Doctoral Thesis

Structure-based design, synthesis, and in vitro evaluation of bisubstrate inhibitors for catechol O-methyltransferase (COMT)

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STRUCTURE-BASED DESIGN, SYNTHESIS, AND IN VITRO EVALUATION OF BISUBSTRATE INHIBITORS FOR CATECHOL O-METHYLTRANSFERASE (COMT)

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
DOCTOR OF NATURAL SCIENCES

Presented by

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Zurich 2000
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ABSTRACT

Inhibition of the enzyme catechol O-methyltransferase (COMT) is an important approach for developing new therapeutic treatments for Parkinson's disease. COMT is a well studied enzyme. It catalyzes methyl group transfer from the cofactor S-adenosylmethionine (SAM) to the hydroxy group of catechol-type substrates such as dopamine. Its biological function in plants, animals, and human beings has been elucidated to a good extent, and the kinetics of the mechanism of methylation have been studied in detail. X-ray crystal structures have been solved for recombinant COMT in the apo form and cocrystallized with SAM, Mg^{2+} ions, and 3,5-dinitrocatechol.

In this thesis, we designed and synthesized a new class of COMT inhibitors which are bisubstrate inhibitors according to their inhibition mechanism. Previously, only single-substrate analog inhibitors of COMT were known. The lead structure 7 was developed employing computer-aided structure-based design. A synthetic pathway was developed for 7 that allows facile introduction of different chemical functionalities into the molecule. A key step in the synthesis was the nucleosidation reaction for the coupling of natural and modified bases to the ribose moiety in a regio- and stereoselective fashion. In vitro kinetic studies on the inhibitory activity of 7 were performed revealing that 7 occupies both the substrate and cofactor binding sites with $K_i$ values of $0.55 \pm 0.1 \, \mu M$ and $0.3 \pm 0.1 \, \mu M$, respectively. It exhibits competitive kinetics with regard to SAM and noncompetitive kinetics with regard to catechol substrates. For comparison, the same kinetic studies were carried out on catechol 14. It shows a competitive inhibition mechanism for the catechol binding site and uncompetitive inhibition of the cofactor binding site as would be expected for a derivative of benzene-1,2-diol which does not bind to the SAM binding site. Cocrystallization of the bisubstrate inhibitor 7 and COMT is currently underway to further clarify the exact complexation mode.
N-Succinimidyl 2,3-dihydroxy-5-nitrobenzoate 8, a precursor in the synthesis of 7 exhibited a surprisingly high inhibitory activity. Therefore, its inhibition mechanism was determined and found to be competitive with the substrate for an incubation time of one minute with COMT and noncompetitive with the substrate after 15 min preincubation of 8 with the enzyme. Such an inhibition mechanism was observed with tight-binding inhibitors like Tolcapone (Tasmar®), which is a pharmaceutical used in the therapy of Parkinson’s disease. In this respect, the N-succinimidyl functionality in 8 seems to be a good replacement for the ortho nitro group in catecholic inhibitors combined with an electron-withdrawing group in para position. If stable analogs of 8 exhibited the same inhibitory activity, these compounds could be interesting follow-ups for Tolcapone.

Structural variations were introduced into lead 7 to determine how much each chemical functionality contributes to its binding affinity for COMT, and to investigate different subunits for the binding sites, which were designed by molecular modeling, in order to enhance the inhibitory potency of the lead. We synthesized and tested two molecules (69 and 70), containing pyrimidine bases, with regard to their binding in the base pocket. These molecules were more than 100-fold less active than the lead 7. The molecules 107, 108, and 109 with alkanediyl and 3-azaalkanediyl chains between adenine and catechol rather than ribose which is a subunit of 7 were synthesized to study the structural influence of the ribose and its function as a H-bond donor. Compounds 107, 108, and 109 were found to be 25-50
times less active than the lead. To investigate whether the catechol moiety in the lead 7 coordinates to the Mg$^{2+}$ ion, compound 88, which does not contain a nitro group on the catechol moiety, was synthesized. It exhibited a more than 100-fold decrease in inhibitory activity, confirming that the lead does bind to the Mg$^{2+}$ ion. In order to study the influence of the length of the connecting bridge between ribose and catechol, molecule 117 was synthesized, which lacks one oxygen atom between ribose and catechol as compared to 7, and found to be about six times more potent than the original lead. Inhibitor 117 exhibits a more rigid scaffold than the lead preventing a collapse of the aromatic side chains, which is favorable for binding to the enzyme in terms of entropy. NOE conformational studies on 7, 69, and 117 in solution revealed that in all three molecules the bases have the anti-conformation which is favorable for binding to the base pocket. In compound 69 a hydrophobic collapse of the two terminal chromophores is observed and reorganizational energy is required for binding, which accounts for the decrease in binding affinity of 69 compared to 7 and 117. In vitro assays were carried out on all compounds, and kinetic studies were performed to gain insight into the inhibition mechanisms and structure-activity relationships.
ZUSAMMENFASSUNG

Inhibitoren des Enzyms Catechol-O-methyltransferase (COMT) werden als Pharmazeutika gegen die parkinsonsche Krankheit eingesetzt.


In dieser Arbeit beschreiben wir den Entwurf und die Synthese von neuartigen Inhibitoren von COMT, welche nach ihrem Bindungsmechanismus an das Enzym als Bissubstratinhibitoren bezeichnet werden können. Die bekannten, im nanomolaren Bereich an das Enzym bindenden Inhibitoren sind Catecholanaloge. Die Leitstruktur 7 wurde mit Hilfe von Computer-gestütztem, stukturbasierten Design gefunden. Der für 7 ausgearbeitete Syntheseweg ermöglicht es, auch andere chemische Funktionalitäten in das Molekül einzuführen. Ein Schlüsselschritt in der Synthese war die Nukleosidierungsreaktion, mit der verschiedene natürliche und modifizierte Basen regio- und stereoselektiv mit der Ribosennucleoideinheit verknüpft wurden. In vitro durchgeführte kinetische Studien mit der Verbindung 7 zeigten, dass diese die Substrat- und die Kofaktorbindestelle im Enzym besetzt, wobei $K_I$ Werte von $0.55\pm0.1 \mu M$, bzw. $0.3\pm0.1 \mu M$ gemessen wurden. Im Vergleich dazu ergab sich in analogen Kinetikexperimenten mit der catecholischen Vergleichsverbindung 14, dass diese kompetitiv zum Substrat an die catecholische Bindestasche bindet und unkompetitiv zum Kofaktor SAM ist. Dieses Ergebnis entspricht den Erwartungen für eine substratanaloge, catecholische Verbindung, die nicht in die Kofaktorbindetasche bindet. Um zusätzliche Informationen über die genauen Wechselwirkungen zwischen 7 und den Aminosäuren im aktiven Zentrum von COMT zu erhalten, werden derzeit Versuche zur Kokristallisation der Verbindung mit rekombinantem COMT unternommen.

Im folgenden wurden Strukturvarianten der Leitstruktur 7 synthetisiert, um abschätzen zu können, wie viel jeder einzelne Teil der Verbindung zur Inhibitorstärke beiträgt und um einen Einblick zu bekommen, welche anderen Struktureinheiten in die jeweiligen Bindetaschen passen, um so die Aktivität der Leitstruktur zu verbessern. Es wurden zwei Moleküle 69 und 70 synthetisiert, welche Pyrimidinbasen enthalten. Diese Moleküle waren 100 mal weniger aktiv als die Leitstruktur 7. Die Verbindungen 107, 108 und 109, die Alkandiyl- und 3-Azaalkandiylketten zwischen Adenin und Ribose enthalten, wurden synthetisiert, um den strukturellen Einfluss der Ribose und ihre Bedeutung als Wasserstoffbrückenbildner im Hemmer 7 zu

Um den Einfluss der "Brückenlänge" für das Verbindungsstück zwischen Ribose und Catechol zu studieren, haben wir die Verbindung 117 synthetisiert, die wegen des fehlenden Sauerstoffs eine im Vergleich zu 7 kürzere Brücke hat. Wie sich in den Enzymtests zeigte, ist 117 sechs mal aktiver als die Leitstruktur 7. Wegen der kürzeren Brückenlänge verliert 117 konformationelle Freiheitsgrade gegenüber 7, weil das Zusammenfalten der aromatischen Seitenarme verhindert wird, d.h. bei der Bindung an das Enzym ist der Entropieverlust geringer. Die Verbindungen 7, 69 und 117 wurden mit Hilfe von NOE spektroskopischen Untersuchungen auf ihre Konformation in Lösung untersucht. In allen drei Molekülen ist die Base in der richtigen anti Orientierung (anti bzgl. der Ribose), um in die Basentasche zu binden entsprechend den "Modelling" Voraussagen. Beim Molekül 69 wurde ein hydrophober Kollaps gefunden, d.h. ein Zusammenfalten der aromatischen Seitenarme, so dass der freie Zustand des Moleküls stabilisiert ist und zusätzlich Energie aufgewandt werden muss, um das Molekül in eine für die Bindung an das Enzym günstige Konformation zu bringen, welches ein Grund für die geringe Affinität dieser Verbindung zum Enzym sein könnte verglichen mit 7 und 117. Mit allen Verbindungen wurden in vitro Enzymtests durchgeführt und zum Teil wurde die Inhibitionskinetik bestimmt, um mehr über den Inhibitionsmechanismus und die Struktur-Aktivitäts-Beziehungen für Bisubstratinhibitoren des Typs 7 zu erfahren.
ZUSAMMENFASSUNG

**69**

IC$_{50}$ (ohne Präinkubation) = 522 μM

**70**

IC$_{50}$ (ohne Präinkubation) > 100 μM

**117**

IC$_{50}$ (ohne Präinkubation) = 600 nM

**88**

IC$_{50}$ (ohne Präinkubation) > 100 μM

<table>
<thead>
<tr>
<th>X</th>
<th>a</th>
<th>IC$_{50}$</th>
</tr>
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<tbody>
<tr>
<td>CH$_2$</td>
<td>1</td>
<td>55 μM</td>
</tr>
<tr>
<td>NH</td>
<td>1</td>
<td>77 μM</td>
</tr>
<tr>
<td>NH</td>
<td>2</td>
<td>86 μM</td>
</tr>
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1 GENERAL BACKGROUND

1.1 THERAPY FOR PARKINSON'S DISEASE

Inhibition of catechol O-methyltransferase (COMT) is an important approach for developing new therapeutic treatments of Parkinson's disease. Today about 5 million people suffer from Parkinson's disease. The symptoms of the disease are a consequence of reduced levels of dopamine in the brain due to degeneration of the dopaminergic neurons (brain cells producing dopamine). The current therapy consists of an artificial increase of the dopamine concentration by oral administration of the amino acid L-DOPA. In the brain, L-DOPA is decarboxylated by the enzyme aromatic amino acid decarboxylase (AAD) to give dopamine. To avoid the degradation of L-DOPA in the periphery, it is given in conjunction with an inhibitor of AAD. Although L-DOPA can cross the blood brain barrier,[1] dopamine and AAD inhibitors cannot. When AAD is inhibited, deactivation by COMT becomes the major metabolic pathway for L-DOPA in the periphery. COMT catalyzes the methylation of L-DOPA to 3-O-methoxydopa (3-OMD) (Scheme 1). Inhibition of COMT reduces the peripheral degradation of L-DOPA and increases its supply into the brain (Figure 1).[2]

Dopamine is further catabolized by the enzyme monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetic acid (DOPAC) and by COMT to 3-methoxytyramine (3-MT). DOPAC is methylated by COMT to homovanillic acid (HVA). Inhibition of the enzymes AAD and COMT therefore lowers the concentration of these metabolites in the periphery, which cause side effects in Parkinsonian patients treated with L-DOPA only.
Figure 1. L-DOPA is decarboxylated by AAD to give dopamine. COMT catalyzes the methylation of L-DOPA to 3-OMD. a) Orally administered L-DOPA is metabolized in the periphery and the blood circulation by the enzymes AAD and COMT. Only 1% of the L-DOPA reaches the brain. b) If the degradation of L-DOPA by AAD in the periphery and in the blood circulation is inhibited, 10% of the L-DOPA reaches the brain. c) If L-DOPA is given with inhibitors of AAD and COMT, about 30% of the orally administered L-DOPA is supplied to the brain.
1.2 THE ENZYME CATECHOL O-METHYLTRANSFERASE (COMT)

1.2.1 The natural function of COMT

COMT is an ubiquitous enzyme which occurs in various plants, microorganisms, and animals. The sequences of COMT enzymes from different mammalian species are highly conserved. The soluble human COMT is 81% identical to the respective rat enzyme. COMT is largely involved in the catabolism of L-DOPA, catecholamines (dopamine, noradrenaline, and adrenaline), and their hydroxylated metabolites such as DOPAC. It is distributed throughout various peripheral organs, with the highest activities in liver and kidney, and is also present in central nervous system (CNS) neurons and glial cells.[2-5]

COMT catalyzes a major deactivation pathway for catechol-based neurotransmitters such as dopamine. After release, the main catabolic processes terminating the signal transfer by dopamine between the synaptic clefts of nerve cells
include uptake into nerve terminals (presynaptic membrane) in the peripheral and central nervous system (uptake 1), or uptake into neuronal cells (postsynaptic membrane) and non-neuronal cells (uptake 2), and subsequent deamination by the enzyme monoamine oxidase (MAO) and/or O-methylation by COMT. The specificity of uptake 1 for dopamine over the catecholamines norepinephrine and epinephrine is higher by a factor of 833 than that of uptake 2.\textsuperscript{[6]} On the other hand, the capacity of the latter is 100 times higher compared to uptake 1.\textsuperscript{[2,7]} In this respect, COMT inhibitors which cross the blood brain barrier might further reduce the degradation of dopamine in the brain.

1.2.2 The kinetic reaction mechanism for the methylation of catechols by COMT

Methyltransferases are enzymes that catalyze the transmethylation reactions involving the transfer of the S-methyl group of S-adenosyl-L-methionine (SAM) to nitrogen, oxygen, or carbon atoms of a wide variety of nucleophiles. Methyltransferases can modify DNA,\textsuperscript{[8]} RNA, proteins, and small molecules such as catechol.

COMT is a Mg\textsuperscript{2+}-dependent enzyme which catalyzes the transfer of the methyl group from SAM to a hydroxy group of a catecholic substrate. From kinetic studies,\textsuperscript{[7,9]} computational studies,\textsuperscript{[10,11]} and the X-ray crystal structures\textsuperscript{[12,13]} it is known that the cofactor SAM binds to the active site of COMT first. The Mg\textsuperscript{2+} ion binds to the enzyme only after SAM. Both SAM and the Mg\textsuperscript{2+} ion organize the active site for the binding of the catechol substrate, which binds last.\textsuperscript{[14]} For the methylation to proceed, the catechol must first be deprotonated. It is proposed in the literature that the side chain NH\textsubscript{2} group of Lys 144 acts as the base to abstract the proton from the catechol.\textsuperscript{[9]} The transfer of the methyl group is the rate determining step of the reaction, with an S\textsubscript{N}2-like transition state in which the methyl fragment is located symmetrically and tightly between the leaving group and the nucleophile.\textsuperscript{[15]} Of the two hydroxy groups in substituted catechols, methylation occurs preferentially on the hydroxy group meta to the third substituent. The orientation of the catechol substrates in the active site is mainly determined by steric factors: the methylation of the para hydroxy group would cause the third substituent to orient in an unfavorable manner within the
After the reaction, the methylated catechol leaves the active site first and S-adenosylhomocysteine (SAH) dissociates last (Scheme 2).

\[ \text{COO} - \text{NH}_3^+ + \text{B (Lys)} \rightarrow \text{H}_3\text{cqpH}^+ + \text{Q}^+ \]

\[ \text{R} \]

Scheme 2. SAM, a Mg$^{2+}$ ion, and the catechol substrate bind to the active site of COMT. The transfer of the methyl group from SAM to the catechol is an $S_N$2-type reaction.

1.2.3 Soluble and membrane-bound COMT

COMT exists in two forms: a soluble cytoplasmatic form (S-COMT) and a membrane-bound (MB-COMT) form. In humans, 90% of the peripheral COMT is S-COMT, which contains 221 amino acids and has a molecular weight of about 24.7 kD. MB-COMT has a molecular weight of 26 kD and is located on the rough endoplasmatic reticulum. MB-COMT might also be localized on postsynaptic membranes. The MB form differs from the soluble form by an N-terminal stretch of 50 (human) or 43 (rat) amino acids containing the membrane anchor domain. Both COMT isoforms are encoded by the same gene (human chromosome 22q11.2) and are thought to derive from initiation of transcription at two alternative sites. The MB-COMT displays a higher affinity for the substrates but a lower capacity than the soluble form: $K_M$ (MB-COMT for SAM) = 3.9 $\mu$M; $K_M$ (S-COMT for SAM) = 23 $\mu$M; $K_M$ (MB-COMT for dopamine) = 3.3 $\mu$M; $K_M$ (S-COMT for dopamine) =
280 μM; $V_{\text{max}}$ (S-COMT in skeletal muscle) = 50 pmol/min/mg protein; $V_{\text{max}}$ (S-COMT in liver) = 14690 pmol/min/mg protein; $V_{\text{max}}$ (MB-COMT) = 2-4 pmol/min/mg protein.[2] The same reaction mechanism is reported for S-COMT and MB-COMT.[14]

### 1.2.4 Current inhibitors of COMT

For decades, medicinal chemists in both industry and academia have searched for inhibitors of the catechol binding site of COMT.[19-27] Two inhibitors containing a 3-nitrocatechol group as the central structural unit, Tolcapone (Tasmar®)[21,28,29] and Entacapone (Comtan®)[30], have recently been introduced to the market.

All compounds exhibiting inhibitory activity in the submicromolar range contain a 3-nitrocatechol group. The catechol motif provides the crucial recognition element between the enzyme and natural substrates such as dopamine. The nitro group ortho to one of the hydroxy groups in the catechol lowers the $pK_A$ of this hydroxy group to a value of 3-4 and thus it is deprotonated under physiological conditions. The less acidic hydroxy group in the 3-nitrocatechols makes a hydrogen bond to the carboxy group of the side chain of Glu199 (Figure 2). Therefore, the more acidic hydroxy group is pointing towards SAM (inhibitor orientation). However, the acidic hydroxy group cannot be methylated by SAM, because of the stabilization of the oxyanion due to charge delocalization and the resulting reduced nucleophilicity. In this substrate orientation the less acidic hydroxy group of the 3-nitrocatechol is pointing at the S-methyl group of SAM and methylation occurs. This orientation is less favorable for 3-nitrocatechols, because the deprotonated hydroxy group cannot make a hydrogen bond to Glu199. The disadvantages of such inhibitors, limiting their use in Parkinson therapy, is a relatively fast metabolism.

Analogs of SAM have also been synthesized and their inhibitory activity on COMT studied, although to date no inhibitors with a $K_i < 10 \mu M$ have been found.[31-35] Of course, in this approach enzyme selectivity would be a major issue, since the cofactor SAM exhibits a multitude of roles in the body.
Tolcapone (Tasmar®); IC₅₀ = 36 nM
Entacapone (Comtan®); IC₅₀ = 18 nM

a) Inhibitor orientation of the catechol:

\[
\begin{align*}
\text{Mg}^{2+} & \quad \text{OH} \quad \text{R} \\
\text{O}_2\text{N} & \quad \text{O} \\
\text{H} & \quad \text{O} \\
\text{Glu199} & \\
\text{R} & \quad +\text{SAM}
\end{align*}
\]

b) Substrate orientation of the catechol:

\[
\begin{align*}
\text{Mg}^{2+} & \quad \text{OH} \quad \text{R} \\
\text{O}_2\text{N} & \quad \text{O} \\
\text{H} & \quad \text{O} \\
\text{SAM} \quad \text{SAH} & \\
\text{R} & \quad +\text{SAM} \quad -\text{SAH} \\
\text{H}_3\text{CO} & \quad \text{O} \\
\text{R} & \quad \text{NO}_2
\end{align*}
\]

Figure 2. Tolcapone and Entacapone are substrate analog inhibitors. The orientation of the inhibitors in the active site of COMT is crucial for the mode of inhibition. Two orientations are possible. a) Inhibitor orientation: the deprotonated hydroxy group is oriented towards the S-methyl group of SAM and methylation is very slow in view of the reduced nucleophilicity of the phenoxide anion. b) Substrate orientation: the less acidic hydroxy group is pointing at the S-methyl group of SAM and methylation occurs.
1.2.5 The X-ray crystal structure of COMT cocrystallized with 3,5-dinitrocatechol and SAM

In 1994, the X-ray crystal structure of recombinant rat liver COMT cocrystallized with 3,5-dinitrocatechol, SAM, and a Mg$^{2+}$ ion was solved to 2.0 Å resolution (Figure 3).[12,13]

![Figure 3](image)

Figure 3. Connolly surface[16] (the surface of the enzyme which can be approached by water molecules) of the 3,5-dinitrocatechol-SAM-COMT complex as seen in the X-ray crystal structure.[12]

COMT is a monomer and consists of eight α-helices and seven β-strands and has a typical α/β fold (Figure 4). The helices α4–α8 and the parallel β-strands β1–β5 are very similar to the nucleotide binding motif (the Rossmann fold) found in alcohol dehydrogenase (ADH).[8] The adenosine of SAM superimposes well on the adenosine moiety of NAD$^+$ in ADH. The last residue of β1 in COMT is Gly66,
which corresponds to a conserved glycine residue in all dehydrogenases (Gly199 in ADH). The last residue in the β2-strand is Glu90, which binds to the hydroxy groups of the ribose unit in SAM. In dehydrogenases, this acidic residue is conserved (Asp223 in ADH). The C-terminal part of the COMT structure consists of two antiparallel β-strands (β6 and β7).

![Figure 4. Topological diagram of secondary structure elements of COMT. The position of the bound SAM (A = adenine, M = methionine) is shown.][13]

The active site of COMT includes the coenzyme binding pocket and the catalytic site situated in the vicinity of the Mg$^{2+}$ ion. It is built up by residues from the amino terminal helices in the nucleotide-binding fold to residues from the C-terminal β-strands (β6 and β7) in the loop region for binding of the catechol.

In Figure 3, it can be seen that the cofactor binding site is not open in the bound state, but almost completely covered by amino acid residues of the enzyme. The catechol binding site on the other hand is readily accessible. From the kinetic studies[14] it is known that the cofactor binds to the active site before the catechol. This is only possible, if the binding pocket of the cofactor is accessible in the apoenzyme, and it can be concluded that binding of the cofactor induces conformational changes in the apoenzyme.

Another important piece of information which is revealed by the X-ray crystal structure is that binding of SAM occurs independently from binding of the Mg$^{2+}$ ion. SAM does not make any contacts to the Mg$^{2+}$ ion.
1.3 THE CONCEPT OF MULTISUBSTRATE INHIBITORS

Multisubstrate analog inhibitors (MAI) offer opportunities for specificity not available to single substrate analog inhibitors. The combination of two or more substrates required by the target enzyme into a single molecule makes it likely that neither component will be recognized by other enzymes using either substrate, so that a very high order of enzyme specificity may be expected. Essentially, any enzymatic reaction in which two or more substrates (cofactors are considered to be substrates in this context) are simultaneously bound to the enzyme is a candidate for the design of MAI. This includes methyl, formyl, and acetyl transferases, dehydrogenases, hydroxylases, kinases, and various synthetic enzymes.[37]

1.3.1 Examples for bisubstrate inhibitors

As the first designed MAI to reach clinical trials, phosphonoacetyl-L-aspartate (PALA) should be mentioned. PALA was designed to inhibit carbamoyl transfer from carbamoyl phosphate to L-aspartate to give N-carbamoyl-L-aspartate. PALA binds to L-aspartate carbamoyl transferase some 3 orders of magnitude more tightly than carbamoyl phosphate, the more tightly bound of the two substrates. Based on the observed kinetics of binding, which were competitive *versus* carbamoyl phosphate and noncompetitive against aspartate, ordered substrate binding with carbamoyl phosphate binding first was proposed. It was further proposed that a major conformational change took place in the enzyme upon interaction with PALA or during normal transcarbamoylation (Figure 5).[38,39]
A great deal of attention has been paid in the 1980s to the SAM dependent methyl transferases. These carry out a large variety of specific heteroatom methylations of substrates, ranging from small molecules such as catechols and indoles to macromolecules such as mRNA. Because these reactions are thought to proceed through a colinear $S_N2$ transition state with the nucleophile attacking the sulfonium-bound methyl group, they have provided the impetus for a number of studies on MAI design and synthesis. Bisubstrate inhibitors of enzymes such as spermidine synthase, spermine synthase, farnesyl transferase, fucosyltransferase, vaccinia RNA guanine 7-methyltransferase and indole $N$-methyltransferase have been shown to be highly selective inhibitors with better affinities than those mimicking only one substrate (Figure 6).
Figure 6. Examples of multisubstrate inhibitors. Transition state structure A and multisubstrate adduct B for a) vaccinia RNA guanine 7-methyltransferase\cite{46} b) indole N-methyltransferase\cite{47} c) spermine synthase\cite{42,43}
1.3.2 Bisubstrate inhibitors of COMT

Reports from the Coward group have described MAI approaches to bisubstrate inhibitors of COMT. Coward demonstrated that the sulfonium derivatives 1 and 2 were weak inhibitors in the mM range. The corresponding uncharged thioethers 3 and 4 were not inhibitory.[48] Lever and colleagues synthesized some simpler catechol derivatives (5 and 6) and found them to be essentially devoid of inhibitory activity, regardless of whether a sulfonium ion or a thioether linkage was present (Figure 7).[49] No bisubstrate inhibitor of COMT in the submicromolar range has been reported.[48-50]

It seems likely that conformational changes in enzymes will frequently be required in multisubstrate reactions, especially when ordered binding is observed. This phenomenon is often ignored in the design of MAIs. Since conformational changes seem to be triggered by strong, frequently ionic interactions between the substrate and the active site, failure to consider the role of such interactions may account for the relatively weak inhibitory properties of many putative MAIs.

\[
\begin{align*}
1 & : & \text{K}_i = 5.8 \text{ mM} & & \text{1 and 2 are weak inhibitors in the mM range.}\[
3 & : & \text{inactive} & & \text{3 and 4 are not inhibitory.}\[
5 & : & \text{n = 2, inactive} & & \text{5 and 6 are essentially devoid of inhibitory activity.}\[
6 & : & \text{n = 2, inactive} & & \text{No bisubstrate inhibitor of COMT in the submicromolar range has been reported.}\[
2 & : & \text{K}_i = 0.8 \text{ mM} & & \text{2 is a lead structure reported.}\[
4 & : & \text{inactive} & & \text{4 is inactive.}\[
\end{align*}
\]

Figure 7. In the 1980s, approaches towards bisubstrate inhibitors of COMT were reported. However, no lead structure was discovered.[48-50]
The determination of the X-ray crystal structure of COMT as a complex with SAM, 3,5-dinitrocatechol, and a Mg$^{2+}$ ion opened the possibility for the rational design of bisubstrate inhibitors.

1.4 RATIONAL DESIGN OF ENZYME INHIBITORS

1.4.1 The role of rational drug design in the drug discovery process

The methods that are used in the drug discovery process are becoming more and more manifold. Whereas the first drug discoveries were serendipitous findings, today a vast array of methods is at scientists' disposal:

- Gene technology for the identification of new targets and the production of human proteins for testing and structural analysis.
- Combinatorial chemistry for the synthesis of large numbers of compounds for lead structure search and optimization.
- High-throughput screening for the rapid identification of new leads from large in-house, external or combinatorial libraries.
- X-ray crystallography and NMR spectroscopic analysis for the determination of protein 3D structures and the identification of ligands.
- Computational chemistry for the calculation and modeling of molecular properties.
- Structure-based and computer-aided drug design for the search for new leads and their rational optimization.

A crucial decision for scientists in drug discovery, therefore, is the choice of the appropriate methods depending on the problem and on available information.

Rational, structure-based design became a method in drug research when more and more X-ray protein structures were solved, and computer programs based on force field methods were developed for visualization and calculation of protein-ligand interactions.
interactions. The success of computer-assisted, structure-based design of enzyme inhibitors\cite{60,62,63} depends on many parameters such as the quality of the X-ray crystal structure analysis, the conformational homogeneity of the enzyme active site, and a detailed knowledge of the catalytic mechanism.\cite{58}

The following Sections provide examples of the application of structure-based design in drug research and describe some of the computational methods that are used nowadays for the identification of hit and lead structures and their optimization.

### 1.4.2 Protein 3D structure-based design

The anti-glaucoma agent Dorzolamide (Merck & Co), introduced to the market in 1995, was the first drug in human therapy that resulted partly from a protein 3D structure-based design (Figure 8).\cite{59}

![Figure 8](image)

**Figure 8.** Benzene and thiophene sulfonamides are moderately active inhibitors of carbonic anhydrase. Acetazolamide has only systemic activity. Dorzolamide, being about three thousand times more active than the original lead, resulted from structure-based design. It is topically applied in the form of eye drops.

Also, the development of the HIV inhibitors Saquinavir (Hoffmann-La Roche), Indinavir (Merck & Co), Ritonavir (Abbot Laboratories), and Nelfinavir (Agouron Pharmaceuticals) was assisted by structure-based design.\cite{62,64} These were introduced into human therapy in the years 1995-1997.

Neuraminidase inhibitors were developed for the treatment of influenza. Analysis of the 3D structure of the complex of neuraminidase with the weak inhibitor Neu5Ac2en ($K_i = 1 \mu M$) showed that the introduction of a guanidino group into the 4-position should enhance inhibitory activity. This was indeed the case;
Zanamivir (Monash University) (Figure 9) is not only highly active in vitro \( (K_i = 0.1 \text{ nM}) \) but also systemically available after nasal application.\(^{[65,66]} \) The drug is now on the market (Relenza®, Glaxo-Wellcome). Aromatic analogs of Zanamivir suggested that removal or replacement of the glycerol side chain could yield active analogs.\(^{[67]} \) Synthesis of a carboxylic Neu5Ac2en analog with a branched alkoxy residue produced the nanomolar neuraminidase inhibitor GS 4071 (IC\(_{50} = 1 \text{ nM})\); its orally active prodrug GS 4104 (Tamiflu®) was also introduced to the market recently (Gilead Sciences/Hoffmann-La Roche) (Figure 9).\(^{[68]} \) Due to its bioavailability, sales of Tamiflu have rapidly surpassed those of Zanamivir in the US despite later introduction to the market.

![Design of the anti-influenza drugs Zanamivir (Relenza®) and GS 4104 (Tamiflu®).](image)

Protein 3D structure-based approaches have been applied in the design and optimization of inhibitors for various enzymes, e.g. aldose reductase, carbonic anhydrase, cysteine protease, dihydrofolate reductase, elastase, factor Xa, HIV protease, β-lactamase, matrix metalloprotease, neuraminidase (sialidase), protein kinase, purine nucleoside phosphorylase, renin, reverse transcriptase, thrombin, and
1.4.2.1 Design of a lead structure and optimization by structure-based design

For the design of a lead structure for an enzyme inhibitor, the X-ray crystal structure of the enzyme is needed.[53] Usually, the enzyme is co-crystallized with a substrate analog and required cofactors (Figure 10). Sometimes, the X-ray crystal structure of the apoenzyme is also available. An NMR structure of the enzyme is also very useful, because it shows the protein conformation in aqueous solution, which is the natural state of the enzyme.[54,55,72] For some enzymes, the conformation is different in the crystal compared to that in solution or in the apoenzyme.[73] Because these conformational changes mostly occur at the surface of an enzyme, where the active site is often located, it is very likely that the active site and hence the binding of an inhibitor will be affected.[74] Thus, the more structural information available, the better the rational design approach.[75]

Generally, the X-ray crystal structure of the active site is analyzed for binding interactions between the amino acid residues of the enzyme and the bound substrate analog and cofactor. Such interactions are electrostatic in nature and are described in Section 1.4.2.2.

Next, the co-crystallized ligands are virtually removed from the active site, and new scaffolds which are complementary to the charge distribution, size, and shape of the active site are fitted. This fitting process is usually done with the help of computer programs. Some programs do the analysis of the active site and the fitting process for new ligands automatically, picking molecular structures from a data base, and refining them in the active site.[76,77] The results of such a search are a few "good" scaffolds, and it is up to the eye of the chemist to validate the results, and to decide whether it is worth synthesizing a particular molecule. While the limits of such programs are defined by the data bases they access, they are useful for generating ideas for new structures, which can be further modified by the chemist. Other programs leave it up to the creativity of the chemist to find proper hit structures complementary to the active site. In that case, the program evaluates the proposed structure by calculating its optimal position in the active site according to electrostatic interactions between the amino acid residues of the active site and the
molecule.\textsuperscript{78,79} Sometimes, a hit from high-throughput screening that can be fitted into the active site can be further improved to a lead structure by molecular modeling. All these programs offer the possibility of visualizing the 3D structure of the enzyme which cannot be achieved by a CPK or other type of molecular model. The computer simulations which can be used for the modeling process are described in Section 1.4.2.3.

\textbf{structural information:} X-ray, NMR  
\textbf{screening hits:} natural compounds, compound libraries  

\textbf{rational design of a lead structure:}  
analysis of the active site  
molecular modeling to generate a synthesizable potential inhibitor  

\textbf{synthesis of potential inhibitors}  

\textbf{biological testing for inhibitory activity}  

lead  
\textbf{cocrystallization} with the enzyme

\textbf{lead optimization:}  
high affinity  
low toxicity  
bioavailability  
metabolic stability

\textbf{Figure 10.} Schematic outline of the steps in the rational design process.
Of all the criteria that need to be fulfilled for a compound to be useful as a pharmaceutical (like synthesizability, low degree of toxicity, metabolic stability and bioavailability), only synthesizability really applies at this stage. If the structure that looks most promising in the modeling is synthesized and has a decent inhibitory activity, it could ideally be cocrystallized with the enzyme to compare the real X-ray crystal structure with the modeling hypothesis. Based on this new information, the lead can be further optimized towards higher affinity for the enzyme and/or for criteria like toxicity, metabolic stability, and bioavailability (Figure 10).

