2O_5Cl$: $[M+H]^+$: 263.04286 found; 263.04346 calculated.



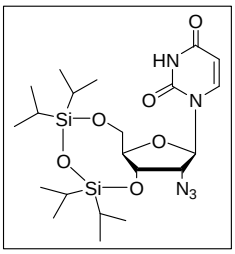

**1-(2-Chloro-2-deoxy-3, 5-*O*-TIPDS-β-D-ribofuranos-1-yl)uracil (4.35a)**

To a solution of **4.34** (1.6 g, 6.1 mmol) and imidazole (2.1 g, 30.5 mmol) in DMF (20 mL) was added $TIPDSCl_2$ (2.14 mL, 6.7 mmol) at 0 ℃. After 3h at room temperature, the reaction was stirred overnight at 30 ℃. Water was added and extraction with ethyl acetate was performed. The organic layer was dried over $MgSO_4$ and purified by silica gel chromatography ($CH_2Cl_2$:MeOH 99:1) to obtain compound **4.35a** in 78% yield (2.4 g).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.93-1.04 (m, 28H, 4x isopropyl), 3.91-3.96 (dd, 1H, *J* = 2.4 Hz and 13.5 Hz, H-5'A), 4.12-4.15 (dd, 1H, *J* = 2.1 Hz and 9.4 Hz, H-2'), 4.18-

4.27 (m, 2H, H-4' and H-5'B), 4.35-4.39 (dd, 1H, *J* = 5.1 en 9.3 Hz, H-3'), 5.62 (dd, 1H, *J* = 2.01 en 8.21 Hz, H-6), 5.88 (s, 1H, H-1'), 7.82 (d, 1H, *J* = 8.2 Hz, H-5), 8.52 (br s, 1H, N-H).

**HRMS** (ESI-MS) $C_{21}H_{38}N_2O_6ClSi_2$: $[M+H]^+$: 505.1957 found; 505.1956 calculated.

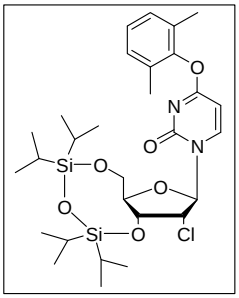


**1-(2-Azido-2-deoxy-3,5-*O*-TIPDS-β-D-ribofuranos-1-yl)uracil (4.35b)**

Compound **4.35b** was prepared as described for the synthesis of **4.35a** from **4.30** (300 mg, 1.1 mmol); yield 720 mg (79%) of compound **4.35b**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.92-1.04 (m, 28H, 4 x isopropyl), 3.90-3.96 (dd, 1H, *J* = 2.6 Hz and 13.8 Hz, H-5'A), 4.03-4.07 (m, 2H, H-2' and H-4'), 4.17 (d, 1H, *J* = 13.8 Hz, H-5'B), 4.41-4.45 (dd, 1H, *J* = 5.3 Hz and 9.4 Hz, H-3'), 5.50 (s, 1H, H-1'), 5.62 (d, 1H, *J* = 8.1 Hz, H-6), 7.72 (d, 1H, *J* = 8.1 Hz, H-5), 8.36 (br s, 1H, N-H).

**HRMS** (ESI-MS) $C_{21}H_{37}N_5O_6Si_2$ $[M+H]^+$: 512.2351 found; 512,23606 calculated.

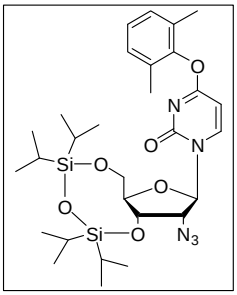


**1-(2-Chloro-2-deoxy-3, 5-*O*-TIPDS-β-D-ribofuranos-1-yl)-4-*O*-(2,6-dimethylfenyl)uracil (4.36a)**

Compound **4.35a** (300 mg, 0.6 mmol) was dissolved in $CH_2Cl_2$ (12 mL) and a solution of tetrabutylammoniumbromide (77 mg, 0.24 mmol) and 2,4,6-triisopropylbenzene sulfonylchloride (234 mg, 0.8 mmol) in 0.2 M $Na_2CO_3$ (24 mL) was added. The mixture was stirred vigorously for 15 h, the organic fraction was isolated and the aqueous fraction was extracted twice with $CH_2Cl_2$. After drying over $MgSO_4$ and concentration *in vacuo*, the residue was dissolved in $CH_3CN$. 2,6-Dimethylphenol (73 mg, 0.6 mmol), triethylamine (248 µl; 1.8 mmol) and 1,4-diaza-bicyclo[2,2,2]octane (7 mg, 0.06 mmol) were added to the solution. After stirring at room temperature for 30 min, sat. aq. $NaHCO_3$ was added. The mixture was extracted with $CH_2Cl_2$, dried over $MgSO_4$ and purified by silica gel chromatography (EtOAc:Hex 90:10) to yield 320 mg (88 %) of compound **4.36a**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.83-1.05 (m, 28H, 4x isopropyl), 2.05 (s, 6H, 2x $CH_3$), 3.91-3.97 (dd, 1H, *J* = 2.6 Hz and 13.5 Hz, H-5'A), 4.17-4.25 (m, 2H, H-2' and H-4'), 4.32-4.41 (m, 2H, H-3' and H-5'B), 5.89 (s, 1H, H-1'), 5.97 (d, 1H, *J* = 7.6 Hz, H-6), 6.98 (s, 3H, Ph), 8.20 (d, 1H, *J* = 7.6 Hz, H-5).

**HRMS** (ESI-MS) $C_{29}H_{46}N_2O_6ClSi_2$: $[M+H]^+$: 609.2585 found; 609.2582 calculated.

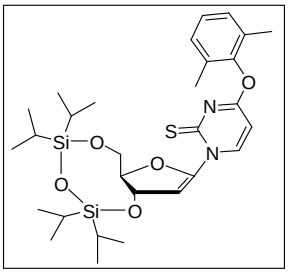


**1-(2-Azido-2-deoxy-3,5-*O*-TIPDS-β-D-ribofuranos-1-yl)-4-*O*-(2,6-dimethylphenyl) uracil (4.36b)**

Compound **4.36b** was prepared as described for the synthesis of **4.36a** from **4.35b** (218 mg, 0.4 mmol); yield: 226.8 mg (84 %) of compound **4.36b**.

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.88-1.05 (m, 28H, 4x isopropyl), 2.05 (s, 6H, 2 x $CH_3$), 3.91-3.97 (dd, 1H, *J* = 13.5 and 2.6 Hz, H-5'A), 4.08-4.12 (dd, 1H, *J* = 2.4 and 9.1 Hz, H-2'), 4.16-4.22 (m, 2H, H-5'B and H-4'), 4.22-4.41 (dd, 1H, *J* = 4.8 and 9.1 Hz, H-3'), 5.54 (s, 1H, H-1'), 5.99 (d, 1H, *J* = 7.5 Hz, H-6), 6.98 (s, 3H, H-Ph), 8.11 (d, 1H, *J* = 7.5 Hz, H-5).

