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Biocatalytic Friedel-Crafts Alkylation

DISSERTATION

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Abstract

The focus on ecologic aspects of industrial processes becomes more and more important. Many established classic chemical processes produce problematic by-products and use precarious catalysts. In many cases the requirement for elevated temperature increases the energy consumption. So the implementation of green processes will play an important role in the next years.
Biocatalysis can offer many interesting reaction characteristics. Typically the reactions are carried out in aqueous media and at ambient temperature. The biocatalysts are biodegradable and environmentally benign. They are extremely efficient and highly selective. The formation of just one single product simplifies the workup, product isolation and purification.
Since many years a special interest in biocatalysis has existed in Graz. In particular biocatalytic C-C-bond formation was investigated intensively. C-C-bond formation is one of the most challenging fields of organic chemistry.

The Friedel-Crafts alkylation is a classical chemical reaction. It is a C-C-bond forming reaction which belongs mechanistically to the electrophilic aromatic substitution reactions. A strong Lewis acid has to be applied as catalyst. In some cases also elevated temperatures are necessary.
In this work a biocatalytic variant of the Friedel-Crafts alkylation is presented. *S*-adenosyl-L-methionine dependent methyltransferases were chosen as biocatalysts. Several special methyltransferases were investigated for their substrate and cofactor specificity.

Different behaviour was observed concerning the substrate promiscuity of the methyltransferases. While some of the investigated methyltransferases showed relaxed substrate specificity, some methyltransferases were restricted to their natural substrates and structurally very closely related compounds and some only accepted their natural substrates. Nearly all investigated methyltransferases readily accepted synthetic cofactor analogs for alkyl transfer instead of methyl transfer.

A new biocatalytic variant of the Friedel-Crafts reaction could be introduced. Some biocatalysts offer broad substrate promiscuity and cofactor acceptance. This is a first step to a general method for the regioselective alkylation of aromatic substrates.

Kurzfassung

Die Beachtung von ökologischen Gesichtspunkten in Produktionsprozessen wird immer wichtiger. Viele etablierte klassisch-chemische Prozesse bilden problematische Nebenprodukte und benötigen gefährliche Katalysatoren. In vielen Fällen muss erhöhte Temperatur verwendet werden, was zu einem erhöhten Energieverbrauch führt. Deshalb wird der Einsatz von „grünen“ Prozessen in den kommenden Jahren eine wichtige Rolle spielen.
Biokatalyse bietet interessante Charakteristika. Üblicherweise werden die Reaktionen in wässrigen Systemen und bei Raumtemperatur durchgeführt. Die Biokatalysatoren sind biologisch abbaubar und umweltverträglich. Des Weiteren sind Biokatalysatoren äußerst effizient und selektiv. Die Bildung von nur einem Produkt vereinfacht die Aufarbeitung, Isolierung und Reinigung.
Seit vielen Jahren gibt es in Graz einen Forschungsschwerpunkt im Bereich der Biokatalyse. Besonders die biokatalytische C-C-Bindungsknüpfung war Gegenstand intensiver Untersuchungen. Das Gebiet der C-C-Bindungsknüpfung stellt eine besondere Herausforderung in der organischen Chemie dar.

Die Friedel-Crafts Alkylierung ist eine klassische chemische Reaktion. Diese Reaktion zur C-C-Bindungsknüpfung ist mechanistisch eine elektrophile aromatische Substitutionsreaktion. Als Katalysator dient eine starke Lewis-Säure. In einigen Fällen sind zusätzlich erhöhte Temperaturen von Nöten.
In dieser Arbeit wird eine biokatalytische Variante der Friedel-Crafts Alkylierung vorgestellt. Als Biokatalysatoren wurden *S*-Adenosyl-L-methionin abhängige Methyltransferasen ausgewählt. Einige spezielle Methyltransferasen wurden auf ihre Substratbreite und Cofaktorakzeptanz untersucht.

In Bezug auf die Substratbreite wurde ein unterschiedliches Verhalten der Methyltransferasen festgestellt. Einige Methyltransferasen zeigten eine geringe Substratspezifität, andere boten nur eine geringe Substratbreite. Eine weitere Gruppe von Methyltransferasen akzeptierte nur ihr natürliches Substrat. Die meisten Methyltransferasen verwendeten die synthetischen Analoga als Cofaktoren für den Transfer von Alkylgruppen anstelle der Methylgruppe wie in der natürlichen Reaktion.

Eine biokatalytische Variante der Friedel-Crafts Alkylierung konnte gefunden werden. Die Substratbreite variierte stark zwischen den untersuchten Methyltransferasen. Die Substratbreite und Cofaktorakzeptanz von einigen Methyltransferasen ist der erste Schritt zu einer allgemeinen, regioselektiven Alkylierungsreaktion von aromatischen Substraten.

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Abbreviations

3h5m*O*mTyr	3-hydroxy-5-methyl-*O*-methyltyrosine
4DPMC	4-demethylpremithramycinone
Ac	acetyl
ACP	acyl carrier protein
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATCC	American type culture collection
Boc	*tert*-butyloxycarbonyl
BSA	bovine serum albumin
CDI	carbonyl diimidazole
CE	cell extract
CL	crude lysate
CoA	coenzyme A
COMT	catechol-*O*-methyltransferase
*Ct*XR	*Candida tenuis* xylose reductase
CouBz	*N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide
CouOHBz	*N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-4-hydroxybenzamide
CouPy	*N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-pyrrol-2-carboxamide
DAD	diode array detector
DAPA	7,8-diaminopelargonic acid
dcSAM	decarboxylated *S*-adenosyl-L-methionine
deion.	deionized
DHBA	3,4-dihydroxybenzoic acid
DHCAA	6,7-dihydroxycoumarin-4-ylacetic acid
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	2´-deoxyribonucleic acids
dopa	3,4-dihydroxyphenylalanine

DPBQ	2,3-dimethyl-5-phytyl-1,4-benzoquinol
DPMA3	9-demethylpremithramycin A3
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German collection of microorganisms and cell cultures)
DTT	dithiothreitol
ec	endcapped
EC	enzyme class
Ed.	Edition
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation
Et	ethyl
ev	empty vector
FAD	flavin adenine dinucleotide
FDH	formate dehydrogenase
FMN	flavin mononucleotide
GDH	glucose dehydrogenase
HAA	3-hydroxyanthranilic acid
HKyn	3-hydroxykynurenine
HPLC	high pressure (or performance) liquid chromatography
HMBA	4-hydroxy-3-methoxybenzoic acid
IPTG	isopropyl-D-thiogalactopyranoside
Ile	isoleucine
KAPA	7-keto-8-aminopelargonic acid
Lit.	literature
MAT	methionine adenosyltransferase
Me	methyl
MeMet	*S*-methyl methionine
MeHAA	3-hydroxy-4-methylanthranilic acid
MeHKyn	3-hydroxy-4-methylkynurenine
MeTyr	3´-methyltyrosine
MOM	methoxymethyl
MPBQ	6-methyl-2-phytyl-1,4-benzoquinol
MS	mass spectroscopy
MTA	*S*-methyl-5´-thioadenosine
MTase	methyltransferase

MTHF	N^5-methyl-5,6,7,8-tetrahydrofolate
MTR	*S*-methyl-5´-thioribose
MWD	multiwavelength detector
NAD(P)	nicotinamide-adenine dinucleotide (phosphate)
NMR	nuclear magnetic resonance
Nu	nucleophile
n°	number
ORF	open reading frame
PLP	pyridoxal phosphate
PMA1	premithramycin A1
PMP	pyridoxamine phosphate
PP	polyphosphate
rt	room temperature
RNA	ribonucleic acids
tRNA	transfer ribonucleic acids
tRNA-preQ$_1$	7-aminomethyl-7-deazaguanosine
rpm	rotations per minute
S_EAr	electrophilic aromatic substitution
S_N2	bimolecular nucleophilic substitution
SAH	*S*-adenosyl-L-homocysteine
SAM	*S*-adenosyl-L-methionine
SIM	single ion monitoring
Tf	triflate
THF	5,6,7,8-tetrahydrofolate
Thr	threonine
Tic	total ion current
TMS	trimethylsulfonium
TMSO	trimethylsulfoxonium
TLC	thin layer chromatography
Trp	tryptophan
Tyr	tyrosine

1 Introduction

It is estimated that about 90 % of all chemical products are produced employing at least one catalysed step.[1] The application of a catalyst accelerates a reaction by lowering the activation energy via following an alternative reaction pathway with a different transition state. Addition of a catalyst only affects the reaction rate. Because the energy difference between the substrate and the product stays untouched, the equilibrium is the same as in the uncatalysed reaction.