There are still many unsolved problems in structure-based computer-aided drug design. Our knowledge of the effects of solvation and desolvation of the ligands and the enzyme does not allow us to estimate the strength of newly formed intermolecular hydrogen bonds. The same applies to entropy changes due to the freezing of conformational degrees of freedom in the inhibitor and in the enzyme and to the release of water molecules at hydrophobic sites of the protein surface. While different approaches have been developed for the estimation of binding energies of protein ligands, minor changes of the chemical structure of a ligand may change its binding mode, and even in favorable cases, where high-resolution protein 3D structures are available, surprising results are sometimes obtained. After all, it is not yet possible to make a reliable prediction about conformational changes in the protein. In the discipline of supramolecular chemistry one aims at making models of enzymes to study the host-guest interactions in smaller systems, an approach which contributes to improving applicability and reliability of computer-aided drug design.

1.4.2.2 Molecular recognition in enzymes

The weak non-covalent electrostatic forces existing between molecules with a nonhomogeneous charge distribution are responsible for the binding of a ligand to an enzyme. Besides electronic complementarity, the ligand must be sterically complementary to the shape of the active site. Emil Fischer postulated this 'lock and key' principle more than 100 years ago. Although they are all electrostatic in nature, one differentiates between the following categories of binding forces between molecules:
Coulombic interactions:

These interactions occur between two oppositely charged ionic groups.[94-96] Positively charged groups in proteins include the guanidinium side-chain of arginine, the ammonium group in the side-chain of lysine, and depending on the pH of the microenvironment, sometimes the imidazolium group in the side-chain of histidine; negatively charged groups include the carboxylate groups in the side-chains of aspartate and glutamate. These groups can make salt bridges to charged groups in the ligand. The energy of Coulombic forces is inversely proportional to the distance between the charges (1/r) and to the dielectric constant of the surrounding medium (1/ε). Typically, such ionic interactions are worth 1.25 ± 0.25 kcal/mol in energy depending on the microenvironment.[97-99] If one of the interacting groups is a dipole, the attractive forces are lower in energy and more strongly distance-dependent (1/r^4). An example of a dipole is the π-face of an aromatic ring which is negatively charged and therefore attracts cations.[100] If both of the charges are dipoles, the attractive forces between them are even weaker and their energy falls off as 1/r^6.

Hydrogen bonds:

These interactions occur between a proton donor (Brönsted acid) and a proton acceptor group (Brönsted base).[101-103] In contrast to all other binding forces hydrogen bonds are directed. Typical hydrogen bond donor groups are O–H and N–H. C–H can be a hydrogen bond donor if it is directed towards an oxygen or the π-system of an aromatic ring and in particular if it is acidified by neighboring σ-or π-acceptor groups in the molecule. Typical hydrogen bond acceptors are C=O, COO-, -OR, and -NR2. The distance between the donor and acceptor heteroatoms is called the length of the hydrogen bond and it is typically between 2.5 and 3.5 Å. Because the resolution of the X-ray crystal structure of a protein does not allow determination of the exact position of the hydrogen atoms in the protein (the positions of the hydrogen atoms are calculated), it is not possible to define the exact position of a proton that is involved in a hydrogen bridge. Ideally, hydrogen bonds are linear, but angles up to 160° are found.[104] The free binding energy (ΔG°) for a hydrogen bond varies in the gas phase from ΔG° = -3 to -9 kcal/mol,[105] in solution ΔG° = -1 to -2 kcal/mol.[106] In proteins, hydrogen bonds contribute to the secondary
structure (α-helix and β-sheet). Therefore, most acidic and basic groups in the apoprotein are involved in hydrogen bonding anyway, many of them with water molecules. The acidic and basic groups of the free ligand are also involved in hydrogen bonds with water molecules. The free energy of hydrogen bonds between a ligand and a protein arises mainly from the entropy gain when these water molecules are released.[107]

**Van-der-Waals interactions or dispersion forces:**

These forces arise from a fluctuating, nonsymmetrical electron distribution in molecules induced by neighboring molecules.[91,108,109] The attraction of these induced dipoles is described by the Lennard-Jones potential energy function and is proportional to \((1/r^{12} - 1/r^6)\). The forces are only present at short distances - basically in the range of the van-der-Waals radii of the atoms - and are weak. At very short distances they become repulsive. Only the sum of many surface contacts between ligand and protein can contribute significantly to the binding free energy of a ligand.[108] These forces are called favorable van-der-Waals contacts. If two atoms get too close, repulsive interactions arise from the positively charged nuclei. These unfavorable van-der-Waals contacts lower the binding energy of a ligand and should therefore be prevented in the inhibitor design.[110]

**Hydrophobic interactions:**

Lipophilic molecules have the tendency to aggregate in aqueous solution.[111,112] Upon aggregation the water molecules which were involved in the solvation of the nonpolar solutes are released into the bulk aqueous medium. This is entropically and enthalpically favorable, as the released water molecules gain orientational freedom and make new hydrogen bonds with other water molecules.[113-115] Also, the dispersion forces between the apolar molecules are larger than those between a lipophilic solute and water. An often observed hydrophobic all driven interaction is the association of two aromatic rings in a face-to-face or edge-to-face mode. \(\pi-\pi\) Stacking (face-to-face) between two aromatic surfaces occurs often in a parallel-shifted way and is favored if the two aromatic rings differ in their electron affinity (and ionization potential). \(C-\pi-\pi\) edge-to-face interactions involve an electrostatic component
which can be described by H-bonding of the C-H of one ring to the \( \pi \)-face of the other.\[^{[116]} \]

1.4.2.3 Computer simulations for rational design

Computer simulations provide the possibility of quantifying the binding forces described above that exist between a protein and a particular ligand. However, all calculations are approximations, models of the real state, and the choice of the computational method, together with other criteria, determines the significance of the result. For the modeling of potential inhibitors of an enzyme, molecular mechanics,\[^{[117-121]} \] molecular dynamics,\[^{[122-127]} \] and Monte Carlo methods\[^{[128-131]} \] are used. If ground states are described, the laws of classical mechanics can give very good results. They ignore the electronic motions and consider the atoms as points which interact with each other through a given potential energy function. Energy minimization generates a model of the structure representative of the actual structure. In Chapter 2.2, the method of molecular mechanics is described in more detail.

Molecular dynamics\[^{[122-127]} \] is a technique to study the behavior of molecules by means of computer simulation. Both methods use the same kind of force field to describe the potential energy of a system, but molecular dynamics simulates the motion of the atoms in the system, whereas molecular mechanics only minimizes the energy of a molecule by geometry optimization. The molecular dynamics method aims at describing the time evolution of a molecular system by solving the classical equations of motion. At the beginning, the system is heated by assigning velocities randomly to the atoms according to a Maxwell-Boltzmann distribution. The equations are integrated starting at a certain time \( t \) and predict the coordinates of the system at time \( t+\Delta t \). Again the forces acting on the atoms are calculated and a new set of atomic coordinates is created after another time step \( \Delta t' \). The trajectory which is generated by this procedure shows the dynamics of the system. In the end, the system is brought back to the desired temperature.

In molecular dynamics, the molecule can cross an energy barrier due to the additional kinetic energy. The probability that a certain atomic configuration will occur is proportional to \( e^{-E/kT} \), where \( E \) is the energy of the molecular conformation (\( k = \) Boltzmann constant; \( T = \) temperature). When the simulation covers a long enough period of time at sufficiently high temperatures, the most important possible
conformations of the system can be found. This is particularly useful when one tries to scan the conformational space of a larger molecule, which has too many rotatable bonds to be studied by classical systematic search techniques.

The Monte Carlo method aims at generating a series of atomic configurations for a molecule sampling from a chosen statistical ensemble. This statistical ensemble must have a Boltzmann distribution. The procedure to get to a series of configurations representative of the total configurational space available to the molecular system consists of millions of steps. Each step means a random selection of an atom and its movement by a random displacement. The energy for the new position is calculated and the new state is accepted if favorable in energy.

1.4.3 SAR by NMR

A highly attractive NMR method has been developed for the stepwise construction of ligands from building blocks: structure-activity relationship (SAR) by NMR. In this approach, libraries of small molecules are screened against a certain protein. Binding of ligands to a subsite is observed by shifts of the corresponding amide proton signals of the $^{15}$N-labeled protein. In the next step, the protein is saturated with the highest affinity ligand for this site and a different library is screened to search for ligands, which bind to a proximal subsite. Both ligands are then combined with an appropriate linker to obtain a high-affinity ligand (Figure 11). This method is certainly based on experience and knowledge gained from bisubstrate inhibitor approaches. It combines screening results with the rationality of the linker design.
Figure 11. SAR by NMR discovers ligands that bind to the proximal subsites of a protein. Acetohydroxamic acid and 3-(cyanomethyl)-4'-hydroxybiphenyl are low-affinity ligands of the matrix metalloprotease stromelysin; combining them with an appropriate linker produces a high-affinity stromelysin inhibitor.\[134\] 

1.4.4 Computational combinatorial docking methods

Virtual computational library screening and combinatorial approaches are the latest trends in rational design. Structure-based drug design is supported by computer programs for the automated superposition of molecules, for flexible docking of ligands, and for de novo design of ligands that fit a binding site in shape and complementarity of their physicochemical properties.\[56,135-139\] FlexS\[140\] and FlexX\[141\] are such programs for the flexible superposition and docking of ligands. A molecule is dissected into rigid fragments that are reassembled by a tree-search procedure to achieve the best mutual alignment to another molecule (FlexS) or to obtain the best fit to a protein binding site (FlexX).

The multiple copy simultaneous search (MCSS) method is another interesting realization of the concept of combinatorial docking.\[142,143\] This approach searches for preferred locations of certain functional groups or small ligands in the binding site. The corresponding positions are analyzed and selectively oriented ligands are connected with linkers to build molecules whose structures are optimized within the binding site.
2 DESIGN OF A LEAD STRUCTURE

2.1 SCOPE OF THE THESIS

The known substrate analog inhibitors of COMT already in use in the therapy of Parkinson's disease, e.g. Tolcapone (Tasmar®),[21,28,29] exhibit disadvantages such as relatively fast metabolism and elevated liver enzymes in some patients, problems that are currently under investigation. In practice, the fast metabolism means that Parkinsonian patients must take their medicine three times daily.

The development of a new class of inhibitors which exhibits a different mode of action could therefore be very valuable in the therapy of Parkinson's disease, if these inhibitors could be further developed into pharmaceuticals. From kinetic studies on the methylation mechanism of COMT, it has been known that cofactor and substrate must be located at neighboring binding sites.[7,9] Consequently, bisubstrate inhibitors replacing both the cofactor and the substrate seemed adequate. In the 1980s, Coward and coworkers tried to make bisubstrate inhibitors for COMT,[37] but abandoned this approach because they could not find a lead structure for an inhibitor which would occupy the binding sites for cofactor and substrate.

When the X-ray crystal structure of COMT cocrystallized with 3,5-dinitrocatechol and SAM was solved in 1994,[12,13] the door to a protein 3D structure-based design was opened (see Section 1.4.2). The X-ray crystal structure of the apoenzyme was solved by F. Winkler (Hoffmann-La Roche) and available for the studies outlined in this thesis.[12]

The aim of this thesis was the design of a lead structure for a bisubstrate inhibitor of COMT applying the method of computer-aided structure-based design. The compound should be synthetically accessible in a reasonable number of steps. An efficient synthesis should be developed for the target molecule that would allow the introduction of different functionalities on the core unit of the molecule for optimization of its inhibitory affinity. The bisubstrate nature of the compound
should be verified in kinetic studies, which might also allow the elucidation of the binding mechanism of the compound. If the compound displayed a significant affinity for the enzyme, a X-ray crystal structure of the inhibitor cocrystallized with COMT might be obtained which would allow comparison of the predictions from the modeling with the X-ray crystal structure.

Based on the new information obtained from the analysis of the binding mechanism and the interactions between the lead structure and COMT, another design cycle would follow to introduce structural variations into the lead compound, and to observe the effects on binding affinity and kinetics. Finally, the results would be interpreted to obtain a structure-activity relationship for the lead compound which is essential for the further development of the lead into potential pharmaceuticals.

2.2 THE METHOD OF MOLECULAR MECHANICS

2.2.1 Principle

For the molecular modeling, force field methods were employed. These methods ignore electronic motions and calculate the energy of a system as a function of nuclear positions only. To this end, molecules are represented as a collection of spheres (atoms) joined by springs (bonds). The motions of these atoms can be described by the laws of classical physics and simple potential functions can be used to describe the overall molecular behavior. Ignoring the electronic movements saves precious computing time and allows much larger systems to be investigated.

Once the molecular representation has been fixed, the next step is to define a potential energy function consistent with the preceding concept of vibrating spheres, allowing accurate calculation of the molecular properties. This function can be more or less sophisticated depending on the employed force field method. It is generally based on the assumption that the total energy of a system can be divided into several terms, arising from bond stretching ($E_b$), angle bending ($E\theta$), torsional interactions ($E_{tor}$), van-der-Waals interactions ($E_{vdW}$), and electrostatic interactions ($E_{elec}$), as expressed in the following equation:
This equation represents a typical force field used to calculate the total energy of various types of compounds. The schematic representations of these key contributions are given in Figure 12.

One functional form of an equation that can be used to model assemblies of atoms is that given below (more advanced force fields may have additional terms). It shows that the various terms can be ascribed to changes in specific internal coordinates such as bond lengths (l), angles (θ), torsion angles (ω), or movements of atoms relative to each other (r).

\[
E_{tot} = \sum k_i (l-l_0)^2 + \sum k_\theta (\theta-\theta_0)^2 + \sum V_n (1 + s \cdot \cos(n\omega)) + \sum \frac{q_i q_j}{D_{ij}^\epsilon} + \sum \frac{\epsilon[(r_{mi})^6 - 2(r_{mi})^3]}{r_{mi}}
\]

From this equation, it becomes clear that, in order to define a force field, one must specify not only its functional form but also the parameters \(k_i\) = stretching force constant describing the deformation, \(l_0\) = equilibrium bond length, \(k_\theta\) = force constant for bond angle, \(\theta_0\) = equilibrium value for the bond angle, \(V_n\) = rotational barrier height, \(n\) = periodicity of rotation, \(s = 1\) for staggered minima, \(s = -1\) for eclipsed minima, \(\epsilon\) = well depth, \(r_{mi}\) = minimum energy interaction distance, \(D\) = dielectric constant, \(q\) = partial charge). This set of parameters, together with the potential energy function, constitutes the force field and is developed and tested on a
relatively small number of known situations. It is obvious that the quality of a given force field depends crucially on the parameters which are derived as constants in the potential function, and that the choice of the employed force field must be based on the type of system to be studied. Indeed, each force field has been parameterized to describe a particular system in the most appropriate way. The AMBER, GROMOS, CVFF, and OPLS force fields are particularly adapted for peptide systems, CFF9 and MM2/MM3 are more suitable for small organic molecules, whereas the relatively recent ESFF covers the entire periodic table.

2.2.2 The MAB force field

The MAB force field used with the program MOLOC is a generally applicable molecular force field for structure modeling in medicinal chemistry. Its major characteristic is the introduction of a purely geometrical energy term for hydrogen bonding interactions. This is realized by strict adherence to the united-atom approximation (omitting the hydrogen atoms), directionality of the hydrogen bonding geometry (from the hydrogen donor towards the lone pair in the acceptor site), and the evaluation of an optimal hydrogen bonding pattern.

2.2.3 Energy minimizations

To obtain the actual geometries and energies corresponding to the minima on the energy surface, the energy of a model of a molecule generated by using molecular graphics must be minimized. To perform the minimization, an algorithm is applied which usually employs derivatives. The first derivative of the energy with respect to the atomic coordinates, or gradient, shows the direction of the minimum, and its magnitude indicates the steepness of the local slope. Thus, the energy of a system can be lowered by calculating the negative gradient of each atom, and then moving the latter along this negative gradient. This process is carried out in an iterative fashion until the function passes through a minimum (where the first derivative is zero).
The most common derivative methods are those of steepest descent and conjugate gradients. The former moves in the direction parallel to the negative gradient, until the function passes through a minimum, where the gradient of the energy is recalculated. This new gradient will be perpendicular to the preceding one, and so the directions of the successive steps will be orthogonal. The resulting path oscillates, leading to undesirable behavior in the vicinity of a minimum. Therefore, this method, which is particularly efficient far from a minimum, is generally used to tidy up model geometries prior to further refinement by another algorithm.

The first derivative method used in the program MOLOC\textsuperscript{[78]} is that of conjugate gradients. In this method, the information of previous steps is used to modify the move in the next step. Including the history of movement into the minimization path improves the convergence properties of the procedure.

### 2.2.4 Molecular modeling procedure

The starting geometries for the molecular mechanics studies were constructed with the program MOLOC\textsuperscript{[78]} from standard molecular fragments and from crystallographic data. The proposed inhibitor was minimized separately and docked manually into its expected binding site. The coordinates of COMT and the Mg\textsuperscript{2+} ion were constrained. The inhibitors were then minimized inside the enzyme. Energy minimizations were performed \textit{in vacuo} by MOLOC\textsuperscript{[78]} with the MAB force field. The energy was minimized to a final value of the sum of the squares of the components of the gradient of less than the accuracy (0.1 or a relative value of 1), i.e. to a final root mean square (RMS) gradient of less than 0.316 kcal/mol. The structures were visualized in Insight II\textsuperscript{[79]} as shown in Figures 13a, 14, and 15a.
2.3 ANALYSIS OF THE COMT ACTIVE SITE

As a prelude to the rational design of enzyme inhibitors, a thorough analysis of the active site of COMT was performed.

The X-ray crystal structure of COMT complexed with a Mg$^{2+}$ ion, 3,5-dinitrocatechol, and SAM$^{12}$ (Figure 13a) was analyzed for intermolecular bonding contacts between the amino acid residues of the enzyme and the three bound components. Figure 13b shows a schematic representation of the H-bonding and Coulombic interactions in the quaternary complex. Three binding pockets were defined. The one that binds the catechol, situated on the surface of the enzyme, will be referred to as the catechol pocket. It contains the Mg$^{2+}$ ion octahedrally coordinated to Asp141, Asp169, Asn170, one localized H$_2$O molecule, and the two HO-groups of the catechol, one of which is deprotonated under physiological conditions (pH ≈ 7). The 3-nitro group of the inhibitor has favorable van-der-Waals interactions with Trp38. Trp38 is located edge-to-face with the planar structure of 3,5-dinitrocatechol, which is a hydrophobic contact that probably contributes to the specificity for aromatic 1,2-diols.

The second binding pocket contains the ribose moiety, and the third the adenine base of the SAM cofactor; they will be referred to as the ribose and base pockets, respectively, and are more deeply embedded in the enzyme. In particular, Trp143 shields the bound nucleoside portion of SAM from the enzyme surface. The two ribose HO-groups interact with the COO$^-$ residue of Glu90, and the ribose ring lies side by side with Trp143, making favorable hydrophobic interactions.

The adenine moiety is bound via a characteristic H-bonding array to the protein. Its NH$_2$ group forms H-bonds to Gln120 and a fixed H$_2$O molecule. Ser119 also forms an essential H-bond with its backbone N–H group to N(1) of the nucleobase. Moreover, adenine has favorable edge-to-face van-der-Waals contacts with Trp143 which is located on the surface of COMT. His142 is situated deeper in the enzyme behind the adenine ring with which it has a favorable H-bonding between a C–H of the imidazole ring and the π-face of the nucleobase. His142 and Met91 have van-der-Waals interactions with opposite sides of the base, which is in a sandwich position between the two amino acids.
Figure 13. a) SAM cofactor, 3,5-dinitrocatechol, and Mg$^{2+}$ ion situated in the active site of COMT as found in the X-ray crystal structure of recombinant rat COMT.\textsuperscript{[12]} The Connolly surfaces of SAM and 3,5-dinitrocatechol are shown to indicate the surface shape of the ligands available for van-der-Waals contacts to the enzyme. b) Schematic drawing of the active site of COMT and its H-bonding and Coulombic interactions with the two substrates and the Mg$^{2+}$ ion. Distances are given in pm.
The methionine part of SAM binds to a channel-like opening in the enzyme made from polar amino acid residues. The COO\(^-\) group of methionine makes a H-bond with the backbone N–H of Val42 and a fixed H\(_2\)O molecule. Its NH\(_2\) group forms H-bonds to the OH-group of Ser72, to the COO\(^-\) residue of Asp141, and to the backbone C=O group of Gly66.

2.4 THE X-RAY CRYSTAL STRUCTURE OF THE APOENZYME

Figure 14 shows an overlay of the X-ray crystal structures of COMT bound to 3,5-dinitrocatechol, a Mg\(^{2+}\) ion, and SAM with the apoenzyme. The overlay visualizes some distinct conformational differences existing between the apoenzyme and the enzyme in the bound state. Basically, the binding pockets for the methionine moiety of SAM and the catechol do not exist in the apoenzyme. These form only by the rearrangement of an entire region in the apoenzyme triggered upon binding of SAM. On the other hand, the adenine pocket and the ribose pocket are already developed - with minor deviations - in the apostructure.

These structural findings are in accordance with what is known about the kinetic mechanism and the order of binding of the catecholic substrate and SAM. Since the catechol binding site does not exist in the apoenzyme, SAM must bind first, inducing the conformational changes in the protein to make the catechol binding site.

The X-ray crystal structure of the apoenzyme of COMT proves the conformational flexibility of some regions in the protein, which could only be assumed from previous kinetic studies.\[^{37}\] Because the active site lacks conformational homogeneity, a protein structure-based de novo design of a potential bisubstrate inhibitor is not feasible. In particular, the design of complementary scaffolds for the methionine binding channel is not possible on a rational basis, because no reliable predictions can be made about whether its structure will be preserved upon binding of an inhibitor.
2.5 DESIGN OF THE LEAD STRUCTURE

The design of the bisubstrate inhibitors was performed by computational methods, using the programs MOLOC, Insight II, and MacroModel. The hypothesis was made that the highly polar methionine channel would be favorably filled with water in the presence of an inhibitor, which would only occupy the remaining three binding pockets. Therefore, in the design of a bisubstrate inhibitor, occupation of the methionine channel was neglected. We subsequently chose to construct the potential inhibitor by connecting the C(5')-OH group of adenosine (as in SAM) via an appropriate spacer to a suitable catechol moiety, thereby filling the adenine, ribose, and catechol binding pockets. We hoped to preserve in the complex formed by the bisubstrate inhibitor in the presence of the Mg$^{2+}$ ion all the directional H-bonding and Coulombic interactions schematically shown in Figure 13b.
Potent enzyme inhibition by catechols is known to require that the $pK_A$ of one of the two HO-groups is sufficiently lowered to ensure deprotonation at physiological pH.\textsuperscript{[19]} Therefore, we chose a catechol derivative in which one HO-group is acidified by a $p$-NO$_2$ group and an $o$-carboxamide group which, at the same time, acts as the anchor for the connection to the nucleoside moiety.

To ensure that the catechol and nucleoside moieties of the bound bisubstrate inhibitor would benefit from all the directional interactions seen in the X-ray crystal structure of the quaternary complex (COMT, SAM, 3,5-dinitrocatechol, Mg$^{2+}$ ion), the structure of the bridge connecting these moieties needed to be optimized. Extensive modeling showed the ribose–C(5')–O–CH$_2$–CH$_2$–NH–CO–catechol linker in 7 (Figure 15a), with a planar trans amide group, to meet the sensitive length and shape requirements for this bridge. Figure 15a displays the most favorable conformation of 7 docked into the X-ray crystal structure of COMT. The picture displays the Connolly surface of 7 visualizing the complementarity between the shape of the molecule and its binding site in the enzyme. This structure was obtained by energy minimization of the inhibitor inside the enzyme whose coordinates (including the Mg$^{2+}$ ion) were fixed. Figure 15b provides a schematic view of the bonding interactions in the modeled ternary complex between COMT, Mg$^{2+}$ ion, and 7. As is readily apparent from a comparison between Figures 13 and 15, all directional H-bonding and Coulombic interactions seen in the X-ray crystal structure of the quaternary complex with SAM and 3,5-dinitrocatechol are maintained in the modeled structure with the bound potential bisubstrate inhibitor. The N–CH$_2$–CH$_2$–O–CH$_2$ bridge between catechol and ribose features a sequence of favorable $ap$ (antiperiplanar)–$ap$–$sc$ (synclinal)–$sc$ torsional angles. The computational analysis revealed 25 short van-der-Waals contacts ($< 3.7$ Å) and no repulsive interactions between enzyme and inhibitor.
Figure 15. a) Computer-modeled ternary complex between COMT, Mg$^{2+}$ ion and bisubstrate inhibitor 7. The Connolly surface of the inhibitor is shown. b) Schematic drawing of the directional interactions in the modeled ternary complex. Distances are given in pm.
3  SYNTHESIS OF THE LEAD COMPOUND

3.1  FIRST RETROSYNTHETIC APPROACH TO THE LEAD STRUCTURE

Scheme 3. General retrosynthetic route for the potential bisubstrate inhibitor 7 of COMT.
Compound 7 can be retrosynthetically dissected into four substructures, complementary to defined binding sites: base, ribose, catechol, and the linker moiety between ribose and catechol. A synthetic strategy was chosen that allows facile introduction of different chemical functionalities for each binding site. In our first retrosynthetic approach (Scheme 3), N-succinimidyl 2,3-dihydroxy-5-nitrobenzoate (8), which is synthesized in three steps from commercially available 2-methoxy-3-hydroxybenzoic acid (9), is coupled to the adenosine derivative 11. The nucleosidic part 11 is built from adenosine derivative 10 by functionalization at C(5')-OH which involves synthetic methodology known from the literature: the Mitsunobu reaction\textsuperscript{[153]} and other reactions that activate the C(5')-OH either by deprotonation\textsuperscript{[154]} or by tosylation\textsuperscript{[155]} provide an array of possibilities for the selective functionalization of adenosine. In this approach, the linker moiety can be modified in length and structure, and coupling to different catechols is also an option. The possibility of introducing such modifications into the original lead is necessary to explore the range of substructures which fit into the various binding sites and allow lead optimization.

3.2 FIRST SYNTHETIC APPROACH TO THE LEAD STRUCTURE

3.2.1 Synthesis of the catechol unit

For the preparation of N-succinimidyl 2,3-dihydroxy-5-nitrobenzoate (8), commercially available 3-hydroxy-2-methoxybenzoic acid (9) was nitrated under standard conditions to give 12 regioselectively in 94% yield (Scheme 4). Demethylation with 40%aq. HBr afforded nitrocatechol 13\textsuperscript{[156]} which was purified by sublimation (79%). Reaction of 13 with N-hydroxysuccinimide (HOSu) in the presence of N,N'-dicyclohexylcarbodiimide (DCC) afforded the N-succinimidyl benzoate 8 (53% yield). Coupling of 8 with 2-methoxyethylamine provided comparison compound 14 in 75% yield.
For preparing amide 14 from dihydroxybenzoate 13, different methods for activating the carboxylic acid were investigated (Scheme 5). Synthesis of the acid chloride 15 and synthesis of the activated pivaloyl ester 16 could not be achieved with dihydroxybenzoate 13. Reaction of 13 with N,N,N',N'-tetraethyldiaminocarbodiimide (EDC), N-hydroxybenzotriazole (HOBt), N,N-diisopropylethylamine (DIPEA), and 2-methoxyethylamine afforded 14 in 48% yield. Activation of the carboxylic acid with 1,1'-carbonyldiimidazole and reaction with 2-methoxyethylamine yielded 85% of amide 14.

The two latter methods were also employed for coupling of 13 to the nucleosidic unit of the target molecule, however, purification of the products from the reagents was not feasible. Therefore, only N-succinimidyl benzoate 8 was used for the coupling of the catechol moiety to the nucleosidic part of 7.
3.2.2 Synthesis of the nucleosidic unit of the lead starting from adenosine

The nucleosidic unit of 7 was synthesized starting from adenosine (17) as shown in Scheme 6. Adenosine was benzoylated selectively at the NH$_2$ group by treatment with TMSCl and benzoyl chloride in pyridine to give 18 in 74% yield.[165] For the protection of the C(2')-OH and C(3')-OH groups of the ribose, 18 was reacted with 2,2-dimethoxypropane in acetone to yield 10 (72% yield).[166] Compound 10 was then tosylated at C(5')-OH with tosyl chloride to afford 19 in 40% yield.[155,167-169]
1. TMSCI, pyridine, r.t., 1 h  
2. BzCl, 4°C, 12 h  

NHBz  
74%  

TsO  
19  

NHBz  
NaH, TsCl, DMF, r.t., 5 h, 40%  

2,2-Dimethoxypropane, TsOH (1 eq.), acetone, r.t., 12 h, 72%  

Scheme 6. Selective protection of the NH₂ group and the ribose C(2',3')-OHs of adenosine 17. Compound 10 was tosylated at the C(5')-OH for its activation as a leaving group.

Several attempts to alkylate 10 at C(5')-OH were made. Reaction with 2-chloroethylamine led to decomposition of 10 (Scheme 7). Linker 20, synthesized in 95% yield by tosylation of N-(2-hydroxyethyl)phthalimide (21), gave similar results.[154,171] Upon reaction of 10 with 22, synthesized in 89% yield by tosylation of N-benzyloxy carbonylethanolamine (23), the expected product 24 could not be isolated.[172]

The Mitsunobu reaction has been successfully applied to the functionalization of C(5')-OH of adenosine derivatives.[153,173] However, our attempts to alkylate adenosine derivative 10 with 21 under these conditions failed to provide the desired product 25 (Scheme 7d).
Finally, we tried to employ the tosylated adenosine derivative 19 for the desired functionalization. Compound 19 was reacted with 21 and 23 under the conditions shown in Scheme 8, which, however, did not yield the respective products 25 or 24.
SYNTHESIS OF THE LEAD COMPOUND

Because functionalization of the adenosine was not successful, the retrosynthetic approach to target molecule 7 described in Section 3.1 was abandoned.

3.3 SECOND RETROSYNTHETIC APPROACH TO THE LEAD STRUCTURE

In Scheme 9, an alternative retrosynthetic approach for the synthesis of the nucleosidic unit 26 required to prepare 7 is outlined. In this approach, N-benzyolated adenine is glycosylated with the ribose derivative 27. The sequence starts from commercially available 2,3-O-isopropylidene-β-D-ribose (28) which is transformed into the protected acetylated ribose derivative 27. Coupling of deprotected 26 with N-succinimidyl benzoate 8 to yield the target compound 7 is performed at the end of the synthesis, because nucleosidation of ribose derivatives already containing the catechol moiety was not successful.

The latter retrosynthetic pathway is more versatile than the one discussed in Section 3.1, because natural and modified bases[174-178] can be introduced into the ribose moiety in a regio- and stereoselective fashion.
Scheme 9. Alternative retrosynthetic route for the potential bisubstrate inhibitor 7 of COMT.
3.4 SECOND SYNTHETIC APPROACH TO THE LEAD STRUCTURE

3.4.1 Synthesis of the nucleosidic unit of the lead starting from the ribosyl unit: Synthetic pathway with the trifluoroacetyl protecting group

For the synthesis of the nucleosidic unit 26, ribose derivative 28 was alkylated with 2-chloroethylamine to give 29 in 88% yield. 2-Chloroethylamine is known to undergo intramolecular $S_n2$-reactions with formation of the aziridine, which is opened by nucleophiles,[179-181] in this particular case by the deprotonated C(5')-OH of the ribose derivative 28. Protection of the amino group in 29 with trifluoroacetic anhydride yielded 30 (90% yield). For the stereoselective nucleosidation of the ribose, the peracetylated precursor 31 was synthesized. Treatment of 29 with aqueous $\text{H}_2\text{SO}_4$ effected cleavage of the isopropylidene and methoxy groups, affording ribose 32 in 95% yield. Subsequent peracetylation provided the acetylated derivative 31 in 85% yield (Scheme 10).

For the one-step nucleosidation of 31, N-benzoylated adenine$^{[182]}$ 33 was silylated in situ using N,O-bis(trimethylsilyl)acetamide (BSA), yielding in all cases the $\beta$-anomer exclusively due to neighboring group participation of the $\alpha$-acetoxy group at C(2').$^{[174-178]}$ However, the use of TMSOTf in the nucleosidation with N-benzoylated adenine led to the exclusive formation of the wrong regioisomer 34 with bond formation occurring between N(7) of adenine and C(1') of the ribose unit (38% yield). In contrast, the use of SnCl$_4$ as Lewis acid led to the desired nucleosidation product 35 in 85% yield (Scheme 11). Regio- and stereoselectivity of the nucleosidation reactions are discussed in Section 3.5.
Scheme 10. Synthesis of the nucleosidation precursor 31 from ribose 28.

Scheme 11. Nucleosidation of 31 using a) TMSOTf and b) SnCl₄ as Lewis acids afforded constitutionally isomeric β- anomers.
Constitution and configuration of products 34 and 35 were confirmed by NOE difference spectroscopy (Figure 16). The NOE which is observed between the ribosyl protons H-C(1') and H-C(4') confirms the configuration (β-anomer) of 34. The NOEs between the adenine proton H-C(8) and the ribosyl protons H-C(1'), H-C(2'), and H-C(3') indicate that the adenine is connected to the ribose at N(7) or N(9). The observed NOE between the aromatic H(Bz) and the ribosyl H-C(1') is only possible in the N(7)-nucleoside 34 where the adenine is linked to the ribose via N(7). In 35, this latter NOE is missing, which shows that the adenine is connected to the ribose at N(9).
Treatment of nucleoside 35 with piperidine (5%) in dioxane/H_2O yielded fully deprotected 36 (100% yield) which was then coupled to the N-succinimidyl benzoate 8 to afford the target molecule 7 in a mixture with the starting materials and reagents. Recrystallization and reversed phase HPLC did not allow a separation of the mixture (Scheme 12).

We figured that purification of 7 should be possible, if the C(2')-OH and C(3')-OH groups of the ribose unit were acetylated because of better solubility in organic solvents as compared to compound 7. However, it was not possible to remove the trifluoroacetyl protecting group from 35 without simultaneous cleavage of the acetates (2-10% piperidine in dioxane/H_2O (1:1)). Therefore, we chose to switch
from trifluoroacetyl to (9H-fluoren-9-ylmethoxy)carbonyl (Fmoc) as a protecting group for the amino function.