**HRMS** (ESI-MS) $C_{29}H_{46}N_5O_6Si_2$ $[M+H]^+$: 616.29847 found; 616.29864 calculated.



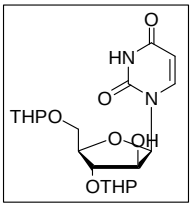
**Attempted synthesis of 1-(2-chloro-2-deoxy-3,5-O-TIPDS-β-D-ribofuranos-1-yl)-4-*O*-(2,6-dimethylphenyl)-2-thiouracil: synthesis of compound 4.37**

Lawesson's reagent (303 mg, 0.8 mmol) was added to a solution of **4.36a** (300 mg, 0.53 mmol) in dry toluene. The mixture was refluxed for 1h and cooled to room temperature. Insoluble materials were filtered off and the filtrate was subjected to silica gel chromatography (EtOAc:hexane 90:10) to obtain **4.37** in 20 % yield (94 mg).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 0.83-1.05 (m, 28 H, 4x isopropyl), 2.05 (s, 6 H, 2x $CH_3$), 3.54 (t, 1 H, *J* = 11.14 Hz, H-5'A), 4.02 (dd, 1 H, *J* = 4.1 and 10.9 Hz, H-5'B), 4.43 (ddd, 1 H, *J* = 11.2, 4.1 and 5.0 Hz, H-4'), 5.20 (dd, 1 H, *J* = 1.2 and 5.0 Hz, H-

3'), 6.34 (d, 1 H, *J* = 6.0 Hz, H-6), 6.54 (d, 1 H, *J* = 1.2 Hz, H-2'), 7.02 (s, 3H, Ph), 8.22 (d, 1H, *J* = 5.7 Hz, H-5).

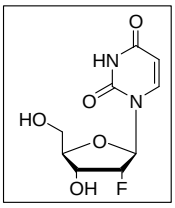
**MS** (m/z) $[M + H]^+$ = 589.3 found; 589.3 calculated.




**1-[3,5-*O*-(Tetrahydropyran-2-yl)-β-D-arabinofuranos-1-yl]uracil (4.39)**

2,3-Dihydro-4*H*-pyrane (22.6 mL, 247.5 mmol) and *p*-toluenesulfonic acid (1.8 g, 9.2 mmol) were added to a solution of 2,2'-anhydrouridine (**4.23**, 2g, 8.84 mmol) in dry DMF (40 mL). The suspension was stirred at 0 °C and after 4h the obtained clear solution was neutralized by adding triethylamine. After addition of sat. aq. $NaHCO_3$, an extraction with EtOAc was performed and the organic fraction was dried over $MgSO_4$ and concentrated *in vacuo*. The resulting white solid was dissolved in EtOH:$H_2O$ (150 mL, 1:1), dioxane (150 mL) and 11 mL of a 1.0 M NaOH solution. After stirring for 3h at room temperature, diluted acetic acid was added until pH 7 and after solvent evaporation, the residue was purified on a silica gel column (EtOAc:hexane, 70:30). Compound **4.39** was obtained in 89 % yield (3.3 g).

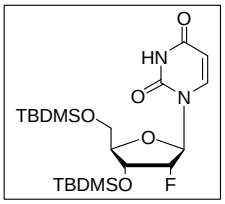
**MS** (m/z) $C_{19}H_{28}N_2O_8Na$ $[M+Na]^+$: 435.8 found; 435.2 calculated.




**1-(2-Deoxy-2-fluoro-β-D-ribofuranos-1-yl)uracil (4.41)**

Compound **4.39** (3.0 g, 7.9 mmol) was dissolved in $CH_2Cl_2$ (45 mL) and pyridine (9 mL) and diethylaminosulfur trifluoride (2.9 ml, 24.5 mmol) was added at -60 °C. The mixture was allowed to warm to room temperature and was subsequently heated for 4h at 40 °C. After addition of sat. aq. $NaHCO_3$ and extraction with $CH_2Cl_2$, the organic layer was dried over $MgSO_4$ and concentrated to dryness. The residue was dissolved in methanol (16 mL) and *p*-toluenesulfonic acid (1.3 g, 7.0 mmol) was added. After 15 min, the mixture was neutralized with triethylamine and evaporated to dryness. Silica gel purification ($CH_2Cl_2$:MeOH, 97:3 → 95:5) yielded compound **4.41** in 71 % yield (985 mg).

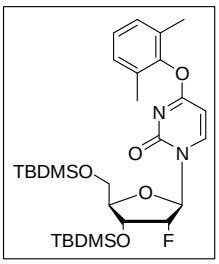
**$^1$H NMR** (300 MHz, $CDCl_3$): δ 3.53-3.60 (m, 1H, H-5'A), 3.71-3.77 (m, 1H, H-5'B), 3.85 (m, 1H, H-4'), 4.06-4.20 (m, 1H, H-3'), 4.92-5.11 (ddd, 1H, *J* = 53.3, 4.4 and 2.1 Hz, H-2'), 5.17 (t, 1H, *J* = 5.3 Hz, 5'-OH), 5.60 (m, 1H, 2H, H-6 and 3'-OH), 5.90 (dd, 1H, *J* = 2.1 and 17.6 Hz, H-1'), 7.92 (dd, 1H, *J* = 1.2 and 8.1 Hz, H-5), 11.38 (s, 1H, N-H). ). (The $^1$H NMR spectrum is accordance with the literature report.[251])
**HRMS** (ESI-MS) $C_9H_{11}N_2O_5FNa$ $[M+Na]^+$: 269.05442 found; 269.05499 calculated.




**1-(2-Deoxy-2-fluoro-3,5-O-TBDMS-β-D-ribofuranos-1-yl) uracil (4.42)**

Compound **4.41** (900 mg, 3.6 mmol) and imidazole (961 mg, 16 mmol) were dissolved in 35 mL DMF and TBDMSCl (2.3 g, 15 mmol) was added at 0 ℃. After stirring for 24 h at room temperature, the mixture was poured into sat. aq. $NaHCO_3$, extracted with EtOAc, dried over $MgSO_4$ and purified on a silica gel column ($CH_2Cl_2$: MeOH 99:1) to obtain compound **4.42** in 61% yield.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 0.00 (s, 12H, Si-$(CH_3)_2$), 0.80 (s, 9H, *t*-Bu), 0.82 (s, 9H, *t*-Bu), 3.75-3.80 (dd, 1H, *J* = 1.8 and 11.7 Hz, H-5'A), 4.03-4.09 (m, 2H, H-5'B and H-4'), 4.23-4.33 (ddd, 1H, *J* = 4.1, 7.3 and 19.1 Hz, H-3'), 4.67-4.87 (ddd, 1H, *J* = 53.2, 4.1 and 1.8 Hz, H-2'), 5.68 (dd, 1H, *J* = 8.2 Hz, H-6), 6.03-6.08 (dd, 1H, *J* = 1.8 and 15.3 Hz, H-1'), 7.93 (d, 1H, *J* = 8.2 Hz, H-5), 8.67 (br s, 1H, N-H).
**HRMS** (ESI-MS) $C_{21}H_{39}N_2O_5FSi_2Na$ $[M+Na]^+$: 497.22754 found; 497.22793 calculated.