In the field of organic chemistry four catalyst classes compete:

- Homogeneous catalysis
- Heterogeneous catalysis
- Organocatalysis
- Biocatalysis

The classes of homogeneous and heterogeneous catalysis are already known for a long time. Many industrial processes using homogeneous or heterogeneous catalysts are established. They are by far the most relevant catalyst classes in industry.
The difference between homogeneous and heterogeneous catalysts is: Homogeneous catalysts are in the same phase as the reactants, usually the catalysts and reactants are in solution. Heterogeneous catalysts are in a different phase than the reactants, eg. a solid catalyst in a liquid or gaseous reaction mixture.[2, 3]
A great number of different catalysts can be found within these two classes. Transition metals and complexes thereof can appear in homogeneous and in heterogeneous catalysts and offer very interesting opportunities. Catalysts with chiral ligands open the door to stereoselective processes.
Transition metal catalysts are able to catalyse very special reactions, for example osmium tetraoxide catalysed syn dihydroxylation of a double bond, but many transition metals have been banned from synthetic applications for pharmaceutical and nutritional products because of their toxicity and still others might follow.

Organocatalysis is a young field compared to the other classes. The reaction is catalysed by an organic compound. If the organic catalyst contains a stereocenter, the induced stereopreference can lead to an enantioenriched or even enantiopure product.

Biocatalysis can offer many interesting reaction characteristics. Typically the reactions are carried out in aqueous media and at ambient temperature. The biocatalysts are biodegradable and environmentally benign. They are extremely efficient and highly selective regarding chemo-, regio- and stereoselectivity. The formation of just one single product simplifies the workup, product isolation and purification.

The importance of ecologic issues increases constantly. The requirement for elevated temperature increases the energy consumption. Many established classical chemical processes produce problematic by-products and use precarious catalysts. Supplementary reactions have to be introduced for many processes to meet modern reaction characteristics and general requirements. Biocatalysis is one opportunity to establish ecologically and economically up-to-date processes.

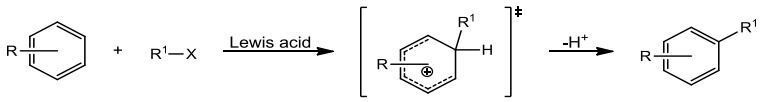
Avoidance of energy consuming processes and toxic byproducts paired with outstanding reaction characteristics are forward looking benefits of the application of biocatalytic methods.

The discovery of new biocatalytic reactions and biocatalysts is an important field of research activities in Graz. In this work a new biocatalytic alternative to a classic chemical reaction will be presented.

2 Friedel-Crafts reaction

C-C-bond formation is one of the most challenging reaction classes in the field of organic chemistry. A variety of reactions has been introduced to form C-C-bonds. Some very popular examples are Aldol type reactions, Pd catalysed cross-coupling reactions and Wittig type reactions. A much smaller number of reactions are used to form aromatic C-C-bonds. Again Pd catalysed cross-coupling can be used and also reactions that belong to the class of electrophilic aromatic substitution S_EAr.[4, 5]

The most prominent S_EAr reaction is the Friedel-Crafts reaction.[6, 7] In a typical Friedel-Crafts reaction an alkyl or acyl halide is activated with a strong Lewis acid. This activated species adds to the aromatic substrate under formation of a σ-bond and as a result the interruption of aromaticity. Subsequent deprotonation leads to the regeneration of the aromatic system (Scheme 1).[4, 5]



Scheme 1 Mechanism of the Friedel-Crafts reaction

Friedel-Crafts alkylation is associated with some major drawbacks:

Usually harsh reaction conditions are needed, for example strong Lewis acid catalysis and elevated temperature. The alkylation occurs at a position with increased electron density which is determined by the substitution pattern. The introduction of an alkyl group into an aromatic substrate forms a product that is more nucleophilic and so further activated towards the uptake of another alkyl group than the starting material. Thus over-alkylation is a common problem. For many substrates also the poor regioselectivity of this reaction is an issue. Furthermore lewis acid activation leads to the formation of cationic species, so Wagner-Meerwein rearrangement can take place in the alkyl chain. The consequence of these three factors is that Friedel-Crafts alkylation is very likely to yield an unwanted product mixture.[4, 5]

Many common Friedel-Crafts catalysts decompose hydrolytically under formation of aggressive agents. Water free conditions are required for these catalysts.

The tendency to by-product formation is also accompanied with the need for time consuming and potentially expensive purification procedures.

Especially when complex substrates are applied at late stages of multi step synthetic routes, the loss of precious material over by-product formation or decomposition under extreme reaction conditions must be avoided. In such a case Friedel-Crafts alkylation is not a proper option.

3 Enzymes

In biocatalysis enzymes are the most relevant catalysts. Enzymes are highly specialised proteins that exhibit reaction parameters that are unreached with any other type of catalyst. Enzymes are highly functionalised macromolecules. They possess the ability to exactly recognize their substrates. Also the reactions themselves run under very defined conditions and usually give only the desired product.

What makes enzymes so special is their composition of just 20 amino acids. All amino acids except glycin contain at least one stereocenter with (*S*) (or L) configuration. This also means that all enzymes are chiral catalysts. Together with the exact substrate recognition the intense stereo induction of the enzymes usually leads to highly enantioenriched products.
The complex structure and composition of just L-amino acids is a drawback as well. A stereoselective enzyme that converts a substrate to a chiral product can be changed in its stereospecificity to form the other enantiomer as a product only with great effort. To get the other enantiomer in many cases a different enzyme with stereocomplementary preference has to be applied.

Enzymes are categorized into six classes according to the reaction they catalyse.

Oxidoreductases	EC 1
Transferases	EC 2
Hydrolases	EC 3
Lyases	EC 4
Isomerases	EC 5
Ligases	EC 6

3.1 Oxidoreductases

Oxidoreductases catalyse the oxidation or reduction of a substrate. All oxidoreductases are cofactor dependent because the enzymes need partners that act as an electron donor or electron acceptor. The cofactor can either be a metal ion or an organic compound. It can shift electrons directly or uses hydrid or oxygen as a redox equivalent. Beneath the cofactors for oxidoreductases some are not independently reformed in vitro. Because cofactors usually are extremely expensive these cofactors need to be recycled for a feasible industrial application. Especially the important cofactors

nicotinaminde adenine dinucleotide (NAD) and the corresponding equivalent with a phosphate at the 2´-position of the ribose of adenosine (NADP) have been intensively investigated and many promising approaches to recycling have been published in the last years.[8, 9]

3.2 Transferases

Transferases catalyse the transfer of a group from a cofactor to a substrate. The class of transferases also shares the problem of cofactor dependence.[10] In comparison to hydrolases and oxidoreductases the enzymes that belong to the class of transferases have been investigated less intensively.[11]

3.3 Hydrolases

The synthetically and industrially most relevant class are the hydrolases. Hydrolases catalyse the hydrolysis of many different substrates and moieties. The most prominent members belong to the subclasses of proteases, lipases and esterases. Proteases catalyse the hydrolysis of the amide bonds in proteins and are found in many commercially available detergents for example.[12]
Some characteristics make hydrolases perfect both for lab scale and industrial scale applications. Hydrolases do not need cofactors. In most cases they offer high stereoselectivity. The reactions catalysed by hydrolases can be run in the reverse direction. Some hydrolases are compatible with organic solvents. Many hydrolases show rather relaxed substrate specificity. Some are used to catalyse unnatural reactions. These characteristics make hydrolases attractive for synthetic purposes.[13, 14]

3.4 Lyases

Lyases are enzymes that catalyse the formation of a double bond such as C=C, C=N or C=O by eliminating a small molecule from the substrate. Synthetically and industrially of bigger interest is the back reaction, the addition of a molecule to a double bond under formation of two new bonds. With lyases two stereogenic centers can be generated in just one reaction.[10, 12]