Scheme 12. Deprotection of the trifluoroacetate group of 35 yielded 36 which was coupled to 8 to afford 7 which could not be separated from starting materials and reagents.
3.4.2 Synthesis of the nucleosidic moiety of the lead starting from the ribosyl unit: Synthetic pathway with the Fmoc protecting group

The Fmoc protecting group\textsuperscript{[183]} is stable under the acidic conditions required for removal of the isopropylidene and methyl protecting groups in 37. Also, it is stable under the conditions of the nucleosidation reaction and could be selectively removed under mild basic conditions in the presence of the acetyl protecting groups on the ribose unit and the \(N\)-benzoyl protecting group of the adenine moiety in 38. Therefore, Fmoc protection of the primary aliphatic amino group in 29 was crucial for the success of the synthesis.

Protection of 29 with Fmoc-\(O\)-succinimide ester (FmocOSu)\textsuperscript{[183]} yielded 37 (65% yield). The reaction conditions were optimized to avoid formation of the by-product 39 arising from Fmoc deprotection of 37 by unreacted amine 29. This by-product was formed at room temperature at reaction times longer than 15 min. Therefore, the reagent FmocCl\textsuperscript{[183]} which is usually used in peptide chemistry to introduce the Fmoc group but requires longer reaction times (4-6 hours), could not be used (Scheme 13).

![Scheme 13](image-url)

*Scheme 13*. Fmoc protection of the amine functionality in 29 with the reagent FmocCl gave rise to the unwanted by-product 39.
Treatment with aqueous HOAc effected cleavage of the isopropylidene and methoxy groups, affording ribose 40 in 96% yield. Subsequent peracetylation provided the acetylated derivative 41 (83% yield) (Scheme 14).


The use of SnCl₄ as Lewis acid led to the desired nucleosidation product 38 in 65% yield (Scheme 15), although the Fmoc group in 41 was partly cleaved by SnCl₄. The constitution and configuration of the product was again confirmed by NOE difference spectroscopy (Figure 17). Fmoc deprotection of 38 and coupling with the activated N-succinimidyl dihydroxybenzoate ester 8 (Scheme 4) were performed in one step (Scheme 15). When piperidine or HNMe₂ (0.5-20%) in DMF/H₂O were used at varying temperatures for the removal of the Fmoc group, intermolecular deacylation by the liberated free amines occurred readily to give a mixture of products 42, 43, 44, 45, 46, and 47 as judged by mass spectrometry (cf. Scheme 16). Under high dilution conditions in DMF/HNEt₂ 1:1, the deprotection step was, however, effected without deacylation of the ribose unit. The base HNEt₂ was readily removed by evaporation at reduced pressure, leading to a solution of the free
amine in DMF, which was used directly in the coupling to the \( N \)-succinimidyl benzoate 8, affording 48 in a yield of 30%. The synthesis of the target compound 7 was completed by complete deprotection. One-step deprotection of 48 using MeNH\(_2\) in EtOH for 20 min at 20 °C afforded 7 in 82% yield.

**Scheme 15.** Nucleosidation of the peracetylated ribose 41 followed by *in situ* cleavage of the Fmoc group and coupling to the dihydroxybenzoate 8.
The NOE that is observed between the ribosyl protons H-C(1') and H-C(4') confirms the β-anomer configuration of 38. The NOEs between the adenine proton H-C(8) and the ribosyl protons H-C(1') and H-C(2') confirm that adenine is connected to the ribose via N(7) or N(9). In comparison to Figure 16, there are no NOEs between the aromatic H(Bz) and ribosyl protons, indicating that adenine is linked to the ribose unit via N(9) (Figure 17).
SYNTHESIS OF THE LEAD COMPOUND

b)

\[ H-C(1') \]

\[ H-C(8) \]

\[ H-C(4) \]

\[ H-C(2') \]

\[ H-C(4') \]

9.0 8.0 7.0 6.0 5.0 4.0

c)

\[ H-C(2') \]

\[ H-C(8) \]

\[ H-C(3') \]

\[ H-C(1') \]

9.0 8.0 7.0 6.0 5.0 4.0
Figure 17. $^1$H NMR (CDCl$_3$, 500 MHz) NOE spectrum of 38.  

- a) Irradiation at H-C(8) (8.40 ppm). Amplified signals (from left to right): H-C(1') (6.42 ppm), H-C(2') (6.03 ppm).  
- c) Irradiation at H-C(2') (6.03 ppm). Signals which are amplified: H-C(8) (8.42 ppm), H-C(1') (6.42 ppm), H-C(3') (5.67 - 5.68 ppm).  
- d) Irradiation at H-C(4') (4.35 - 4.37 ppm). Amplified signals: H-C(Fmoc) (7.55-7.59), H-C(1') (6.42 ppm), H-C(3') (5.67 - 5.68 ppm), H-C(5') (3.90-3.70 ppm).

Scheme 16. Cleavage of the Fmoc protecting group in 38 led to a mixture of compounds 42 - 47 arising from intermolecular deacylation.
3.4.3 Conformational studies on the lead compound

For determining the conformation of 7 in solution, an NOE spectrum was measured in CD$_3$OD. The observed NOEs between H-C(8) of adenine and H-C(2'), H-C(3') of the ribose demonstrate that adenine is in the right "anti" orientation for binding to the active site as predicted by modeling.[184] On the other hand, no NOEs are observed between any of the catechol hydrogens and H-C(8) which indicates that the catechol moiety adopts a conformation where it does not come close to the base which would be an unfavorable conformation for the binding of 7 to COMT (Figure 18).
3.4.4 Synthesis of comparison compounds

Two compounds (14 and 49) that contain substructures of the lead molecule were synthesized. They were expected to provide more information on the influence that the different components of the lead have on its inhibitory activity. In Section 3.2.1 (Scheme 4) we described the synthesis of comparison compound 14 which bears the catechol and linker moieties of the lead structure 7. Comparison compound 49 on the other hand contains the catechol, linker, and ribosyl units, but lacks the base. For the preparation of 49, the ribose derivative 29 was coupled to N-succinimidyl benzoate 8 (Scheme 4) to provide 50 (75% yield), which was deprotected with aqueous H$_2$SO$_4$ in 91% yield (Scheme 17).
3.5 MECHANISTIC DISCUSSION OF THE NUCLEOSIDATION REACTION

There are two major approaches to the synthesis of nucleosides.[185] The first approach involves glycosidation of an appropriate sugar with an intact purine or pyrimidine derivative. The second approach involves the construction of the purine or pyrimidine system from a simple N-glycosylated precursor. The first approach has been frequently applied, and especially nucleosidations with the natural bases adenine, cytosine, guanine, thymine and uracil have been studied in detail.[178] Nucleosidation reactions employing the "silyl method" have been widely used since its invention in 1963.[186] The key step in the synthesis of nucleosides by the silyl method is the reaction between silylated heterocyclic bases and sugar derivatives. In principle, silylation of the base improves its solubility and nucleophilicity for the glycosylation reaction. An advantage of the method is that the remaining trimethylsilyl groups can be readily removed during workup by treatment with water or alcohol.[185]

In our synthesis, we employed the in situ silylation and glycosylation method reported by Vorbrüggen et al. in the 1980s,[174-177] in which the base is silylated
with N,O-bis(trimethylsilyl)acetamide (BSA) in acetonitrile or dichloroethane. For
the nucleosidation, a 1,2,3-tris(acyloxy)ribose derivative is employed, usually an
acetate or a benzoate. A Lewis acid such as SnCl₄ or TMSOTf is added to activate
the 1-acyloxy group on the ribose 51 (Scheme 18) as a leaving group.[187] The 1,2-
acyloxonium ion 52 is formed via neighboring group participation and is then
selectively attacked from above by the silylated base, generating the β-anomer 53 of
the ribose stereoselectively (Scheme 18). If the sugar component contains polar
groups (such as the trifluoroacetyl or Fmoc groups in our case), the normal reaction
course requires an excess of Lewis acid catalyst, part of which is neutralized to give a
stable complex with the sugar residue.[188]

Scheme 18. Stereoselectivity in nucleosidation reactions.

3.5.1 Nucleosidation with adenine and SnCl₄

For adenine, the following mechanism is generally agreed upon: N-
benzoyladenine 33 is silylated at N(9) and at the NH₂Bz group to give 54 (Scheme
19). The Lewis acid SnCl₄ forms a complex at N(1) with the silylated adenine,
thus constituting an obstacle to glycosylation at N(1), and enabling the preferential
formation of other products.[188] The most nucleophilic nitrogen atom N(3) attacks
the 1,2-acetoxonium ion 55 to give intermediate 56, which is stabilized under loss of a
trimethyl silyl cation at N(9) and formation of 57. The following N(3) to N(9)
transglycosylation is supposedly induced by an electron imbalance in the purine
system owing to the electron-withdrawing N-benzoyl group. It affords the N(9)-nucleoside 58 which is the thermodynamic reaction product (Scheme 19). The
products 35 (Scheme 11) and 38 (Scheme 15) likely arise from such a reaction. The
nature of the base, catalyst, solvent, and the temperature play a vital role in this
process.
Scheme 19. Mechanism of the nucleosidation with adenine and 1,2,3-triacetoxyribose in CH$_3$CN with SnCl$_4$. 
3.5.2 Nucleosidation with adenine and TMSOTf

If TMSOTf is used as the Lewis acid, the N(9)-silylated N-benzoyladenine 54 (Scheme 20) is additionally silylated at N(1) to give intermediate 59 which is stabilized by cleavage of the TMS group at N(9). The N(1)-silylated base 60 can undergo initial glycosylation at N(3) to give the kinetically preferred product, which can be observed after short reaction times. This N(3)-nucleoside 57 can rearrange to the thermodynamically more stable N(9)-nucleoside 58. Concurrently, 60 can immediately interact with the acetoxonium ion 55 to yield the N(9)-nucleoside or the N(7)-nucleoside 61 in analogy to our compound 34. Again solvent, temperature, and reaction time have a strong impact on the outcome of the reaction (Scheme 20).

\[
\begin{align*}
\text{Me}_3\text{SiNBz} & \quad \text{+TMSOTf} & \quad \text{Me}_3\text{SiNBz} \\
54 & \quad \text{Me}_3\text{SiNBz} & \quad \text{Me}_3\text{SiNBz} \\
\quad \text{SiMe}_3 & \quad \text{SiMe}_3 & \quad \text{SiMe}_3 \\
\text{Me}_3\text{SiNBz} & \quad -\text{TMSOTf} & \quad \text{Me}_3\text{SiNBz} \\
59 & \quad 60 & \quad 61
\end{align*}
\]

**Scheme 20.** a) TMSOTf affects silylation at N(1) in 54. b) Possible reaction products arising from glycosylation with 60.
3.5.3 Nucleosidation with pyrimidine bases and TMSOTf

Silylation of pyrimidine bases such as benzoylated cytosine 62 affords the O-silylated products. Interaction of 63 with the 1,2-acetoxonium ion 55 leads to the formation of intermediate 64 which subsequently reacts with OTf\(^-\) to give the stable reaction product 65 (Scheme 21).[185]
4 IN VITRO EVALUATION OF THE LEAD COMPOUND

4.1 AN INTRODUCTION TO ENZYME KINETIC STUDIES

The mechanism of inhibitor binding to an enzyme in the presence of substrate is investigated by application of the Michaelis-Menten equation\cite{91,189} to determine whether the experimental data are in accord with a distinct inhibitory mechanism. If the equation applies, the inhibitory mechanism could be competitive, noncompetitive, or uncompetitive.

The interaction between a competitive inhibitor and an enzyme resembles that between a substrate and the enzyme.\cite{92,190-193} When both substrate and inhibitor are present, the following reaction equation applies:

```
Substrate + Inhibitor + Enzyme \rightleftharpoons Substrate-Enzyme complex
```

At equilibrium,

\[
K_M = \frac{s(e - p - q)}{p} \quad \text{and} \quad K_i = \frac{i(e - p - q)}{q}
\]

where \(K_M\) is the dissociation constant for the substrate-enzyme complex (Michaelis-Menten constant), \(K_i\) is the dissociation constant for the inhibitor-enzyme complex, \(i = \) concentration of inhibitor, \(p = \) concentration of substrate-enzyme complex, \(q = \) concentration of inhibitor-enzyme complex, \(e = \) concentration of enzyme originally present, and \((e-p-q) = \) amount of free enzyme.
These equations can be transformed into the following equation

\[
\frac{1}{v} = \frac{1}{V} + \frac{(1 + i / K_i) K_M}{V} \times \frac{1}{s}
\]

where \( V \) = maximal value of reaction rate, \( s \) = substrate concentration, \( v \) = reaction rate, and \( K_M \) = Michaelis-Menten constant. A graphical procedure for data analysis is the Lineweaver-Burk plot which is the graph of \( 1/v \) against \( 1/s \) at varying inhibitor concentrations. These plots should be straight lines intersecting at the same point on the ordinate. The intercept on the abscissa (\( 1/v = 0 \)) is \(-1/K_M\) in the absence of inhibitor and \(-1/[K_M (1 + i/K_i)]\) in its presence. Further information can be obtained by plotting the linear relationship between \( 1/v \) and \( i \) (Dixon plot). If the substrate concentration is changed, the slope changes and the intercept on the \( 1/v \) axis (\( i = 0 \)) changes, but if the lines are extrapolated they intersect at a point whose coordinates correspond to the value of \(-1/K_i\) on the abscissa (\( i \) axis) and \( 1/V \) on the ordinate (\( 1/v \) axis). The lower the value of \( K_i \), the higher the affinity of the inhibitor for the enzyme.

In the case of noncompetitive inhibition, it is assumed that the inhibitor combines equally well with the free enzyme or the substrate-enzyme complex, and in either case, it inhibits the reaction. In this case, the following equation may be derived:

\[
\frac{1}{v} = \frac{1}{V} (1 + K_M / s)(1 + i / K_i)
\]

In the Lineweaver-Burk plot, the lines intersect at the same point on the abscissa. In the corresponding Dixon plot, all lines for different substrate concentrations intersect at the same point on the abscissa (-1/\( K_i \)).

A third type of inhibition, termed uncompetitive, arises when the inhibitor combines only with the substrate-enzyme complex. The following equation may be derived:

\[
\frac{1}{v} = \frac{K_M}{sV} + \frac{1}{V} (1 + iK_i)
\]

Parallel lines in the Lineweaver-Burk and the Dixon plot indicate an uncompetitive mechanism.\[^{[189]}\]
4.2 THE BIOLOGICAL ASSAY

The synthesized compounds were tested for in vitro inhibitory activity with COMT obtained from rat liver. The IC$_{50}$ values (concentration of inhibitor at which 50% activity of the enzyme is observed) were determined in a radiochemical assay developed by Zürcher et al.$^{[194]}$

In the assay, the substrate benzene-1,2-diol 66, the cofactor, and varying concentrations of inhibitor are incubated with the enzyme in a buffered aqueous solution containing Mg$^{2+}$ ions at 37 °C. The cofactor SAM carries a tritiated methyl group, which is transferred to the substrate upon incubation with the enzyme. After a certain time, the reaction is quenched by the addition of acetic acid and an organic scintillation fluid is added. The product ($^3$H)-2-methoxyphenol is extracted into the organic phase, whereas ($^3$H)-SAM remains in the aqueous phase. The product concentration is measured by counting the decays per min (dpm) in the organic phase. The dpm are proportional to the concentration of the product 67, which is a measure for the enzymatic activity (Scheme 22).

![Scheme 22](image)

Scheme 22. Principle of the radiochemical assay developed by Zürcher et al.$^{[194]}$

In the case of the potential bisubstrate inhibitors, the assays were performed with and without preincubation of the enzyme with the inhibitor in the absence of both substrates (benzene-1,2-diol, SAM). Binding of an inhibitor to an enzyme requires some time until an equilibrium is established. If an inhibitor is preincubated
with an enzyme, this equilibrium can be established and the inhibitor will exhibit its full effect when the substrate is added and the enzymatic reaction is measured. If the inhibitor is not preincubated with the enzyme, the equilibrium might not be fully established (if the process is slow) and a weaker inhibitory activity is measured. Tight-binding inhibitors of COMT exhibit different inhibition kinetics with and without preincubation.\[25,195\] Figure 19 shows a logarithmic plot of dpm vs logarithmic inhibitor concentration for inhibitor 7. The inflection point of the sigmoidal curve corresponds to the IC$_{50}$ value. For the determination of the IC$_{50}$ value (concentration of inhibitor at which 50% activity of the enzyme is observed) of an inhibitor, the enzymatic activity is measured for at least seven different inhibitor concentrations within a concentration range that covers the steep part of the curve.

\begin{align*}
\text{curve fitting:} \\
y := & a[1] + (a[2] - a[1])/(1 + \exp(-a[3](x-a[4]))) \\
\text{without preincubation:} \\
& IC_{50} = 10^{a[4]} = 4.7 \ \mu M \\
\text{with preincubation:} \\
& IC_{50} = 10^{a[4]} = 2.6 \ \mu M
\end{align*}

Figure 19. Plot of dpm vs logarithmic inhibitor concentration for inhibitor 7 (Scheme 15). The concentration of the inhibitor at the turning point of the sigmoid curve corresponds to the IC$_{50}$ value of the compound.
4.3 IC\textsubscript{50} VALUES FOR THE LEAD AND COMPARISON COMPOUNDS

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cartoon}
\caption{IC\textsubscript{50} values of potential bisubstrate inhibitor 7, precursor 48, and model compounds 14, 17, 8, 50, and 49 determined with and without preincubation in the presence of inhibitor. Preincubation was performed in the absence of both benzene-1,2-diol and SAM.}
\end{figure}
The results (Figure 20) show that molecule 7 is a 10-fold more potent inhibitor than catechol 14 and more than 50-fold more potent than adenosine 17. This could be the result of its bisubstrate nature, which enables it to occupy both the catechol and SAM binding sites. For a bisubstrate inhibitor one would expect tighter binding than for the single substructures 14 and 17, confirming the synergistic effect arising from linking two molecules, each of which has a rather weak affinity for the enzyme. The nearly identical IC$_{50}$ values with (2 µM) and without preincubation (4 µM) support similar inhibition modes in the two assays. Compounds 49 and 50 have an inhibitory activity in the same range as catechol 14. This indicates that addition of a ribose fragment alone does not improve the inhibitory effect of the catechol moiety. The IC$_{50}$ value of compound 48 is 50 times higher compared to molecule 7. These results clearly confirm that the nucleosidic unit of 7 does contribute to its binding affinity for COMT which is another indication of its bisubstrate nature. They encouraged us to further investigate the binding mechanism of 7 to COMT by performing kinetic studies.

Molecule 8 was found to be surprisingly potent, inhibiting COMT in the nanomolar range, similar to inhibitors such as Tolcapone (Figure 2 in Section 1.2.4). One interpretation of these findings could be that $N$-succinimidyl 2,3-dihydroxy-5-nitrobenzoate (8) reacts with an amino acid side-chain functionality in the active site of COMT and blocks it irreversibly. Another explanation might be that the succinimide ester functionality exhibits similar electrostatic interactions with the enzyme as the nitro group in Tolcapone. These hypotheses were further examined in studies on the inhibition kinetics of 8 (cf. Section 4.4.4).
4.4 INHIBITION KINETICS

4.4.1 Inhibition kinetics of the lead compound

To investigate the mechanism of enzyme inhibition by molecule 7, kinetic studies were performed. To determine the inhibition mode with respect to the catechol pocket, the concentration of benzene-1,2-diol was varied at saturating SAM concentrations for different inhibitor concentrations (Figure 21). The incubation time was varied between 1 min and 15 min. Assays were performed with and without 15 min of preincubation of the enzyme in the presence of inhibitor. Lineweaver-Burk plots were used to illustrate the inhibition kinetics and to calculate the $K_M$ values of the substrates. The $K_M$ value is the concentration of substrate at which the reaction rate $v$ is half that of the maximum value.\[^{189}\] The two $K_M$ values of $357 \pm 30 \mu M$ for benzene-1,2-diol and $33 \pm 5 \mu M$ for SAM are in good agreement with literature results.\[^{25,194}\] $K_I$ values were calculated from the corresponding Dixon plots (graphs not shown). The $K_I$ value is the concentration of inhibitor at which the $K_M$ value of the enzyme for the corresponding substrate is doubled (cf. Section 4.4.3, Table 3).

An error analysis was performed on the data from the radioactivity measurements and the program proFit was used to apply a weighted linear regression employing the Levenberg-Marquardt algorithm to generate the Lineweaver-Burk plots.\[^{196}\]

Inhibitor 7 shows a noncompetitive inhibitory behavior with respect to the catechol binding site (Figure 21) in all experiments (Table 1).

<table>
<thead>
<tr>
<th>incubation time / min</th>
<th>preincubation time / min</th>
<th>inhibition mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0</td>
<td>noncompetitive</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>noncompetitive</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>noncompetitive</td>
</tr>
</tbody>
</table>
To determine the inhibition mechanism with respect to the SAM binding site, the concentration of SAM was varied at saturating concentrations of benzene-1,2-diol for different inhibitor concentrations. The Lineweaver-Burk plots for inhibitor 7 (Figure 22) reveal a competitive inhibition pattern in all experiments (Table 2).
Figure 22. Lineweaver-Burk plot of reciprocal enzymatic activity vs reciprocal SAM concentration for varying concentrations of inhibitor 7 at saturating benzene-1,2-diol concentrations ([benzene-1,2-diol] = 2.5 mM) for an incubation time of 1 min.

Table 2. Summary of the results from all kinetic experiments which were performed with 7 at varying concentrations of SAM to determine the inhibition mechanism with respect to the SAM binding site.

<table>
<thead>
<tr>
<th>incubation time / min</th>
<th>preincubation time / min</th>
<th>inhibition mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>competitive</td>
</tr>
<tr>
<td>1</td>
<td>15 (with SAM)</td>
<td>competitive</td>
</tr>
<tr>
<td>1</td>
<td>15 (with substrate)</td>
<td>competitive</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>competitive</td>
</tr>
</tbody>
</table>
An important piece of information about the kinetic behavior of an inhibitor is its off-rate from the enzyme, which is determined in a dialysis experiment: The inhibitor 7 was incubated with the enzyme preparation at 37 °C for 15 min in the presence of SAM and MgCl₂. Dialysis of the incubation mixture against 20 l KHPO₄/KH₂PO₄ buffer, pH = 7.6, was performed, and the relative inhibition was determined after 0, 0.5, 2, 4, 6, 8, and 24 h. The loss of activity owing to degradation of the enzyme was measured in the same experiment (Figure 23). We measured a decrease in inhibition by 50% within 5 h. This indicates that the inhibitor binds reversibly but its dissociation from the enzyme is rather slow and in the same range as that of tightly binding inhibitors of COMT. [25]

![Chemical Structure](image)

**Figure 23.** Plot of enzymatic activity (dpm) vs time of dialysis for a preparation of inhibitor 7 preincubated with the enzyme and then dialyzed against a buffered solution for 24 h.
4.4.2 Inhibition kinetics of the comparison compound

The same studies were performed on catechol 14 for comparison. Catechol 14 shows a competitive inhibition pattern for the catechol pocket as would be expected for a derivative of benzene-1,2-diol (Figure 24).

![Diagram of catechol 14](image)

**Figure 24.** Lineweaver-Burk plot of reciprocal enzymatic activity vs reciprocal benzene-1,2-diol concentration for varying concentrations of inhibitor 14 at saturating SAM concentrations ([SAM] = 183 μM) for an incubation time of 15 min.

Model compound 14 features an uncompetitive inhibition mechanism with respect to the SAM binding site, which was found in the equivalent experiment when
the concentration of SAM was varied at saturating benzene-1,2-diol concentrations for different concentrations of inhibitor. This result is expected for a compound that does not bind to the SAM binding site. It can be understood considering that SAM binds to the enzyme complex prior to the substrate: the amount of enzyme which is in the right conformation to bind the catechol substrate is dependent on the concentration of SAM only (in the concentration range below saturating concentrations) if the inhibitor does not interfere with SAM binding site. Therefore, the lines for different inhibitor concentrations in the Lineweaver-Burk plot are parallel as depicted in Figure 25.

![Chemical Structure](HO\_OH\_O\_N\_H\_OMe

**Figure 25.** Lineweaver-Burk plot of reciprocal enzymatic activity vs reciprocal SAM concentration for varying concentrations of inhibitor 14 at saturating benzene-1,2-diol concentrations ([benzene-1,2-diol] = 2.5 mM) for an incubation time of 1 min.
4.4.3 Interpretation of the kinetics for the lead compound

It is known that binding of the cofactor SAM to COMT occurs prior to the binding of the catechol substrate.\(^{[9]}\) Complexation of SAM actually induces the formation of the catechol binding site as can be seen by comparison of the X-ray crystal structure of the apoenzyme\(^{[12]}\) with the structure of the quaternary complex containing SAM, a Mg\(^{2+}\) ion, and 3,5-dinitrocatechol;\(^{[12,13]}\) the catechol substrate does not bind to the enzyme without previous complexation of SAM.

Table 3 gives a summary of the results found for the inhibition kinetics of the lead 7 and the comparison compound 14. Compound 14 is a competitive inhibitor with regard to the catecholic substrate and an uncompetitive inhibitor for the cofactor SAM, which means that it does not bind to the cofactor binding site. Both results are in accord with our expectations for a substrate analog inhibitor.

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>inhibition mechanism with regard to benzene-1,2-diol</th>
<th>inhibition mechanism with regard to SAM</th>
<th>(K_i \ [\mu M]) with regard to benzene-1,2-diol</th>
<th>(K_i \ [\mu M]) with regard to SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>competitive</td>
<td>uncompetitive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>noncompetitive</td>
<td>competitive</td>
<td>(0.55 \pm 0.1)</td>
<td>(0.3 \pm 0.1)</td>
</tr>
</tbody>
</table>

Bisubstrate inhibitor 7 is a competitive inhibitor with regard to the cofactor SAM and, therefore, competes with SAM for the cofactor binding site in a first equilibrium (Figure 26). These findings are in accord with what is known about the inhibition kinetics for analogs of SAM that have been tested as inhibitors of COMT, and exhibit a competitive inhibition mechanism for the SAM binding site.\(^{[32-34,197]}\) So far the kinetic studies confirm that 7 does bind to the SAM binding site which would be expected for a bisubstrate inhibitor.

The binding of inhibitors of the catechol binding site has been thoroughly studied for many catechols.\(^{[14,25]}\) Most catechols that inhibit the enzyme in the \(\mu M\) range exhibit a competitive inhibition mechanism such as model compound 14, whereas inhibitors with high affinity to COMT like Tolcapone\(^{[21]}\) (IC\(_{50} = 40 \text{nM}\))
and Entacaponel\(^{30}\) (IC\(_{50} = 18\) nM) bind in a noncompetitive fashion to the enzyme after preincubation with the enzyme\(^{195}\) and in a competitive mechanism without preincubation.

![Figure 26. Hypothetical mechanism of competitive binding of SAM or of bisubstrate inhibitor 7 to the active site of COMT. Binding of SAM induces conformational alterations in the active site which creates the binding pocket for the catechol substrate. If 7 binds to the active site of COMT, the enzyme is inhibited. Binding of 7 might also induce conformational changes in the enzyme.](image)

For compound 7, a noncompetitive inhibition mechanism was found with respect to the catechol binding site. Because model compound 14 does bind to the catechol binding site in a competitive mechanism, it is unlikely that the catechol residue in the bisubstrate inhibitor 7 binds to a different binding site in the enzyme. Based on the order of binding of the natural cofactor and substrate, a possible interpretation of the observed kinetics is the following: in the initial equilibrium, 7
competes with SAM for the cofactor binding site. Only if SAM binds to the enzyme is the latter in the right conformation for binding substrate. Therefore, the concentration of inhibitor 7 directly affects the amount of enzyme which is in the active conformation for catalysis. In this respect, 7 behaves as a classical noncompetitive inhibitor which binds to an enzyme (not necessarily to the active site) inducing conformational changes in the enzyme which, as a consequence, cannot bind the substrate and perform catalysis (Figure 26).

Binding of the bisubstrate inhibitor to the SAM binding pocket might also induce alterations in the binding characteristics of the catechol site. The synergistic effect arising from the bisubstrate nature of the inhibitor might enhance the affinity of its catechol residue for the catechol binding site. This could result in a situation where the catechol residue of the inhibitor 7 is locked in the binding site, a situation that might resemble the noncompetitive kinetics found for tight-binding inhibitors after preincubation. The latter hypothesis is supported by the slow dissociation of the inhibitor from the enzyme as observed in the dialysis experiments.

In both cases, the bisubstrate inhibitor must dock into the SAM binding site before it binds to the catechol binding pocket (Figure 27). Co-crystallization of the bisubstrate inhibitor 7 and COMT is currently underway to further clarify the exact complexation mode.

**Figure 27.** Summary of the kinetic studies: bisubstrate inhibitor 7 binds to the SAM pocket prior to binding to the catechol pocket in COMT.
4.4.4 Inhibition kinetics for \( N \)-succinimidyl 2,3-dihydroxy-5-nitrobenzoate (8)

In order to prove or to disprove the hypothesis that the activated \( N \)-succinimidyl 2,3-dihydroxy-5-nitrobenzoate (8) reacts with an amino acid side-chain functionality in the active site of COMT and, therefore, blocks its active site irreversibly, we performed the same dialysis experiment with 8 as described in Section 4.4.1 with 7. If 8 were an irreversible inhibitor, enzyme inhibition should remain constant over a couple of hours. When a preparation of the inhibitor 8, preincubated with the enzyme was dialyzed, the inhibition decreased by 50% within 4.5 h. This indicates that the inhibitor binds reversibly, but its dissociation from the enzyme is rather slow and in the same range as that of tight-binding inhibitors of COMT (Figure 28).\(^{25}\)

![Chemical structure of 8](image)

**Figure 28.** The inhibitor 8 was incubated with the enzyme preparation at 37 °C for 15 min in the presence of SAM and MgCl\(_2\). Dialysis of the incubation mixture against 20 litres of \( \text{KHPO}_4/\text{K}_2\text{PO}_4 \) buffer, pH = 7.6, was performed, and the relative inhibition was determined after 0, 0.5, 2, 4, 6, 8, and 24 h. The loss of activity owing to degradation of the enzyme was measured in the same experiment.
To elucidate the inhibition mode of 8, the concentration of benzene-1,2-diol was varied at saturating SAM concentrations for different inhibitor concentrations. The incubation time was 1 min. Assays were performed with and without preincubation of the enzyme (15 min) in the presence of inhibitor 8 (Figure 29).
Catechol 8 displays a competitive inhibition mode at an incubation time of 1 min ($K_i = 100 \pm 5 \text{ nM}$) as would be expected for a derivative of benzene-1,2-diol. On the other hand, 8 exhibits noncompetitive inhibition kinetics after 15 min of preincubation ($K_i = 150 \pm 10 \text{ nM}$). These findings are in accordance with the kinetics of tight-binding inhibitors like Tolcapone or 3,5-dinitrocatechol which exhibit competitive inhibition kinetics for an incubation time of 1 min and noncompetitive inhibition kinetics if preincubated with the enzyme for 15 min. Generally, it is known that tight-binding inhibitors (IC$_{50}$ values in the nanomolar range, combined with a slow dissociation from the active site of the enzyme) tend to block the active site. If the incubation or preincubation time is long, the initial equilibrium between inhibitor and substrate gets very slow, because the inhibitor does not dissociate fast enough from the enzyme. Thus, a compound that might be expected to act as a linear
competitive inhibitor with respect to the variable substrate could appear to act as a noncompetitive inhibitor.\textsuperscript{[195]}

Because the kinetic behavior of 8 resembles the inhibition kinetics of 3,5-dinitrocatechol, and because of the structural similarities between the two molecules, 8 most probably binds to the catechol binding pocket similar to 3,5-dinitrocatechol and Tolcapone.\textsuperscript{[12,13]} This suggests that the N-succinimidyl functionality has similar interactions with the amino acids in the active site as the nitro group, and perhaps it can be used as a replacement for the nitro functionality. It would be interesting to look at stable analogs of the N-succinimidyl functionality and test their inhibitory activity, or to substitute the N-succinimidyl functionality for nitro groups in other enzyme inhibitors since nitro groups tend to cause toxicity problems, and, therefore, such compounds are rarely used as pharmaceuticals. However, such studies go beyond the scope of this thesis.
5 STRUCTURAL VARIATIONS OF THE LEAD

5.1 VARIATION OF THE FUNCTIONALITY FOR BINDING IN THE BASE POCKET

5.1.1 Design of new target molecules

In order to explore the range of chemical functionality that fit into the adenine binding site, we considered introducing cytosine (68) and modified bases into the lead compound 7 (Figure 20). Molecules 69, 70, and 71 (Figure 30) containing cytosine, 6-amino-3,4-dihydropyrimidin-4-one (72), and 4-aminopyrazolo[3,4-<i>d</i>]pyrimidine (73) (cf. Figure 31) as adenine substitutes were chosen as new targets.

![Figure 30. Potential inhibitors 69, 70 and 71 containing cytosine and the modified bases 72 and 73, respectively.](image)

Figure 31 shows a schematic drawing of the H-bonding interactions of the three bases docked into COMT. While the H-bonding arrays resemble those seen in the modeled complex with 7 (Figure 15b), an unfavorable contact between the carbonyl O-atom of Gly117 and cytosine was observed in the modeled complex with 69. In
the complex with 70, an additional H-bond was found between the pyrimidinone C=O group and the backbone N–II of Trp143. This N–II group can also participate in an H-bond with N(2) in the complex with 71. The intermolecular interactions involving the ribose and catechol moieties are similar for 7 and the predicted complexes with all three potential bisubstrate inhibitors 69, 70, and 71. Figure 32a is an overlay showing the relative positions of the two pyrimidine bases and adenine docked into the base pocket of the active site of COMT. The 4-aminopyrazolo[3,4-d]pyrimidine (73) is located in the same position as adenine. Figure 32b depicts an overlay of 7, 69, and 70 and shows the conformational differences in the molecules due to the different ring sizes of pyrimidine as compared to adenine. The bridge N-CH2-CH2-O-CH2 between catechol and ribose features a sequence of favorable sc-ap-ap-sc torsional angles for 69 and 70. The sequence of torsional angles for that bridge found in the modeled compound 7 is ap-ap-sc-sc (Section 2.5, Figure 15a).