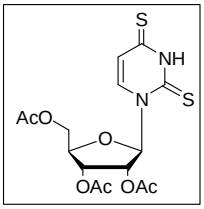

**1-(2-Deoxy-2-fluoro-3,5-*O*-TBDMS-β-D-ribofuranos-1-yl)-4-*O*-(2,6-dimethylphenyl) uracil (4.43)**

The reaction was performed as described for the synthesis of **4.36a** and **4.36b**. 1.03 g of compound **4.42** yielded 1.027 g (82%) of compound **4.43**.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 0.00 (s, 12H, Si-$(CH_3)_2$), 0.80 (s, 9H, *t*-Bu), 0.82 (s, 9H, *t*-Bu), 2.04 (s, 6H, 2 x $CH_3$-Ph), 3.71-3.75 (dd, 1H, *J* = 1.5 and 12.3 Hz, H-5'A), 4.03-4.06 (m, 2H, H-5'B and H-4'), 4.13-425 (ddd, 1H, *J* = 3.8, 8.5 and 23.2 Hz, H-3'),

4.66-4.84 (dd, 1H, *J* = 3.8 and 51.9 Hz, H-2'), 5.92-5.99 (d, 1H, *J* = 22.3 Hz, H-1') 5.97 (d, 1H, *J* = 7.2 Hz, H-6), 8.31 (d, 1H, *J* = 7.3 Hz, H-5).

**HRMS** (ESI-MS) $C_{29}H_{47}N_2O_5FSi_2Na$ $[M+Na]^+$: 601.29072 found; 601.29053 calculated.

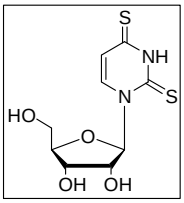



**2',3',5'-Tri-*O*-acetyl-2,4-dithiouridine (4.44)**

To a solution of **4.10** (290 mg, 0.75 mmol) in dry toluene (10 mL), Lawesson's reagent (304 mg, 0.75 mmol) was added. After heating the reaction mixture at 80 ℃ overnight, insoluble materials were filtered off and the filtrate was purified by silica gel chromatography (Hex:EtOAc 65:35) yielding compound **4.44** as a yellow foam (250 mg, 83%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 2.06 (s, 3H, $CH_3$), 2.07 (s, 3H, $CH_3$), 2.09 (s, 3H, $CH_3$), 4.25-4.30 (dd, 1H, *J* = 3.5 and 13.5 Hz, H-5'A), 4.34-4.40 (m, 2H, H-5'B and H-4'), 5.16 (app t, 1H, *J* = 5.7 Hz, H-3'), 5.37 (dd, 1H, *J* = 4.1 and 5.6 Hz, H-2'), 5.98 (d, 1H, *J* = 8.2 Hz, H-6), 6.76 (d, 1H, *J* = 4.1 Hz, H-1'), 7.65 (d, 1H, *J* = 8.2 Hz, H-5) 9.97, (br s, 1H, N-H).

**HRMS** (ESI-MS) $C_{15}H_{19}N_2O_7S_2$: $[M+H]^+$: 403.06338 found; 403.06335 calculated.

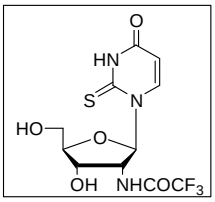



**2,4-Dithiouridine (4.45)**

Compound **4.44** (240 mg, 0.60 mmol) was dissolved in dry methanol (15 mL) and the warmed solution was treated with 130µL of a solution of NaOMe (30% w/w) in methanol. The reaction was refluxed for 4h and then treated with diluted acetic acid to pH 5, evaporated to dryness and purified on a silica gel column ($CH_2Cl_2$:MeOH 93:7) to obtain compound **4.45** as a yellow foam (150 mg, 91%).

**$^1$H NMR** (300 MHz, DMSO-$d_6$): δ 3.58-3.64 (m, 1H, H-5'A), 3.72-3.78 (m, 1H, H-5'B), 3.93 (m, 2H, H-3' and H-4'), 4.08 (m, 1H, H-2'), 5.11 (d, 1H, *J* = 5.9 Hz, 3'-OH), 5.29 (t, 1H, *J* = 5.0 Hz, 5'-OH), 5.53 (d, 1H, *J* = 5.3 Hz, 2'-OH), 6.40 (d, 1H, *J* = 2.7 Hz, H-1'), 6.63 (d, 1H, *J* = 7.7 Hz, H-6), 8.07 (d, 1H, *J* = 7.7 Hz, H-5), 13.80 (br s, 1H, N-H).

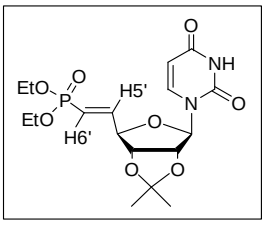
**HRMS** (ESI-MS) $C_9H_{11}N_2O_4S_2$: $[M-H]^-$: 275.01637 found; 275.01602 calculated.



**2'-Trifluoroacetylamino-2'-deoxy-2-thiouridine (4.46)**

*N*,*N*-Diisopropylethylamine (0.006 mL, 0.035 mmol) and ethyl trifluoroacetate (0.004 mL, 0.035 mmol) were added sequentially to a solution of **4.33** (6 mg, 0.023 mmol) in DMF (1 mL). The reaction mixture was stirred for 13 h at room temperature. The solvent was removed *in vacuo*, and the residue was purified by preparative thinlayer chromatography ($CH_2Cl_2$:MeOH, 90:10) to afford **4.46** (7.2 mg, 88%) as a white solid.

**$^1$H NMR** ($CD_3OD$): $\delta$ 3.84 (m, 2H, H-5'A and H-5'B), 4.14 (dd, *J* = 5.4, 2.7 Hz, 1H, H-4'), 4.34 (dd, *J* = 5.7, 3.0 Hz, 1H, H-3'), 4.58 (dd, *J* = 6.6, 6.3 Hz, 1H, H-2'), 6.01 (d, *J* = 8.1 Hz, 1H, H-6), 7.12 (d, *J* = 6.9 Hz, 1H, H-1'), 8.25 (d, *J*= 8.1 Hz, 1H, H-5).
**HRMS** (ESI-MS) $C_{11}H_{13}N_3O_5F_3S$: $[M+H]^+$: 356.0520 found; 356.0528 calculated.



**1-[5,6-Dideoxy-6-(diethoxyphosphinyl)-2,3-*O*-isopropylidene-β-D-*ribo*-hex-5-enofuranosyl]uracil (4.47)**

**1$^{st}$ method**: Trifluoroacetic acid (18 µL) was added to a solution of **4.14** (100 mg, 0.33 mmol), DCC (275 mg, 1.33 mmol) and pyridine (36 µL) in anhydrous DMSO (2 mL). The mixture was stirred at room temperature for 22h and freshly prepared [(diethoxyphosphinyl)methylidene] triphenylphosphorane (**4.57**) in DMSO (2 mL) was added. After stirring for 24h, excess carbodiimide was hydrolyzed by a careful addition of a solution of oxalic acid (90 mg, 1 mmol) in methanol (365 µL). After 10 min, the volatiles were removed under reduced pressure and the formed DCU was removed by centrifugation. After adding water and extraction with EtOAc, the combined organic layers were dried over $MgSO_4$, filtered and concentrated to dryness. The residue was lyophilized to remove the remaining DMSO and purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5 and acetone:pentane 50:50) yielding 51 mg (34%) of **4.47**.