3.5 Isomerases

Isomerases are enzymes that catalyse the racemisation, epimerisation or rearrangement of a substrate. Especially the potential of isomerases has long been underestimated. In the recent years the interest in isomerases constantly increased. Racemisation is part of dynamic kinetic resolution of chiral compounds. Industry and academia have great interest in methods that can provide an enantiomerically pure product with up to 100% conversion starting from a racemic substrate. Enzymatic racemisation with isomerases coupled with a second enzyme that consumes just one enantiomer of the substrate would be a very potent system for dynamic kinetic resolution as it leads to a theoretical yield of 100%.[10, 12]

3.6 Ligases

Enzymes that catalyse the C-X bond formation under consumption of ATP as an energy source belong to the class of ligases. This means all ligases need ATP as a cofactor. The ATP dependence is the reason that ligases were not able to make their way into industrial processes. Also their use in lab scale is limited to few applications because of the high costs for the ATP or the complexity of setting up of a suitable recycling system.[12]

4 Cofactor dependence

4.1 Definition

According to Collins English dictionary a cofactor is defined as follows: "A cofactor is a nonprotein substance that forms a complex with certain enzymes and is essential for their activity. It may be a metal ion or a coenzyme."[15]

Many enzymes are known to be cofactor dependent. Cofactors can be roughly classified into inorganic cofactors, which means metal ions complexed in the enzyme, and organic cofactors. Organic cofactors are termed coenzymes. A further classification distinguishes between coenzymes that are tightly bound to the enzyme, called prosthetic groups, and loosely bound coenzymes. Some structures can be seen as mixtures of both classes, they are organic cofactors with a metal ion complexed that is necessary for activity. Porphyrin and corrin based cofactors like heme and cobalamin are examples for cofactors combining both categories.[16]
A list of common cofactors is shown in Table 1.[12]

Table 1. List of the most important cofactors and representative reactions they are involved in. [a] Recycling of cofactor is necessary (+) or not required (-).[12]

Coenzyme	Reaction type	Recycling[a]
ATP	energy, phosphorylation	+
SAM	C_1-alkylation	+
NAD^+/NADH	removal or addition of hydrid	+
$NADP^+$/NADPH		+
Coenzyme A	C_2-alkylation	+
Flavins	electron transfer, oxygenation	-
Pyridoxal-phosphate	transamination	-
Biotin	carboxylation	-
Metal-porphyrin complexes	peroxidation, oxygenation	-

4.2 Important cofactors

4.2.1 Adenosine triphosphate

The most prominent cofactor is adenosine triphosphate ATP. It is nature´s energy source and storage. By cleavage of one, two or all three phosphate units the energy can be released and used for thermodynamically unfavoured reactions. It is also used as a phosphate donor for the phosphorylation of various substrates.

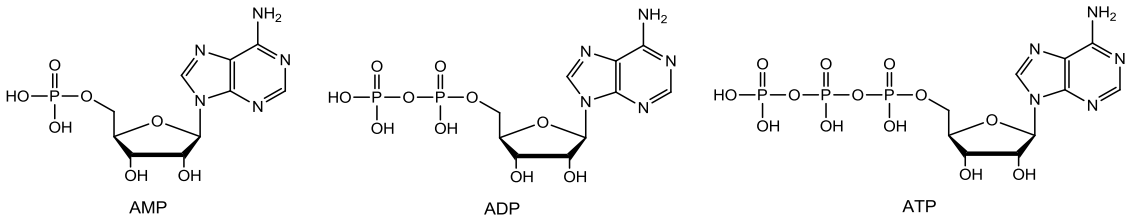


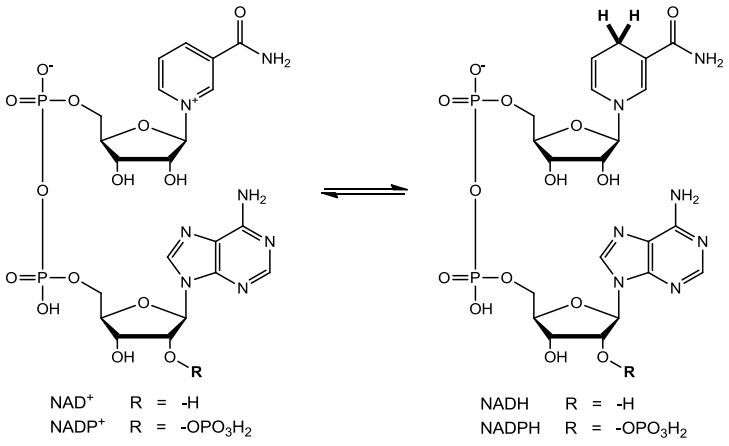
Figure 1. Structure of AMP/ADP/ATP

ATP is not reformed during the reaction, recycling is necessary.

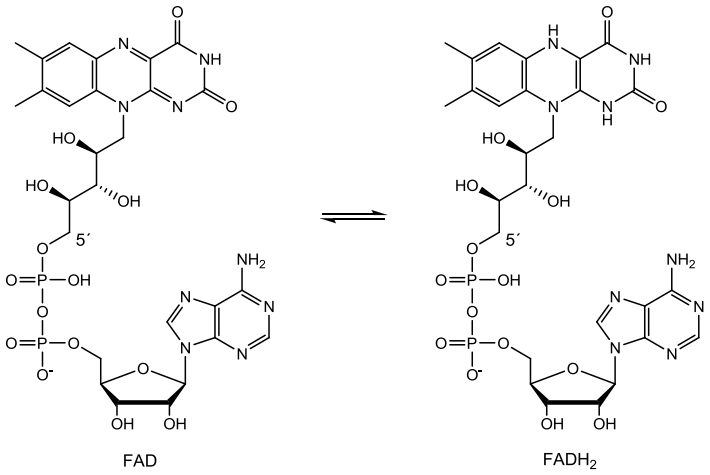
The coenzyme SAM is object of investigations in this work and is described in detail in chapter 5.5.

4.2.2 Nicotinamide adenine dinucleotide (phosphate) and flavin adenine dinucleotide

As mentioned before the whole enzyme class of oxidoreductases requires cofactors as an electron shuttle. They can either transfer electrons directly or they use a hydrid or oxygen as redox equivalent. Common cofactors are NAD(P)H / NAD(P)$^+$ (Scheme 2) or FAD / $FADH_2$ (Scheme 3).



Scheme 2. Structure of the cofactors NAD^+/NADH and $NADP^+$/NADPH.



Scheme 3. Structure of the cofactor FAD/$FADH_2$.

A cofactor that is closely related to FAD is flavinmononucleotide FMN. Structurally it is a phosphate condensed to a riboflavin unit at 5´-hydroxyl and acts similar to FAD.

A big difference between NAD(P) and FAD is that whereas NAD(P) has to be recycled, for reactivation of FAD no recycling is needed.

Another difference is that NAD(P)H / $NAD(P)^+$ transfers a hydrid as an electron equivalent. This means that always two electrons are transferred. In contrast to that FAD also has the ability to transfer single electrons and is also able to activate molecular oxygen.

4.2.3 Coenzyme A

Coenzyme A (Figure 2) is an important coenzyme in many anabolic and catabolic pathways. It is able to activate a carboxylic acid by formation of a thioester. Acetyl-CoA is used for introduction of C_2-units, in the reverse direction coenzyme A can catalyse the removal of a C_2-unit.

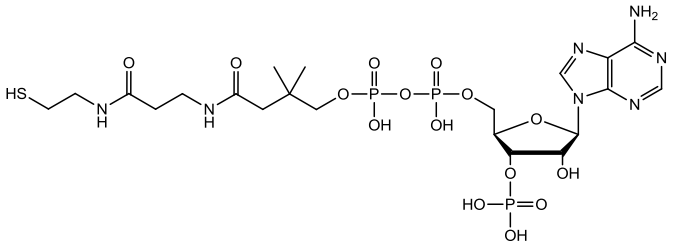


Figure 2. Structure of Coenzyme A. The terminal thiol is activating carboxylic acids via formation of a thioester.

4.2.4 Pyridoxal phosphate

Pyridoxal phosphate (PLP) is a cofactor that is involved in various reactions. It participates in reactions catalysed by type I aldolases, transaminase reactions, decarboxylation, racemisation and elimination reactions.[17] It occurs in an oxidised form as PLP and a reduced amino form as pyridoxamine phosphate (PMP, Figure 3).