Figure 31. a) Schematic drawing of the interactions between the cytosine base (68) of 69 and COMT in a modeled complex. b) Schematic drawing of the interactions between the 6-amino-3,4-dihydropyrimidin-4-one (72) moiety of 70 and COMT in a modeled complex. c) Schematic drawing of the interactions between 4-aminopyrazolo[3,4-d]pyrimidine (73) in 71 and COMT in a modeled complex. Distances are in pm.
Figure 32. a) The bases 72 (cf. Scheme 23) (blue), cytosine (green), and adenine (orange) overlaid in the base pocket of COMT as seen in the molecules 70, 69, and 7 in a complex with COMT. Dotted lines indicate hydrogen bonds. b) Overlay of the potential bisubstrate inhibitors 69, 70, and the lead 7 in the modeled complex with COMT.
5.1.2 Synthesis of potential inhibitors with different bases

5.1.2.1 Synthesis of the target compounds 69 and 70 with the pyrimidine bases

The synthesis of the target molecules 69 and 70 was carried out in analogy to that of the lead compound 7 during the diploma work of P. Ballmer.\textsuperscript{[198]} Thus, the nucleosidation precursor 41 was coupled to \( N \)-benzoylated cytosine 62 and to 6-benzoylamino-3,4-dihydropyrimidin-4-one (74) (cf. Scheme 24). The synthesis of 62 has been reported.\textsuperscript{[182]} Compound 74 was obtained in three steps by reacting thiourea and ethyl cyanoacetate in the presence of base to give 75,\textsuperscript{[199]} followed by desulfurization with Raney-Ni which afforded 72\textsuperscript{[200]}, and benzoylation (Scheme 23).

\[
\text{NaOEt, EtOH,} \quad \Delta, 2 \text{h, 93\%}
\]

\[
\text{H}_2\text{N}^+\text{NH}_2 \quad \text{A, 2h, 93\%}
\]

\[
\text{ Ra-Ni, aq. NH}_3, \quad \Delta, 1 \text{h, 47\%}
\]

\[
\text{Benzoic anhydride, EtOH,} \quad 140 ^\circ \text{C, 4h, 83\%}
\]

Scheme 23. Synthesis of 6-benzoylamino-3,4-dihydropyrimidin-4-one (74).

In the nucleosidation reactions, the best results were obtained with trimethylsilyl triflate (TMSOTf) as the Lewis acid, which gave the nucleosides 76 and 77 in 79\% and 58\% yield, respectively (Scheme 24). The use of SnCl\(_4\) in the nucleosidation with 74 gave rise to a dimeric by-product 78, which was identified by \(^1\text{H}\) NMR spectroscopy and mass spectrometry, resulting from nucleosidation at the Fmoc-protected N-atom in 41 and at C(1') of the ribose. Scheme 25 shows a possible structure of 78 which is in accordance with the available spectroscopic data.
Scheme 24. Nucleosidation of the peracetylated ribose 41 with the pyrimidinone bases \(N\)-benzoylcytosine and 74.

Scheme 25. The nucleosidation reaction with SnCl\(_4\) gave rise to a dimeric by-product with the proposed structure 78.

The constitution and configuration of products 76 and 77 were confirmed by NOE difference spectroscopy (Figures 33 and 34).
The NOE (Figure 33) observed between the ribosyl protons H-C(1') and H-C(4') confirms the β-configuration of 76. The NOEs between the base proton H-C(6) and the ribosyl protons H-C(1'), H-C(2'), and H-C(3') confirm that 62 is connected to the ribose via N(1).
Figure 33. $^1$H NMR (CDCl$_3$, 500 MHz) NOE spectrum of 76. a) Irradiation at H-C(6) (8.20 ppm). Amplified signals (from left to right): H-C(1') (6.37 ppm), H-C(2') (5.55-5.48 ppm), H-C(3') (5.55-5.48 ppm). b) Irradiation at H-C(1') (6.37 ppm). Amplified signals: H-C(6) (8.20 ppm), H-C(1') (6.37 ppm), H-C(2') (5.55-5.48 ppm), H-C(3') (5.55-5.48 ppm), H-C(4') (4.33 ppm). c) Irradiation at H-C(2') (5.55-5.48 ppm). Amplified signals: H-C(6) (8.20 ppm), H-C(1') (6.37 ppm), H-C(4') (4.33 ppm). d) Irradiation at H-C(3') (5.55-5.48 ppm). Amplified signals: H-C(6) (8.20 ppm), H-C(1') (6.37 ppm), H-C(4') (4.33 ppm), H-C(5') (3.77-3.75 ppm).
The NOE (Figure 34) observed between the ribosyl protons H-C(1') and H-C(4') confirms the $\beta$-configuration of 77. The NOEs between the base proton H-C(2) and the ribosyl protons H-C(1'), H-C(2'), and H-C(5') confirm that 74 is connected to the ribose via N(1).
Fmoc deprotection of 76 and 77, and coupling with the N-succinimidyl benzoate 8 (Scheme 4) were performed in one pot. Cleavage of the Fmoc group was effected under high dilution conditions in DMF/HNEt₂ 1:1. The HNEt₂ was readily removed by evaporation at reduced pressure to provide a solution of the free amine in DMF, which was used directly in the coupling to 2,3-dihydroxybenzoate 8 (Scheme 4), affording 79 and 80 in yields of 49 and 56%, respectively (Scheme 26). The synthesis of the target compounds 69 and 70 was completed by global deprotection. One-step deprotection using MeNH₂ in EtOH for 20 min at 20 °C afforded 69 from 79 in 82% yield. The same reaction conditions led only to cleavage of the acetate groups in 80 to give compound 81 (80%). However, compound 80 or
81 could be transformed into 70 in 82% yield by reaction with 25% aq. NH$_3$/MeOH 1:1 at 55 °C for 18 h.

Scheme 26. Coupling of the nucleosides 76 and 77 with the dihydroxybenzoate 8 (Scheme 4) afforded 79 and 80, respectively, which were then deprotected to the target molecules 69 and 70.

5.1.2.2 Conformational studies on 69

An NOE spectrum of 69 in D$_2$O was obtained to analyze its conformation in water (Figure 35). The NOEs between the cytosine proton H-C(6) and the ribosyl protons H-C(3') and H-C(2') indicate that cytosine adopts the favorable anti-conformation with respect to the ribose (and therefore is exposed for H-bonding on the "Watson-Crick" H-bonding site)$^{[184]}$ in the free state which is also needed to bind to the active site of COMT as predicted by modeling. However, there is a weak
NOE between one of the catecholic protons and H-C(5) of the cytosine, indicating that the unbound state of the molecule is stabilized by hydrophobic collapse. Hydrophobic collapse could stabilize the free ligand in two ways. On the one hand, it is favorable enthalpically, because the two terminal chromophores cytosine and catechol are brought into proximity for attractive interactions. On the other hand, this phenomenon is accompanied by desolvation of the molecule resulting in an entropy gain. In the collapsed conformation molecule 69 adopts in solution, the catechol unit is not in the right orientation to bind to the active site of COMT and, therefore, upon binding significant reorganizational energy would be required, which is unfavorable for the binding free enthalpy of the ligand.
Figure 35. $^1$H NMR ($\text{D}_2\text{O}, 500 \text{ MHz}$) NOE spectrum of 69. a) Irradiation at H-C(cat.) (8.44 ppm). Amplified signals (from left to right): H-C(5) (5.60 ppm) (weak), b) Irradiation at H-C(6) (7.72 ppm). Amplified signals: H-C(1') (5.88 ppm), H-C(5) (5.60 ppm), H-C(2') (4.40-4.37), H-C(3') (4.30-4.27 ppm). c) Irradiation at H-C(5) (5.60 ppm). Amplified signals: H-C(cat.) (8.44 ppm) (weak), H-C(6) (7.72 ppm).
5.1.2.3 Nucleosidation with the base 4-aminopyrazolo[3,4-d]pyrimidine (73)

B. Laukas attempted the synthesis of target molecule 71 (Figure 30) in his semester project. Nucleosidation of 41 was carried out with 4-N-benamidopyrazolo[3,4-d]pyrimidine (82). Compound 82 was obtained by benzylation of 4-aminopyrazolo[3,4-d]pyrimidine (73) with one equivalent of benzoic anhydride in 74% yield. With two equivalents of benzoic anhydride the product of double benzylation at N(9) and NH2 (83) was isolated in 51% yield (Scheme 27). The conditions of the nucleosidation reaction were varied as shown in Table 4, but all attempts failed to provide the desired product.

### Scheme 27. Benzylation of 73.

### Table 4. Reaction conditions tested for the nucleosidation of 41 with 82.

<table>
<thead>
<tr>
<th>Lewis acid \ base:41:LA:BSA</th>
<th>solvent</th>
<th>reaction time (min)</th>
<th>T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SnCl\textsubscript{4} 1.2:1:4:2:4</td>
<td>CH\textsubscript{3}CN</td>
<td>15</td>
<td>55</td>
</tr>
<tr>
<td>SnCl\textsubscript{4} 2:1:4:4</td>
<td>CH\textsubscript{3}CN</td>
<td>5, 15, 25</td>
<td>55</td>
</tr>
<tr>
<td>SnCl\textsubscript{4} 2:1:4:2</td>
<td>CH\textsubscript{3}CN</td>
<td>10, 15, 25, 35</td>
<td>55</td>
</tr>
<tr>
<td>TMSOTf 2:1:5:4</td>
<td>CH\textsubscript{3}CN</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>TMSOTf 2:1:5:4</td>
<td>ClCH\textsubscript{2}CH\textsubscript{2}Cl</td>
<td>40, 150</td>
<td>55</td>
</tr>
<tr>
<td>TMSOTf 2:1:2.1:1.1</td>
<td>ClCH\textsubscript{2}CH\textsubscript{2}Cl</td>
<td>100, 120, 150, 180, 240, 1080</td>
<td>60</td>
</tr>
</tbody>
</table>
In a different approach to 71, 41 was reacted with allopurinol (84) employing SnCl₄ as Lewis acid. This reaction gave nucleoside 85 in 10% yield. Chlorination at C(6) to afford 86, followed by coupling of the latter to the N-succinimidyl benzoate 8 (Scheme 4), would provide 87. Deprotection of the acetylated sugar OH-groups and simultaneous replacement of the chlorine substituent by NH₂ with NH₃ would provide target molecule 71 (Scheme 28). However, this sequence was not completed within the scope of this thesis.

Scheme 28. Synthetic pathway leading to 71 via nucleoside 85 obtained by nucleosidation of 41 with allopurinol (84).
5.1.3 *In vitro* studies on inhibitor affinities

The following IC50 values were measured for the potential bisubstrate inhibitors 69 and 70, and their precursor molecules 79, 80, and 81 (Figure 36).

![Chemical structures](image)

<table>
<thead>
<tr>
<th>R1</th>
<th>R2</th>
<th>IC50 (without / with preincubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>79 Ac</td>
<td>Bz</td>
<td>147 µM / 193 µM</td>
</tr>
<tr>
<td>69 H</td>
<td>H</td>
<td>522 µM</td>
</tr>
</tbody>
</table>

![Chemical structures](image)

<table>
<thead>
<tr>
<th>R1</th>
<th>R2</th>
<th>IC50 (without / with preincubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 Ac</td>
<td>Bz</td>
<td>24 µM / 28 µM</td>
</tr>
<tr>
<td>81 H</td>
<td>Bz</td>
<td>231 µM / 255 µM</td>
</tr>
<tr>
<td>70 H</td>
<td>H</td>
<td>&gt; 100 µM</td>
</tr>
</tbody>
</table>

Figure 36. IC50-values of potential bisubstrate inhibitors 69 and 70, and precursors 79, 80, and 81 determined with and without preincubation in the presence of inhibitor. Preincubation was performed in the absence of both benzene-1,2-diol and SAM.

Neither the potential bisubstrate inhibitors 69 and 70 nor their precursors 79, 80, and 81 exhibited significant inhibitory activity. They are less active than compound 7 by orders of magnitude. Although 69 and 70 contain the same catechol motif as model compounds 14, 50, and 49 (Figure 20), they exhibit lower activity. Apparently, their catechol moieties cannot bind efficiently to the catechol pocket.

The potential bisubstrate inhibitor 80 unexpectedly exhibited higher inhibitory potency than 70. Therefore, its kinetic behavior with respect to the SAM binding
site was studied by varying the concentration of SAM at saturating benzene-1,2-diol concentrations for different inhibitor concentrations. The Lineweaver-Burk plot of inhibitor 80 (Figure 37) reveals an uncompetitive inhibition mechanism, which implies that it does not bind to the cofactor site and is not a bisubstrate inhibitor.

![Inhibitor 80](image.png)

Figure 37. Lineweaver-Burk plot of reciprocal enzymatic activity vs reciprocal SAM concentration for varying concentrations of inhibitor 80 at saturating benzene-1,2-diol concentrations for an incubation time of 1 min.

Comparison of 7, 69, and 70 demonstrates that the structure of the base is crucial for biological activity of compounds designed as bisubstrate inhibitors. It can be speculated that the bridge N-CH$_2$-CH$_2$-O-CH$_2$ connecting the nucleosidic and
catechol moieties is only of suitable length in 7, but not in 69 and 70 with their smaller bases, although the modeling studies do not provide conclusive support for such an assumption. The bridge between catechol and ribose features a sequence of sc-ap-ap-sc torsional angles for both compounds 69 and 70 in the modeled complex with the enzyme (Figure 32b), which is as favorable as the ap-ap-sc-sc sequence of torsional angles found in the modeled compound 7 (Section 2.5, Figure 15a). The advantage of 7 vs 69 presumably arises from different stabilities of the free states. Compound 69 undergoes hydrophobic collapse and reorganizational energy is required for binding. In contrast, the NOE data obtained for 7 (Section 3.4.3, Figure 18) do not indicate a hydrophobic collapse explaining the huge differences in activity between the two compounds. It can be speculated that the same argument holds for 70 which exhibits a similar structure as 69.

It would be interesting to finish the synthesis of 71 (Scheme 28) and determine its inhibitory activity, because it is similar in length and conformation to 7, but can potentially exploit an extra H-bond (Figure 31c).

5.2 VARIATION OF THE CATECHOL UNIT

5.2.1 Synthesis of a potential bisubstrate inhibitor bearing a catechol moiety without a nitro group

In lead compound 7, one hydroxy group is deprotonated at physiological pH, because of the \( \pi \)-acceptor properties of the nitro group which stabilizes the phenoxide ion. This enhances the affinity of the catecholic moiety in 7 for its binding site, because no energy is needed for the deprotonation of this hydroxy group before coordination to the \( \text{Mg}^{2+} \) ion and the enthalpic gain (more negative enthalpy) is increased. To gain deeper insight into the contribution of this effect to the inhibitory activity of 7, we decided to synthesize an analog (88) lacking a nitro group in the catechol unit.

The synthesis of 88 was achieved by coupling the \( \text{N} \)-succinimidyl benzoate 89 to nucleoside 38. The synthesis of 38 has been discussed in Section 3.4.2, Scheme 14. Compound 89 was synthesized in 63\% yield from commercially available 2,3-dihydroxybenzoic acid (90) which was reacted with HOSu in the presence of DCC.
(Scheme 29). Deprotection of 38 under high dilution conditions in DMF/HNEt$_2$ 1:1 led to a solution of the free amine in DMF after removal of HNEt$_2$ by evaporation. It was used directly in the coupling with catechol 89, affording 91 in a yield of 25%. The synthesis of target compound 88 was completed by global deprotection with MeNH$_2$ in EtOH at 20 °C for 20 min, yielding 88 in 90% yield.

**Scheme 29.** Synthesis of dihydroxybenzoate 89 and coupling to nucleoside 38 (Scheme 14) afforded the target molecule 88.
5.2.2 *In vitro* studies of inhibitor affinity

To determine their inhibitory effects with respect to COMT, the IC₅₀ values of molecules 88 and 89 were measured (Figure 38). Both molecules were found to be about 500 times less potent than the corresponding molecules 8 and 7 (Figure 20) bearing a nitro group *para* to one of the hydroxy groups of the catechol unit.

\[
\text{IC₅₀ (without preincubation) = 138 μM} \quad \text{IC₅₀ (without preincubation) > 100 μM}
\]

*Figure 38. IC₅₀ values of potential bisubstrate inhibitor 88 and precursor 89.*

Compound 89 is a substrate for COMT, because both hydroxy groups at the catechol are protonated at physiological pH and, therefore, can be methylated upon reaction with SAM in the active site of COMT.

Because of steric encumbrance by the enzyme it is not likely that 88 is oriented like a substrate in the active site of COMT (Section 1.2.4, Figure 2), but rather like the bisubstrate inhibitor 7 (Figure 20). In inhibitor 7, the catechol OH-group *para* to the nitro group is deprotonated at physiological pH. In the analog 88 lacking a nitro group at the catechol, the OH-group is protonated. This results in a smaller enthalpic gain upon coordination of 88 to the Mg²⁺ ion compared to 7, because deprotonation of the hydroxy group in 88 prior to complexation to the Mg²⁺ ion costs energy and, hence, results in a decrease of inhibitory activity. In this respect, the results confirm that the catechol unit in the bisubstrate inhibitor 7 does bind to the catechol binding site which was not clear from the kinetic studies described in Section 4.4.1 only.
5.2.3 Synthesis of catechols with a sulfonamide linker

Because the sulfonamide group is more strongly electron-withdrawing than the carboxamide function and, therefore, may be a better substitute for a nitro group, we considered a sulfonamide as a replacement for the carboxamide group on the catechol unit. The torsional angle CSO$_2$-NHCH$_2$ is 90° for a sulfonamide, whereas a carboxamide CCO-NHCH$_2$ is planar. In compound 92 the torsional angles for the bridge N-CH$_2$-CH$_2$-O-CH$_2$ exhibit a less favorable sequence of sc-sc-sc-sc as compared to 7 (Figure 15a). The C=O group in carboxamides is reported to be a better hydrogen bond acceptor than the S–O group in sulfonamides, although conformational effects and the use of different H-bond acceptors might have influenced this result.[209,210] The latter aspect does not play a role for the binding enthalpy of our compounds, because according to the models neither the carboxamide in 7 nor the sulfonamide in 92 are involved in hydrogen bonding with amino acid residues from the enzyme.

In analogy to the synthesis of 7 as described in Section 3, the synthetic strategy toward molecules such as 92 included the preparation of the sulfonyl chloride 93 and its coupling to the nucleosidic unit 38 after deprotection of the Fmoc group (Scheme 30).

Scheme 30. Sulfonamide 92 can be obtained by coupling of chlorosulfonated catechol 93 to the nucleosidic part 38 after Fmoc deprotection of the latter.
5.2.3.1 Sulfonation of the catechol unit

For the synthesis of the sulfonated catechol 93, we tried to perform a sulfonation on a nitrocatechol derivative. Sulfonation of 1,2-dihydroxybenzene, 1,2-dimethoxybenzene, and 1,2-ethylenedioxybenzene has been described in literature;[211-215] it usually occurs para to one of the hydroxy groups. Depending on the reaction conditions, products of double sulfonation (with one sulfonic acid group ortho and the other one para to one hydroxy or alkoxy group), polysulfonates, and polysulfates[211] were isolated from the reaction mixtures. However, all our efforts to sulfonate the nitro derivatives 1,2-dihydroxy-4-nitrobenzene (94), 1,2-dimethoxy-4-nitrobenzene (95), and 1,2-methyleneedioxy-4-nitrobenzene (96) failed. Table 5 summarizes the results.

We finally succeeded in sulfonating 6-nitro-2,3-dihydro-1,4-benzodioxine (97),[216] obtained in 94% yield from 94 and 1,2-dibromoethane,[217] by reaction with sulfuric acid (95%) at 140 °C to give 98 in a yield of 31%.[218,219] The corresponding sulfonyl chloride 99 was synthesized by reaction of 98 with PCl₅ in 80% yield. Compound 99 was reacted with methylamine to yield 100 (84%) which could be used as a model compound to investigate the cleavage of the ethylenedioxy protecting group (Scheme 31).

![Scheme 31. Sulfonation of 97 and synthesis of the sulfonamide 100.](image)
Table 5. Attempts to sulfonate the nitrated catechol derivatives 94, 95, and 96.

<table>
<thead>
<tr>
<th>compound</th>
<th>reagent</th>
<th>T [°C]</th>
<th>t [min]</th>
<th>product</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>ClSO₂H</td>
<td>0</td>
<td>60</td>
<td>no desired product</td>
</tr>
<tr>
<td>95</td>
<td>ClSO₂H</td>
<td>20</td>
<td>15</td>
<td>no desired product</td>
</tr>
<tr>
<td></td>
<td>ClSO₂H in CH₂Cl₂</td>
<td>60</td>
<td>160</td>
<td>no desired product</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (95%)</td>
<td>0</td>
<td>15</td>
<td>no desired product</td>
</tr>
<tr>
<td>96</td>
<td>ClSO₂H</td>
<td>0</td>
<td>15</td>
<td>starting material</td>
</tr>
<tr>
<td></td>
<td>ClSO₂H in CH₂Cl₂</td>
<td>20</td>
<td>60</td>
<td>no desired product</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (95%)</td>
<td>60</td>
<td>240</td>
<td>no desired product</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (95%)</td>
<td>20</td>
<td>4320</td>
<td>starting material</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (95%)</td>
<td>60</td>
<td>30</td>
<td>no desired product</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (95%)</td>
<td>140</td>
<td>15</td>
<td>no desired product</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (95%)</td>
<td>120</td>
<td>360</td>
<td>no desired product</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄ (95%)</td>
<td>20</td>
<td>15</td>
<td>starting material</td>
</tr>
</tbody>
</table>
Table 6 summarizes the results of our efforts toward cleavage of the ethylenedioxy protecting group. In general, Lewis acids such as BBr$_3$\cite{220} and (CH$_3$)$_3$Si\cite{221-223} can be used for the cleavage of alkyl ethers.\cite{220-224} The reaction of 100 with Me$_3$SiI afforded 7-amino-N$_5$-methyl-2,3-dihydro-1,4-benzodioxine-5-sulfonamide (101) through reduction of the nitro group by iodide. Because the cleavage of the ethylenedioxy group was not successful in model compound 100, this approach toward the synthesis of sulfonamide 92 was abandoned.

Table 6. Results of the efforts to cleave of the ethylenedioxy group in 100.

<table>
<thead>
<tr>
<th>reagent</th>
<th>solvent</th>
<th>$T$ [°C]</th>
<th>$t$ [min]</th>
<th>product</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBr$_3$ (5 eq.)</td>
<td>CH$_2$Cl$_2$</td>
<td>20</td>
<td>720</td>
<td>starting material</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>CH$_2$Cl$_2$</td>
<td>40</td>
<td>720</td>
<td>starting material</td>
<td>23%</td>
</tr>
<tr>
<td>CH$_3$SiI (4 eq.)</td>
<td>CH$_2$Cl$_2$</td>
<td>20</td>
<td>120</td>
<td>101</td>
<td>95%</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>20</td>
<td>720</td>
<td>101</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>20</td>
<td>2880</td>
<td>101</td>
<td>60%</td>
<td></td>
</tr>
<tr>
<td>CH$_2$Cl$_2$</td>
<td>20</td>
<td>120</td>
<td>101</td>
<td>20%</td>
<td></td>
</tr>
</tbody>
</table>
5.2.3.2 Synthesis of 2,3-dihydroxy-5-nitrosulfonic acid by deamination of 3-amino-2-hydroxy-5-nitrosulfonic acid

3-Amino-2-hydroxy-5-nitrosulfonic acid (102) is commercially available and can be transformed into the desired sulfonic acid via diazotation and treatment with sulfuric acid or Cu(I)/Cu(II) (Scheme 32). Such reactions of substrates bearing sulfonic acid and hydroxy groups have been reported in the literature. Nevertheless, 102 could not be converted into 103 under the conditions shown in Scheme 32.

We figured that protection of the sulfonic acid and the hydroxy group might increase the chances for a successful conversion of amine 102 to catechol 103. Therefore, we tried to prepare the methyl ether of the hydroxy and the methyl ester of the sulfonic acid in one step by reaction with diazomethane. The reaction was not successful, probably because of solubility problems. We then decided to protect the amino group as an acetamide, which could be selectively cleaved before the diazotation reaction. The hydroxy group could be protected as TBDMS ether or methyl ether. Acetamide 104 was synthesized from 102 by reaction with acetic anhydride in 42% yield. Acetylation and silylation of the hydroxy group failed, but reaction with dimethyl sulfate gave 105 in 47% yield. Reaction of 105 with diazomethane, however, did not afford the sulfonic acid ester 106 (Scheme 33).
Finally, we tried unsuccessfully to prepare the N-methylsulfonamide of 105 under the reaction conditions listed in Table 7.[233,234] The methyl ether function in 105 appeared to be unstable under the reaction conditions. Its cleavage occurred easily, because the corresponding oxynion is stabilized by the electron-withdrawing sulfonic acid and nitro groups. No further efforts were made toward the synthesis of 93 (Scheme 30).

**Scheme 33.** Synthesis of 105. Methyl ester 106 could not be synthesized.

<table>
<thead>
<tr>
<th>reagent</th>
<th>T [°C]</th>
<th>t [min]</th>
<th>product</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PCl₅ (2 eq.)</td>
<td>1.50</td>
<td>1.10</td>
<td>104</td>
<td>80%</td>
</tr>
<tr>
<td>2. MeNH₂ (40% in H₂O)</td>
<td>2.60</td>
<td>2.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. PCl₅ (2 eq.)</td>
<td>1.40</td>
<td>1.180</td>
<td>104</td>
<td>100%</td>
</tr>
<tr>
<td>2. MeNH₂ (40% in H₂O)</td>
<td>2.60</td>
<td>2.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. SOCl₂ (1 eq.)</td>
<td>1.80</td>
<td>1.180</td>
<td>104</td>
<td>100%</td>
</tr>
<tr>
<td>2. MeNH₂ (40% in H₂O)</td>
<td>2.60</td>
<td>2.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. SOCl₂ (2 eq.)</td>
<td>1.40</td>
<td>1.180</td>
<td>104</td>
<td>100%</td>
</tr>
<tr>
<td>2. MeNH₂ (40% in H₂O)</td>
<td>2.60</td>
<td>2.60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3 SUBSTITUTES FOR THE RIBOSE MOIETY

5.3.1 Design of the linker between adenine and the catechol unit

To gather more information on the structural importance of the ribose moiety in lead 7 and its importance as a hydrogen bond donor for the carboxylate acceptor functionality of Glu90, we decided to substitute the ribose between adenine and the catechol moiety by alkyl chains. The length of the linker was designed by molecular modeling. For a linker containing only methylene units, the optimal length was found to be a hexane-1,6-diyl chain as in molecule 107. To mimic the hydrogen bond donors C(2')-OH and C(3')-OH of the ribose, amino functionality was incorporated in the chains of 108 and 109, which would be protonated at physiological pH and could undergo ion pairing with the carboxylate of Glu90 (Figure 39).

![Molecules 107, 108, and 109](image)

Figure 39. Molecules 107, 108, and 109 were chosen as target molecules to investigate the structural importance of the ribose unit in 7 and its importance as hydrogen bond donor for the carboxylate function of Glu90.

Figure 40 depicts an overlay of 107, 108, and 109 in the active site of COMT and reveals the relative conformations of the chains between adenine and the catechol unit. The chain N-CH2-CH2-CH2-CH2-CH2 in 107 features a sequence of sc-ap-ap-ap-sc-sc torsional angles. In 108 the chain N-CH2-CH2-CH2 has a sequence of sc-sc-sc torsional angles and in 109 the sequence for the torsional angles in the chain N-CH2-CH2-CH2-CH2 is sc-sc-ap-ap.
5.3.2 Synthesis of N1-[6-(6-amino-9H-purin-9-yl)hexyl]-2,3-dihydroxy-5-nitrobenzamide (107)

For the synthesis of 107, 1,6-dibromohexane (110) was reacted with potassium phthalimide to afford N-(6-bromohexyl)phthalimide (111) in 70% yield.[235,236] Reaction of sodium adenide with 111 provided 65% of 9-(6-phthalimidohexyl)adenine (112). The phthalimide unit in 112 was cleaved with hydrazine yielding 65% of 9-(6-aminohexyl)adenine (113).[237] Subsequent coupling to N-succinimidyl benzoate 8 (Scheme 4) afforded 55% of 107 after recrystallization from EtOAc/propan-2-ol (Scheme 34).
5.3.3 Synthesis of the inhibitors with the 3-azahexane-1,6-diyd and 3-azaheptane-1,7-diyd linkers 108 and 109

Molecules 108 and 109 were synthesized as part of the semester project of B. Laukas[201] starting from adenine which was reacted with 1,2-dibromoethane to yield 35% of 6-amino-9-(2-bromoethyl)-9H-purine (114). Compound 114 was then heated to reflux in 1,3-diaminopropane affording \( N1-[2-(6\text{-amino-9H\text{-purin-9-yl}})\text{ethyl}]\text{propane-1,3-diamine (115) (53% yield)}. \)\(^{[238]} \) Coupling between adenine derivative 115 and \( N\text{-succinimidyl benzoate 8 (Scheme 4) gave 108 in 54% yield after recrystallization from propan-2-ol. Heating 114 to reflux in 1,4-diaminobutane provided 33% of \( N1-[2-(6\text{-amino-9H\text{-purin-9-yl}})\text{ethyl}]\text{butane-1,4-diamine (116)}. \) 116 was then coupled to 2,3-dihydroxybenzoate 8 (Scheme 4) and purified by recrystallization from propan-2-ol yielding 109 in 70% yield (Scheme 35).
5.3.4 In vitro studies of inhibitor affinities

Figure 41 lists the IC_{50} values measured for molecules 107, 108, and 109. The inhibitory activity of all three molecules is lower by 2-3 times than that of model compound 14, which contains the same catechol moiety. Therefore, it is unlikely that any of the molecules 107, 108, or 109 occupies the cofactor binding site. The binding affinities indicate that only the catechol unit of these molecules binds to the catechol binding site in the active site and the alkyl chain with the attached adenine does not fit into the cofactor binding site causing steric hindrance for the binding of the catechol unit. Therefore, compounds 107, 108, and 109 most probably are no bisubstrate inhibitors. It can be speculated that molecules 107, 108, and 109 undergo
hydrophobic collapse because of the flexible alkyl chains, which decreases the binding free enthalpy by orders of magnitude due to a lack of preorganization as observed for molecule 69 (Section 5.1.2.2, Figure 35).

From these results, it can be deduced that the ribose moiety in 7 does contribute to its inhibitory activity. However, they do not differentiate between its structural influence in preorganizing the conformation of 7 and orienting the other units into their binding pockets, and its noncovalent interactions with the amino acid residues of the active site.
5.4 CHANGING THE LENGTH OF THE LINKER BETWEEN THE RIBOSE AND CATECHOL UNITS IN THE LEAD COMPOUND

5.4.1 Design of a shorter alkyl linker

To investigate the importance of the length of the linker between the ribose and catechol units in 7, we decided to look at the corresponding molecules with a shorter chain. According to molecular modeling studies, molecules with a chain shorter by one C-atom (CH₂ unit) as compared to 7 also fit in the active site of COMT, but would not be stable due to the acylated N, O-acetal structure. Compound 117 (cf. Scheme 37), which lacks one O-atom, would be stable and provides an interesting target. Figure 42 depicts inhibitor 117 in the active site of COMT after minimization with the program MOLOC. The bridge N-CH₂-CH₂-CH₂ features a sequence of favorable sc-ap-ap torsional angles.

![Computer-modeled ternary complex between COMT, Mg²⁺ ion, and bisubstrate inhibitor 117. The Connolly surface of the inhibitor is shown.](image_url)

**Figure 42.** Computer-modeled ternary complex between COMT, Mg²⁺ ion, and bisubstrate inhibitor 117. The Connolly surface of the inhibitor is shown.
5.4.2 Synthesis of N1-{2-[(2R,3R,4S,5R)-5-(6-Amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl]propyl}-2,3-dihydroxy-5-nitrobenzamide (117)

P. Wyss at Hoffmann-La Roche provided the nucleoside 118. Scheme 36 outlines the synthesis of 118.[239]

Scheme 36. Synthetic pathway toward the nucleosidic precursor 118 as reported by Hoffmann-La Roche.
The synthesis started from commercially available 28, which was oxidized in a Swern oxidation to afford 119 in 87% yield. Wittig reaction of 119 with 120 provided 121 (98% yield), and catalytic hydrogenation of the latter gave 122 (95% yield). The ester 122 was transformed into amide 123 by reaction with ammonia in 85% yield, and reduction with lithium aluminum hydride yielded amine 124. Introducing a trifluoroacetyl protecting group into 124 provided 125 in 77% yield. Nucleosidation with N-benzoylated adenine was carried out in analogy to compound 35 (Schemes 10 and 11) to give 118 (Scheme 36).