**2$^{nd}$ method**: Compound **4.14** (100 mg, 0.33 mmol) was dissolved in $CH_3CN$ (3 mL) and dry pyridine (27 µL, 0.33 mmol) was added. The solution was treated dropwise with 0.3 M Dess-Martin periodinane in $CH_2Cl_2$ (2.7 mL, 0.53 mmol) and stirring was continued for 1h at room temperature. The mixture was diluted with $CH_2Cl_2$ to 20 mL and sat. aq. $NaHCO_3$ (9 mL) and sat. aq. $Na_2S_2O_3$ (1.8 mL) were added. The organic fraction was isolated and the aqueous fraction was extracted with $CH_2Cl_2$. The

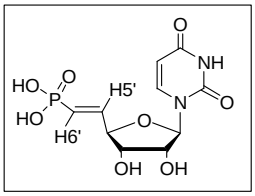
combined organic fractions were dried over $MgSO_4$, filtered and evaporated to dryness. The residue was dissolved in anhydrous DMSO (1 mL) and freshly prepared **4.57** in DMSO (1 mL) was added. After 20h, the mixture was poured into water and extracted with $CH_2Cl_2$. The combined layers were dried over $MgSO_4$, filtered and concentrated to dryness. The residue was lyophilized to remove the remaining DMSO and purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5 and acetone:pentane 50:50) yielding 48 mg (35%) of **4.47**.

**3rd method**: Compound **4.14** (100 mg, 0.33 mmol) was dissolved in $CH_3CN$ (3 mL) and IBX was added (275 mg 45 % wt, 0.49 mmol). The resulting suspension was stirred vigorously open to the atmosphere at 80 ℃. After 4h, the reaction was cooled to room temperature and filtered through a medium glass frit. The filter cake was washed 3 times with $CH_3CN$ (3 mL) and the combined filtrates were concentrated to dryness. The residue was dissolved in anhydrous DMSO (1 mL) and freshly prepared **4.57** in DMSO (1 mL) was added. After 20h, the mixture was poured into water and extracted with $CH_2Cl_2$. The combined layers were dried over $MgSO_4$, filtered and concentrated to dryness. The residue was lyophilized to remove the remaining DMSO and purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5 and acetone:pentane 50:50) yielding 60 mg (44%) of **4.47**.

**$^1$H NMR** (DMSO-$d_6$): $\delta$ 1.22 (t, 6H, *J* = 7.1 Hz, 2 x $OCH_2CH_3$), 1.30 (s, 3H, $CH_3$), 1.51 (s, 3H, $CH_3$), 3.93-3.99 (m, 4H, 2 x $OCH_2CH_3$), 4.60 (dd, 1H, *J* = 4.7 and 6.3 Hz, H-4'), 4.85 (dd, 1H, *J* = 4.2 and 6.3 Hz, H-3'), 5.13 (ddd, 2H, *J* = 1.3 and 6.3 Hz, H-2'), 5.63 (d, 1H, *J* = 8.1 Hz, H-6), 5.82 (d, 1H, *J* = 1.6 Hz, H-1'), 5.96-6.02 (dt, 1H, $J_{H6'-H4'}$ = 1.1 Hz, $J_{H6'-H5'}$ 17.0 Hz, H-6'), 6.66-6.73 (ddd, 1H, $J_{H5'-H4'}$ = 6.3 Hz, $J_{H5'-H6'}$ = 17.0 Hz, $J_{H5'-P}$ = 21.8 Hz, H-5'), 7.73 (d, 1H, *J* = 8.1 Hz, H-5), 11.43 (s, 1H, N-H).

**$^{31}$P NMR** ($D_2O$) $\delta$ 17.84.

**HRMS** (ESI-MS) $C_{17}H_{25}N_2O_8PNa$ $[M+Na]^+$: 439.12395 found; 439.12463 calculated.

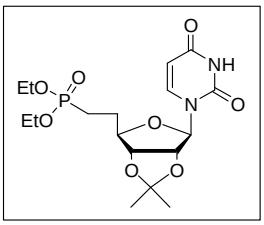



**1-[5,6-Dideoxy-6-(dihydroxyphosphinyl)-β-D-*ribo*-hex-5-enofuranosyl]uracil (4.48)**

To a solution of **4.47** (150 mg, 0.36 mmol) in $CH_2Cl_2$ (10 mL) was added TMSBr (480 µL, 3.6 mmol). After stirring overnight,

the volatiles were removed *in vacuo*. The residue was dissolved in water, washed with diethylether and lyofilized to give pure product **4.48** in 62 % yield.

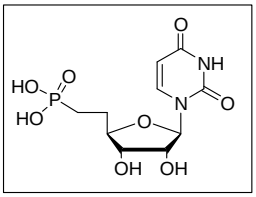
**$^1$H NMR** ($D_2O$): δ 4.03 (app t, 1H, *J* = 5.5 Hz, H-3'), 4.23 (app t, 1H, *J* = 5.5 Hz, H-2'), 4.43 (m, 1H, H-4'), 5.73 (d, 1H, *J* = 8.2 Hz, H-5), 5.74 (d, 1H, *J* = 3.8 Hz, H-1'), 5.98-6.10 (app t, 1H, *J* = 17.5 Hz, H-6'), 6.48-6.63 (ddd, 1H, $J_{H5'-H4'}$ = 5.3 Hz, $J_{H5'-H6'}$ 17.3 Hz, $J_{H5'-P}$ = 22.3 Hz, H-5'), 7.49 (d, 1H, *J* = 8.2 Hz, H-5);
**$^{31}$ P NMR** ($D_2O$) δ 13.80
**HRMS** (ESI-MS) $C_{10}H_{14}N_2O_8P$ $[M+H]^+$: 323.04897 found; 321,04878 calculated.



**1-[5,6-Dideoxy-6-(diethoxyphosphinyl)-2,3-*O*-isopropylidene-β-D-*ribo*-hexofuranosyl]uracil (4.49)**

To a solution of compound **4.47** (100 mg, 0.27 mmol) in methanol (5 mL) was added Pd/C (10%wt, 10 mg). The reaction mixture was stirred under hydrogen atmosphere. After 20h, the mixture was filtered through Celite to remove the catalyst and purified on a silica gel column ($CH_2Cl_2$:MeOH 95:5) yielding 85 mg (84%) of compound **4.49**.

**$^1$H NMR** (DMSO-$d_6$): δ 1.22 (t, 6H, *J* = 7.1 Hz, 2 x $OCH_2CH_3$), 1.29 (s, 3H, $CH_3$), 1.48 (s, 3H, $CH_3$), 1.72-1.85 (m, 4H, H-5' A-B and H-6' A-B), 3.92-4.02 (m, 5H, H-4' and 2 x $OCH_2CH_3$), 4.64 (dd, 1H, *J* = 4.7 and 6.5 Hz, H-3'), 5.00 (dd, 1H, *J* = 2.3 and 6.5 Hz, H-2'), 5.64 (dd, 1H, *J* = 2 and 7.9 Hz, H-6), 5.75 (d, 1H, *J* = 2.9 Hz, H-1'), 7.70 (d, 1H, *J* = 7.9 Hz, H-5), 11.40 (s, 1H, N-H).