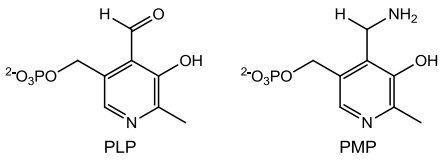
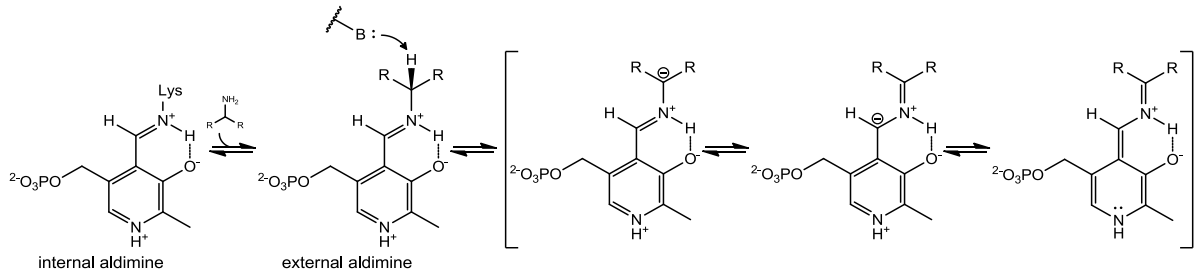


Figure 3. Free pyridoxal phosphate PLP and its reduced amino form pyridoxamine phosphate PMP

In all PLP dependent enzymes PLP is bound to a lysine as a Schiff base (internal aldimine, Scheme 4) in its resting state. When a substrate attacks an external aldimine is formed. The external aldimine is also common in all PLP dependent reactions. From this point the reaction specificity differs. The great majority of reactions proceed via an anionic intermediate, but also radical-based reactions are known. The external aldimine is highly activated towards cleavage of one of the α-C bonds (except the C-N bond) under formation of a carbanionic species. This intermediate is resonance stabilized by the neighbouring π-electron system. The cleavage reactions can be deprotonation (shown as an example in Scheme 4), decarboxylation or C_α-C_β cleavage.[18]



Scheme 4. Mechanism of substrate activation of PLP dependent enzymes and the most significant resonance forms of anionic aldimine derived from deprotonation of the external aldimine.

The key catalytic step in the reactions in all PLP dependent enzymes is the formation of an external Schiff base with the substrate. Because PLP is reformed during the catalytic cycle, it need not be recycled.

4.2.5 Biotin

Biotin is a compound that carries many names. It is called vitamin B_7 or B_8, vitamin H and coenzyme R. It plays an important role for carboxylase reactions. Biotin is covalently bound in the enzyme and is able to fix carbon dioxide which is then incorporated into the acceptor substrate.[19]

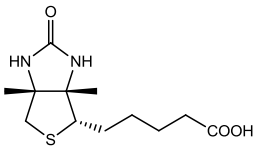


Figure 4. Structure of unbound biotin

4.2.6 Heme

Heme is a very important cofactor for oxygen fixation and activation. Heme consists of a substituted porphyrin core complexing an iron atom over the porphyrin nitrogen atoms (Figure 5).

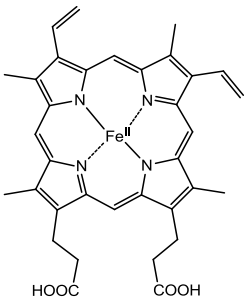


Figure 5. Structure of heme b

There exist several biologically relevant derivatives with differing substitution pattern.

As an example heme is found as prosthetic group in cytochrome P450 enzymes, nitric oxide synthase or heme oxygenase.[16] In the last years the class of cytochrome P450 enzymes was intensively investigated. These enzymes are able to catalyse the introduction of oxygen to a great variety of substrates.

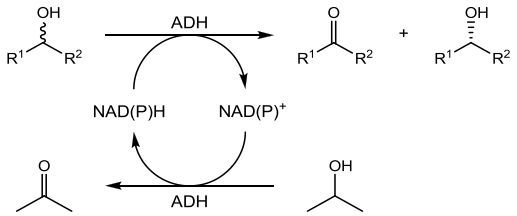
Other porphyrin based biologically relevant substances are chlorophyll with Mg^{2+} as complexed metal, and factor F_{430} with Ni^{2+}. The corrin based cofactor cobalamin containing a Co^{2+} is a tetrapyrrol structure with high similarity to the prophyrin based cofactors and is described in chapter 5.7.1.

4.3 Cofactor Recycling

The great majority of cofactors is very expensive and a feasible process in not possible if the cofactor has to be applied in stoichoimetric quantities. Recycling of the cofactor can overcome the problem of high costs in cofactor dependent reactions.
The recycling of NAD(P) has been very intensely studied during the last years and is chosen to explain the different methods of recycling systems.
In general there exist two different approaches to enzymatic recycling.

4.3.1 Substrate coupled recycling systems

The idea of this approach is to use the substrate promiscuity of the applied enzyme. A cheap co-substrate is added to the reaction in great excess. Using the co-substrate the enzyme performs the reaction in the reversed direction under reformation of the needed cofactor species. This way of cofactor regeneration can be used for NAD(P) dependent alcohol dehydrogenases for example.



Scheme 5. Principle of a substrate coupled cofactor recycling system for recycling of NAD(P).

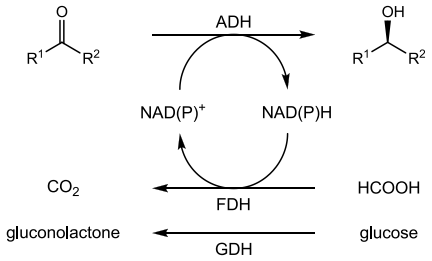
Some points have to be considered to get a well working process: Both the co-substrate and the co-product shall not inhibit the enzyme. If the co-product can be removed from the reaction mixture, the equilibrium of reaction is shifted to the co-product side, and thus to the regenerated cofactor. This can be done by precipitation or vaporisation of the co-product. A very popular co-substrate is isopropanol. In this case the co-product formed is acetone. If the reaction can be run at slightly elevated temperature or under reduced pressure, the acetone that is formed is easily removed from the reaction mixture and the equilibrium is shifted to the co-product side.[9, 12, 20]

4.3.2 Enzyme coupled recycling systems

The principle of this approach is to use a second enzyme (Scheme 6) for regeneration. The auxiliary enzyme has the single role to regenerate the cofactor. This methodology offers the opportunity to

pick an enzyme whose substrate and product do not interfere with the desired reaction. It can be used for the regeneration of NAD(P) or ATP.

Two widely used enzymes for the regeneration of NAD(P)H are formate dehydrogenase (FDH) and glucose dehydrogenase (GDH).

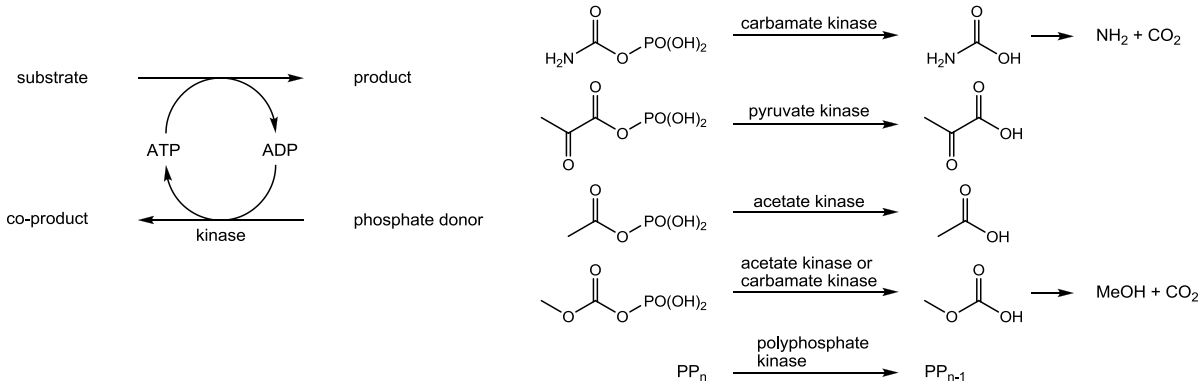


Scheme 6. Principle of enzyme coupled recycling systems showing two recycling systems as an example.