118 was then deprotected with piperidine to afford 126 in 100% yield. Coupling of 126 with N-succinimidyl benzoate 8 (Scheme 4) gave 117 in 14% yield after recrystallization from propan-2-ol (Scheme 37).

\[
\text{Scheme 37. Completion of the synthesis of 117.}
\]

5.4.3 Conformational studies on 117

To determine the conformation of 117, an NOE spectrum was measured in CD$_3$OD. The observed NOEs between H-C(8) of the adenine and H-C(2'), H-C(3') of the ribose units demonstrate that the adenine heterocycle adopts the favorable anti-geometry with respect to the ribose[184] in the free ligand which is also needed to
bind to the active site of COMT as predicted by modeling. On the other hand, no NOEs are observed between any of the catechol hydrogen atoms and H-C(8). This indicates that the free state of the molecule is not stabilized by hydrophobic collapse in CD$_3$OD. Hydrophobic collapse might be prevented in 117 by the shorter N-CH$_2$-CH$_2$-CH$_2$-CH$_2$ chain which cannot fold back due to its distinct preference for the "zig-zag" anti-conformation as compared to the more flexible N-CH$_2$-CH$_2$-O-CH$_2$ bridge in 69.
b) H-C(8)

H-C(1')

H-C(2')

c) H-C(cat.)
5.4.2 *In vitro* studies on inhibitor activity

For compound 117 an IC\textsubscript{50} value of 600 (± 200) nM was determined in an experiment without preincubation with the enzyme. This is a significant improvement of inhibitory activity compared to the lead compound 7 (IC\textsubscript{50} (without preincubation) = 4 µM, Figure 20). Therefore, 117 is most probably a bisubstrate inhibitor with inhibition kinetics similar to 7. Nevertheless, kinetic studies would have to be carried out to confirm the inhibition mode of 117. This result confirms our hypothesis that length and structure of the linker between the nucleosidic unit and the catechol moiety have a significant influence on the inhibitory affinity of the bisubstrate inhibitors.
The huge advantage of 117 vs 69 (117 exhibits a 900-fold higher inhibitory activity than 69) presumably arises from the different stabilization of the free state. Compound 69 undergoes hydrophobic collapse and significant reorganizational energy is required for binding. In contrast, 117 is more preorganized for complexation due to the reduced ability of the shorter alkyl chain to fold and to bring the two terminal chromophores in proximity for attractive interactions under desolvation (hydrophobic collapse). The smaller, six-fold advantage of 117 vs 7 might arise from the higher preorganization of 117 as compared to the lead, because the longer N-CH₂-CH₂-O-CH₂ linker in 7 is more flexible and exhibits a sequence of \( ap-ap-sc-sc \) torsional angles, whereas the alkyl chain in 117 has a more distinct preference for the anti-conformation featuring a sequence of \( sc-ap-ap \) torsional angles for the N-CH₂-CH₂-CH₂ chain.
6 CONCLUSIONS AND PERSPECTIVES

6.1 SUMMARY OF RESULTS

Inhibition of the enzyme COMT is an important approach for developing new therapeutic treatments for Parkinson's disease. For the development of a new class of inhibitors occupying both the substrate and the cofactor binding sites of COMT, molecule 7 was designed employing computational molecular force field methods. A synthetic pathway that allows facile introduction of different chemical functionalities into the molecule was developed for 7. Its inhibition mechanism was elucidated by in vitro kinetic studies, which revealed that 7 is indeed a bisubstrate inhibitor. The $K_i$ values determined for each binding site ($K_i$ (with regard to benzene-1,2-diol) = 0.55±0.1 μM, $K_i$ (with regard to SAM) = 0.3±0.1 μM) demonstrate tighter binding of the bisubstrate inhibitor than of the single substructures 14 and 17 (Figure 20), confirming the synergistic effect arising from linking two molecules, each of which has a rather weak affinity for the enzyme.

Structural variations were introduced into the lead structure 7 to determine how much its different chemical groups contribute to the binding affinity for COMT, to investigate different subunits for the binding sites as designed by molecular modeling and, ultimately, to enhance its inhibitory activity. We synthesized and tested two molecules (69 and 70) containing pyrimidine bases with regard to their binding in the base pocket, and three molecules (107, 108, and 109) with oligomethylene linkers between adenine and catechol rather than ribose, which is a subunit of 7. Compound 88 which has a catechol moiety without a nitro group was synthesized to investigate the binding of the catechol subunit in the bisubstrate inhibitors to the Mg$^{2+}$ ion in the active site of COMT. Compound 117 in which the linker between ribose and catechol lacks one O-atom turned out to be a remarkable improvement on the original lead in terms of inhibitory activity (Figure 44). The biological activity of these compounds revealed further information about the structure-activity relationship for the lead 7 (cf. Section 6.2).
Figure 44. Lead structure 7 and a number of structural variations synthesized in the context of this thesis and their IC₅₀ values.
**CONCLUSIONS**

*N*-succinimidy 2,3-dihydroxy-5-nitrobenzoate 8 exhibited a surprisingly high inhibitory activity. Therefore, its inhibition mechanism was determined and found to be competitive with the substrate without and noncompetitive with the substrate with preincubation of 8 with COMT. Such an inhibition mechanism occurs with tightly binding inhibitors like Tolcapone, a pharmaceutical used in the therapy of Parkinson's disease.\(^{195}\)

### 6.2 STRUCTURE-ACTIVITY RELATIONSHIPS

The biological studies reveal a strong structure-activity relationship for the binding of bisubstrate inhibitors to COMT. In the rational design of bisubstrate inhibitor 7 (Figure 45), it was assumed that occupation of the "methionine binding channel" is not necessary for inhibition, a hypothesis that was confirmed by the kinetic results.

![Figure 45. CPK model of the bisubstrate inhibitor 7 in the active site of COMT. The Connolly surface of the amino acids of the active site of the enzyme is shown.](image-url)
In the studies with inhibitors 69 and 70, we found that the choice of the base for the adenine pocket is essential for inhibitory activity (Section 5.1). Pyrimidinone bases like cytosine and 6-amino-3,4-dihydropyrimidin-4-one (72) exhibit a more than 100-fold weaker inhibitory activity than 7. This could be due to the nature of the base, as well as to the fact that the corresponding molecules 69 and 70 are shorter than 7, which leads to conformational differences upon binding to the active site. NOE conformational studies on 69 in solution revealed that the cytosine moiety does have the anti-orientation favorable for binding to the base pocket, but it undergoes hydrophobic collapse with the catechol unit of 69 stabilizing the free state of the molecule and significant reorganization energy would be required for binding to the enzyme, which might account for its weak inhibitory activity. Compound 71 containing the base 4-aminopyrazolo[3,4-d]pyrimidine (73), which is conformationally identical to 7, would be a worthwhile target. From the modeling studies it also appeared that cyanuric acid\[^{[240,241]}\] (127) could be a very good subunit for the base pocket (Figure 46).

Furthermore, we showed that the inhibitory activity of the bisubstrate inhibitor 7 is strongly dependent on the catechol moiety. Compound 88, which lacks the electron-withdrawing nitro group on the catechol, is 100 times less active than the lead (Section 5.2). Because both hydroxy groups are protonated, the enthalpic gain which arises upon coordination of its catechol moiety to the Mg\(^{2+}\) ion is less favorable, since energy for the deprotonation is required first. These findings confirm that the lead 7 binds to the Mg\(^{2+}\) ion with its catechol unit. Moreover, they suggest that other electron-withdrawing groups on the catechol para to one of the hydroxy groups should make equally potent inhibitors, e.g. a ketone functionality as in compound 128. Such compounds might be superior to the existing pharmaceuticals in terms of toxicity. This hypothesis has yet to be investigated.

Molecules 107, 108, and 109 with hexane-1,6-diyl, 3-azahehexane-1,6-diyl, and 3-azaheptane-1,7-diyl linkers, respectively, between adenine and catechol exhibit a 25-50-fold lower activity as compared to the lead 7 (Section 5.3). This shows that the ribose moiety in 7 does have a structural function in orienting the base and catechol towards their binding pockets. Therefore, it cannot be replaced by an oligomethylene chain, even, if it contains H-bond donors which could mimic the hydroxy groups of the ribose. Nevertheless, the ribose might be replaced successfully by a carbacycle such as 129, which would increase the metabolic stability of 7, or by other more rigid scaffolds.\[^{[242]}\] In order to strengthen the H-
bonds between the ribose and the carboxylate of Glu90 without changing the rigid scaffold, protonated aminoribose (130) could replace the ribose in 7.

Shortening the chain length between the ribose and catechol moiety as in 117 was a remarkably successful modification of the original lead. Inhibitor 117 is about six times more potent than 7. The removal of the O-atoms in the chain reduces the conformational flexibility, presumably preventing the approach of the two subchromophores catechol and cytosine (hydrophobic collapse), which would result in an undesired stabilization of the unbound state. This could be an explanation for the inhibitory activity of 117 being 900 times higher than that of 69 which is stabilized in the free state because of hydrophobic collapse. The better activity of 117 compared to 7 could also be due to a higher preorganization of the alkyl chain in 117 adopting a "zig-zag" anti-conformation as compared to the longer alkyl chain containing an O-atom in 7 which is more flexible. Of course, it is of interest to verify this notion by synthesizing and investigating the corresponding molecules 131 and 132, which contain a linker that is shorter or longer by one methylene unit than 117, respectively. It certainly is also worthwhile exploring more rigid linkers which have been designed by molecular modeling, such as the unsaturated linker in 133 or the double amide in 134. Introducing more rigidity into the lead might also enhance its binding affinity (Figure 46).

The N-succinimidyl functionality in 8 (IC$_{50} = 106$ nM) appears to be a good replacement for the ortho nitro group in Tolcapone (Figure 2) (IC$_{50} = 36$ nM). If stable analogs of 8 such as 135 exhibited the same inhibitory activity, these compounds could be interesting follow-ups for Tolcapone. Moreover, incorporation of stable analogs of the N-succinimidyl functionality in the chain between catechol and ribose provide additional interesting linkers for the bisubstrate inhibitors, like compounds 136 and 137. Replacement of the nitro group in 8 by a N-succinimidyl functionality might lead to a similarly active inhibitor 138.
Figure 46. Suggestions for molecules which would give further insight into the structure-activity relationship of the bisubstrate inhibitors and might provide improvements in terms of binding affinity, toxicity, and metabolic stability.
6.3 A FINAL REMARK

It is natural for scientists to have working hypotheses and to perform experiments that either give the expected results or lead to unexpected discoveries, which are the real breakthroughs in science. In both cases, it is rational human thought and the application of the scientific method that lead to discovery. They also drive the ongoing development of new approaches to old problems. It is fascinating to observe the evolution of new ideas based on the success or failure of old ones. Rational drug design includes all of this, yet remains an unrealized goal today. That is what will keep it difficult at times, but always interesting.
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7 EXPERIMENTAL PART

7.1 SYNTHESIS

7.1.1 General methods

Reagents and solvents were purchased reagent-grade and used without further purification. Solvents (DMF, pyridine, MeCN) were dried for 12 h over molecular sieves (4 Å). N-Benzoyladenine and N-benzoylcytosine were prepared as described.\textsuperscript{182} Evaporation and concentration \textit{in vacuo} was done at water aspirator pressure. Drying under vacuum was done at 0.05 Torr.

Thin layer chromatography was performed on glass-backed sheets coated with either SiO$_2$ 60 F$_{254}$ or neutral Al$_2$O$_3$ from Merck, containing fluorescence indicator UV$_{254}$.

Column chromatography (CC) was performed using Fluka SiO$_2$ 60, 40-63 mesh, or Fluka SiO$_2$ H, 5-40 mesh. Flash column chromatography (FC) was performed at a pressure of 0-0.4 bar (SiO$_2$ 60) and of 1 bar (SiO$_2$ H).

Analytical HPLC was performed with a Knauer HPLC system with WellChrom Maxi-Star K-1000 pumps with a flow of 1 mL min$^{-1}$ using a Nucleosil RP18 (240 mm x 4 mm, 100 Å/5) column from Macherey-Nagel and UV detection (220 nm, 254 nm).

Melting points were determined on a Büchi SMP-20 apparatus and are uncorrected.

Optical rotations were measured on a Perkin Elmer 241-Polarimeter at $\lambda = 589$ nm.

Infrared spectra were recorded on a Perkin Elmer 1600-FT-IR spectrometer.

$^1$H and $^{13}$C NMR spectra were recorded on Varian Gemini-200 and -300 and Bruker AMX-500 instruments.
NOE spectra were measured by the NMR service of the Laboratorium für Organische Chemie at ETH Zürich on a Bruker WM-300 (300 MHz).

Mass spectra were recorded by the MS service in the Laboratorium für Organische Chemie at ETH Zürich. FAB mass spectra were recorded on a VG-ZAB-2SEQ instrument using 3-nitrobenzyl alcohol as a matrix. A VG Tribrid instrument was used for EI and a Finnigan MAT TSQ 7000 instrument for electrospray ionization (ESI) mass spectrometry. MALDI-TOF spectra were recorded on a Bruker Reflex spectrometer with 2-(4-hydroxyphenylazo)benzoic acid (HABA), α-cyano-4-hydroxycinnamic acid (CCA), 2,4,6-trihydroxyacetophenone/diammonium citrate 2:1 or 1,8,9-trihydroxyanthracene (dithranol) as matrix; positive ion mode.

Elemental analyses were made by the Mikrolabor of the Laboratorium für Organische Chemie at ETH Zürich.
### 7.1.2 Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Ac</td>
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<tr>
<td>ap</td>
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<td>aqueous</td>
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<td>br.</td>
<td>broad (resonance)</td>
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<tr>
<td>BSA</td>
<td>N,O-bis(trimethylsilyl)acetamide</td>
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<td>butyl-PDB</td>
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<td>N,N'-diisopropylethylamine</td>
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<td>dimethylacetamide</td>
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<td>dimethylformamide</td>
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<td>dpm</td>
<td>decays per minute</td>
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<tr>
<td>EDC</td>
<td>N,N,N',N'-tetraethylidiaminocarbodiimide</td>
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<td>Abbreviation</td>
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<tr>
<td>EI</td>
<td>electron impact (mass spectrometry)</td>
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<td>ESI</td>
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<td>Fmoc</td>
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<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<tr>
<td>Hz</td>
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<td>J</td>
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<td>m</td>
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<td>MALDI-TOF</td>
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<tr>
<td>M.p.</td>
<td>melting point</td>
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<td>triethylamine</td>
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<td>NMR</td>
<td>nuclear magnetic resonance (spectroscopy)</td>
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<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<tr>
<td>Ph</td>
<td>phenyl</td>
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7.1.3 Experimental details on the compounds described in Chapter 3

2-Hydroxy-3-methoxy-5-nitrobenzoic acid (12)\textsuperscript{[243]}

![Chemical structure of 2-Hydroxy-3-methoxy-5-nitrobenzoic acid (12)](image)

To 9 (5.00 g, 29.73 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (33 mL) was added a mixture of 98\% H\textsubscript{2}SO\textsubscript{4} (3.27 mL) and 68\% HNO\textsubscript{3} (2.73 mL) over 15 min at 0 °C with stirring. The solution was stirred for 1 h at 0 °C, warmed to 20 °C, and stirred for another 2 h. The mixture was poured into ice water (50 mL), and the white precipitate was filtered off, washed (H\textsubscript{2}O), and dried under vacuum at 40 °C for 14 h to give 12 (5.95 g, 94\%).

M.p. 220 °C (Lit. \textsuperscript{[243]}: 220 °C); IR (KBr): 3108, 1667, 1526, 1455, 1336, 1262, 1170, 1095, 1060, 867, 774, 741, 696, 478 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (200 MHz, CD\textsubscript{3}OD): \(\delta = 8.38 \text{ (d, } J = 2.5 \text{ Hz, 1 H, H-C(6))}, 7.94 \text{ (d, } J = 2.5 \text{ Hz, 1 H, H-C(4))}, 3.97 \text{ (s, 3 H, OCH}_3\text{)}; \textsuperscript{13}C NMR (50 MHz, CD\textsubscript{3}OD): \(\delta = 171.12, 157.56, 148.96, 139.25, 117.63, 109.99, 109.89, 55.61\); MS (EI): \textit{m/z} (%): 213 (\textit{M}^+, 41), 195 ([\textit{M} - \text{H}_2\text{O}]^+, 100); HR-MS (EI): calcd. for C\textsubscript{8}H\textsubscript{7}NO\textsubscript{6}\textsuperscript{+} (\textit{M}^+): 213.0273; found: 213.0268.

2,3-Dihydroxy-5-nitrobenzoic acid (13)\textsuperscript{[156]}

![Chemical structure of 2,3-Dihydroxy-5-nitrobenzoic acid (13)](image)

A solution of 12 (7.30 g, 34.28 mmol) in HBr (48\% in H\textsubscript{2}O) (200 mL) was heated for 4 h at 135 °C. The volatiles were removed by distillation (0.02 Torr). The remaining
dark solid was twice redissolved in a mixture of toluene (100 mL) and H$_2$O (100 mL) and distilled again to remove residual HBr. The solid residue was purified by recrystallization from toluene and sublimed to yield 13 as a light yellow solid (5.40 g, 79%).

M.p. 222 °C (Lit. [156]: 223-224 °C); IR (KBr): 3511, 3400, 3096, 2844, 2567, 1672, 1526, 1470, 1345, 1278, 1223, 1156, 1081, 985, 900, 798, 769, 743, 696, 489 cm$^{-1}$; $^1$H NMR (200 MHz, CD$_3$OD): $\delta = 8.28$ (d, $J = 3.0$ Hz, 1 H, H-C(6)), 7.81 (d, $J = 3.0$ Hz, 1 H, H-C(4)); $^{13}$C NMR (50 MHz, CD$_3$OD): $\delta = 171.34$, 156.17, 146.74, 139.44, 116.49, 113.67, 112.2; MS (EI): $m/z$ (%): 199 (M$,^+$, 29), 181 ([M - H$_2$O]$^+$, 100); C$_7$H$_5$NO$_6$·0.5 H$_2$O (208.13): calcd.: C 40.40, H 2.91, N 6.71; found: C 40.52, H 2.80, N 6.51.

2,5-Dioxotetrahydro-1H-pyrrol-1-yl 2,3-dihydroxy-5-nitrobenzoate (8)

![Structure of 8]

To 13 (300 mg, 1.51 mmol) in THF (3 mL) were added N-hydroxysuccinimide (173 mg, 1.51 mmol) and DCC (312 mg, 1.51 mmol) at 0 °C. The mixture was stirred for 1 h at 0 °C during which time a white precipitate formed. The suspension was left in the refrigerator at 4 °C overnight, after which the white precipitate was filtered off and washed with THF (5 mL). Evaporation of the filtrate in vacuo and recrystallization from propan-2-ol gave 8 as cream-colored needles (232 mg, 53%).

M.p. 209 °C; IR (KBr): 3402, 1736, 1542, 1473, 1346, 1306, 1200, 1111, 1072, 972, 922, 783, 739, 650, 606 cm$^{-1}$; $^1$H NMR (200 MHz, (CD$_3$)$_2$SO): $\delta = 8.18$ (d, $J = 2.8$ Hz, 1 H, H-C(6)), 7.85 (d, $J = 2.8$ Hz, 1 H, H-C(4)), 2.90 (s, 4 H, CH$_2$); $^{13}$C NMR (125 MHz, CD$_3$OD): $\delta = 171.59$, 162.79, 156.69, 148.62, 140.96, 118.32, 115.13, 111.10, 26.60; MS (EI): $m/z$: 296 (M$^+$); C$_{11}$H$_8$N$_2$O$_8$ (296.19): calcd.: C 44.61, H 2.72, N 9.46; found: C 44.48, H 2.90, N 9.23.
**N-(2-Methoxyethyl)-2,3-dihydroxy-5-nitrobenzamide (14)**

![Chemical structure of 14](image)

To 8 (0.15 g, 0.50 mmol) in DMF (1 mL) was added 2-methoxyethylamine (92 µL, 1.06 mmol) and NEt₃ (0.18 mL, 1.29 mmol), and the mixture was stirred for 4 h at 20 °C. Evaporation in vacuo and FC (SiO₂; CH₂Cl₂/acetone/HCOOH 80:19:1) gave 14 (97 mg, 75%) as a yellow powder.

M.p. 143 °C; IR (KBr): 3389, 3089, 2933, 2833, 1644, 1606, 1556, 1511, 1472, 1339, 1172, 1100, 1011, 944, 900, 833, 783, 744, 711, 661, 583, 450 cm⁻¹; ¹H NMR (200 MHz, CDCl₃): δ = 8.03 (d, J = 2.4 Hz, 1 H, H-C(6)), 7.92 (d, J = 2.4 Hz, 1 H, H-C(4)), 6.94 (s, 1 H, NH), 6.06 (s, 1 H, OH), 3.72-3.62 (m, 4 H, OCH₂, CH₂NH), 3.46 (s, 3 H, OCH₃); ¹³C NMR (75 MHz, CD₃OD): δ = 170.03, 157.22, 148.60, 140.70, 116.52, 116.44, 113.22, 71.95, 59.15, 40.55; MS (EI): m/z (%): 256 (M⁺, 56), 182 ([M – MeO(CH₂)₂NH]⁺, 100); C₁₀H₁₂N₂O₆ (256.21): calcd.: C 46.87, H 4.72, N 10.93; found: C 46.92, H 4.73, N 10.71.

**N-Benzoyladenosine (18)**[165a]

![Chemical structure of 18](image)

Trimethylsilyl chloride (20 mL, 160.00 mmol) was added to a solution of adenosine (5.34 g, 20.00 mmol) in pyridine (100 mL) at 4 °C. The mixture was stirred for 1 h at 20 °C, then benzoyl chloride (7 mL, 60.00 mmol) was added at 4 °C. After stirring for 17 h at 20 °C, ice water (40 mL) was added to the mixture, which was stirred for...
EXPERIMENTAL PART

15 min at 4 °C. Aq. ammonia (40 mL) was added, and the mixture was stirred for 30 min at 4 °C. The solvents were evaporated in vacuo, and toluene (100 mL) was added and evaporated twice to remove rests of pyridine. The crude product was dissolved in H₂O (30 mL), and the mixture was extracted with CH₂Cl₂ (3 x 100 mL). The combined organic phases were dried (MgSO₄), and evaporation in vacuo afforded 18 (5.50 g, 74%).

M.p. 150 °C (decomposition) (Lit. [165a]: 152 °C); ¹H NMR (200 MHz, (CD₃)₂SO): δ = 8.68 (s, 1 H, H-C(4)), 8.64 (s, 1 H, H-C(8)), 8.05-8.01 (m, 2 H, arom. H(Bz)), 7.60-7.47 (m, 3 H, arom. H(Bz)), 6.05-5.95 (m, 1 H, H-C(1’)), 5.55-5.45 (m, 1 H, H-C(2’)), 5.35-5.19 (m, 2 H, H-C(3’), H-C(4’)), 4.63-4.59 (m, 2 H, H-C(5’)), 4.17 (br. s, 1 H, OH), 3.95 (br. s, 1 H, OH), 3.64-3.58 (m, 1 H, OH).

N₆-Benzoyl-2’,3’-O-isopropylidenadenosine (10)[154]

Acetone dimethylacetal (16.04 g, 154.00 mmol) and toluene sulfonic acid monohydrate (1.54 g, 8.10 mmol) were added to a suspension of 18 (3.00 g, 8.10 mmol) in acetone (100 mL). The mixture was stirred at 20 °C for 12 h, a saturated aq. solution of NaHCO₃ (20 mL) was added, the mixture was filtered and the filtrate was evaporated in vacuo. CC (SiO₂; CH₂Cl₂/MeOH 9:1) provided 10 (2.39 g, 72%) as a white powder.

M.p. 112 °C (Lit. [154]: 113-114 °C); IR (CHCl₃): 3677, 3011, 1711, 1611, 1589, 1489, 1455, 1327, 1244, 1150, 1111, 1077, 1000, 911, 850, 700 cm⁻¹; ¹H NMR (200 MHz, (CD₃)₂SO): δ = 8.75 (s, 1 H, H-C(4)), 8.65 (s, 1 H, H-C(8)), 8.04-8.00 (m, 2 H, arom. H(Bz)), 7.63-7.46 (m, 3 H, arom. H(Bz)), 6.26 (d, J = 3.0 Hz, 1 H, OCH₃, 7.61 (d, J = 3.0 Hz, 1 H, OCH₃).
H-C(1’)), 5.42 (dd, J = 6.0 Hz, 3.0 Hz, 1 H, H-C(2’)), 5.12 (d, J = 6.0 Hz, 1 H, H-C(3’)), 5.00-4.96 (m, 1 H, H-C(4’)), 4.26-4.23 (m, 1 H, H-C(5’)), 3.56-3.50 (m, 1 H, H-C(5’)), 1.54 (s, 3 H, CH3), 1.32 (s, 3 H, CH3); 13C NMR (75 MHz, CDCl3): δ = 164.84, 152.69, 150.83, 150.60, 142.85, 133.73, 133.23, 129.18, 128.79, 128.16, 114.51, 94.36, 86.47, 83.31, 81.76, 63.40, 27.66, 25.28; MS (FAB): m/z: 412 (MH+); HR-MS (FAB): caleld. for C20H22N5O5+(MH+): 412.1621; found: 412.1621.

2-(1,3-Dioxo-2,3-dihydro-1H-isoxindol-2-yl)ethyl 4-methylbenzene-1-sulfonate (20)[170]

![Image of 2-(1,3-Dioxo-2,3-dihydro-1H-isoxindol-2-yl)ethyl 4-methylbenzene-1-sulfonate (20)]

Toluene-4-sulfonyl chloride (1.00 g, 5.25 mmol) was added to a solution of N-(2-hydroxyethyl)phthalimide (1.00 g, 5.23 mmol) in pyridine (10 mL) at 0-5 °C. The mixture was stirred for 15 min at 0-5 °C. After standing at 0-5 °C for 1 h, the solution was filtered and the precipitate washed with water to afford 20 (1.72 g, 95%).

M.p. 144 °C (Lit. [170]: 144.5 °C); 1H NMR (200 MHz, CDCl3): δ = 7.84-7.68 (m, 6 H, arom. H), 7.19-7.15 (m, 2 H, arom. H), 4.32 (t, J = 6.0 Hz, 2 H, OCH2), 3.93 (t, J = 6.0 Hz, 2 H, CH2NH), 2.33 (s, 3 H, CH3).

2-[(Benzyloxy)carbonyl]amino]ethyl 4-methylbenzene-1-sulfonate (22)[172]

![Image of 2-[(Benzyloxy)carbonyl]amino]ethyl 4-methylbenzene-1-sulfonate (22)]
Toluene-4-sulfonylchloride (1.56 g, 8.18 mmol) was added to a solution of N-benzzyloxy carbonyl ethanolamine (1.60 g, 8.20 mmol) in pyridine (10 mL) at 0 °C. The reaction mixture was allowed to stand at 0-4 °C for 14 h, then poured on ice, and extracted with CH2Cl2 (3 x 100 mL). The organic phases were combined, washed with HCl (2 N, 100 mL) and H2O (100 mL), and dried (MgSO4). CC (SiO2; hexane/EtOAc 1:1) provided 22 as white powder (2.46 g, 89%).

M.p. 80 °C (Lit. [172]: 79-80 °C); 1H NMR (200 MHz, CDCl3, ): δ = 7.80-7.76 (m, 2 H, arom. H), 7.37-7.31 (m, 7 H, arom. H), 5.07 (s, 2 H, OCOCH2), 4.13-4.08 (m, 2 H, OCH2), 3.51-3.43 (m, 2 H, CH2NH), 2.44 (s, 3 H, CH3).

2- [[(3aR,4R,6R,6aR)-6-Methoxy-2,2-dimethylperhydrofuro[3,4-d][1,3]dioxol-4-yl]methoxy]ethylamine (29)

Sodium hydride (55-65% dispersion in mineral oil, 12.00 g, 250.00 mmol) was added over 1 h to a solution of 28 (4.99 g, 24.41 mmol) in DMF (150 mL) at 0 °C under Ar. The mixture was stirred for 1 h at 20 °C, then 2-chloroethylamine hydrochloride (17.40 g, 150 mmol) was added over 1 h while the temperature was allowed to rise from -5 to 0 °C. After stirring for 5 h at 20 °C, MeOH (100 mL) was added, the solvents were evaporated in vacuo, and CC (SiO2; CH2Cl2/MeOH/NEt3 90:10:5) provided 29 as a colorless oil (5.32 g, 88%).

[α]D20 = -63.0 (c = 1.0, CHCl3); IR (CHCl3): 3377, 3200, 2938, 1681, 1584, 1456, 1383, 1267, 1238, 1160, 1110, 1014, 962, 869, 660 cm⁻¹; 1H NMR (200 MHz, CDCl3): δ = 4.99 (s, 1 H, H-C(1′)), 4.71 (d, J = 6.2 Hz, 1 H, H-C(2′)), 4.61 (d, J = 6.2 Hz, 1 H, H-C(3′)), 4.36-4.33 (m, 1 H, H-C(4′)), 3.58-3.47 (m, 4 H, OCH2), 3.36 (s, 3 H, OCH3), 2.90 (t, J = 5.4 Hz, 2 H, CH2NH), 1.52 (s, 3 H, CH3), 1.36 (s, 3 H, CH3); 13C NMR (50 MHz, CDCl3): δ = 110.05, 106.97, 82.81, 82.71,
79.73, 71.04, 69.54, 52.37, 39.36, 24.00, 22.54; HR-MS (El): calcd. for C_{13}H_{22}NO_{5}^{+} (MH^{+}): 248.1498; found: 248.1503.

\textit{N-}2-\{(3aR,4R,6R,6aR)-6-Methoxy-2,2-dimethylperhydrofuro[3,4-\textit{d}][1,3]-dioxol-4-yl)methoxy\}ethyl\textit{trifluoroacetamide (30)}

\begin{center}
\includegraphics[width=0.2\textwidth]{30.png}
\end{center}

Trifluoroacetic anhydride (105 mg, 0.50 mmol) and pyridine (39 mg, 0.50 mmol) were added to a solution of 29 in CH$_2$Cl$_2$ (2 mL). After stirring for 4 h at 20 °C, the solvents were evaporated in vacuo and CC (SiO$_2$; CH$_2$Cl$_2$/MeOH 99:1) provided 30 as a colorless oil (133 mg, 95%).

[α]$^{20}_{D}$ = -45.0 (c = 1.0, CHCl$_3$); IR (CHCl$_3$): 3433, 3277, 3011, 2933, 1716, 1544, 1450, 1383, 1261, 1161, 1111, 1089, 1011, 961, 866 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ = 7.18 (br. s., 1 H, NH), 4.99 (s, 1 H, H-C(1')), 4.67 (d, $J$ = 5.7 Hz, 1 H, H-C(2')), 4.58 (d, $J$ = 5.7 Hz, 1 H, H-C(3')), 4.35 (t, $J$ = 5.7 Hz, 1 H, H-C(4')), 3.66-3.45 (m, 6 H, H-C(5'), OCH$_2$, CH$_2$NH), 3.34 (s, 3 H, OCH$_3$), 1.49 (s, 3 H, CH$_3$), 1.33 (s, 3 H, CH$_3$); $^{13}$C NMR (50 MHz, CDCl$_3$): 234.44, 154.74, 110.21, 107.23, 83.00, 82.71, 79.38, 69.83, 66.24, 52.59, 37.42, 24.03, 22.47; MS (El): $m/z$: 328 (MH$^+$); HR-MS (El): calcd. for C$_{13}$H$_{21}$NO$_5$F$_3^{+}$ (MH$^+$): 328.1372; found: 328.1376.

\textit{N-}2-\{(2R,3R,4R)-3,4,5-Trihydroxytetrahydrofuran-2-yl)methoxy\}-ethyl\textit{trifluoroacetamide (32)}

\begin{center}
\includegraphics[width=0.2\textwidth]{32.png}
\end{center}
A solution of 30 (100 mg, 0.34 mmol) in H₂SO₄ (0.1 N in H₂O, 1 mL) and H₂O (2 mL) was heated for 1 h at 100 °C. After cooling and neutralization with a saturated aqueous solution of BaCO₃ (pH control), the precipitated BaSO₄ was filtered off and the solvent was evaporated in vacuo to give 32 as a colorless oil (mixture of anomers) (76 mg, 90%).

¹H NMR (300 MHz, CD₃OD, mixture of anomers): δ = 5.25 (d, J = 5.7 Hz, 1 H, H-C(1')), 5.14 (d, J = 5.7 Hz, 1 H, H-C(1')), 4.13-4.07 (m, 1 H, H-C(2')), 4.01-3.94 (m, 1 H, H-C(3')), 3.84 (dd, J = 6.6 Hz, 1.8 Hz, 1 H, H-C(4')), 3.67-3.48 (m, 6 H, H-C(5'), OCH₂, CH₂NH).

(3R,4R,5R)-5-[(2-[(2,2,2-Trifluoroacetyl)amino]ethoxy)methyl]tetrahydrofuran-2,3,4-triyl triacetate (31)

To 32 (100 mg, 0.34 mmol) in pyridine (1 mL) was added Ac₂O (0.2 mL, 2.20 mmol), and the mixture was stirred for 24 h at 20 °C. After addition of a saturated aqueous solution of NaHCO₃ (4 mL), the mixture was extracted with CHCl₃ (3 x 10 mL) and the combined organic phases were dried (MgSO₄) and evaporated in vacuo. FC (SiO₂; hexane/EtOAc 2:1) gave 31 as a colorless oil (134 mg, 94%).

IR (CHCl₃): 3689, 3433, 3011, 2956, 1744, 1605, 1544, 1444, 1367, 1211, 1166, 1100, 1011, 961, 900 cm⁻¹; ¹H NMR (300 MHz, CDCl₃, mixture of anomers): δ = 8.63 (s, 1 H, NH), 6.40 (d, J = 4.5 Hz, 1 H, H-C(1')), 6.16 (d, J = 4.5 Hz, 1 H, H-C(1')), 5.44 (dd, J = 6.6 Hz, 4.5 Hz, 1 H, H-C(2')), 5.33-5.22 (m, 1 H, H-C(3')), 4.35-4.24 (m, 1 H, H-C(4')), 3.71-3.59 (m, 6 H, H-C(5'), OCH₂, CH₂NH), 2.14-2.08 (m, 9 H, CH₃); ¹³C NMR (50 MHz, CDCl₃, mixture of anomers): δ = 170.36, 169.86, 169.52, 169.37, 111.21, 109.82, 98.34, 94.20, 92.19, 83.28, 80.79, 74.38, 70.56, 70.06, 69.33, 39.74, 21.36, 21.01, 20.75, 20.61, 20.54, 20.36; MS (FAB):
\[ m/z: 438 (MNa^+) \]; HR-MS (FAB): calcd. for \( C_{15}H_{21}NO_9F_3 \) (MH\(^+\)): 416.1168; found: 416.1164.