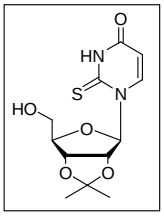
**1-[5,6-Dideoxy-6-(dihydroxyphosphinyl)-β-D-*ribo*-hexofuranosyl]uracil (4.50)**

This compound was synthesized by the procedure described for the synthesis of **4.48** from 80 mg (0.21 mmol) of **4.50** in 65% yield (44 mg).

**$^{1}$H NMR** (300 MHz, $D_2O$) : δ 1.68-1.87 (m, 4H, H-5' A-B and H-6' A-B), 3.86-3.93 (m, 2H, H-3' and H-4'), 4.20 (app t, 1H, *J* = 4.5 Hz, H-2'), 5.67 (d, 1H, *J* = 4.4 Hz, H-1'), 5.72 (d, 1H, *J* = 8.0, H-6), 7.49 (d, 1H, *J* = 8.2, H-5).

**$^{31}$P NMR** ($D_2O$) δ 30.13.

**HRMS** (ESI-MS) $C_{10}H_{16}N_2O_8P$ $[M+H]^+$: 323.06373 found; 323.04639 calculated.

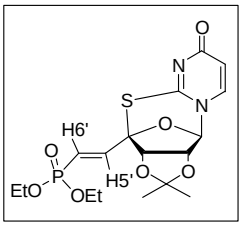


**2',3'-O-Isopropylidene-2-thiouridine (4.51)**

The reaction was performed as described for the synthesis of 2-thiouridine from 2-ethoxyuridine. 2-Thiouridine (**4.12**) (5g, 1.6 mmol) yielded 4.3 g (88%) of **4.51**.

**$^{1}$H NMR** (DMSO-$d_6$) δ 1.29 (s, 3H, $CH_3$), 1.51 (s, 3H, $CH_3$), 3.58-3.72 (m, 2H, H-5'A and H-5'B), 4.14 (dd, 1H, *J* = 3.5 and 7.0 Hz, H-4'), 4.75-4.83 (m, 2H, H-2' and H-3'), 5.30 (t, 1H, *J* = 5.0 Hz, 5'-OH), 6.02 (d, 1H, *J* = 8.2 Hz, H-6), 6.86 (d, 1H, *J* = 2.6 Hz, H-1'), 7.98 (d, 1H, *J* = 8.2 Hz, H-5), 12.70 (s, 1H, N-H).

**HRMS** $C_{12}H_{16}N_2O_5SNa$ $[M+Na]^+$: 323.06722 found; 323.06778 calculated.

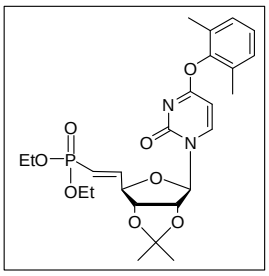


**Attempted synthesis of 1-[5,6-Dideoxy-6-(diethoxyphosphinyl)-2,3-*O*-isopropylidene-β-D-*ribo*-hex-5-enofuranosyl]-2-thiouracil, yielding compound 4.52**

This compound was synthesized from **4.51** (380 mg, 1.27 mmol) by the 1$^{st}$ method described for the synthesis of **4.47**, to afford compound **4.52** in 56 % yield (308 mg).

**$^{1}$H NMR** (300 MHz, DMSO-$d_6$) : δ 1.22 (t, 6H, *J* = 7.1 Hz, 2x $CH_2CH_3$), 1.29 (s, 3H, $CH_3$), 1.36 (s, 3H, $CH_3$), 3.96-4.07 (m, 4H, 2 x $CH_2CH_3$), 5.24 (d, 1H, *J* = 5.3 Hz, H-3'), 5.31 (d, 1H, *J* = 5.6 Hz, H-2'), 5.98 (d, 1H, *J* = 7.6 Hz, H-6), 6.23-6.34 (t, *J* = 17.3 Hz, H-6'), 6.26 (s, 1H, H-1'), 6.80-6.93 (dd, 1H, $J_{H5'-H4'}$ = 17.0 Hz, $J_{H5'-P}$ = 22.0 Hz, H-6'), 7.88 (d, 1H, *J* = 7.9 Hz, H-5);

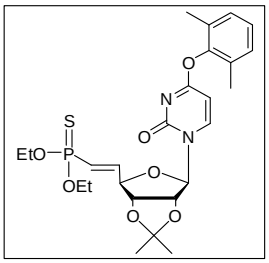
**$^{13}$C NMR** (75 MHz, DMSO-$d_6$): δ 16.80, 16.88 (2 x $CH_2\mathit{CH_3}$), 25.66, 26.31 (2 x $CH_3$), 62.45 (2 x $\mathit{CH_2}CH_3$), 85.53 (C-3'), 87.88 (C-2'),110.48 (C-6), 113.68 (C-4'), 120.93, 123.34 (C-5'), 93.34 (C-1'), 142.29 (C-6'), 156.33 (C-2), 166.82 (C=O).
**HRMS** $C_{17}H_{24}N_2O_7PS$ $[M+H]^+$: 431.0934 found; 431.10418 calculated.



**1-[5,6-Dideoxy-6-(diethoxyphosphinyl)-2,3-*O*-isopropylidene-β-D-*ribo*-hex-5-enofuranosyl]uracil-4-*O*-(2,6-dimethylphenyl)uracil (4.53)**

Compound **4.47** (40 mg, 0.106 mmol) was dissolved in $CH_2Cl_2$ (1 mL) and a solution of tetrabutylammoniumbromide (14 mg, 0.021mmol) and 2,4,6-triisopropylbenzene sulfonyl chloride (40 mg, 1.32 mmol) in 0.2M $Na_2CO_3$ (4 mL) was added. The mixture was stirred vigorously for 15 h, the organic fraction was isolated and the aqueous fraction was extracted twice with $CH_2Cl_2$. After drying over $MgSO_4$ and concentration *in vacuo*, the residue was dissolved in $CH_3CN$. 2,6-Dimethylphenol (13 mg, 0.106 mmol), triethylamine (22 µl, 0.34 mmol) and 1,4-diaza-bicyclo[2,2,2]octane (0.6 mg, 0.106 mmol) were added to the solution. After stirring at room temperature for 30 min, sat. aq. $NaHCO_3$ was added. The mixture was extracted with $CH_2Cl_2$, dried over $MgSO_4$ and purified by silica gel chromatography ($CH_2Cl_2$ 98:2) to yield 40 mg (73 %) of compound **4.53**.