FDH uses formate as a substrate and carbon dioxide is formed as product. It is used for lab scale applications. Glucose acts as a substrate for glucose dehydrogenase. This enzyme forms 1,5-gluconolactone as product. GDH is used for cofactor recycling from lab scale to industrial scale.[12]

Also for recycling of ATP this strategy can be applied. ATP appears as a cofactor in many reactions. Because it is not reformed during the enzymatic reaction, a recycling system is needed to run a feasible process with ATP dependent enzymes.

Different kinases can be used for the regeneration of ATP. A phosphorylated substrate acts as phosphate donor for the enzyme, common substrates and enzymes are shown in Scheme 7. ATP is reformed from ADP in a transphosphorylation reaction.[12]



Scheme 7. Strategy for the recycling of ATP from ADP via enzyme coupled recycling method. On the left side common substrates and enzymes for ATP recycling are listed.[12]

The recycling systems shown are only applicable for reactions which form ADP from ATP. The reassembly of ATP from AMP or adenosine is much more complicated and the upper strategy cannot be applied. To establish a recycling system from AMP an additional enzyme has to be employed for

the formation of ADT. ATP is then formed from ADP with one of the kinases already mentioned or with adenylate kinase, an enzyme that catalyses the disproportionation of two ADP molecules under formation of AMP and ATP.

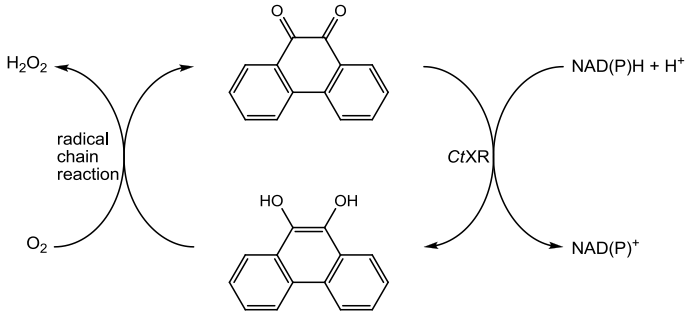
An example from literature for ATP reformation from AMP is using polyphosphate as sole substrate. The formation of ADP from AMP is catalysed by the enzyme polyphosphate:AMP phosphotransferase from *Acinetobacter johnsonii* strain 210A. ATP is then reformed from ADP with adenylate kinase. An alternative for the second step would be the application of a polyphosphate kinase for ATP formation.[21]

4.3.3 Chemical recycling

Besides the enzymatic recycling also a chemical reaction can be applied for the regeneration of a cofactor. Three variations can be used: Electrochemical, photochemical and chemical cofactor regeneration. This is an interesting alternative to enzymatic recycling systems.

Electrochemical recycling can be performed directly on the electrode for the reduction/oxidation of NAD(P). A similar method is the use of a mediator molecule which is reduced/oxidised on the electrode. The mediator is then used for an enzymatic recycling of NAD(P).[8]

A very interesting way of NAD(P) recycling was introduced by Pival *et al*. Phenanthrene-9,10-dione is reduced to phenanthrene-9,10-diol by xylose reductase from *Candida tenuis* under regeneration of $NAD(P)^+$ which is then used in the desired reaction. The mediator itself is regenerated by molecular oxygen under formation of H_2O_2.[22]



Scheme 8. A $NAD(P)^+$ recycling system that uses molecular oxygen as oxidising agent and 9,10-phenanthrenedione as mediator and substrate for the enzyme *Candida tenuis* xylose reductase (*Ct*XR) which reforms $NAD(P)^+$.

5 Methyltransferases

Methyltransferases (MTases) EC[2.1.1] belong to the class of transferases (EC[2]). MTases are member of the subgroup of one-carbon group transferring enzymes (EC[2.1]) together with hydroxymethyl-, formyl and related transferases [2.1.2], carboxy- and carbamoyl transferases [2.1.3] and amidinotransferases [2.1.4]. These enzymes are able to transfer a methyl group from a cofactor to a substrate.

5.1 Sources for methyltransferases

MTases are found in nearly every organism. MTases are involved in metabolic and anabolic pathways. For example DNA and RNA MTases are necessary for gene regulation, DNA repair or protection against foreign DNA. Catechol-O-MTase is an enzyme class that is found in many different organisms. In mammals COMT occurs in two different forms: The soluble COMT and the membrane bound MTase. The SAM dependent Glycine *N*-MTase is responsible for the formation of sarcosine by methylation of glycine.

Very important sources for special MTases are actinomycetes, especially streptomycetes. Actinomycetes are able to produce an incredible variety of secondary metabolites. Diverse structures are formed and the core structures are decorated with carbohydrates, peptides and other substituents. Many highly specialised enzymes that are involved in the biosynthetic pathways to antibiotics can be isolated from these organisms.

In antibiotics biosynthesis MTases participate in different modes. MTases are employed in the formation of the core structures. Methyl groups also occur in the substitution pattern of antibiotics and modify the steric demand and the electronic surface of the antibiotic. Frequently MTases are involved in the formation of unusual sugars that are linked to the antibiotic core.

Methyltransferases can be divided into different classes pointing out two aspects. They can be classified according to their substrates and also the atom species that is methylated.

5.2 Classification of MTases according to substrate classes

- DNA MTases
- RNA MTases
- Protein MTases
- Small molecule MTases

5.2.1 DNA & RNA Mtases

The class of DNA methyltransferases catalyses the transfer of a methyl group to a DNA strand. The methylation site is encoded by a specific nucleobase sequence. Methylation of a nucleotide in the sequence is important for the recognition of a specific position.
DNA MTases from prokaryotes target the positions *C*5 or *N*4 in cytosine or *N*6 in adenine. In eukaryotes and higher organisms exclusively cytosine *C*5 methylation has been proven.[23]
When using SAM analogs as cofactors the DNA MTases can be used for the sequence specific labelling of a DNA strand.[24]

Like DNA MTases also RNA MTases are able to catalyse the methylation of RNA. Again the sequence pattern is used for recognition of the methylation site.

5.2.2 Protein MTases

Few examples of MTases are known which catalyse the methylation of an amino acid within a protein. Lysine and arginine act as potential methyl group acceptors in a protein. Histone lysine-*N*-MTase [25] and SAM dependent protein arginine-*N*-MTase [26] are two examples for protein *N*-MTases.

5.2.3 Small molecule MTases

MTases are involved in many metabolic pathways. For instance MTases play an important role in detoxification or they participate in the biosynthesis of secondary metabolites.
Lots of MTases accept small molecules as substrates. Prominent examples are Catechol-*O*-MTase, various homocysteine MTases and glycine-*N*-MTase.
Glycine-*N*-MTase is used for the formation of sarcosine from glycine upon methylation. The role of this MTase is assumed to be the regulation of the cellular SAM/SAH ratio.[27]

5.3 Classification of MTases according to target atom

The classification with a focus on the target atom species distributes the MTases into several subclasses.

- *O*-MTases
- *N*-MTases
- *C*-MTases
- *S*-MTases
- Other Mtases

5.3.1 *O*-MTases

The MTases of this class methylate a hydroxyl group under formation of a methyl ether. A very prominent member of this class is the SAM dependent catechol-3-*O*-MTase.[28-30] Also in the formation of secondary metabolites *O*-MTases are often employed for the formation of methyl ether moieties.

5.3.1.1 Catechol-O-MTase

Catechol-*O*-MTase (COMT) is one of the best investigated *O*-MTases. It occurs in many organisms and is usually found in two different forms. It can either appear as soluble or membrane bound form. The crystal structure of the soluble COMT from rat liver is known.[31, 32] This enzyme was also crystallised in its apo and its holo form.[33]

The mechanistic studies showed that a Mg^{2+} ion has to be present in the active site to allow methyl transfer to the phenolic substrate.[34] The dependence of a metal ion is exceptional within the class of methyltransferases.