**N6-Benzoyladenine (33)**

![Diagram of N6-Benzoyladenine (33)](image)

A mixture of adenine (2.50 g, 18.50 mmol) and benzoic anhydride (10.00 g, 44.20 mmol) was heated at 140 °C for 2 h. EtOH (70 mL) was added and the solution held at 90 °C for 1 h. After cooling to 20 °C 33 readily crystallized from the solution as white crystals (2.90 g, 65%).

M.p. 242 °C (Lit. [244]: 242-242.5 °C); \(^1\)H NMR (200 MHz, (CD\(_3\))\(_2\)SO): \( \delta = 10.40 \) (s, 1 H, NH-Bz), 8.75 (s, 1 H), 8.66 (s, 1 H), 8.09-8.05 (m, 2 H, arom. H(Bz)), 7.65-7.47 (m, 3 H, arom. H(Bz)).

**\((2S,3R,4R,5R)-2-[6-(Benzoylamino)purin-7-yl]-5-[2-[(2,2,2-trifluoroacetyl)amino]ethoxy]methyl]tetrahydrofuran-3,4-diyl diacetate (34)**

![Diagram of (2S,3R,4R,5R)-2-[6-(Benzoylamino)purin-7-yl]-5-[2-[(2,2,2-trifluoroacetyl)amino]ethoxy]methyl]tetrahydrofuran-3,4-diyl diacetate (34)](image)
BSA (0.20 mL, 0.82 mmol) was added at 20 °C under Ar to a stirred suspension of N6-benzoyladenine (98 mg, 0.41 mmol) in MeCN (2 mL). After 30 min, a colorless solution formed, to which 31 (142 mg, 0.34 mmol) in MeCN (1 mL) and TMSOTf (71 mg, 0.34 mmol) were added. Stirring was continued at 20 °C for 15 min, then saturated aqueous solution of NaHCO₃ (10 mL) and EtOAc (10 mL) were added and the mixture was extracted three times with EtOAc (30 mL). The organic phases were evaporated in vacuo, and FC (SiO₂; EtOAc) provided 34 as a white powder (131 mg, 65%).

M.p. 115 °C; [α]D²⁰ = -56.0 (c = 1.0, CHCl₃); IR (CHCl₃): 3678, 3011, 1750, 1722, 1638, 1594, 1500, 1483, 1422, 1367, 1311, 1283, 1261, 1216, 1172, 1127, 1089, 1016, 911 cm⁻¹; ¹H NMR (500 MHz, CDCl₃): δ = 9.51 (s, 1 H, H-C(8)), 8.42 (br. s, 1 H, NH), 8.23-8.20 (m, 2 H, arom. H(Bz)), 8.16 (s, 1 H, H-C(4)), 7.51-7.40 (m, 3 H, arom. H(Bz)), 6.91 (d, J = 1.4 Hz, 1 H, H-C(1')), 5.72 (dd, J = 5.0 Hz, 1.4 Hz, 1 H, H-C(2')), 5.57 (dd, J = 5.0 Hz, 3.2 Hz, 1 H, H-C(3')), 4.52-4.49 (m, 1 H, H-C(4')), 4.13-4.08 (m, 1 H, H-C(5')), 3.90-3.50 (m, 5 H, H-C(5'), OCH₂, CH₂NH), 2.17 (s, 3 H, CH₃), 2.06 (s, 3 H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ = 175.29, 169.46, 168.96, 158.80, 158.50, 157.34, 148.60, 143.71, 141.77, 137.28, 132.33, 129.77, 128.04, 114.81, 90.59, 80.94, 75.23, 69.69, 67.62, 67.48, 40.47, 20.45, 20.40; MS (FAB): m/z: 595 (M+H⁺); HR-MS (FAB): calcd. for C₂₅H₂₆N₆O₈F₃⁺ (MH⁺): 595.1764; found: 595.1764.

(2S,3R,4R,5R)-2-[(6-(Benzoylamino)-9H-purin-9-yl)-5-([2-(2,2,2-trifluoroacetyl)amino]ethoxy)methyl|tetrahydrofuran-3,4-diyl diacetate (35)
BSA (0.20 mL, 0.82 mmol) was added at 20 °C under Ar to a stirred suspension of 6-N-benzyoladenine (98 mg, 0.41 mmol) in MeCN (2 mL). After 30 min, a colorless solution formed, to which 31 (142 mg, 0.34 mmol) in MeCN (1 mL) and SnCl₄ (0.16 mL, 1.36 mmol) were added. Stirring was continued at 20 °C for 15 min, then saturated aqueous solution of NaHCO₃ (10 mL) and EtOAc (10 mL) were added and the mixture was extracted three times with EtOAc (30 mL). The organic phases were evaporated in vacuo, and FC (SiO₂; EtOAc) provided 35 as a white powder (171 mg, 85%).

M.p. 128 °C; [α]D²⁰ = -35.0 (c = 1.0, CHCl₃); IR (CHCl₃): 3677, 3344, 3011, 1750, 1716, 1611, 1550, 1489, 1455, 1372, 1222, 1172, 1138, 1089, 922, 900 cm⁻¹; ¹H NMR (300 MHz, CDCl₃): δ = 8.77 (s, 1 H, H-C(4)), 8.76 (s, 1 H, H-C(8)), 8.03-8.00 (m, 2 H, arom. H(Bz)), 7.67-7.49 (m, 3 H, arom. H(Bz)), 6.32 (d, J = 6.0 Hz, 1 H, H-C(1')), 6.00 (t, J = 6.0 Hz, 1 H, H-C(2')), 5.65 (dd, J = 6.0 Hz, 3.6 Hz, 1 H, H-C(3')), 4.38-4.37 (m, 1 H, H-C(4')), 3.90-3.58 (m, 6 H, OCH₂, CH₂NH), 2.15 (s, 3 H, CH₃), 2.08 (s, 3 H, CH₃); ¹³C NMR (CDCl₃, 125 MHz): δ = 171.71, 169.97, 166.78, 165.78, 158.33, 158.03, 151.54, 149.74, 142.58, 141.29, 133.08, 132.60, 132.18, 122.63, 85.41, 75.40, 73.56, 71.00, 69.80, 69.05, 39.28, 20.57, 20.19; MS (FAB): m/z: 595 (M⁺); HR-MS (FAB): calcd. for C₂₅H₂₆N₆O₈F₃⁺ (MH⁺): 595.1764; found: 595.1765.

9H-Fluoren-9-ylmethyl N-{2-[(3aR,4R,6R,6aR)-6-methoxy-2,2-dimethylperhydrofuro[3,4-d][1,3]-dioxol-4-yl]methoxy}ethyl]carbamate (37)

To 29 (1.00 g, 4.04 mmol) in DMF (50 mL) were added NEt₃ (1.8 mL, 12.12 mmol) and FmocOSu (4.35 g, 12.12 mmol) at 0 °C under stirring. After 10 min, H₂O (50 mL) was added and the mixture was extracted with CH₂Cl₂ (3 x 100 mL). The combined organic phases were washed with H₂O (4 x 100 mL) and dried (MgSO₄).
Evaporation in vacuo and FC (SiO2; hexane/EtOAc 3:2) afforded 37 as a white powder (1.23 g, 65%).

M.p. 94-95 °C; [α]D20 = -41.0 (c = 1.0, CHCl3); IR (neat): 3442, 3027, 1715, 1515, 1231, 1110, 803 cm⁻¹; ¹H NMR (300 MHz, CDCl3): δ = 7.78 (d, J = 7.5 Hz, 2 H, arom. H), 7.62 (d, J = 7.5 Hz, 2 H, arom. H), 7.43-7.29 (m, 4 H, arom. H), 5.48 (br. s, 1 H, NH), 4.98 (s, 1 H, H-C(1')), 4.70 (d, J = 6.3 Hz, 1 H, H-C(2')), 4.60 (d, J = 6.3 Hz, 1 H, H-C(3')), 4.42 (d, J = 6.9 Hz, 2 H, CH₂(Fmoc)), 4.35 (t, J = 6.0 Hz, 1 H, H-C(4')), 4.22 (t, J = 6.9 Hz, 1 H, CH(Fmoc)), 3.57-3.40 (m, 6 H, H-C(5'), C(5)₂NH, OCH₂), 3.31 (s, 3 H, OCH₃). 13C NMR (50 MHz, CDCl₃): δ = 154.26, 141.72, 139.03, 125.35, 124.71, 122.74, 117.63, 110.08, 107.6, 83.07, 82.81, 79.61, 69.70, 67.35, 64.24, 52.56, 44.85, 38.50, 24.06, 22.57; MS (FAB): m/z: 470 (M⁺); C₂₆H₃₁NO₇ (469.53): calcd.: C 66.51, H 6.65, N 2.98; found: C 66.58, H 6.75, N 2.91.

*N-(9H-Fluoren-9-ylmethyl)-N-{2-[(6-methoxy-2,2-dimethylperhydrofuro[3,4-d][1,3]dioxol-4-yl)methoxy]ethyl}amine (39)*

To 29 (1.00 g, 4.04 mmol) in DMF (50 mL) were added NEt₃ (1.8 mL, 12.12 mmol) and FmocOSu (4.35 g, 12.12 mmol) at 20 °C under stirring. After 30 min, H₂O (50 mL) was added and the mixture was extracted with CH₂Cl₂ (3 x 100 mL). The combined organic phases were washed with H₂O (4 x 100 mL) and dried (MgSO₄). Evaporation in vacuo and FC (SiO2; hexane/EtOAc 3:2) afforded 39 as a colorless oil (0.65 g, 38%).

IR (neat): 3622, 2922, 1672, 1444, 1383, 1261, 1233, 1105, 1050, 955, 911 cm⁻¹; ¹H NMR (200 MHz, CDCl₃): δ = 7.80-7.78 (m, 2 H, arom. H), 7.70-7.63 (m, 2 H, arom. H), 7.41-7.29 (m, 4 H, arom. H), 4.99 (s, 1 H, H-C(1')), 4.68 (d, J = 5.8 Hz, 1 H, H-C(2')), 4.59 (d, J = 5.8 Hz, 1 H, H-C(3')), 4.35 (t, J = 7.8 Hz, 1 H, H-C(4')), 4.22 (t, J = 6.9 Hz, 1 H, CH(Fmoc)), 3.57-3.40 (m, 6 H, H-C(5'), C(5)₂NH, OCH₂), 3.31 (s, 3 H, OCH₃). 13C NMR (50 MHz, CDCl₃): δ = 154.26, 141.72, 139.03, 125.35, 124.71, 122.74, 117.63, 110.08, 107.6, 83.07, 82.81, 79.61, 69.70, 67.35, 64.24, 52.56, 44.85, 38.50, 24.06, 22.57; MS (FAB): m/z: 470 (M⁺); C₂₆H₃₁NO₇ (469.53): calcd.: C 66.51, H 6.65, N 2.98; found: C 66.58, H 6.75, N 2.91.
4.12 (t, J = 6.6 Hz, 1 H, CH(Fmoc)), 3.66-3.36 (m, 4 H, H-C(5'), OCH₂), 3.30 (s, 3 H, OCH₃), 3.08 (d, J = 6.6 Hz, 2 H, CH₂(Fmoc)), 2.91 (t, J = 4.6 Hz, 2 H, CH₂NH), 1.53 (s, 3 H, CH₃), 1.36 (s, 3 H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ = 146.27, 141.42, 127.47, 127.19, 124.96, 124.93, 120.17, 112.60, 109.50, 85.39, 85.25, 82.32, 72.13, 70.91, 54.90, 53.39, 49.27, 47.78, 26.57, 25.14; MS (FAB): m/z: 426 (M+).

9H-Fluoren-9-ylmethyl N-{2-[(2R,3R,4R)-3,4,5-trihydroxytetrahydrofuran-2-yl]methoxy}ethyl carbamate (40):

A solution of 37 (1.50 g, 3.19 mmol) in HOAc (70% in H₂O, 100 mL) was heated for 5 h at 90 °C, then the mixture was evaporated in vacuo. Toluene was added twice, followed each time by evaporation in vacuo, yielding 40 as a white solid which was used without further purification (1.27 g, 96%).

M.p. 118-120 °C; IR (KBr): 3323, 3056, 2933, 1690, 1543, 1444, 1265, 1120, 1017, 739 cm⁻¹; ¹H NMR (200 MHz, CDCl₃, mixture of anomers): δ = 7.79 (d, J = 8.0 Hz, 2 H, arom. H), 7.62 (d, J = 8.0 Hz, 2 H, arom. H), 7.45-7.17 (m, 4 H, arom. H), 5.50-5.20 (m, 2 H, H-C(1'), NH), 4.43 (d, J = 6.0 Hz, 2 H), 4.30-3.90 (m, 4 H), 3.70-3.20 (m, 6 H); ¹³C NMR (75 MHz, CDCl₃, mixture of anomers): δ = 157.09, 144.18, 141.58, 141.48, 127.97, 127.34, 127.23, 125.29, 120.24, 108.75, 102.26, 96.76, 82.20, 81.86, 71.70, 70.72, 70.09, 67.30, 66.84, 47.26, 41.50, 40.79; HR-MS (FAB): calcd. for C_{22}H_{26}NO_{7}⁺ (M⁺): 416.1702; found: 416.1702.
(3R,4R,5R)-5-[[2-[[(9H-Fluoren-9-ylmethoxy)carbonyl]amino]ethoxy)methyl]tetrahydrofuran-2,3,4-triyl triacetate (41):

To 40 (1.20 g, 2.89 mmol) in pyridine (4 mL) was added Ac₂O (2.40 mL, 25.40 mmol), and the mixture was stirred for 24 h at 20 °C. After addition of a saturated aqueous solution of NaHCO₃ (20 mL), the mixture was extracted three times with EtOAc (50 mL) and the combined organic phases were dried (MgSO₄) and evaporated in vacuo. FC (SiO₂; hexane/EtOAc 1:1) gave 41 as a white powder (1.3 g, 83%).

M.p. 65 °C; IR (KBr): 3400, 2933, 1750, 1528, 1450, 1367, 1222, 1106, 1022, 961, 894, 739 cm⁻¹; ¹H NMR (200 Hz, CDCl₃, mixture of anomers): δ = 7.80 (d, J = 8.0 Hz, 2 H, arom. H), 7.66 (d, J = 8.0 Hz, 2 H, arom. H), 7.36-7.27 (m, 4 H, arom. H), 6.19 (s, 1 H, H-C(1′)), 5.55-5.35 (m, 3 H, H-C(2′), H-C(3′), NH), 4.50-4.20 (m, 4 H, CH₂(Fmoc), H-C(4′), CH(Fmoc)), 3.75-3.30 (m, 6 H, H-C(5′), OCH₃, Cr72NH), 2.14, 2.11, 2.10, 2.09, 2.07 (5 x s, 9 H, CH₃); ¹³C NMR (75 MHz, CDCl₃, mixture of anomers): δ = 170.35, 169.92, 169.54, 169.16, 156.63, 143.98, 141.31, 127.81, 127.73, 127.68, 127.58, 127.19, 126.93, 125.20, 125.07, 120.00, 199.96, 98.33, 98.30, 81.17, 80.84, 74.50, 74.21, 70.90, 70.65, 47.33, 47.17, 40.72, 20.22, 20.56, 21.07, 21.25, 21.51, 21.73; MS (FAB): m/z (%): 482 ([M – OAc]⁺, 100); C₂₈H₃₁NO₁₀ (540.55): calcd.: C 62.22, H 5.59, N 2.59, O 29.60; found: C 62.01, H 5.80, N 2.53, O 29.83.
(2S,3R,4R,5R)-2-[6-(Benzoylamo)-9H-purin-9-yl]-5-{[2-{{(9H-fluoren-9-ylmethoxy)carbonyl}amino}ethoxy)methyl]tetrahydrofuran-3,4-diyl diacetate (38)

To a stirred suspension of N6-benzoyladenine (196 mg, 0.82 mmol) in MeCN (2 mL) was added BSA (0.40 mL, 1.64 mmol) at 50 °C under Ar. After 10 min, a colorless solution formed, to which 41 (0.37 g, 0.68 mmol) in MeCN (1 mL) and SnCl4 (0.32 mL, 2.7 mmol) were added. Stirring was continued at 55 °C for 15 min, saturated aqueous solution of NaHCO3 (20 mL) and EtOAc (20 mL) were added and the mixture was extracted three times with EtOAc (50 mL). The organic phases were evaporated in vacuo, and FC (SiO2; CH2Cl2/MeOH 99:1) provided 38 as a white powder (0.318 g, 65%).

M.p. 100 °C; [α]D20 = −25.0 (c = 1.0, CHCl3); IR (KBr): 3420, 2922, 2367, 1750, 1717, 1694, 1611, 1578, 1517, 1450, 1372, 1244, 1094, 794, 761, 739, 711, 650, 567 cm⁻¹; 1H NMR (500 MHz, CDCl3): δ = 8.93 (s, 1 H, NH(Bz)), 8.81 (s, 1 H, H-C(4)), 8.40 (s, 1 H, H-C(8)), 7.91 (d, J = 7.5 Hz, 2 H, arom. H(Fmoc)), 7.69 (d, J = 7.5 Hz, 2 H, arom. H(Fmoc)), 7.69-7.55 (m, 3 H, arom. H(Bz)), 7.47-7.43 (m, 2 H, arom. H(Bz)), 7.33-7.18 (m, 4 H, arom. H(Fmoc)), 6.42 (d, J = 6.6 Hz, 1 H, H-C(1′)), 6.03 (t, J = 6.6 Hz, 1 H, H-C(2′)), 5.68-5.67 (m, 1 H, H-C(3′)), 5.57 (t, J = 5.4 Hz, 1 H, NH(Fmoc)), 4.41-4.40 (m, 1 H, H-C(4′)), 4.37-4.35 (d, J = 7.4 Hz, 2 H, CH2(Fmoc)), 4.22 (t, J = 7.4 Hz, 1 H, CH(Fmoc)), 3.85 (d, J = 9.3 Hz, 1 H, H-C(5′)), 3.73 (d, J = 5.2 Hz, 2 H, OCH2), 3.62-3.61 (m, 1 H, H-C(5′)), 3.50-3.48 (m, 2 H, CH2NH), 2.17 (s, 3 H, CH3), 2.07 (s, 3 H, CH3); 13C NMR (125 MHz, CDCl3): δ = 167.63, 167.47, 162.26, 154.36, 150.68, 149.69, 147.34, 141.76, 141.69, 138.87, 131.15, 130.42, 126.49, 125.50, 125.28, 124.65, 124.55, 122.81, 117.57, 82.65, 80.08, 71.54, 69.42, 68.40, 67.96, 64.62, 44.69, 38.53, 18.22, 17.96; MS (FAB): m/z
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(%) 743 ([M + Na]+, 22), 721 (MH+, 100); HR-MS (FAB): calcd. for C38H37N6OCP1 (MH+): 721.2622; found: 721.2625.

(2S,3R,4R,5R)-2-[6-(Benzoylamino)-9H-purin-9-yl]-5-{2-[2,3-dihydroxy-5-nitrobenzoylamino]ethoxy}methyl)tetrahydrofuran-3,4-diyl diacetate (48)

To 38 (100 mg, 0.14 mmol) in DMF (40 mL) was added HNEt2 (40 mL), and the solution was stirred under Ar for 2.5 h at 20 °C. After evaporation of HNEt2 in vacuo, 8 (41 mg, 0.14 mmol) and NEt3 (0.06 mL, 0.43 mmol) were added and the mixture was stirred for 24 h at 20 °C. Evaporation in vacuo and recrystallization from propan-2-ol gave 48 as an orange powder (28 mg, 30%).

M.p. 127 °C; [α]D20 = −21.0 (c = 1.0, THF); IR (KBr): 3411, 2922, 2344, 1744, 1700, 1639, 1611, 1583, 1516, 1460, 1336, 1241, 1073, 904, 792, 747, 708 cm⁻¹; ¹H NMR (500 MHz, CDCl3): δ = 8.71 (s, 1 H, H-C(4)), 8.69 (s, 1 H, H-C(8)), 8.43 (d, J = 2.7 Hz, 1 H, arom. H(cat.)), 8.06 (d, J = 7.2 Hz, 2 H, arom. H(Bz)), 7.67-7.53 (m, 3 H, arom. H(Bz)), 7.43 (d, J = 2.7 Hz, 1 H, arom. H(cat.)), 6.37 (d, J = 5.7 Hz, 1 H, H-C(1')), 5.98 (t, J = 5.7 Hz, 1 H, H-C(2')), 5.81-5.78 (m, 1 H, H-C(3')), 4.29-4.28 (m, 1 H, H-C(4')), 3.94-3.59 (m, 6 H, H-C(5'), OCH2, CH2NH), 2.15 (s, 3 H, CH3), 2.00 (s, 3 H, CH3); ¹3C NMR (125 MHz, CD3OD): δ = 170.09, 169.74, 168.52, 165.86, 152.33, 151.93, 151.53, 149.55, 146.74, 141.51, 133.22, 132.61, 128.44, 127.89, 126.78, 122.53, 115.39, 113.88, 111.31, 85.45, 82.34, 73.15, 71.35, 69.81, 69.74, 39.09, 20.19, 19.87; HR-MS (FAB): calcd. for C30H30N7O12⁺ (MH⁺): 680.1952; found: 680.1952.
A solution of 48 (48 mg, 0.07 mmol) in MeNH₂ (25% in EtOH, 5 mL) was stirred for 30 min at 20 °C. After evaporation in vacuo, the orange residue was dissolved in H₂O (10 mL) and extracted with Et₂O (3 x 10 mL), toluene (10 mL), and CH₂Cl₂ (10 mL). After lyophilization from H₂O, 7 (29 mg, 82%) was obtained as a yellow powder and shown by analytical HPLC (H₂O/MeCN/CF₃COOH 90:10:0.1 → 40:60:0.1) to be pure.

M.p. 141 °C; [α]D²⁰ = -16.0 (c = 1.0, H₂O); IR (KBr): 3422, 2944, 1639, 1556, 1500, 1472, 1422, 1333, 1261, 1122, 1072, 989, 828, 806, 700, 650, 556, 478, 406 cm⁻¹; ¹H NMR (300 MHz, CD₃OD): δ = 8.48 (d, J = 3.3 Hz, 1 H, arom. H(cat.)), 8.45 (s, 1 H, H-C(4)), 8.18 (s, 1 H, H-C(8)), 7.50 (d, J = 3.3 Hz, 1 H, arom. FT(cat.)), 6.06 (d, J = 5.0 Hz, 1 H, H-C(1')), 4.69 (t, J = 5.4 Hz, 1 H, H-C(2')), 4.49 (t, J = 5.4 Hz, 1 H, H-C(3')), 4.22-4.20 (m, 1 H, H-C(4')), 3.84-3.65 (m, 6 H, OCH₂, CH₂NH); ¹³C NMR (125 MHz, D₂O + 1 drop of CD₃OD): δ = 169.39, 156.83, 155.78, 153.12, 149.14, 148.47, 140.33, 133.03, 121.31, 119.21, 116.00, 108.74, 89.08, 84.70, 75.34, 71.37, 70.38, 70.18, 39.79; HR-MS (FAB): calcd. for C₁₉H₂₂N₇O₉⁺ (MH⁺): 492.1479; found: 492.1460.
2,3-Dihydroxy-N-\{2-[(3aR,4R,6R,6aR)-6-methoxy-2,2-dimethylperhydrofuro[3,4-d][1,3]dioxol-4-yl]methoxy\}ethyl\}-5-nitrobenzamide (50)

![Structure of 50](image)

A mixture of 29 (0.10 g, 0.41 mmol), 8 (0.12 g, 0.41 mmol), and NEt₃ (0.18 mL, 1.20 mmol) in DMF (5 mL) was stirred under Ar for 4 h at 20 °C. Evaporation in vacuo and FC (SiO₂; CH₂Cl₂/aceton/HCOOH 79:20:1) provided 50 (0.13 g, 75%) as a yellow powder.

M.p. 137 °C; [α]D²⁰ = -62.0 (c = 1.0, CHCl₃); IR (KBr): 3400, 2989, 2944, 2856, 2344, 1650, 1561, 1511, 1472, 1350, 1322, 1278, 1161, 1100, 1039, 872, 816, 739, 656, 594 cm⁻¹; ¹H NMR (300 MHz, CDCl₃): δ = 8.24 (d, J = 2.7 Hz, 1 H, H-C(6)), 7.90 (d, J = 2.7 Hz, 1 H, H-C(4)), 7.62 (br. s, 1 H, NH), 5.12 (s, 1 H, H-C(1')), 4.72 (d, J = 5.7 Hz, 1 H, H-C(2')), 4.64 (d, J = 5.7 Hz, 1 H, H-C(3')), 4.42 (t, J = 5.1 Hz, 1 H, H-C(4')), 3.71-3.50 (m, 6 H, H-C(5'), OCH₂, CH₂NH), 3.38 (s, 3 H, OCH₃), 1.48 (s, 3 H, CH₃), 1.31 (s, 3 H, CH₃); ¹³C NMR (50 MHz, CDCl₃): 226.30, 166.80, 152.87, 143.88, 137.22, 126.04, 111.19, 110.33, 107.89, 83.10, 82.84, 79.19, 69.93, 66.43, 52.63, 37.71, 24.00, 22.47; MS (FAB): m/z (%): 429 (MH⁺, 74), 397 ([M–MeO⁺], 100); C₁₈H₂₄N₂O₁₀ (428.40): calcd.: C 50.47, H 5.65, N 6.54; found: C 50.31, H 5.90, N 6.24.

2,3-Dihydroxy-5-nitro-N-\{2-[(2R,3R,4R)-3,4,5-trihydroxytetrahydrofuran-2-yl]methoxy\}ethyl\}-benzamide (49)

![Structure of 49](image)
A solution of 50 (0.10 g, 0.23 mmol) in H₂SO₄ (0.1 N in H₂O, 1 mL) and H₂O (2 mL) was heated for 1 h at 100 °C. After cooling and neutralization with a saturated aqueous solution of BaCO₃ (pH control), the precipitated BaSO₄ was filtered off and the solvent was evaporated in vacuo. The solid residue was dissolved in H₂O (containing 1% of HCOOH, 10 mL) and extracted three times with EtOAc (20 mL). The combined organic layers were evaporated in vacuo to give 49 (mixture of anomers) (0.08 g, 91%) as a yellow solid.

M.p. 87-90 °C; IR (KBr): 3400, 2933, 1639, 1556, 1517, 1472, 1339, 1283, 1089, 894, 783, 744, 711, 656 cm⁻¹; ¹H NMR (300 MHz, CD₃OD, mixture of anomers): δ = 8.36 (d, J = 2.7 Hz, 1 H, H-C(6)), 7.74 (d, J = 2.7 Hz, 1 H, H-C(4)), 5.14 (d, J = 4.2 Hz, 1 H, H-C(1')), 4.14-4.09 (m, 1 H, H-C(2')), 4.02-3.95 (m, 2 H, H-C(3'), H-C(4')), 3.86-3.84 (m, 1 H, H-C(5')), 3.74-3.59 (m, 5 H, H-C(5'), OCH₂, CH₂NH); ¹³C NMR (125 MHz, CD₃OD, mixture of anomers): δ = 170.16, 156.70, 148.45, 141.09, 116.37, 116.08, 113.56, 103.57, 98.30, 83.37, 82.88, 77.24, 74.00, 72.77, 72.53, 72.48, 70.93, 70.86, 58.50, 40.90, 40.76; MS (FAB): m/z (%): 375 (M⁺, 100); HR-MS (FAB): calcd. for C₁₄H₁₈N₂NaO₁₀⁺ ([M + Na]⁺): 397.0859; found: 397.0824.
7.1.4 Experimental details on the compounds described in Chapter 5

6-Amino-2-sulfanyl-3,4-dihydropyrimidin-4-one (75)[199]

To EtONa (4.29 g, 63.00 mmol) in EtOH (44 mL) was added thiourea (4.56 g, 60.00 mmol) and ethyl cyanoacetate (6.40 mL, 60.00 mmol), and the mixture was heated to reflux for 2 h. The formed precipitate was filtered off, and the solvents were evaporated *in vacuo*. The resulting solid residue and the initial precipitate were dissolved in H2O (50 mL), then HOAc was added until precipitation of 75 as a white powder (8.00 g, 93%) was complete.

M.p. 295 °C (decomposition); IR (KBr): 3322, 1556, 1298, 1187, 1000, 922, 833, 790, 613, 574, 525 cm⁻¹; ¹H NMR (300 MHz, (CD₃)₂SO): δ = 11.55 (s, 1 H, NH), 6.38 (s, 2 H, NH₂), 4.69 (s, 1 H, arom. H); HR-MS (FAB): calcd. for C₄H₆N₃O⁺ (MH⁺): 144.0232; found: 144.0231.

6-Amino-3,4-dihydropyrimidin-4-one (72)[200]

An aqueous suspension of Ra-Ni (12.00 g) was added to a vigorously stirred mixture of 75 (3.00 g, 20.96 mmol) in H₂O (30 mL) and NH₃ (25% in H₂O, 1.82 mL) at reflux. After 1 h, the Ra-Ni was filtered off and washed with boiling H₂O. Evaporation and recrystallization (H₂O) yielded 72 (1.10 g, 47%) as a white powder.
M.p. 263-264 °C (Lit. [200]: 263-264 °C); IR (KBr): 3322, 3142, 2922, 2767, 1939, 1661, 1614, 1544, 1495, 1447, 1367, 1305, 1242, 1200, 1094, 990, 914, 808, 767, 609, 565, 526, 449, 418 cm⁻¹; ¹H NMR (300 MHz, (CD₃)₂SO): δ = 11.38 (s, 1 H, NH), 7.74 (s, 1 H, H-C(2)), 6.38 (s, 2 H, NH₂), 4.94 (s, 1 H, H-C(5)); MS (FAB): m/z (%): 111.9 (MH⁺, 100); HR-MS (FAB): calcd. for C₄H₆N₃O⁺ (MH⁺): 112.0511; found 112.0506; C₄H₅ON₃ (111.10): calcd.: C 43.24, H 4.54, N 37.82; found: C 43.42, H 4.73, N 37.85.

N-(6-Oxo-1,6-dihydropyrimidin-4-yl)benzamide (74)

A mixture of 72 (0.61 g, 5.47 mmol) and benzoic anhydride (2.96 g, 13.08 mmol) was heated to 140 °C. After 2 h, EtOH (18 mL) was added at 90 °C and stirring was continued for 1 h. After standing at 20 °C for 4 h, 74 (0.98 g, 83%) precipitated from the solution as a white powder.

M.p. 275-276 °C; IR (KBr): 3390, 3064, 1687, 1656, 1591, 1526, 1460, 1405, 1336, 1258, 1214, 1178, 985, 853, 720, 694, 636, 594, 516, 466 cm⁻¹; ¹H NMR (300 MHz, (CD₃)₂SO): δ = 12.35 (s, 1 H, NH), 10.69 (s, 1 H, NHBz), 8.13 (s, 1 H, H-C(2)), 8.00-7.80 (m, 2 H, arom. H), 7.70-7.30 (m, 3 H, arom. H), 7.05 (s, 1 H, H-C(5)); ¹³C NMR (75 MHz, (CD₃)₂SO): δ = 166.50, 162.50, 156.50, 149.50, 133.50, 131.90, 128.27, 128.04, 98.50; HR-MS (FAB): calcd. for C₁₁H₁₀N₃O₂⁺ (MH⁺): 216.0773; found: 216.0782.
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(2S,3R,4R,5R)-2-[4-(Benzoylamino)-2-oxo-1,2-dihydropyrimidin-1-yl]-5-[[9H-fluoren-9-ylmethoxy]carbonyl]amino]ethoxy)methyl|tetrahydrofuran-3,4-diyl diacetate (76)

To a stirred suspension of N4-benzoylcytosine (72 mg, 0.33 mmol) in MeCN (2 mL) was added BSA (0.16 mL, 0.66 mmol) at 50 °C. After 10 min, a colorless solution formed to which 41 (150 mg, 0.28 mmol) in MeCN (1 mL) and TMSOTf (0.25 mL, 1.38 mmol) were added under Ar. The mixture was stirred at 55 °C for 20 min, then quenched with a saturated aqueous solution of NaHCO₃ (20 mL) and EtOAc (20 mL), and extracted with EtOAc (3 x 50 mL). The combined organic layers were evaporated in vacuo, and FC (SiO₂ H; CH₂Cl₂/MeOH 98.5:1.5) afforded 76 as a white powder (0.152 g, 79%).

M.p. 184-185 °C; [α]D²⁰ = +29.0 (c = 1.0, CHCl₃); IR (KBr): 3370, 3067, 2956, 2878, 1746, 1695, 1670, 1615, 1555, 1525, 1488, 1374, 1309, 1248, 1139, 1090, 790, 738, 706 cm⁻¹; ¹H NMR (500 MHz, CDCl₃): δ = 8.65 (s, 1 H, NH(Bz)), 8.21-8.18 (m, 1 H, H-C(6)), 7.78 (d, J = 5.7 Hz, 2 H, arom. H(Fmoc)), 7.67 (d, J = 7.5 Hz, 2 H, arom. H(Bz)), 7.65-7.55 (m, 4 H, arom. H(Fmoc), arom. H(Bz), H-C(5)), 7.48-7.44 (m, 2 H, arom. H(Bz)), 7.36-7.32 (m, 2 H, arom. H(Fmoc)), 6.37 (d, J = 5.4 Hz, 1 H, H-C(1')), 5.55-5.48 (m, 3 H, H-C(2'), H-C(3'), NH(Fmoc)), 4.41 (d, J = 7.3 Hz, 2 H, CH₂(Fmoc)), 4.33 (m, 1 H, H-C(4')), 4.23 (t, J = 7.3 Hz, 1 H, CH(Fmoc)), 3.89 (d, J = 10.4 Hz, 1 H, H-C(5')), 3.77-3.75 (m, 1 H), 3.68 (d, J = 10.4 Hz, 1 H, H-C(5')), 3.62-3.60 (m, 1 H), 3.50-3.45 (m, 2 H, CH₂), 2.12 (s, 3 H, CH₃), 2.11 (s, 3 H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ = 169.88, 166.04, 162.35, 156.58, 144.34, 144.01, 143.98, 141.21, 133.11, 128.91, 127.56, 126.94, 125.22, 125.19, 119.84, 87.44, 81.84, 74.22, 70.91, 70.71, 69.71, 66.94, 47.18, 40.97, 20.64, 20.51 (3 peaks missing); MS (FAB): m/z (%): 697.2 (MH⁺, 100); HR-MS (FAB): calcd. for C₃⁷H₃⁷N₄O₁₀⁺ (MH⁺): 697.2509; found:
(2S,3R,4R,5R)-2-[(4-(Benzoylamino)-6-oxo-1,6-dihydropyrimidin-1-yl)-5-[(2-\{(9H-fluoren-9-ylmethoxy)carbonyl\}amino)ethoxy)methyl]tetrahydrofuran-3,4-diyl diacetate (77)

To a stirred suspension of 74 (160 mg, 0.75 mmol) and 41 (168 mg, 0.31 mmol) in MeCN (2 mL) was added BSA (0.38 mL, 1.55 mmol) at 60 °C under Ar, and the resulting colorless solution was stirred for 1 h at this temperature. TMSOTf (0.28 mL, 1.55 mmol) was added, and the mixture was stirred at 60 °C for 50 min, then saturated aqueous solutions of NaHCO₃ (20 mL) and EtOAc (20 mL) were added. The mixture was extracted with EtOAc (3 x 50 mL), and the combined organic phases were evaporated in vacuo. FC (SiO₂ H; CH₂Cl₂ /MeOH 100:1) afforded 77 as a white powder (0.11 g, 58%).