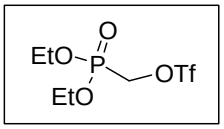
**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.24-1.37 (m, 6H, 2 x $CH_3$ (OEt)), 1.32 and 1.56 (s, 3H, $CH_3$ (isopropylidene)), 2.12 (s, 6H, 2 x $CH_3$), 4.00-4.09 (q, 4H, $J$ = 7.0 Hz, 2 x $CH_2$), 4.64-4.69 (m, 1H, H-4'), 4.89 (dd, 1H, $J$ = 4.4 and 6.4 Hz, H-3'), 5.11 (dd, 1H, $J$ = 1.2 and 6.2 Hz, H-2'), 5.66 (d, 1H, $J$ = 1.2 Hz, H-1'), 5.82-5.95 (dt, 1H, $J_{H6'\text{-}H4'}$ = 1.7 Hz, $J_{H6'\text{-}H5'}$ = 17.2 Hz, H-6'), 6.10 (d, 1H, $J$ = 7.3 Hz, H-6), 6.77-6.92 (ddd, 1H, $J_{H5'\text{-}H4'}$ = 5.6 Hz, $J_{H5'\text{-}H6'}$ 17.3 Hz, $J_{H5'\text{-}P}$ = 22.9 Hz, H-5'), 5.99 (d, 1H, $J$ = 7,5 Hz, H-6), 7.04 (s, 3H, H-Ph), 7.57 (d, 1H, $J$ = 7,3 Hz, H-5).
**HRMS** (ESI-MS) $C_{25}H_{33}N_2O_8PNa$: $[M+Na]^+$ 543,18751 found; 543,18722 calculated.



**Attempted synthesis of 1-[5,6-Dideoxy-6-(diethoxyphosphinyl)-2,3-*O*-isopropylidene-β-D-*ribo*-hex-5-enofuranosyl]uracil-4-*O*-(2,6-dimethylphenyl)-2-thiouracil (4.53): synthesis of compound 4.54.**

Lawesson's reagent (27 mg, 0.067 mmol) was added to a solution of compound **4.53** (35 mg, 0.067 mmol) in dry toluene (1 mL). The mixture was refluxed for 3h, cooled to room temperature, concentrated *in vacuo* and purified by silica gel chromatography ($CH_2Cl_2$:MeOH 98:2) to obtain compound **4.54** in 20 % yield.

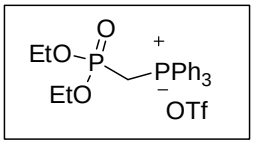
**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.23-1.37 (m, 6H, 2 x $CH_3$ (OEt)), 1.33 and 1.57 (s, 3H, $CH_3$ (isopropylidene)), 2.13 (s, 6H, 2 x $CH_3$), 4.04-4.14 (q, 4H, J = 7.0 Hz, 2 x $CH_2$), 4.69 (m, 1H, H-4'), 4.86 (dd, 1H, *J* = 4.4 and 6.2 Hz, H-3'), 5.11 (dd, 1H, J = 1.3 and 6.4 Hz, H-2'), 5.68 (d, 1H, J = 1.4 Hz, H-1'), 6.02-6.15 (dt, 1H, $J_{H6'-H4'}$ = 1.7 Hz, $J_{H6'-H5'}$ 16.8 Hz, H-6'), 6.10 (d, 1H, J = 7.3 Hz, H-6), 6.77-6.92 (ddd, 1H, $J_{H5'-H4'}$ = 4.8 Hz, $J_{H5'-H6'}$ = 16.6 Hz, $J_{H5'-P}$ = 22.1 Hz, H-5'), 7.04 (s, 3H, H-Ph), 7.57 (d, 1H, *J* = 7,2 Hz, H-6).
**$^{31}$P NMR** ($CDCl_3$): δ 15.41
**MS** (m/z) $C_{25}H_{34}N_2O_7PS$ $[M+H]^+$: 537.4 found; 537,2 calculated.



**(Diethoxyphosphinyl)methyl triflate (4.55)**

To a stirred solution of diethyl(hydroxymethyl)phosphonate (13.6 mmol, 2.28 g) and 2,6-lutidine (1.9 mL, 16.18 mmol) in anhydrous $CH_2Cl_2$ (22 mL) at -50 °C was added trifluoromethanesulfon ic anhydride (2.7 mL, 15.7 mmol) dropwise. The resulting mixture was allowed to warm to 0 °C over 3h and diluted with ether. The precipitates formed were removed by filtration and the ethereal solution was successively washed with water and 1N HCl and then dried over $MgSO_4$. After concentration *in vacuo*, a yellowish oil (3.5 g, 90%) was obtained, which was used in the next step without purification.

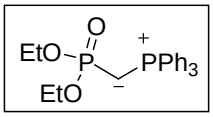
**$^1$H NMR** (300 MHz, $CDCl_3$) : δ 1.38 (t, 6H, *J* = 7.1 Hz, 2 x $OCH_2CH_3$), 4.19-4.29 (m, 4H, 2 x $OCH_2$ $CH_3$), 4.63 (d, 2H, *J* = 8.8 Hz, $CH_2$). (The $^1$H NMR spectrum is accordance with the literature report.[237])



**[(Diethoxyphosphinyl)methyl]triphenylphosphonium triflate (4.56)**

Compound **4.55** (3.5 g, 12.3 mmol) was added dropwise to a stirred solution of triphenylphosphine (3.7 g, 14.1 mmol) in 7 mL anhydrous $CH_2Cl_2$ at 0 ℃. The mixture was allowed to warm to room temperature and then stirred overnight. The solvent was removed under reduced pressure to about one-third of the volume and the remaining oil triturated with ether. The white solid formed was collected by filtration and crystallized from EtOAc/Hexane (6.1 g, 80%).

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.12 (t, 6H, *J* = 7.1 Hz, 2 x $OCH_2\mathit{CH_3}$), 3.91-4.06 (m, 4H, 2 x $O\mathit{CH_2}$ $CH_3$), 4.09-4.22 (dd, 2H, *J* = 16.1 and 20.2 Hz, $CH_2$), 7.65-7.90 (m, 15H, $PPh_3$). (The $^1$H NMR spectrum is accordance with the literature report.[237])



**[(Diethoxyphosphinyl)methylidene]triphenylphosphorane (4.57)**

To a stirred suspension of NaH (221 mg, 9 mmol) in anhydrous THF (9 mL) was added triphenylphosphonium triflate salt **4.56** (2.26 g, 4.1 mmol) in anhydrous THF (9 mL) at 0 ℃. The resulting mixture was stirred at 0 ℃ for 30 min. The solvent was removed under reduced pressure and the residue was extracted with anhydrous $CH_2Cl_2$. After concentration of the extracts, a slightly yellow oil was obtained, that was used immediately as reagent for the synthesis of α,β-unsaturated phosphonate esters.

**$^1$H NMR** (300 MHz, $CDCl_3$): δ 1.12 (t, 6H, *J* = 7.1 Hz, 2 x $OCH_2\mathit{CH_3}$), 1.27 (d, 1H, *J* = 7.5 Hz, CH), 3.81-3.93 (m, 4H, 2 x $O\mathit{CH_2}$ $CH_3$), 7.40-7.74 (m, 15H, $PPh_3$). (The $^1$H NMR spectrum is accordance with the literature report.[237])

# 5.2 Binding Studies

## 5.2.1 Binding studies at the $A_3$AR

The binding studies at the $A_3$AR are performed under the guidance of prof. dr. Kenneth A. Jacobson at the Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.