COMT offers a broad substrate acceptance for the methylation. The substrate has to provide one important recognition pattern: a vicinal dihydroxy moiety has to be situated on an aromatic system. The hydroxyl groups are placed at the 3- and 4-position of an arene in the case of a third substituent.[35] Nearly exclusively the hydroxyl group in *meta* position is methylated.[36] Only minimal side product formation with *para* methylation is observed.

From *Myxococcus xanthus* a specialised catechol-4-*O*-MTase for *para* methylation was isolated which uses L-dopa as natural substrate.[37]

5.3.2 *N*-MTases

The class of *N*-MTases contains a large variety of enzymes. Many DNA and RNA MTases belong to the class of *N*-MTases and methylate the nucleobase at exocyclic or endocyclic nitrogen.[38]
Also many MTases that accept proteins as a substrate transfer a methyl group to a nitrogen, for example the two protein *N*-MTases already mentioned above.

5.3.3 *C*-MTases

The number of *C*-MTases is smaller than *O*-MTases and *N*-MTases. Either aliphatic or aromatic substrates are methylated. This class of MTases is of special synthetic interest because a carbon-carbon bond is formed. The regioselective *C*-methylation provides a powerful tool in the synthesis of complex compounds.
Members of the aliphatic *C*-MTases are for example SAM dependent steroid 24*C*-MTase or methyltransferases that accept carbohydrates as substrates.
A well known member of the aromatic *C*-MTases is the cytosine-*C*5-MTase M.Hha I that methylates the pyrimidine base cytosine at the position 5. The mechanism of this enzyme has been clarified.[39]
The *C*-MTases involved in α-tocopherol biosynthesis are two similar enzymes that methylate aromatic carbon at different positions.[40, 41] These enzymes will be described in more detail in chapter 6.5.

5.3.4 *S*-MTases

The *S*-MTases methylate a thiol under formation of a thioether or methylate a thioether under formation of a sulfonium cation.[42, 43] One group of *S*-MTases has to be highlighted. The homocysteine MTases catalyse the formation of methionine from homocysteine. Whereas the great majority of MTases uses SAM as a cofactor, within the homocysteine MTases a variety of methyl donors act as cofactors. There exist homocysteine MTases that apply betaine, methyl-L-methionine, thetin or N^5-methyl-5,6,7,8-tetrahydrofolate as a methyl source (depicted in Figure 7).

5.3.5 Other MTases

In nature the methylation of selenium or arsenic is made for detoxification.[44, 45] The non canonical amino acid selenocysteine is methylated at the selenol moiety under formation of a non-proteinogenic amino acid.[46]

5.4 Cofactors for MTases

All methyltransferases require a cofactor as methyl source. The most common cofactor is *S*-adenosyl-L-methionine (SAM), formally a conjugate of methionine and adenosine (Figure 6).

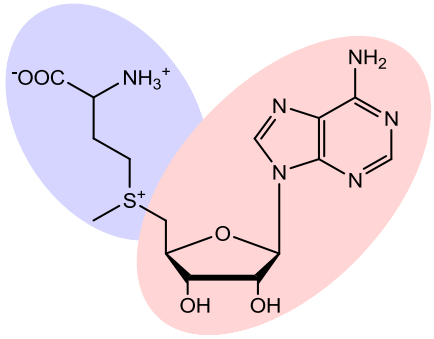


Figure 6. Structure of SAM. The methionine part of SAM is highlighted in light blue, the adenosine part is highlighted in light red.

Other naturally occuring cofactors for MTases are N^5-methyl-5,6,7,8-tetrahydrofolate, methyl cobalamin, betaine, dimethylthetin or *S*-methyl-L-methionine. All these cofactors are used in the methylation of homocysteine for the methionine synthesis. The cofactors are shown in Figure 7.

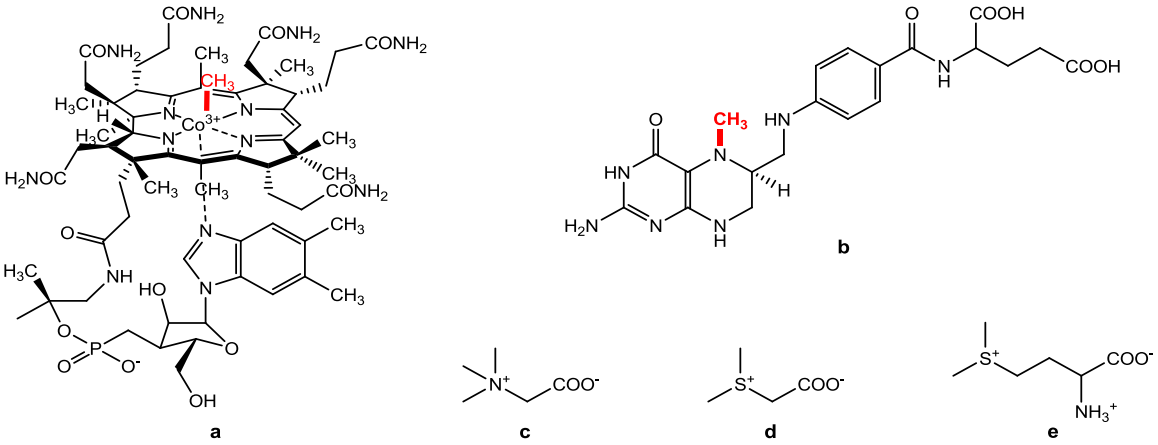


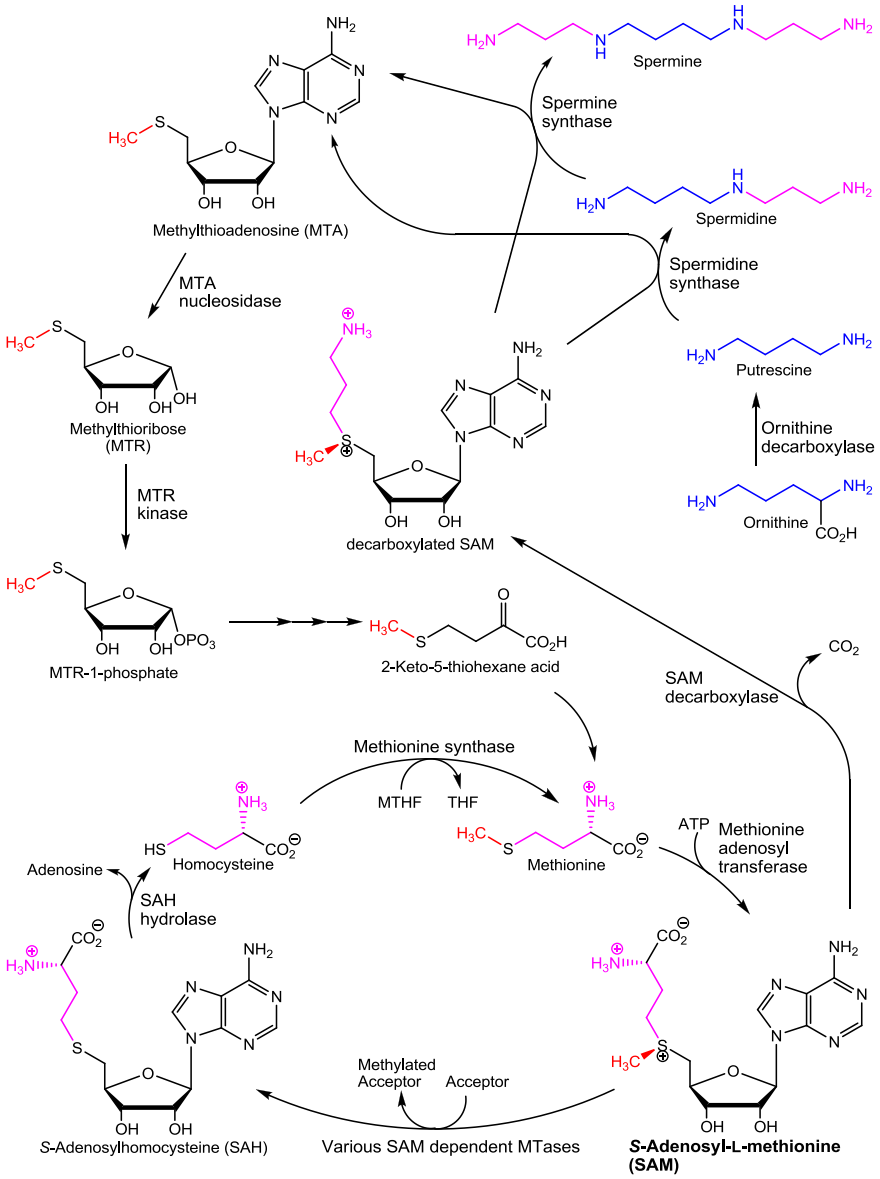
Figure 7. Structures of common cofactors of MTases. a methylcobalamin, b N^5-methyl-5,6,7,8-tetrahydrofolate, c betaine, d dimethylthetin, e *S*-methyl-L-methionine

5.5 *S*-Adenosyl-L-methionine

The cofactor *S*-Adenosyl-L-methionine (SAM) was first discovered in the year 1953 by Giulio L. Cantoni.[47] He called the species that is active in methyltransfer activated methionine. As he already

found out both methionine and ATP are necessary for the production of the active species. He proposed a structure of methionine condensed to the 5´-*C* of the ribosyl moiety of adenosine. The structure was confirmed later.