M.p. 92 °C; [α]D²⁰ = +33.0 (c = 1.0, CHCl₃); IR (KBr): 3342, 3056, 1961, 1750, 1508, 1444, 1372, 1292, 1243, 1094, 849, 760, 741 cm⁻¹; ¹H NMR (300 MHz, CDCl₃): δ = 8.62 (s, 1 H, H-C(2)), 7.97 (s, 1 H, NH(Bz)), 7.70-7.16 (m, 14 H, arom. H(Bz), arom. H(Fmoc), H-C(5)), 6.34 (d, J = 4.2 Hz, 1 H, H-C(1')), 5.82-5.80 (m, 1 H, NH(Fmoc)), 5.60-5.55 (m, 2 H, H-C(2'), H-C(3')), 4.54-4.16 (m, 4 H, CH₂(Fmoc), H-C(4'), CH(Fmoc)), 3.96-3.34 (m, 6 H, H-C(5'), OCH₂, CH₂NH), 2.14 (s, 3 H, CH₃), 2.12 (s, 3 H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ = 169.77, 169.57, 165.45, 161.44, 156.71, 154.01, 147.90, 143.89, 143.86, 141.28, 141.25, 133.14, 132.65, 128.98, 128.85, 127.70, 127.66, 127.18, 126.99, 126.95, 125.06,
Starting from 76 (100 mg, 0.14 mmol) and 8 (41 mg, 0.14 mmol), the procedure described for 48 afforded 79 (46 mg, 49%) as an orange powder.

M.p. 146 °C; [α]D²₀ = +40.0 (c = 1.0, THF); IR (KBr): 3422, 2978, 2933, 1750, 1700, 1656, 1628, 1561, 1372, 1311, 1250, 1128, 1072, 806, 783, 706 cm⁻¹; ¹H NMR (500 MHz, CD₃OD): δ = 8.50 (d, J = 3.0 Hz, 1 H, arom. H(cat.)), 8.39 (d, J = 7.6 Hz, 1 H, H-C(6)), 8.00-7.90 (m, 2 H, arom. H(Bz)), 7.67-7.56 (m, 3 H, arom. H(Bz)), 7.51 (d, J = 7.6 Hz, 1 H, H-C(5)), 7.46 (d, J = 3.0 Hz, 1 H, arom. H(cat.)), 6.24 (d, J = 5.2 Hz, 1 H, H-C(1')), 5.70-5.65 (m, 1 H, H-C(2')), 5.62-5.58 (m, 1 H, H-C(3')), 4.50-4.45 (m, 1 H, H-C(4')), 4.05-3.65 (m, 6 H, OCH₂, CH₂NH), 2.15 (s, 3 H, CH₃), 2.10 (s, 3 H, CH₃); ¹³C NMR (125 MHz, CD₃OD): δ = 171.47, 171.14, 170.04, 168.81, 164.99, 157.96, 150.13, 146.07, 134.67, 134.00, 129.87, 129.74, 129.22, 120.53, 115.91, 108.17, 99.12, 96.76, 89.65, 83.92, 76.00, 72.75, 71.43, 70.73, 40.13, 20.53, 20.34; MS (FAB): m/z (%): 678.1 ([M + Na]⁺, 24), 656.1 (MH⁺, 100); HR-MS (FAB): calcd. for C₂₉H₃₀N₅O₁₃⁺ (MH⁺): 656.1840; found: 656.1843.
(2S,3R,4R,5R)-2-[4-(Benzoylamino)-6-oxo-1,6-dihydropyrimidin-1-yl]-5-[(2-
\[(2,3-dihydroxy-5-nitrobenzoyl)amino]ethoxy)methyl]tetrahydrofuran-3,4-diyl
diacetate (80)

Starting from 77 (100 mg, 0.14 mmol) and 8 (41 mg, 0.14 mmol), the procedure
described for 48 afforded 80 (53 mg, 56%) as an orange powder.

M.p. 147 °C; [α]D\text{20} = +25.0 (c = 1.0, THF); IR (KBr): 3400, 2967, 2867, 1750,
1690, 1558, 1509, 1444, 1372, 1328, 1248, 1133, 1074, 844, 806, 702 cm\textsuperscript{-1}; \textsuperscript{1}H
NMR (500 MHz, CD\textsubscript{3}OD): δ = 8.75 (d, J = 0.7 Hz, 1 H, H-C(2)), 8.50 (d, J = 3.0
Hz, 1 H, arom. H(cat.)), 8.00-7.90 (m, 2 H, arom. H(Bz)), 7.70-7.50 (m, 3 H, arom.
H(Bz)), 7.48 (d, J = 3.0 Hz, 1 H, arom. H(cat.)), 7.29 (d, J = 0.7 Hz, 1 H, H-C(5)),
6.30 (d, J = 5.1 Hz, 1 H, H-C(1')), 5.76-5.70 (m, 1 H, H-C(2')), 5.68-5.62 (m, 1 H,
H-C(3')), 4.48-4.42 (m, 1 H, H-C(4')), 4.05-3.80 (m, 6 H, H-C(5'), OCH\textsubscript{2}, CH\textsubscript{2}NH),
2.10 (s, 3 H, CH\textsubscript{3}), 2.06 (s, 3 H, CH\textsubscript{3}); \textsuperscript{13}C NMR (125 MHz, CD\textsubscript{3}OD): δ = 171.47,
171.14, 170.14, 168.83, 164.47, 157.57, 150.26, 149.67, 149.62, 134.97, 133.65,
129.77, 128.90, 120.67, 116.09, 107.50, 98.42, 88.02, 83.85, 76.12, 72.52, 71.11,
70.64, 40.21, 20.52, 20.30 (1 peak missing); HR-MS (FAB): calcd. for
C\textsubscript{29}H\textsubscript{29}N\textsubscript{5}NaO\textsubscript{13}\textsuperscript{+} ([M + Na]\textsuperscript{+}): 678.1659; found: 678.1646.
Starting from 79 (48 mg, 0.07 mmol), the procedure described for 7 gave 69 (28 mg, 82%) as an orange powder.

M.p. 166 °C; [α]D20 = +15.0 (c = 1.0, CHCl3); IR (KBr): 3412, 3211, 2922, 2878, 1666, 1561, 1494, 1406, 1339, 1273, 1122, 1076, 989, 911, 789, 700, 600, 561 cm⁻¹; ¹H NMR (500 MHz, D2O): δ = 8.44 (d, J = 3.0 Hz, 1 H, arom. H(cat.)), 7.81 (d, J = 7.8 Hz, 1 H, H-C(6)), 7.66 (d, J = 3.0 Hz, 1 H, arom. H(cat.)), 5.88 (d, J = 4.0 Hz, 1 H, H-C(1')), 5.68 (d, J = 7.6 Hz, 1 H, H-C(5)), 4.40-4.37 (m, 1 H, H-C(2')), 4.30-4.29 (m, 1 H, H-C(3')), 4.28-4.24 (m, 1 H, H-C(4')), 4.00 (d, J = 9.5 Hz, 1 H, H-C(5')), 3.90-3.60 (m, 5 H, OCH₂, H-C(5'), CH₂NH); ¹³C NMR (125 MHz, D₂O + 1 drop of CD₃OD): δ = 169.83, 168.64, 166.67, 158.42, 149.10, 141.77, 132.77, 122.15, 116.60, 108.83, 96.62, 90.73, 83.91, 75.58, 70.52, 70.44, 70.03, 39.98; HR-MS (FAB): calcd. for C₁₈H₂₁N₅O₁₀Na⁺ ([M + Na⁺]: 490.1186; found: 490.1176.

N-(2-[(2S,3R,4R,5R)-5-(4-Amino-2-oxo-1,2-dihydropyrimidin-1-yl)-3,4-dihydroxytetrahydrofuran-2-yl]methoxy)ethyl)-2,3-dihydroxy-5-nitrobenzamide (81)
Starting from 80 (30 mg, 0.05 mmol), the procedure described for 7 provided 81 (21 mg, 80%) as an orange powder.

M.p. 142 °C; [α]D20 = +7.0 (c = 1.0, CHCl3); IR (KBr): 3421, 2933, 2867, 1683, 1656, 1550, 1444, 1333, 1261, 1122, 989, 844, 806, 700, 556 cm⁻¹; 1H NMR (300 MHz, D2O): δ = 8.56 (d, J = 0.7 Hz, 1 H, H-C(2)), 8.24 (d, J = 3.0 Hz, 1 H, arom. H(cat.)), 7.80-7.70 (m, 2 H, arom. H(Bz)), 7.63-7.50 (m, 3 H, arom. H(Bz)), 7.28 (d, J = 3.0 Hz, 1 H, arom. H(cat.)), 6.94 (d, J = 0.7 Hz, 1 H, H-C(5)), 5.91 (d, J = 2.1 Hz, 1 H, H-C(1′)), 4.70-4.68 (m, 1 H, H-C(2′)), 4.63-4.60 (m, 1 H, H-C(3′)), 4.36-4.30 (m, 1 H, H-C(4′)), 4.10-3.60 (m, 6 H, H-C(5′), OCH2, CH2NH); 13C NMR (125 MHz, D2O + 1 drop of CD3OD): δ = 169.89, 169.26, 168.82, 165.10, 156.64, 149.32, 148.84, 134.15, 133.27, 131.95, 129.79, 128.40, 122.13, 116.28, 107.74, 98.47, 91.86, 84.64, 76.35, 70.07, 69.95, 68.95, 40.46; HR-MS (FAB): calcd. for C25H26N5O11+ (MH+): 572.1629; found: 572.1645.

N-(2-[(2S,3R,4R,5R)-5-(4-Amino-6-oxo-1,6-dihydropyrimidin-1-yl)-3,4-dihydroxytetrahydrofuran-2-yl]methoxy)ethyl)-2,3-dihydroxy-5-nitrobenzamide (70)

A solution of 81 (20 mg, 0.035 mmol) in NH3 (25% in H2O, 5 mL) and MeOH (5 mL) was stirred for 24 h at 55 °C and then evaporated in vacuo. The orange residue was dissolved in H2O (10 mL) and extracted with Et2O (3 x 10 mL), toluene (10 mL) and CH2Cl2 (10 mL). After lyophilization from H2O, 70 (29 mg, 82%) was obtained as an orange powder, which contained traces of benzamide.

IR (KBr): 3411, 3189, 2911, 2844, 2355, 1650, 1555, 1467, 1389, 1333, 1261, 1122, 1072, 977, 805, 716, 667 cm⁻¹; 1H NMR (500 MHz, D2O): δ = 8.40 (s, 1 H, H-C(2)), 7.90 (d, J = 2.7 Hz, 1 H, arom. H(cat.)), 7.49 (d, J = 2.7 Hz, 1 H, arom.
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H(cat.), 5.90 (s, 1 H, H-C(5)), 5.45 (d, J = 4.9 Hz, 1 H, H-C(1’)), 4.27 (d, J = 5.3 Hz, 1 H, H-C(2’)), 4.22-4.18 (m, 1 H, H-C(3’)), 4.15-4.05 (m, 1 H, H-C(4’)), 3.98-3.50 (m, 6 H, H-C(5’), OCH2, CH2NH); 13C NMR (125 MHz, (CD3)2SO): δ = 169.03, 168.52, 167.32, 166.28, 166.23, 162.89, 160.54, 149.15, 120.80, 114.48, 104.01, 86.97, 82.57, 73.87, 70.07, 69.96, 69.58, 38.19; MS (ESI): m/z (%): 468 (MH+, 10), 467 (M+, 39), 466 ([M – H]+, 100).

NI-(1H-Pyrazolo[3,4-d]pyrimidine-4-yl)benzamide (82)

A mixture of 73 (0.42 g, 3.09 mmol) and benzoic anhydride (0.77 g, 3.40 mmol) was heated under stirring for 2 h at 140 °C. Then EtOH (15 mL) was added at 90 °C and stirring was continued for 1 h. After standing at 20 °C for 2 h, 82 precipitated from the solution as white crystals (0.58 g, 74%).

M.p. 225 °C; IR (KBr): 1715, 1601, 1582, 1527, 1492, 1334, 1263 cm⁻¹; 1H NMR (300 MHz, (CD3)2SO): δ = 8.67 (s, 1 H), 8.39 (s, 1 H), 8.10-8.04 (m, 2 H), 7.67-7.58 (m, 1 H), 7.57-7.50 (m, 2 H); 13C NMR (75 MHz, (CD3)2SO): δ = 166.81, 156.21, 154.64, 152.80, 136.48, 133.26, 132.89, 128.83, 128.62, 103.47; MS (FAB): m/z (%): 239 (M+, 8), 240 (MH+, 100), 241 (MH2+, 27); HR-MS (MALDI-TOF): calcd. for C12H10N5O+ [MH]+: 240.0885; found: 240.0880.
A mixture of 73 (0.50 g, 3.70 mmol) and benzoic anhydride (2.00 g, 8.84 mmol) was heated for 2 h at 140 °C. EtOH (15 mL) was added at 90 °C and stirring was continued for 1 h. After standing at 20 °C for 2 h, 83 (0.66 g, 51%) precipitated from the solution as white crystals.

M.p. 194 °C; IR (KBr): 3420, 2995, 1700, 1640, 1475, 1260 cm⁻¹; ¹H NMR (200 MHz, (CD₃)₂SO): δ = 11.91 (s, 1 H), 9.00 (s, 1 H), 8.70 (s, 1 H), 8.16-8.11 (m, 2 H), 7.98-7.93 (m, 2 H), 7.79-7.57 (m, 6 H); ¹³C NMR (75 MHz, (CD₃)₂SO): δ = 166.86, 166.30, 157.64, 156.86, 153.42, 140.20, 133.26, 133.10, 132.80, 132.68, 130.93, 128.92, 128.62, 128.34, 106.21; MS (FAB): m/z (%): 344 (M⁺, 100), 345 (MH⁺, 30).

To a stirred suspension of allopurinol (202 mg, 1.48 mmol) in MeCN (2 mL) was added BSA (0.36 mL, 1.48 mmol) at 50 °C under Ar. After 10 min, a colorless
solution formed to which 41 (0.40 g, 0.74 mmol) in MeCN (1 mL) and SnCl₄ (0.35 mL, 2.96 mmol) were added. Stirring was continued at 55 °C for 15 min, saturated aqueous solutions of NaHCO₃ (20 mL) and EtOAc (20 mL) were added and the mixture was extracted with EtOAc (3 x 50 mL). The organic phases were evaporated in vacuo, and FC (SiO₂, CH₂Cl₂/MeOH 99:1) provided 85 as a white powder (0.40 g, 4%).

M.p. 145 °C; ¹H NMR (200 MHz, CDCl₃): δ = 8.63 (s, 1 H, H-C(6)), 8.12 (s, 1 H, H-C(3)), 7.79-7.20 (m, 8 H, arom. H(Fmoc)), 6.50 (d, J = 4.0 Hz, 1 H, H-C(1')), 5.90-5.85 (m, 1 H, H-C(2')), 5.60-5.50 (m, 1 H, H-C(3')), 4.41-4.12 (m, 4 H, H-C(4'), CH(Fmoc), CH₂(Fmoc)), 3.81-3.32 (m, 6 H, H-C(5'), OCH₂, CH₂NH), 2.13-2.06 (m, 6 H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ = 169.83, 169.67, 156.82, 152.88, 147.09, 143.99, 143.93, 141.25, 139.67, 136.10, 127.58, 127.02, 126.91, 126.87, 125.04, 119.87, 119.84, 105.04, 86.13, 82.09, 74.70, 71.80, 70.83, 66.62, 47.22, 41.00, 20.64, 20.46; MS (FAB): m/z (%): 618 (M⁺, 100), 617 (M⁺, 30); HR-MS (FAB): calcd. for C₃₁H₃₁N₅O₉Na⁺ ([M + Na⁺]: 640.2019; found: 640.2019.

2,5-Dioxotetrahydro-1H-pyrrol-1-yl 2,3-dihydroxybenzoate (89)

To 90 (300 mg, 6.35 mmol) in THF (12 mL) were added N-hydroxysuccinimide (0.73 g, 6.35 mmol) and DCC (1.31 g, 6.35 mmol) at 0 °C. The mixture was stirred for 1 h at 0 °C during which time a white precipitate formed. The suspension was left in the refrigerator at 4 °C overnight, the white precipitate was filtered off and washed with THF (20 mL). Evaporation of the filtrate in vacuo and recrystallization from propan-2-ol gave 89 as beige powder (1.10 g, 69%).

M.p. 175 °C; IR (KBr): 3320, 2977, 2933, 1727, 1577, 1488, 1427, 1366, 1300, 1277, 1227, 1205, 1166, 1088, 1066, 989, 905, 811, 738, 705, 650, 605, 555, 444, 405 cm⁻¹; ¹H NMR (200 MHz, (CD₃)₂SO): δ = 7.31 (dd, J = 8.0 Hz, 1.6 Hz, 1 H,
H-C(6)), 7.14 (dd, J = 8.0 Hz, 1.6 Hz, 1 H, H-C(4)), 6.83 (t, J = 8.0 Hz, 1 H, H-C(5)), 2.89 (s, 4 H, CH2); 13C NMR (125 MHz, (CD3)2SO): δ = 170.45, 161.47, 148.83, 146.45, 120.84, 120.63, 119.17, 111.03, 25.46; MS (EI): m/z (%): 251 (M+, 27), 137 (100); C11H9N6O6 (251.20): calcd.: C 52.60, H 3.61, N 5.58, O 38.22; found: C 52.55, H 3.76, N 5.51.

(2S,3R,4R,5R)-2-[6-(Benzoylamino)-9H-purin-9-yl]-5-[(2-[(2,3-
dihydroxybenzoyl)amino]ethoxy)methyl]tetrahydrofuran-3,4-diyl
diacetate (91)

To 38 (100 mg, 0.14 mmol) in DMF (40 mL) was added HNEt2 (40 mL), and the solution was stirred under Ar for 2.5 h at 20 °C. After evaporation of HNEt2 in vacuo, 89 (35 mg, 0.14 mmol) and NEF3 (0.06 mL, 0.43 mmol) were added and the mixture was stirred for 24 h at 20 °C. Evaporation in vacuo and recrystallization from propan-2-ol gave 91 as a brownish powder (32 mg, 37%).

M.p. 119 °C; [α]D20 = -25.0 (c = 1.0, THF); IR (KBr): 3678, 3600, 3455, 3322, 3011, 2355, 1750, 1705, 1616, 1577, 1533, 1455, 1378, 1333, 1211, 1133, 1089, 905, 816, 694, 650 cm⁻¹; 1H NMR (200 MHz, CD3OD): δ = 8.70 (s, 1 H, H-C(4)), 8.65 (s, 1 H, H-C(8)), 8.10-8.07 (m, 2 H, arom. H(Bz)), 7.66-7.57 (m, 3 H, arom. H(Bz)), 7.15 (dd, J = 5.4 Hz, 1.2 Hz, 1 H, arom. H-C(6)), 6.85 (dd, J = 5.4 Hz, 1.2 Hz, 1 H, arom. H-C(4)), 6.61 (t, J = 5.4 Hz, 1 H, arom. H-C(5)), 6.37 (d, J = 3.8 Hz, 1 H, H-C(1')), 6.00 (t, J = 3.8 Hz, 1 H, H-C(2')), 5.70 (dd, J = 3.8 Hz, 2.7 Hz, 1 H, H-C(3')), 4.46-4.43 (m, 1 H, H-C(4')), 3.96-3.61 (m, 6 H, OCH2, CH2NH), 2.14 (s, 3 H, CH3), 2.02 (s, 3 H, CH3); 13C NMR (125 MHz, CD3OD): δ = 173.41, 171.54, 171.10, 168.16, 153.41, 153.32, 151.22, 144.05, 135.02, 133.91, 130.47,
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129.78, 129.46, 122.34, 119.09, 118.94, 118.66, 118.01, 87.65, 83.94, 75.30, 72.96, 71.19, 64.75, 40.22, 20.51, 20.48; MS (FAB): m/z (%): 635 (MH⁺, 100), 634 (M⁺, 27); HR-MS (FAB): calcd. for C₃₀H₃₁N₆O₁₀⁺ [MH⁺]: 635.2101; found 635.2110.

\[ N-(2-\{[(2S,3R,4R,5R)-5-(6-Amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl]methoxy\}ethyl)-2,3-dihydroxybenzamide \] (88)

Starting from 91 (31 mg, 0.05 mmol), the procedure described for 7 provided 88 (18 mg, 75%) as a beige powder.

M.p. 135 °C; [α]D²⁰ = -10.0 (c = 1.0, H₂O); IR (KBr): 3411, 2922, 2333, 1633, 1472, 1416, 1333, 1289, 1255, 1100, 900, 800 cm⁻¹; \(^1\)H NMR (500 MHz, CD₃OD): δ = 8.45 (s, 1 H, H-C(4)), 8.23 (s, 1 H, H-C(8)), 7.28 (d, J = 8.5 Hz, 1 H, arom. H-C(6)), 6.86 (d, J = 8.5 Hz, 1 H, arom. H-C(4)), 6.62 (t, J = 8.5 Hz, 1 H, arom. H-C(5)), 6.09 (d, J = 4.9 Hz, 1 H, H-C(1')), 4.70-4.68 (m, 1 H, H-C(2')), 4.47-4.39 (m, 1 H, H-C(3')), 4.25-4.24 (m, 1 H, H-C(4')), 3.94-3.43 (m, 6 H, H-C(5'), OCH₂, CH₂NH); MS (FAB): m/z (%): 447 (MH⁺, 33); HR-MS (FAB): calcd. for C₁₉H₂₂N₆O₇⁺ [M⁺]: 446.1550; found 446.1554.

6-Nitro-2,3-dihydro-1,4-benzodioxine (97)

\[ \text{NO}_2 \]
K₂CO₃ (7.13 g, 51.57 mmol) was added to a solution of 94 (4.00 g, 25.78 mmol) and 1,2-dibromoethane (19.37 g, 103.10 mmol) in ethylene glycol (50 mL). The mixture was heated for 6 h at 120 °C, extracted with CH₂Cl₂ (3 x 150 mL), and the combined organic phases were washed with H₂O (3 x 200 mL) and dried (MgSO₄). Evaporation of the solvent in vacuo afforded 97 as a yellow solid (4.39 g, 94%).

M.p. 120 °C (Lit. [216]: 121-122 °C); IR (KBr): 3089, 1589, 1519, 1469, 1347, 1286, 1215, 1077, 1055, 896, 737, 629 cm⁻¹; ¹H NMR (200 MHz, CDCl₃): δ = 7.81-7.76 (m, 2 H, arom. H), 6.96-6.92 (m, 1 H, arom. H), 4.38-4.30 (m, 4 H, CH₂); ¹³C NMR (50 MHz, CDCl₃): δ = 173.59, 115.32, 114.97, 114.97, 111.19, 62.28, 61.67.

**Sodium 7-nitro-2,3-dihydro-1,4-benzodioxine-5-sulfonate (98):**[219]

![98](image)

Compound 97 (4.39 g, 24.23 mmol) was added to 96-98% H₂SO₄ (4 mL) at 140 °C and heated for 15 min. The mixture was poured into ice water (30 mL) and filtered. An aqueous suspension of Ca(OH)₂ was added to the filtrate at 90 °C until pH = 7. The hot solution was filtered again and saturated aqueous solution of Na₂CO₃ was added until precipitation of CaCO₃ stopped. The solution was filtered, the aqueous phase was concentrated in vacuo, and cooled until 98 precipitated as a white solid (2.15 g, 31%).

M.p. 370 °C (decomposition); IR (KBr): 3490, 3100, 1590, 1519, 1469, 1347, 1286, 1264, 1217, 1077, 1054, 935, 897, 798, 737, 628 cm⁻¹; ¹H NMR (200 MHz, D₂O): δ = 8.28 (d, J = 2.4 Hz, 1 H, arom. H), 8.02 (d, J = 2.4 Hz, 1 H, arom. H), 4.58-4.44 (m, 4 H, CH₂); ¹³C NMR (200 MHz, D₂O): δ = 147.28, 144.17, 139.85, 130.96, 116.65, 116.05, 65.45, 64.18; MS (ESI): m/z (%): 260 ([M - Na]⁺, 100).
**EXPERIMENTAL PART**

7-Nitro-2,3-dihydro-1,4-benzodioxine-5-sulfonyl chloride (99)[219]

![Chemical Structure](image)

Compound 98 (1.91 g, 6.74 mmol) and PCl₅ (4.00 g, 19.20 mmol) were mixed and heated at 150 °C for 15 min. H₂O (20 mL) was added and the mixture was filtered. The precipitate was recrystallized from HOAc to provide 99 as a white powder (1.50 g, 80%).

M.p. 164 °C (Lit. [219]: 170 °C); IR (KBr): 3113, 1590, 1526, 1485, 1457, 1374, 1342, 1287, 1265, 1225, 1193, 1166, 1067, 955, 916, 889, 864, 798, 736, 674 cm⁻¹; 

¹H NMR (200 MHz, CDCl₃): δ = 8.47 (d, J = 2.4 Hz, 1 H, arom. H), 8.10 (d, J = 2.4 Hz, 1 H, arom. H), 4.67-4.63 (m, 2 H, CH₂); ¹³C NMR (50 MHz, CDCl₃): δ = 147.53, 145.27, 140.33, 131.95, 119.24, 117.63, 65.83, 64.04; MS (El): m/z (%): 279 (M⁺, 100); HR-MS (El): calcd. for C₈H₆N₀₆SCl⁺ [M⁺]: 278.9604; found 278.9607.

**N₅-Methyl-7-nitro-2,3-dihydro-1,4-benzodioxine-5-sulfonamide (100)**

![Chemical Structure](image)

MeNH₂ (40% in H₂O, 1 mL) was added to 99 (0.50 g, 1.79 mmol) and heated for 30 min at 60 °C. H₂O (10 mL) was added and the precipitated 100 was filtered off. FC (SiO₂, CH₂Cl₂/MeOH 95:5) provided 100 (411 mg, 84%) as a white solid.

M.p. 148°C; IR (KBr): 3456, 3278, 3089, 2944, 1589, 1517, 1472, 1427, 1339, 1283, 1256, 1189, 1156, 1117, 1061, 889, 800 cm⁻¹; ¹H NMR (200 MHz, CDCl₃): δ = 8.41 (d, J = 2.4 Hz, 1 H, arom. H), 7.99 (d, J = 2.4 Hz, 1 H, arom. H), 4.76-4.75
(m, 1 H, NH), 4.58-4.52 (m, 2 H, CH₂), 4.45-4.40 (m, 2 H, CH₂), 2.72 (d, J = 5.6 Hz, 3 H, H₃CNH); ¹³C NMR (50 MHz, CDCl₃): δ = 143.69, 141.85, 126.04, 125.22, 116.14, 114.9, 63.16, 61.42, 27.07; MS (EI): m/z (%): 274 (M⁺, 100), 181 ([M - CH₃NO₂S]+, 83); HR-MS (EI): calcd. for C₉H₁₀N₂O₆S⁺ [M⁺]: 274.0259; found 274.0261.

7-Amino-N₅-methyl-2,3-dihydro-1,4-benzodioxine-5-sulfonamide (101)

Iodotrimethylsilane (281 mg, 1.41 mmol) was added to a solution of 100 (85 mg, 0.31 mmol) in CH₂Cl₂ (10 mL) under Ar, and the mixture was stirred for 12 h at 20 °C. MeOH (7 mL) was added, the solvents were evaporated in vacuo, and the residue was dissolved in EtOAc (10 mL). The organic phase was washed with saturated aqueous solution of Na₂SO₃ (5 mL), dried (MgSO₄), and evaporated in vacuo. CC (SiO₂, CH₂Cl₂/MeOH 99:1) afforded 101 as a white powder (68 mg, 90%).

M.p. 151°C; IR (KBr): 3422, 3356, 3311, 2956, 2544, 2356, 1617, 1583, 1489, 1383, 1311, 1256, 1217, 1161, 1078, 1044, 933, 800 cm⁻¹; ¹H NMR (500 MHz, (CD₃)₂SO): δ = 6.55 (d, J = 2.7 Hz, 1 H, arom. H-C(6)), 6.30 (d, J = 2.7 Hz, 1 H, arom. H-C(4)), 4.99 (s, 2 H, NH₂), 4.21-4.16 (m, 4 H, CH₂), 2.40 (d, J = 5.0 Hz, 3 H, H₃CNH); ¹³C NMR (125 MHz, (CD₃)₂SO): δ = 144.18 (C(3)), 142.23 (C(5)), 131.43 (C(2)), 126.73 (C(1)), 106.96 (C(6)), 106.13 (C(4)), 64.14 (CH₂), 64.04 (CH₂), 28.74 (CH₃); MS (EI): m/z (%): 244 (M⁺, 85); C₉H₁₂N₂O₄S (244): calcd.: C 44.25, H 4.95, N 11.46, O 26.20, S 13.12; found: C 44.15, H 4.97, N 11.23, O 26.16, S 13.02.
Disodium 3-acetamido-5-nitro-2-oxidobenzenesulfonate (104)

Compound 102 (5.40 g, 23.05 mmol) was dissolved in acetic anhydride (50 mL) and heated for 3.5 h at 100 °C. H₂O (50 mL) was added and the reaction mixture was heated at reflux for 15 min. After evaporation of the solvents in vacuo, the residue was recrystallized from saturated aqueous solution of Na₂CO₃ (4 mL) to provide 102 (3.09 g, 42%) as a yellow powder.

M.p. 350 °C (decomposition); IR (KBr): 3550, 1645, 1595, 1522, 1152, 1375, 1288, 1182, 1082, 1045, 868 cm⁻¹; ¹H NMR (200 MHz, D₂O): δ = 8.71 (d, J = 3.2 Hz, 1 H, arom. H), 8.52 (d, J = 3.2 Hz, 1 H, arom. H), 2.29 (s, 3 H, CH₃CONHN); ¹³C NMR (125 MHz, D₂O): δ = 172.88, 167.38, 129.51, 128.99, 128.80, 123.88, 118.35, 23.35.

Sodium 3-acetamido-2-methoxy-5-nitrobenzenesulfonate (105)

(CH₃)₂SO₄ (5.42 g, 43.00 mmol) was added to a solution of 104 (0.66 g, 2.05 mmol) and Na₂CO₃ (6.07 g, 57.28 mmol) in DMA (80 mL), and the mixture was stirred for 48 h at 20 °C. The reaction mixture was filtered and the filtrate was evaporated in vacuo. The residue was dissolved in H₂O (10 mL), acidified with HCl (1N), and the aqueous phase was extracted with EtOAc (3 x 10 mL). The organic phase was dried (MgSO₄), and the solvents were evaporated in vacuo. The residue was recrystallized from a saturated aqueous solution of Na₂CO₃ (10 mL) to afford 105 (0.30 g, 47%) as a yellow powder.
M.p. 370 °C (decomposition); IR (CHCl₃): 3565, 1690, 1592, 1522, 1338, 1268, 1192, 1156, 1080, 1047, 914, 789, 750, 636 cm⁻¹; ¹H NMR (200 MHz, D₂O): δ = 8.76 (d, J = 3.0 Hz, 1 H, arom. H), 8.47 (d, J = 3.0 Hz, 1 H, arom. H), 3.76 (s, 3 H, CH₃CO), 2.22 (s, 3 H, CH₃CNHCO); ¹³C NMR (125 MHz, D₂O): δ = 172.96, 130.25, 128.5, 126.1, 123.58, 118.99, 117.01, 57.11, 23.12; MS (El): m/z (%): 290 ([M - Na]⁺, 8).

N-(6-Bromohexyl)phthalimide (111)[235]

Potassium phthalimide (23.15 g, 125 mmol) was added in one portion to a stirred refluxing solution of 1,6-dibromohexane (61.00 g, 250.00 mmol) in acetone (250 mL) under reflux. The mixture was heated for 24 h, then KBr was filtered off, and washed with several portions of hot acetone. The combined solutions were evaporated in vacuo and redissolved in hot hexane from which the product 111 crystallized upon cooling as white crystals (17.43 g, 45%).

M.p. 56 °C (Lit. [235]: 52-55 °C); IR (CHCl₃): 3011, 2944, 1767, 1706, 1433, 1394, 1367, 1039 cm⁻¹; ¹H NMR (200 MHz, CDCl₃): δ = 7.87-7.70 (m, 4 H, arom. H), 3.70 (t, J = 7.0 Hz, 2 H, H-C(6)), 3.40 (t, J = 7.0 Hz, 2 H, H-C(1)), 1.90-1.37 (m, 8 H); ¹³C NMR (50 MHz, CDCl₃): δ = 166.17, 131.57, 129.82, 120.83, 35.36, 31.20, 30.12, 25.93, 25.23, 23.55; MS (El): m/z (%): 311 (M⁺, 22), 309 ([M - H]⁺, 22).