### 5.2.1.1 Cell Culture and Membrane Preparation

CHO cells expressing recombinant human ARs or the rat $A_3$AR were cultured in DMEM (Dulbecco's modified Eagle's medium) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/mL glutamine. After harvest and homogenization, the cells were centrifuged at 500*g* for 10 min. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 1 mMEDTA. The suspension was homogenized with an electric homogenizer for 10 s and was then recentrifuged at 20000*g* for 20 min at 4 ℃. The resulting pellets were resuspended in buffer containing 3 units/mL of adenosine deaminase, and the suspension was stored at -80 ℃ prior to the binding experiments. The protein concentration was measured using the Bradford assay.[252]

### 5.2.1.2 Radioligand Binding Studies

For the $A_3$AR binding experiments, the procedures were similar to those previously described.[195] Briefly, each tube contained 100 mL of membrane suspension, 50 mL of [$^{125}$I]I-AB-MECA (final concentration of 0.5 nM), and 50 μL of increasing concentrations of compounds in Tris-HCl buffer (50 mM, pH 7.4) containing 10 mM $MgCl_2$ and 1 mM EDTA. Nonspecific binding was determined using 10 μM NECA. The mixtures were incubated at 25 ℃ for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. The binding of [$^3$H]CCPA (2-chloro-*N*$^6$-cyclopentyladenosine, **[$^3$H]1.33**, Figure 1.15) to the recombinant h$A_1$AR and the binding of [$^3$H]CGS21680 (2-[*p*-(2-

carboxyethyl)phenylethylamino]-5'-*N*-ethylcarbamoyladenosine) to the recombinant $hA_{2A}AR$ was performed as previously described.[196,253]

### 5.2.1.3 Cyclic AMP Accumulation Assay

Intracellular levels of 3',5'-cyclic AMP were measured by the competitive protein binding method.[216] CHO cells expressing of medium/well. After 24 h the medium was removed and cells were washed three times with 1 mL/well of DMEM containing 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.4. Cells were then treated with recombinant human ARs[254] were harvested by trypsinization. After resuspension in the medium, cells were plated in 24-well plates in 0.5 mL agonists and/or test compounds in the presence of rolipram (10 mM) and adenosine deaminase (3 units/mL) and incubated at 37 °C. For the $A_3AR$, after 45 min forskolin (10 μM) was added to the medium, and incubation was continued for an additional 15 min. The reaction was terminated upon removal of the medium, and the cells were lysed with 200 μL/well of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at –20 °C. For determination of cyclic AMP p roduction, protein kinase A (PKA) was incubated with [$^3$H]cyclic AMP (2 nM) in $K_2HPO_4$/EDTA buffer ($K_2HPO_4$, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL of 0.1 M HCl. Bound radioactivity was separated by rapid filtration through Whatman GF/C filters under reduced pressure and washed once with cold buffer. Bound radioactivity was subsequently measured by scintillation spectrometry. Calculation of the relative maximal efficacy at the $A_3AR$ was determined at a fixed concentration of the nucleoside analogue (10 μM) and expressed as a relative percent of the effect of 10 μM NECA determined in each experiment, which typically reached ~50% inhibition of the forskolin stimulated cyclase.

### 5.2.1.4 [$^{35}$S]GTPγS binding assay

[$^{35}$S]GTPγS binding was measured by a variation of the method described.[255] Each assay tube consisted of 200 μL buffer containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 3 U/ml ADA, 0.2 nM [$^{35}$S]GTPγS, 0.004% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS), and 0.5% bovine serum albumin. Incubations were started upon addition of the membrane suspension (CHO cells stably expressing the native human $A_3$AR, 5 μg protein/tube) to the test tubes, and they were carried out in duplicate for 30 min at 25°C. The reaction was stopped by rapid filtration through Whatman GF/B filters, pre-soaked in 50 mM Tris HCl, 5 mM $MgCl_2$ (pH 7.4) containing 0.02% CHAPS. The filters were washed twice with 3 mL of the same buffer, and retained radioactivity was measured using liquid scintillation counting. Non-specific binding of [$^{35}$S]GTPγS was measured in the presence of 10 μM unlabelled GTPγS.

## 5.2.2 Binding Studies at the $P2Y_2$ Receptor

The binding studies at the $P2Y_2$ receptor are performed under the guidance of prof. dr. Kendall Harden at the Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA.

### 5.2.2.1 Assay of PLC Activity Stimulated by $P2Y_2$, $P2Y_4$, and $P2Y_6$ Receptors

Stable cell lines expressing the human $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptor in 1321N1 human astrocytoma cells were generated as described.[123] Agonist-induced [$^3$H]inositol phosphate production was measured in 1321N1 cells grown to confluence on 96-well plates. Twelve hours before the assay, the inositol lipid pool of the cells was radiolabeled by incubation in 200 µL of serum-free inositol-free Dulbecco's modified Eagle's medium, containing 0.4 µCi of *myo*-[$^3$H]inositol. No changes of medium were made subsequent to the addition of [$^3$H]inositol. On the day of the assay, cells were challenged with 50 µL of the five-fold concentrated solution of receptor agonists in 200 mM Hepes (N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid), pH 7.3, containing 50 mM LiCl for 20 min at 37 °C. Incubations were terminated by aspiration of the drug-containing medium and addition of 450 µL of ice-cold 50 mM formic acid. After 15 min at 4 °C, samples were neutralized with 150 µL of 150 mM $NH_4OH$. [$^3$H]Inositol phosphate accumulation was quantified using scintillation proximity assay methodology as previously described in detail.[256]

# 5.3 Molecular modeling and docking

## 5.3.1 Docking Studies of Compound 3.10

The docking studies of compound **3.10** are performed under the guidance of prof. dr. Kenneth A. Jacobson at the Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.

All calculations were performed on a Silicon Graphics Octane2 workstation (600 MHz IP30 processor, MIPS R14000). Compound **3.10** was constructed with the use of the Sketch Molecule of SYBYL 7.1.[257], A grid search was performed in which flexible bonds were rotated by 0° and 180° for t1 ($C_5$–$C_6$–$N^6$–CMe) at the $N^6$-position, t2 (4'O–4'C–5'C–5'OH) at the 5'-position, and t3 ($N_3$–$C_2$–$N_1$'–$N_2$') and by 60°, 180°, and –60° for t4 ($N_3$'–$C_4$'–$C_{Me}$–$C_{Cyc}$) and t5 ($C_4$'–$C_{Me}$–$C_{Cyc}$–$C_{Cyc}$) at the C2-position. The low-energy conformers from the grid search were reoptimized, removing all torsional constraints. Merck molecular force field (MMFF)[258] and charges were applied with the use of distance-dependent dielectric constants and the conjugate gradient method until the gradient reached 0.05 kcal·mol$^{-1}$·Å$^{-1}$. After clustering the low-energy conformers from the result of the grid search were clustered, the representative ones from all groups were reoptimized by semiempirical molecular orbital calculations with the PM3 method in the MOPAC 6.0 package.[259]

A human $A_3$AR model (PDB code 1OEA) constructed by homology to the X-raystructure of bovine rhodopsin with 2.8 Å resolution (PDB codeID 1F88)[222] was used for the docking study. All atom types were assigned by the Amber7_FF99 force field.[260] Amber charges for protein and MMFF charges for ligand were calculated. The starting geometry of the ligand conformation was chosen from the human $A_3$AR complex model with Cl-IB-MECA (**1.9**),[195] which was already validated by point mutation. The ribose binding position was fixed, using an atom-by-atom fitting method for the carbon atoms of the ribose ring. To determine the binding region of the 2-(4-cyclopentylmethyl-1,2,3-triazole) moiety at the adenine 2-position, the flexible bond defining a $\chi$1 (O–$C_1$'–$N_9$–$C_4$) angle was searched while docked within the putative binding cavity through various low-energy conformers with diverse t1–to

t5 angles, rotating by –60°, –110°, and –160°, assu ming an anti conformation. Several conformations without any steric bump were selected for further optimization. The initial structures of all complexes were optimized using the Amber force field with a fixed dielectric constant of 4.0 and a terminating gradient of 0.05 kcal·mol$^{-1}$·Å$^{-1}$.