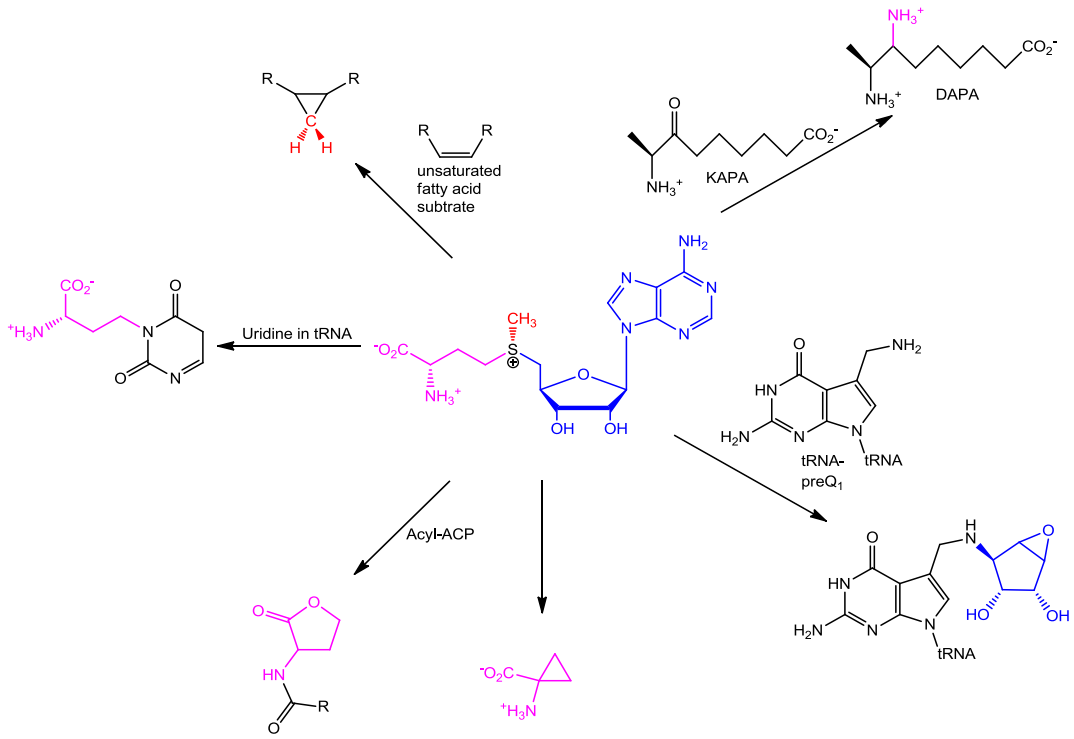
Although the most frequent methyl donor, SAM does not only act as cofactor for MTases, it is also involved in many other reactions and metabolic pathways. Nearly every part of SAM occurs somewhere in a metabolic pathway. The metabolism of SAM is depicted in Scheme 9.[48]



Scheme 9. Biosynthesis and metabolism of SAM.[48]

Besides its role as methyl donor, SAM is also involved in the polyamine pathway. After decarboxylation dcSAM is able to transfer an aminopropyl moiety.

SAM is also applied as substrate or cofactor in less common reactions. A selection of these reactions is depicted in Scheme 10.[49]



Scheme 10. Different reaction types use SAM as substrate or cofactor.[49]

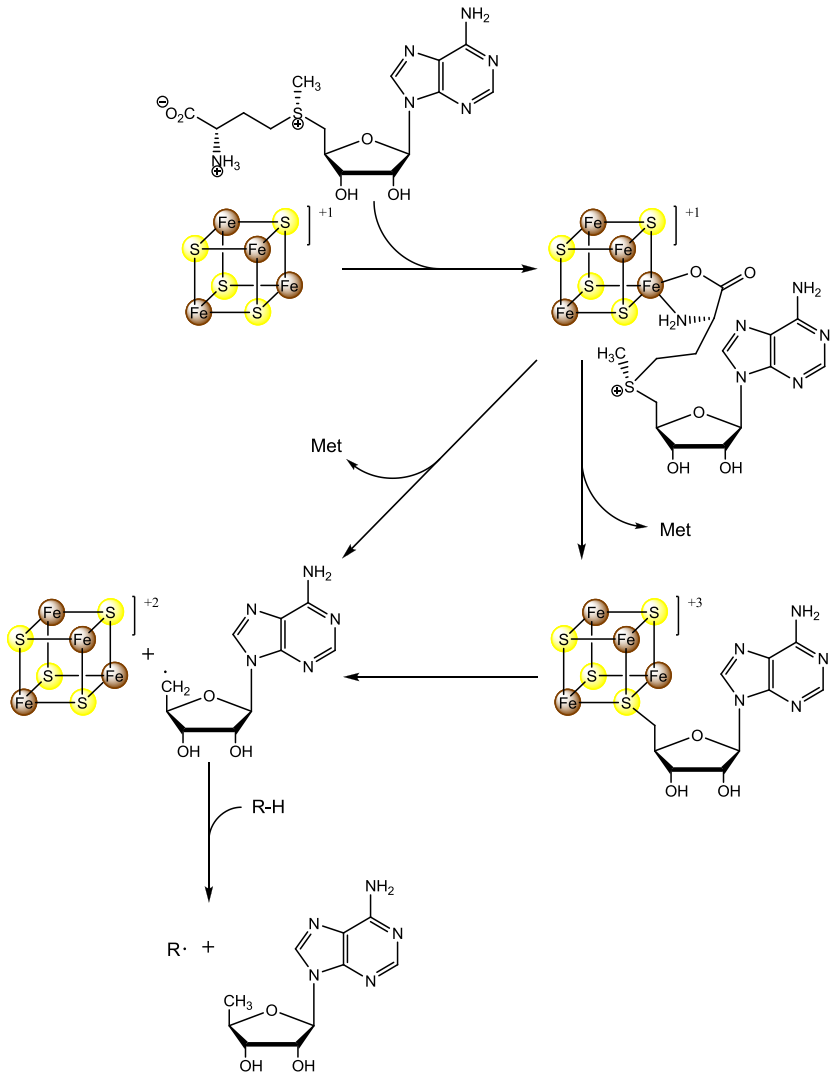
SAM is able to act as a methylene donor for cyclopropane fatty acid synthase.[50] It acts as an amino donor in a PLP dependent transamination reaction for the formation of 7,8-diaminopelargonic acid (DAPA) which is an intermediate in the biotin biosynthesis.[51] The ribosyl moiety of SAM is incorporated into the hypermodified tRNA nucleoside queuosine, a deazaguanosine. The enzyme that catalyses this reaction is the tRNA modifying enzyme *S*-adenosyl-L-methionine:tRNA ribosyl transferase isomerase (QueA).[52-54]

The amino acid chain of SAM is used for 2-aminobutyrate transfer to uridine in tRNA.[55] The unusual amino acid 1-aminocyclopropane-1-carboxylic acid is also formed from SAM. It is a preliminary stage to ethylene in plants.[56, 57] Homoserine lactone and *N*-acylhomoserine lactone are other metabolites derived from SAM. Homoserine lactone is formed upon the cleavage of SAM to 5´-methylthioadenosine and homoserine lactone.[58-61] *N*-acylhomoserine lactone is formed with acetylated acyl carrier protein (acyl-ACP). This compound plays an important role in quorum sensing signal transduction.[62-64]

Decarboxylation of SAM is the initial step for the participation of SAM in the polyamine pathway as an aminopropyl donor.[65-68]

SAM is also involved in enzymatic radical reactions. It acts as a source of 5´-deoxyadenosine radicals. The 5´-deoxyadenosine radical is able to abstract a hydrogen from a substrate and to generate a substrate radical in this way which reacts further.