9-(6-Phthalimidoethyl)adenine (112)[237]
EXPERIMENTAL PART

111 (2.50 g, 8.10 mmol) was added to a suspension of sodium adenine in DMF (30 mL), prepared from adenine (1.10 g, 8.10 mmol) and sodium hydride (55-65% dispersion in mineral oil, 0.71 g, 8.10 mmol) and the mixture was stirred for 7 d. The precipitate was filtered off, and the filtrate was evaporated in vacuo, swirled with Et₂O (100 mL), and filtered. The combined solids were washed successively with H₂O (50 mL), EtOH (50 mL), and Et₂O (50 mL). Recrystallization from MeOH afforded 112 as a white powder (2.19 g, 74%).

M.p. 185 °C (Lit. [237]: 180-187 °C); IR (CHCl₃): 3522, 3411, 2933, 1767, 1711, 1627, 1583, 1472, 1439, 1411, 1394, 1356, 1328, 1300, 1050 cm⁻¹; ¹H NMR (200 MHz, CF₃COOD + 10% C₆D₆): δ = 8.53 (s, 1 H, H-C(4)), 8.28 (s, 1 H, H-C(8)), 7.56-7.35 (m, 4 H, arom. H), 4.02 (t, J = 7.4 Hz, 2 H), 3.41 (t, J = 7.4 Hz, 2 H), 1.67-1.18 (m, 4 H); ¹³C NMR (75 MHz, CF₃COOD + 10% C₆D₆): δ = 171.95, 142.92, 135.40, 131.01, 124.07, 120.40, 116.63, 112.86, 109.10, 46.81, 37.98, 29.03, 27.69, 25.63, 25.37; MS (FAB): m/z (%): 365 (M+H⁺, 100); C₁₉H₂₀N₆O₂·0.5 H₂O (373): calcld.: C 61.11, H 5.67, N 22.51; found: C 61.45, H 5.95, N 22.31.

9-(6-Aminohexyl)adenine (113)[237]

To a stirred solution of 112 (1.00 g, 2.75 mmol) in EtOH (53 mL), heated to reflux under Ar, was added hydrazine hydrate (98% in H₂O, 0.35 mL, 7.53 mmol). The solution was heated at reflux for 17 h, then the solvent was removed in vacuo. To the residue maintained in an inert atmosphere was added HCl (2 N, 8 mL). The precipitated phthalhydrazide was filtered off, washed with water, and the filtrates were evaporated in vacuo. The residue was then recrystallized from MeOH/propan-2-ol (1:1, 10 mL), to afford the dihydrochloride dihydrate salt of 113.[237] It was dissolved in water and stirred for 6 h with Dowex 1-X-8 (OH⁻) ion exchange resin (20.00 g). The resin was filtered off, and the filtrate was passed through a column.
containing fresh Dowex (OH\(^-\)) (10.00 g). The resin beads were washed several times with water and then with MeOH, and the combined filtrates were evaporated in vacuo to provide 113 as a white solid (0.52 g, 80%).

M.p. 165 °C (Lit. [237]: 164-165 °C); IR (CHCl\(_3\)): 3322, 3178, 1644, 1600, 1572, 1478, 1411, 1339, 1311, 1244, 1211, 1144, 1078, 1050, 989, 794, 722, 644 cm\(^{-1}\); \(^1\)H NMR (200 MHz, CF\(_3\)COOD + 10% C\(_6\)D\(_6\)): \(\delta = 8.71\) (s, 1 H, H-C(4)), 8.40 (s, 1 H, H-C(8)), 4.11 (t, \(J = 7.5\) Hz, 2 H), 2.84 (br. s., 2 H), 1.76 (br. s., 2 H), 1.53 (br. s., 2 H), 1.24 (br. s., 4 H); \(^13\)C NMR (50 MHz, CD\(_3\)OD + 10% D\(_2\)O): \(\delta = 154.01, 150.70, 147.31, 140.80, 116.84, 42.25, 37.83, 27.42, 25.14, 23.74, 23.61\); MS (ESI): \(m/z\) (%): 235 (\(M^+\), 100).

\(N1-[6-(6-Amino-9H-purin-9-yl)hexyl]-2,3-dihydroxy-5-nitrobenzamide (107)\)

A mixture of 113 (63 mg, 0.27 mmol), 8 (80 mg, 0.27 mmol), and NEt\(_3\) (0.11 mL, 0.81 mmol) in DMF (4 mL) was stirred under Ar for 17 h at 20 °C. Evaporation in vacuo and recrystallization from EtOAc/propan-2-ol (1:1) gave 107 as a yellow powder (45 mg, 40%).

M.p. 182 °C; IR (CHCl\(_3\)): 3422, 2933, 2844, 1705, 1633, 1517, 1472, 1417, 1339, 1256, 1072, 800 cm\(^{-1}\); \(^1\)H NMR (300 MHz, CD\(_3\)OD): \(\delta = 8.42\) (d, \(J = 3.0\) Hz, 1 H, arom. H(cat.)), 8.19 (s, 1 H, H-C(4)), 8.14 (s, 1 H, H-C(8)), 7.57 (d, \(J = 3.0\) Hz, 1 H, arom. H(cat.)), 4.24 (t, \(J = 7.2\) Hz, 2 H), 3.40 (t, \(J = 7.2\) Hz, 2 H), 1.97-1.89 (m, 2 H), 1.63-1.60 (m, 2 H), 1.43-1.38 (m, 4 H); \(^13\)C NMR (300 MHz, CD\(_3\)OD): \(\delta = 169.71, 163.13, 157.26, 153.58, 150.65, 150.21, 149.51, 142.79, 120.04, 118.65, 116.26, 110.06, 47.93, 44.87, 40.62, 30.93, 30.25, 28.38; MS (FAB): \(m/z\) (%): 416 (\(M^+\), 100), 415 (\(M^+\), 38); HR-MS (FAB): calcd. for C\(_{18}\)H\(_{22}\)N\(_7\)O\(_5^+\) [\(M^+\)]: 416.1682; found 416.1684.
**6-Amino-9-(2-bromoethyl)-9H-purine (114):**

To a mixture of adenine (1.01 g, 7.40 mmol) and 1,2-dibromoethane (6.00 g, 31.96 mmol) in DMF (40 mL), was added K$_2$CO$_3$ (2.40 g, 17.14 mmol), and the mixture was stirred under Ar for 48 h at 20 °C. After filtration, the solvents were evaporated in vacuo, and the residue was washed with H$_2$O (30 mL) to afford 114 as dark yellow solid (1.08 g, 35%).

M.p. 189 °C (Lit. [238]: 195-200 °C); IR (KBr): 3289, 3134, 1672, 1600, 1572 cm$^{-1}$; $^1$H-NMR (200 MHz, CD$_3$OD): $\delta$ = 8.20 (s, 1 H, H-C(4)), 8.15 (s, 1 H, H-C(8)), 4.60 (t, $J$ = 6.0 Hz, 2 H), 3.80 ( t, $J$ = 6.0 Hz, 2 H).

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**N1-[2-(6-Amino-9H-purin-9-yl)ethyl]propane-1,3-diamine (115):**

A mixture of 114 (1.00 g, 4.10 mmol) and 1,3-diaminopropane (10 mL, 120 mmol) in MeOH was heated to reflux for 24 h. After evaporation of MeOH in vacuo, aqueous solution of NaOH (1 N, 12 mL) was added to the residue and the mixture was evaporated in vacuo. The residual slurry was stirred with Et$_2$O (50 mL) overnight and filtered. The precipitate was dissolved in HCl (2 N, 35 mL), filtered, and the filtrate was evaporated in vacuo. After recrystallization from MeOH, the material was dissolved in water and stirred for 6 h with DOWEX 1-X-8 (OH$^-$) ion exchange resin (70 g). The resin was filtered, and the filtrate was passed through a column containing fresh DOWEX (OH$^-$) (60 g). The resin beads were washed several times...
with H$_2$O and subsequently with MeOH, and the combined filtrates were evaporated *in vacuo* to provide 115 as white crystals (0.21 g, 22%).

M.p. 108 °C (Lit. [238]: 108-109 °C); $^1$H NMR (200 MHz, D$_2$O): $\delta = 8.10$ (s, 1 H, H-C(4)), 8.05 (s, 1 H, H-C(8)), 4.30 (t, $J = 6.2$ Hz, 2 H), 3.00 (t, $J = 6.2$ Hz, 2 H), 2.80 (t, $J = 7.0$ Hz, 2 H), 2.60 (t, $J = 7.6$ Hz, 2 H), 1.60 (m, 2 H); MS (FAB): m/z (%): 236 (MH$^+$, 100), 235 (M$^+$, 3), 234 ([M - H]$^+$, 9).

* $N_1$-[2-(6-Amino-9H-purin-9-yl)ethyl]butane-1,4-diamine (116)

A mixture of 114 (1.00 g, 4.10 mmol) and 1,4-diaminobutane (12 mL, 120 mmol) in MeOH was heated to reflux for 24 h. After evaporation of MeOH *in vacuo*, an aqueous solution of NaOH (1 N, 12 mL) was added to the residue and the mixture was evaporated *in vacuo*. The residual slurry was stirred with Et$_2$O (50 mL) overnight and filtered. The precipitate was dissolved in HCl (2 N, 35 ml), filtered, and the filtrate was evaporated *in vacuo*. After recrystallization from MeOH, the material was dissolved in water and stirred for 6 h with DOWEX 1-X-8 (OH$^-$) ion exchange resin (70 g). The resin was filtered, and the filtrate passed through a column containing fresh DOWEX (OH$^-$) (60 g). The resin beads were washed several times with water and subsequently with MeOH, and the combined filtrates were evaporated *in vacuo* to provide 116 as white crystals (0.33 g, 33%).

M.p. 94 °C; IR (KBr): 1678, 1602, 1481, 1416, 1331, 1305, 1246 cm$^{-1}$; $^1$H NMR (200 MHz, D$_2$O): $\delta = 8.29$ (s, 1 H, H-C(4)), 8.20 (s, 1 H, H-C(8)), 4.70 (t, $J = 5.8$ Hz, 2 H), 3.66 (t, $J = 6.2$ Hz, 2 H), 3.20 (t, $J = 5.5$ Hz, 2 H), 3.07 (t, $J = 5.5$ Hz, 2 H), 1.82-1.79 (m, 4 H); $^{13}$C NMR (75 MHz, D$_2$O): $\delta = 158.09$, 155.05, 154.13, 151.49, 121.02, 50.78, 50.09, 46.06, 43.12, 32.04, 28.66; MS (FAB): m/z (%): 251 (MH$_2^+$, 21), 250 (MH$^+$, 100), 249 ($M^+$, 3); HR-MS (FAB): calcd. for C$_{11}$H$_{20}$N$_7^+$ (MH$^+$): 250.1780; found: 250.1776.
**EXPERIMENTAL PART**

*N1-(3-[(2-(6-Amino-9H-purin-9-yl)ethyl)amino]-propyl)-2,3-dihydroxy-5-nitrobenzamide (108)*

![108](image)

A mixture of 115 (0.10 g, 0.43 mmol), 8 (0.13 g, 0.43 mmol), and NEt₃ (0.17 mL, 1.27 mmol) in DMF (4 mL) was stirred under Ar for 17 h at 20 °C. Evaporation *in vacuo* and recrystallization from propan-2-ol gave 108 as orange crystals (96 mg, 54%).

M.p. 185 °C; IR (KBr): 3333, 2962, 1700, 1640, 1475, 1260, 1077 cm⁻¹; ¹H NMR (300 MHz, (CD₃)₂SO): δ = 10.88 (br. s, 1 H), 8.30 (d, J = 2.0 Hz, 1 H, arom. H(cat.)), 8.14 (s, 1 H, H-C(4)), 8.10 (s, 1 H, H-C(8)), 7.21 (d, J = 2.0 Hz, 1 H, arom. H(cat.)), 4.42-4.39 (m, 2 H), 3.97-3.91 (m, 2 H), 3.50-3.43 (m, 2 H), 3.00-2.93 (m, 2 H), 1.84-1.77 (m, 2 H); ¹³C NMR (75 MHz, (CD₃)₂SO): δ = 167.36, 156.23, 152.63, 149.94, 149.16, 141.06, 129.64, 120.67, 118.94, 114.79, 114.20, 104.48, 62.03, 46.16, 45.08, 35.22, 26.55; MS (FAB): m/z (%): 417 (MH⁺, 65), 416 (M⁺, 21), 415 ([M – H]⁺, 9); HR-MS (MALDI-TOF): calcd. for C₁₇H₂₀N₈O₅⁺ (M⁺): 417.1635; found: 417.1630.

*N1-(4-[(2-(6-Amino-9H-purin-9-yl)-ethyl)amino]-butyl)-2,3-dihydroxy-5-nitrobenzamide (109)*

![109](image)
A mixture of 116 (0.10 g, 0.34 mmol), 8 (84 mg, 0.34 mmol), and NEt₃ (0.17 mL, 1.27 mmol) in DMF (4 mL) was stirred under Ar for 17 h at 20 °C. Evaporation in vacuo and recrystallization from propan-2-ol gave 109 as red powder (101 mg, 70%).

M.p. 165 °C; IR (KBr): 1637, 1472, 1439, 1345, 1248 cm⁻¹; ¹H NMR (500 MHz, CDCl₃): δ = 10.75 (br. s, 1 H), 8.53 (br. s, 1 H), 8.32 (d, J = 3.1 Hz, 1 H, arom. H(cat.)), 8.16 (s, 1 H, H-C(4)), 8.10 (s, 1 H, H-C(8)), 7.22 (d, J = 3.1 Hz, 1 H, arom. H(cat.)), 4.43 (t, J = 0.6 Hz, 2 H), 3.43-3.39 (m, 2 H), 2.99 (t, J = 7.2 Hz, 2 H), 2.10-2.08 (m, 2 H), 1.61-1.52 (m, 4 H); ¹³C NMR (125 MHz, CDCl₃): δ = 206.43, 166.38, 156.02, 149.71, 148.87, 140.84, 129.29, 120.55, 118.80, 114.40, 104.05, 46.89, 46.22, 37.41, 30.63, 26.66, 23.30; MS (FAB): m/z (%): 432 (M⁺, 24), 431 (M⁺, 100), 430 ([M-H]⁺, 27); HR-MS (MALDI-TOF): calcd. for C₁₈H₂₂N₈O₅(M⁺): 431.1791; found: 431.1790.

(2R,3R,4S,5R)-2-(3-Aminopropyl)-5-(6-amino-9H-purin-9-yl)tetrahydrofuran-3,4-diol (126)[²³⁹]

Piperidine (0.25 mL, 0.26 mmol) was added to a solution of 118 (0.10 g, 0.26 mmol) in dioxane/H₂O (1:2, 3.75 mL) under Ar, and the mixture was stirred for 20 h at 60 °C. The solvents were evaporated in vacuo and the residue was dissolved in MeOH (10 mL) and filtered over Celite. The solvent was evaporated in vacuo, and the crude product was stirred in CH₂Cl₂ (3 mL) to afford 126 as white crystals which were redissolved in MeOH (5 mL). Evaporation of the solvent in vacuo yielded highly hygroscopic 126 (0.52 g, 70%).
**EXPERIMENTAL PART**

$N1\{-[3\{2R,3R,4S,5R\}-5-(6-Amino-9H-purin-9-y1)-3,4-
dihydroxytetrahydrofuran-2-y1\{propyl\}-2,3-dihydroxy-5-nitrobenzamide (117)**

A mixture of 126 (0.11 g, 0.39 mmol), 8 (0.12 g, 0.39 mmol), and NEt$_3$ (0.16 mL, 1.16 mmol) in DMF (4 mL) was stirred under Ar for 17 h at 20 °C. Evaporation *in vacuo* and recrystallisation from propan-2-ol gave 117 as a yellow powder (26 mg, 14%).

M.p. 80 °C; $[\alpha]_D^{20} = -55.0$ (c = 1.0, THF); IR(KBr): 3340, 1706, 1646, 1475, 1263, 1077 cm$^{-1}$; $^1$H NMR (200 MHz, CD$_3$OD): $\delta =$ 8.44 (d, $J =$ 3.0 Hz, 1 H, arom. H(cat.)), 8.28 (s, 1 H, H-C(4)), 8.19 (s, 1 H, H-C(8)), 7.53 (d, $J =$ 3.0 Hz, 1 H, arom. H(cat.)), 5.97 (d, $J =$ 5.1 Hz, 1 H, H-C(1’)), 4.70 (t, $J =$ 5.1 Hz, 1 H, H-C(2’)), 4.16 (t, $J =$ 5.1 Hz, 1 H, H-C(3’)), 3.92-3.85 (m, 1 H, H-C(4’)), 3.60-3.10 (m, 6 H, H-C(5’), H-C(6’), H-C(7’)); $^{13}$C NMR (125 MHz, CD$_3$OD): $\delta =$ 174.95, 169.78, 157.31, 153.88, 150.71, 141.34, 134.78, 120.57, 120.12, 116.32, 108.56, 90.01, 85.62, 75.72, 64.75, 40.05, 32.17, 27.09. MS (FAB): $m/z$ (%): 476 (MH$^+$, 17); HR-MS (FAB): calcd. for C$_{19}$H$_{21}$N$_7$NaO$_8$ $^+$([M + Na]$^+$): 498.1349; found: 498.1345.
7.2 ENZYMATIC STUDIES

7.2.1 Materials

Reagents were purchased from Fluka and Merck, Darmstadt. S-Adenosyl-L-
[methyl-^{3}H]methionine ([^{3}H]SAM, specific activity: 15 Ci/mmol, The
Radiochemical Centre, Amersham) was diluted with SAM (sulfate, p-
toluenesulfonate, BioResearch, Liscate, Italy) to a specific activity of 3.64 Ci/mol
and to a concentration of 5.5 mmol/L. Dithiothreitol (DTT) was obtained from
Calbiochem-Behring Corp., Luzern, and 2-(4'-tert-butylphenyl)-5-(4"-biphenyl)
1,3,4-oxadiazole (butyl-PBD) from Novartis, Basel.

Wistar rats were killed by decapitation and the liver removed. The tissue was
homogenized in ice-cold water (1:10 w/v) containing 0.2% Triton X-100 and 0.002%
dithiothreitol (triton/DTT) and then centrifuged at 12 000 g for 20 min at 4 °C.
Supernatants were further diluted (1:10) with Triton/DTT solution.

7.2.2 Kinetic measurements

IC_{50} values were determined as follows: the inhibitors were dissolved in
Me_{2}SO as 1.2 mM stock solution and further diluted with 0.001 N HCl. The
reactions were performed in standard polyethylene scintillation vials. 25 µL of the
inhibitor in varying concentrations from 10^{-4} to 10^{-6} mol/L were mixed with 250 µL
freshly prepared buffer-substrate mixture, composed of 200 µL potassium
phosphate buffer (0.1 mol/L, pH = 7.6), 10 µL MgCl_{2} (0.1 mol/L), 15 µL substrate
(benzene-1,2-diol, 0.05 mol/L), 10 µL dithiothreitol (0.065 mol/L), 5 µL deionized
H_{2}O, and 10 µL [^{3}H]SAM (5.5 mmol/L, specific activity: 3.64 Ci/mol). Then 25 µL
of tissue extract were added. The reaction was started by incubating the vials in a
water-bath at 37 °C for 15 min. The incubation was stopped by adding 250 µL of
HOAc (5.7 %) containing guaiacol (0.1 g/L) and 3 mL of scintillation fluid (5 g butyl-
PBD, dissolved in 200 mL toluene, made up to 1 L with n-hexane) was added. The
vials were capped and more than 98 % [^{3}H]guaiacol formed was extracted into the
organic phase by vigorous shaking for 1 min. The samples were counted in a
Beckmann LS 6000 TA scintillation counter. IC_{50} values were determined with and
without preincubation in the presence of inhibitor. Enzyme preparations were
preincubated for 15 min at 37 °C at varying inhibitor concentrations from 10^{-4} to 10^{-6} mol/L in the presence of buffer mixture without the substrate benzene-1,2-diol in the case of the inhibitor 5. In the case of the potential bisubstrate inhibitors, preincubation without the substrates benzene-1,2-diol and SAM was performed.

For the determination of $K_M$ and $K_I$ values, either the concentration of the substrate benzene-1,2-diol was varied between 28 μM and 500 μM at saturating SAM concentration (183 μM) or the concentration of SAM was varied between 10 μM and 183 μM at saturating benzene-1,2-diol concentration (2.5 mM). The volumes of all reagents were doubled, and the incubation time varied between 1 min and 15 min at 37 °C with and without preincubation in the presence of inhibitor. A total of 10 ml of scintillation fluid was added, and the samples were shaken for 5 min and centrifuged. The aqueous phase was frozen by placing the samples in dry ice/acetone, and the organic phase was poured into a scintillation vial and counted.

A dialysis experiment was performed as follows: the inhibitors were incubated with the enzyme preparation at 37 °C for 15 min in the presence of SAM and MgCl₂. Dialysis of the incubation mixture against 0.01 M KH₂PO₄/KH₂PO₄ buffer, pH = 7.6, which contained 0.003 M MgCl₂ and 100 mg of DTT in 10 L of buffer was performed, and the relative inhibition was determined after 0, 2, 4, 6, 8, and 24 h. The loss of activity owing to degradation of the enzyme was measured in the same experiment.
7.2.3 Selected IC\textsubscript{50} curves

Compound 7, 15 min incubation at 37 °C:

\[
\]

\begin{align*}
\text{Chi squared} & \approx 5.6999 \times 10^4 \\
\text{Parameters:} & \\
\text{Standard deviations:} & \\
a[1] & = 64.2988 \quad \Delta a[1] = 87.7767 \\
a[2] & = 1.8340 \times 10^4 \quad \Delta a[2] = 127.8114 \\
a[4] & = 0.6793 \quad \Delta a[4] = 1.0458 \times 10^{-2} \\
\end{align*}

IC\textsubscript{50} = 10\textsuperscript{a[4]} = 4.7 \mu M

\begin{align*}
\text{with preincubation:} & \\
\text{Chi squared} & \approx 1.6626 \times 10^5 \\
\text{Parameters:} & \\
\text{Standard deviations:} & \\
a[1] & = -181.4515 \quad \Delta a[1] = 157.6562 \\
a[2] & = 2.0942 \times 10^4 \quad \Delta a[2] = 320.3554 \\
a[4] & = 0.4158 \quad \Delta a[4] = 2.2800 \times 10^{-2} \\
\end{align*}

IC\textsubscript{50} = 10\textsuperscript{a[4]} = 2.5 \mu M
Compound 14, 15 min incubation at 37 °C:

\[
\]

\[
\text{Chi squared } = 5.5248 \times 10^5
\]

\[
\begin{align*}
\text{Parameters:} & \\
\text{Standard deviations:} & \\
a[1] & = -54.3522, \quad \Delta a[1] = 323.3270 \\
a[3] & = -2.7223, \quad \Delta a[3] = 0.1323 \\
\end{align*}
\]

\[
IC_{50} = 10^{4.8} = 26 \mu M
\]

with preincubation:

\[
\text{Chi squared } = 1.1088 \times 10^5
\]

\[
\begin{align*}
\text{Parameters:} & \\
\text{Standard deviations:} & \\
a[1] & = -48.6935, \quad \Delta a[1] = 143.6641 \\
a[2] & = 2.0352 \times 10^4, \quad \Delta a[2] = 102.7362 \\
\end{align*}
\]

\[
IC_{50} = 10^{4.6} = 25 \mu M
\]
Compound 50, 15 min incubation at 37 °C:

\[
y := a[1]+(a[2]-a[1])/(1+\exp(-a[3]*(x-a[4])))
\]

\[
\text{Chi squared} = 3.7729e+5
\]

Parameters: Standard deviations:
\[
a[1] = 67.9843 \quad \Delta a[1] = 264.0592
\]
\[
a[2] = 2.08286+4 \quad \Delta a[2] = 188.0256
\]
\[
a[3] = -2.9215 \quad \Delta a[3] = 0.1206
\]
\[
\]

\[IC_{50} = 10^{a[4]} = 25 \text{ \mu M}\]

with preincubation:

\[
\text{Chi squared} = 3.0057e+5
\]

Parameters: Standard deviations:
\[
a[1] = 26.0326 \quad \Delta a[1] = 236.9165
\]
\[
\]
\[
a[3] = -2.8844 \quad \Delta a[3] = 0.1111
\]
\[
\]

\[IC_{50} = 10^{a[4]} = 26 \text{ \mu M}\]
Compound 49, 15 min incubation at 37 °C:

Curve fitting:
\[ y := a[1] + \frac{a[2] - a[1]}{1 + \exp(-a[3] \cdot (x - a[4]))} \]

Chi squared = 2.6986e+5
Parameters:
- \( a[1] = -47.2032 \)  \( \Delta a[1] = 227.4516 \)

\( IC_{50} = 10^{a[4]} = 27 \mu M \)

With preincubation:
Chi squared = 2.2468e+5
Parameters:
- \( a[1] = -23.4324 \)  \( \Delta a[1] = 208.7671 \)

\( IC_{50} = 10^{a[4]} = 35 \mu M \)
Compound 48, 15 min incubation at 37 °C:

\[ y = a[1] + \frac{a[2] - a[1]}{1 + \exp(-a[3](x - a[4]))} \]

\[ \text{IC}_{50} = 10^{4} \text{ nM} \]

Chi squared = 1.6792e+5

Parameters: Standard deviations:

\[ a[1] = -47.6155 \quad \Delta a[1] = 189.8105 \]
\[ a[2] = 2.3363e+4 \quad \Delta a[2] = 197.3144 \]
\[ a[4] = 2.0481 \quad \Delta a[4] = 1.4519e-2 \]

Compound 8, 15 min incubation at 37 °C:

\[ y = a[1] + \frac{a[2] - a[1]}{1 + \exp(-a[3](x - a[4]))} \]

\[ \text{IC}_{50} = 10^{4} \text{ nM} \]

Chi squared = 5.1853e+5

Parameters: Standard deviations:

\[ a[1] = -773491 \quad \Delta a[1] = 266.3419 \]
\[ a[2] = 18782e+4 \quad \Delta a[2] = 258.8118 \]
\[ a[3] = -3.0638 \quad \Delta a[3] = 0.1767 \]
\[ a[4] = 2.0267 \quad \Delta a[4] = 2.1005e-2 \]
Compound 79, 15 min incubation at 37 °C:

\[
y := \frac{a[1]+(a[2]-a[1])/(1+\exp(-a[3]*(x-a[4])))}{1+\exp(-a[3]*(x-a[4]))}
\]

\[
\text{Chi squared} = 2.7463 \times 10^5
\]

Parameters:

- \(a[1] = -42.1226\)
- \(\Delta a[1] = 216.4083\)
- \(a[2] = 2.3161 \times 10^4\)
- \(\Delta a[2] = 186.6427\)
- \(a[3] = -2.6717\)
- \(\Delta a[3] = 8.6637 \times 10^{-2}\)
- \(a[4] = 2.1678\)
- \(\Delta a[4] = 1.4389 \times 10^{-2}\)

\(\text{IC}_{50} = 10^{[4]} = 147 \mu\text{M}\)

with preincubation:

\[
\text{Chi squared} = 6.8948 \times 10^5
\]

Parameters:

- \(a[1] = -83.5452\)
- \(\Delta a[1] = 353.9220\)
- \(a[2] = 2.3890 \times 10^4\)
- \(\Delta a[2] = 276.2385\)
- \(a[3] = -2.7252\)
- \(\Delta a[3] = 0.1332\)
- \(a[4] = 2.2873\)
- \(\Delta a[4] = 2.1823 \times 10^{-2}\)

\(\text{IC}_{50} = 10^{[4]} = 193 \mu\text{M}\)
Compound 80, 15 min incubation at 37 °C:

\[ y = a[1] + (a[2] - a[1])/(1 + \exp(-a[3] \times (x - a[4]))) \]

\text{Chi squared} = 8.3462e+5

\begin{align*}
\text{Parameters:} & \quad \text{Standard deviations:} \\
a[1] &= 35.0990 & \Delta a[1] &= 222.4039 \\
\end{align*}

\( \text{IC}_{50} = 10^{a[4]} = 24 \mu M \)

with preincubation:

\text{Chi squared} = 1.2940e+6

\begin{align*}
\text{Parameters:} & \quad \text{Standard deviations:} \\
\end{align*}

\( \text{IC}_{50} = 10^{a[4]} = 28 \mu M \)
Compound 81, 15 min incubation at 37 °C:

\[
\]

Chi squared = 2.9594e+5

Parameters: Standard deviations:

- \(a[1] = -120.7993\) \(\Delta a[1] = 244.3062\)

IC\(_{50}\) = 10\(^{a[4]}\) = 231 µM

with preincubation:

Chi squared = 1.6712e+6

Parameters: Standard deviations:

- \(a[1] = -151.7234\) \(\Delta a[1] = 580.1102\)
- \(a[3] = -2.2323\) \(\Delta a[3] = 0.1781\)

IC\(_{50}\) = 10\(^{a[4]}\) = 255 µM
Compound 69, 15 min incubation at 37 °C:

\[
y := a[1]+(a[2]-a[1])/(1+\exp(-a[3] *(x-a[4]))) \quad IC_{50} = 10^{a[4]} = 522 \, \mu M
\]

Chi squared = 1.5079e+6

Parameters: Standard deviations:

\begin{align*}
a[1] &= -5.1770 \quad \Delta a[1] = 563.9027 \\
a[3] &= -2.4487 \quad \Delta a[3] = 0.2038 \\
a[4] &= 2.7181 \quad \Delta a[4] = 3.5483 \times 10^{-2}
\end{align*}

Compound 89, 15 min incubation at 37 °C:

\[
y := a[1]+(a[2]-a[1])/(1+\exp(-a[3] *(x-a[4]))) \quad IC_{50} = 10^{a[4]} = 138 \, \mu M
\]

Chi squared = 1.6196e+7

Parameters: Standard deviations:

\begin{align*}
a[1] &= -1253.5388 \quad \Delta a[1] = 2380.3433 \\
a[3] &= -1.3749 \quad \Delta a[3] = 0.1363 \\
a[4] &= 2.1405 \quad \Delta a[4] = 8.6376 \times 10^{-2}
\end{align*}
Compound 107, 15 min incubation at 37 °C:

\[
dpm
\]

\[
\text{curve fitting:} \quad y := a[1]+(a[2]-a[1])/(1+\exp(-a[3]*(x-a[4]))) \quad \text{IC}_{50} = 10^{a[4]} = 55 \, \mu\text{M}
\]

Chi squared = 1.7518e+7

Parameters:

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<th>Standard deviation</th>
</tr>
</thead>
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<td>a[1]</td>
<td>1012.7843</td>
<td>1147.7703</td>
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<tr>
<td>a[2]</td>
<td>6.2284e+4</td>
<td>1546.4331</td>
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<tr>
<td>a[3]</td>
<td>-4.2193</td>
<td>0.4543</td>
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</tbody>
</table>

Compound 108, 15 min incubation at 37 °C:

\[
dpm
\]

\[
\text{curve fitting:} \quad y := a[1]+(a[2]-a[1])/(1+\exp(-a[3]*(x-a[4]))) \quad \text{IC}_{50} = 10^{a[4]} = 77 \, \mu\text{M}
\]

Chi squared = 3.8202e+7

Parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>a[1]</td>
<td>-1892.1834</td>
<td>2904.6096</td>
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<tr>
<td>a[2]</td>
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<tr>
<td>a[3]</td>
<td>-2.1953</td>
<td>0.2830</td>
</tr>
</tbody>
</table>
Compound 109, 15 min incubation at 37 °C:

\[ y := a[1] + (a[2] - a[1]) / (1 + \exp(-a[3] \cdot (x - a[4]))) \]

\[ IC_{50} = 10^{4} \text{d} = 86 \mu \text{M} \]

\[ \text{Chi squared} = 2.4537 \times 10^{7} \]

Parameters:
- \( a[1] = -1529.4295 \)
- \( a[2] = 7.7561 \times 10^{4} \)
- \( a[3] = -1.9391 \)
- \( a[4] = 1.9351 \)

Standard deviations:
- \( \Delta a[1] = 2398.1831 \)
- \( \Delta a[2] = 2516.2664 \)
- \( \Delta a[3] = 0.2001 \)
- \( \Delta a[4] = 6.1962 \times 10^{-2} \)

Compound 117, 15 min incubation at 37 °C:

\[ y := a[1] + (a[2] - a[1]) / (1 + \exp(-a[3] \cdot (x - a[4]))) \]

\[ IC_{50} = 10^{4} \text{d} = 605 \text{nM} \]

\[ \text{Chi squared} = 1.6706 \times 10^{6} \]

Parameters:
- \( a[1] = -118.9173 \)
- \( a[2] = 8.0266 \times 10^{4} \)
- \( a[3] = -2.0995 \)
- \( a[4] = -0.2180 \)

Standard deviations:
- \( \Delta a[1] = 407.7456 \)
- \( \Delta a[2] = 2517.8668 \)
- \( \Delta a[3] = 0.1113 \)
- \( \Delta a[4] = 4.2007 \times 10^{-2} \)
8 REFERENCES


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Born in Ahlen, Germany, October 18, 1971.

1978 - 1982
Marienschule, Ahlen, Germany, primary school

1982 - 1991
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1991 - 1996
Westfälische Wilhelms-Universität Münster, Germany: Studies in chemistry and biochemistry.
Vordiplom: October 1993
Diplom: July 1995

1994
University of Toledo, Ohio, USA: Studies and research projects in inorganic chemistry and biochemistry in a study abroad program.

1995 - 1996
University of California at Berkeley, USA: Diploma work in organic chemistry in the laboratory of Prof. K. Peter C. Vollhardt on "Functionalization of pyridones by C-H and N-H activation".

1996
Internship at BASF Bioresearch Corporation, Worcester, USA: Organic synthesis in two ongoing projects in oncology.

1996-2000
Ph.D. project in the Laboratorium für Organische Chemie at the Swiss Federal Institute of Technology (ETH) Zurich in the group of Prof. Dr. François Diederich: "Structure-Based Design, Synthesis, and in vitro Evaluation of Bisubstrate Inhibitors for Catechol O-Methyltransferase (COMT)."

Zürich, May 2000

Birgit Masjost