## 5.3.2 Molecular Modeling of the $P2Y_2$ Receptor

The molecular modeling of the $P2Y_2$ receptor is performed under the guidance of prof. dr. Kenneth A. Jacobson at the Laboratory of Biological Modeling, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.

### 5.3.2.1 Molecular Modeling

The published molecular model of the human $P2Y_2$ receptor[1] was updated by insertion of a part of the C-terminal domain including H8 helix and the N-terminal domain. The $P2Y_2$ C-terminal domain (residues Gly310 – Met346) was modeled by homology to bovine rhodopsin. Based on the published sequence alignment[1], the backbone atoms of the TM domain of the $P2Y_2$ receptor were superimposed on the corresponding atoms of bovine rhodopsin (PDB ID = 1U19)[261] using Sybyl 7.1.[257] The rhodopsin TM domains and its hydrophilic loops (Ala26 - Met309) as well as Gly310 of the $P2Y_2$ receptor (last residue in the published model) subsequently were removed from the receptor structures. The remaining rhodopsin N-terminal (Met1 – Glu25) and C terminal (residues starting from Asn310) domains were connected to Gly22 and Ala309 of the $P2Y_2$ receptor, respectively. The residues of the C-terminal domain were replaced with the corresponding residues of the $P2Y_2$ receptor (residues Gly310 – Met346) using Sybyl 7.1. An attempt to apply the same technique for modeling of the NT domain failed due to overlap of the rhodopsin NT domain and the extracellular loops (EL) of the $P2Y_2$ receptor. For this reason, the configuration of the $P2Y_2$ receptor N-terminal domain (Met1 – Gly22) was predicted using the Loopy program from the Jackal package,[262] and one thousand initial conformations were generated. The option of energy minimization of the generated candidates was used. The $P2Y_2$ receptor model was minimized using Sybyl 7.1 in the Amber7 FF99 force field. The energy minimization of sidechains of the terminal domains initially was performed with constrained positions of all other atoms of the $P2Y_2$ receptor until an energy gradient lower than 0.05 $kcal \cdot mol^{-1} \cdot Å^{-1}$ was reached. The minimization was continued without any constraints in the terminal domains, but with constraint of the atoms of the TM helices and hydrophilic loops. The structure then was minimized fixing only the backbone atoms of the TM $\alpha$-helices until the energy gradient lower

than 0.05 kcal·mol$^{-1}$·Å$^{-1}$ was reached. Finally, an unconstrained energy minimization of the whole $P2Y_2$ receptor model was performed until an energy gradient was lower than 0.05 kcal·mol$^{-1}$·Å$^{-1}$. The formal geometry of the model was tested using the ProTable command of Sybyl 7.1 as well as the Procheck software.[151]

### 5.3.2.2 Molecular Dynamics Simulation of the $P2Y_2$ Receptor

The molecular dynamics (MD) simulation of the $P2Y_2$ receptor was performed on the Biowulf cluster at the NIH (Bethesda, MD) using CHARMM 32a2 software.[263] The protocol used for the system construction and MD simulation was described previously.[264],[265] The constructed system included the $P2Y_2$ receptor surrounded by 100 DOPC lipids, 5964 TIP3 water molecules, 107 Cl and $K^+$ ions, for a total of 37,242 atoms. The MD simulation was performed for 10 ns under conditions of CPT (constant pressure and temperature) as previously described.[151] A hexagonal unit cell (69.3_69.3_85Å) and hexagonal periodic boundary conditions in all directions were used. For the first 4.5 ns of MD simulation, nuclear Overhauser effect (NOE) restraints were applied within TM7 to the distances between each backbone carbonyl oxygen atom of residue *n*, and the backbone NH-group of the residue *n+4*. The restraints were applied to all residues of TM7 with the exception of prolines and residues before and after prolines. MD simulation performed without such restraints led to a disordered secondary structure of TM7. Similar findings were described previously for the $P2Y_6$ receptor.[151] After 4.5 ns the MD simulation was continued without any restraints, and the helical structure of the TM7 remained stable until the end of the simulation. The typical structure of the $P2Y_2$ receptor calculated from the last 100 ps of the MD trajectory was used as final model.

### 5.3.2.3 Manual Molecular Docking

The structure of UTP containing all hydrogen atoms initially was sketched using Sybyl 7.1. The ligand geometry was optimized in the Tripos force field with Gasteiger-Hückel atomic charges.[257] Available site-directed mutagenesis data and previously published results of molecular modeling[123,149,151] were utilized to manually preposition UTP, with an anti-conformation of the uracil ring and a (N)-conformation of the ribose ring, inside the putative binding site of the $P2Y_2$ receptor using the

DOCK command of the Sybyl 7.1 package.[257] The triphosphate chain of the ligand was placed between the R3.29, R6.55, and R7.39, while the nucleobase ring was located near Y1.39, Y2.53, and S7.43. The manual molecular docking procedure subsequently was performed in several stages. During the first stage of the molecular docking process, only the bonds of the ligand were flexible. When the most energetically favorable location and conformation of the ligand was found, the molecular docking procedure was repeated with flexible bonds of the receptor. During each iteration of the docking process the minimization of the binding site with the ligand inside was performed until a RMS of 0.01 $kcal \cdot mol^{-1} \cdot Å^{-1}$ was reached. Finally, the energy of the entire obtained protein-ligand complexes was minimized until an energy gradient lower than 0.01 $kcal \cdot mol^{-1} \cdot Å^{-1}$ was reached.

### 5.3.2.4 Conformational Analysis of UTP, ATP and Their Derivatives

The conformational analysis of the studied mononucleotides located in the putative binding site of the $P2Y_2$ receptor was performed using the Monte Carlo Multiple Minimum (MCMM) and the mixed torsional/low-mode sampling methods implemented in MacroModel 9.0 software.[266] MCMM calculations initially were performed for UTP and all residues located within 5Å of the ligand, using a shell of constrained atoms with a radius of 2Å. The following parameters were used: MMFFs force field; water as an implicit solvent; maximum of 1000 iterations of the Polak-Ribier Conjugate Gradient (PRCG) minimization method with a convergence threshold of 0.05 $kJ \cdot mol^{-1} \cdot Å^{-1}$; number of conformational search steps = 100; energy window for saving structures = 1000 $kJ \cdot mol^{-1}$. The ligand-receptor complex obtained after MCMM calculations was subjected to additional 100 steps of the mixed torsional/low-mode conformational search. The structure of UTP docked in the receptor subsequently was transformed to other studied UTP analogues by substitution of functionalities, and MCMM calculations were performed for each analogue, setting the number of steps to 100 and the energy window for saving structures to 100 $kJ \cdot mol^{-1}$

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