A common motif in the SAM radical superfamily is CXXXCXXC sequence. Each of the cysteines coordinates to an iron of a $[Fe_4\text{-}S_4]^{+1}$ cube that is crucial for the SAM activation and radical formation.[69] The activation of SAM and radical formation is depicted in Scheme 11.



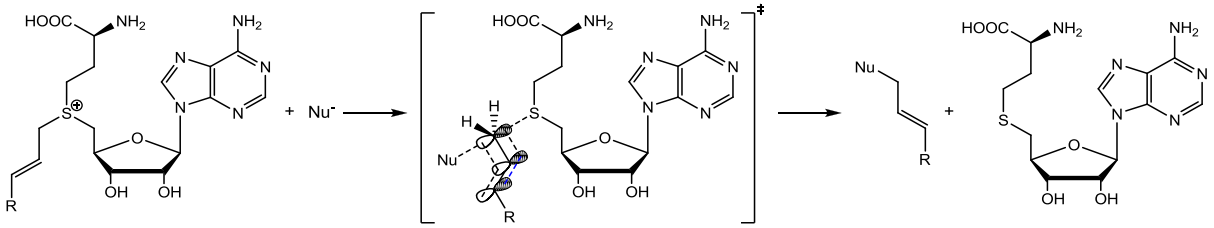
Scheme 11. Mechanism of the enzymes of the SAM radical superfamily.

Also methyltransferases are found within the class of SAM radical superfamily. For example pyrrol-2-carboxylic acid 5-MTase from *Streptomyces roseochromogenes*, which is involved in the formation of chlorobiocin, belongs to the BchE like MTase subgroup of the radical SAM superfamily.[70, 71] The similar enzyme CouN6 from *Streptomyces rishiriensis* catalyzing the equal reaction in coumermycin A1 biosynthesis is assumed to apply the same mechanism.[70, 72]

5.5.1 *S*-Adenosyl-L-methionine analogs

SAM can be modified at different positions. It can be assumed, that a change in the amino acid, the ribose or the adenine moiety affects the binding of the cofactor. Therefore cofactors with a modification at one of these sites are likely to be inactive or exhibit an inhibiting effect.
The introduction of a larger group than methyl does not influence the binding of the cofactor. Thus the variation of the methyl group is a potential modification site.
An exchange of the sulfur to another atom species influences the reactivity and stability. Cofactors with a selenium or tellurium instead of the sulfur gave higher activity, but the synthetic effort to produce these cofactors makes them unattractive compared to other modification strategies.[73]

Methyl transfer in SAM dependent MTases typically follows an S_N2 reaction mechanism concerning the methyl group of SAM. SAH acts as the leaving group in the mechanism. By replacing the methyl group with an ethyl group the reaction slows down due to steric hindrance and electronic effects. An n-propyl group is transferred at very low rates, longer and branched chains are not transferred at a detectable amount.[74]
Mechanistically S_N2 reactions proceed via a monomolecular sp^2 hybridised transition state. Stabilisation of the transition state increases the reaction rate. One way of stabilisation of the transition state is orbital overlap of the reacting p-orbital with a neighbouring π-orbital (Scheme 12). Therefore the reactivity can be restored to a large extent by the introduction of a multiple bond next to the reacting carbon, these cofactors were named double activated cofactors.[75]



Scheme 12. Reaction course and mode of stabilisation of the transition state of the alkyl transfer with SAM analogs.

The class of double activated cofactors was first explained as active cofactors for MTases in 2006. The proof that DNA MTases are able to transfer alkyl groups larger than methyl was an outstanding finding.[24, 76, 77] Sequence specific DNA labeling is possible with the synthetic cofactors and MTases as catalysts. The potential of a method for sequence specific introduction of functionalized alkenes or alkynes broadens the field of application for DNA and RNA MTases.[78] The application of the double activated cofactors is not limited to DNA and RNA MTases. Some very interesting examples with other MTases show the potential of the synthetic cofactors.[79, 80]

A SAM analog carrying an acetonyl side chain instead of the methyl group was shown to be able to transfer an acetonyl moiety to the nucleophilic substrate. The MTases applied were COMT and thiopurine S-MTase.[81] These experiments could show, that also a carbon-heteroatom double bond is able to activate the cofactor for alkyl transfer.

The double activated cofactors are not the only form of artificial cofactors for SAM dependent MTases. A nitrogen based variant of SAM imitating cofactors carries an aziridine moiety instead of the methionine moiety (Figure 8).[82] Vinyl-SAH is closely related to the double activated cofactors carrying a vinyl group instead of the methyl group (Figure 8). Nevertheless the behaviour and the product formed differ from the double activated cofactors.

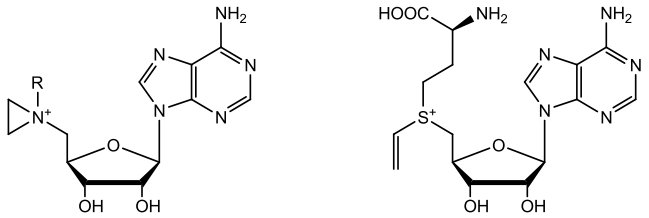


Figure 8. Structures of the synthetic aziridine cofactors (R = alkyl, H) and the vinyl-SAH derived cofactors. Both can be substituted at various positions.

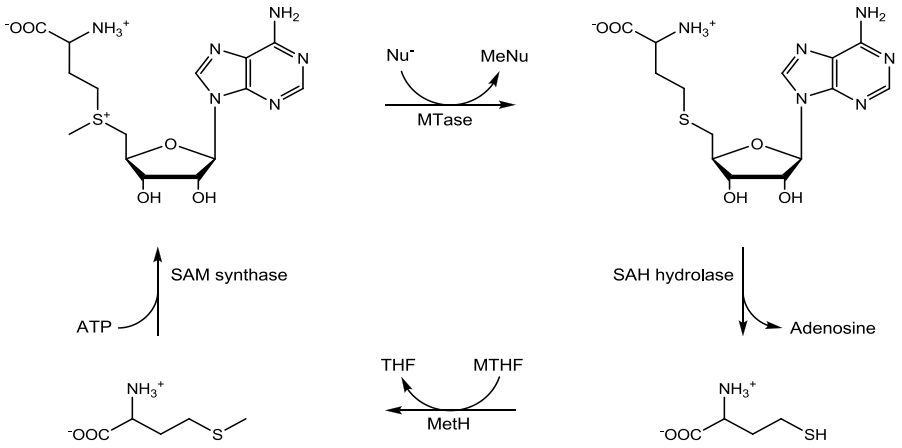
In case of the aziridine cofactors the nucleophile attacks at one of the aziridine ring carbons and the ammonium moiety acts as a leaving group by ring opening. Vinyl-SAH is attacked at the terminal carbon of the vinyl moiety. The negative charge that evolves from bond formation to the nucleophile is compensated by protonation.[83] The major difference between the double activated cofactors and the two other synthetic cofactors is that whereas the double activated cofactors are able to transfer a functionalised alkyl chain, the aziridine cofactors and vinyl-SAH are transferred to the substrate as a whole.[84] This diminishes the field of application in comparison to the double activated cofactors.

5.5.2 SAM recycling

The cofactor dependence of the aromatic *C*-MTases investigated in this work is a handicap for industrial applications. Like many other cofactors SAM is extremely expensive and up to now no practical regeneration system has been developed. There are examples in literature, where organisms are modified to increase the SAM level in the cell.[85-90] These organisms can be used for the production of SAM.

The establishment of a recycling system for SAM is complex. A fully enzymatic recycling system would involve several enzymes (Scheme 13) which would have to work under the same conditions. ATP would need to be recycled as well.

Chemical regeneration of SAM is possible via methylation of SAH, but the required reagents and conditions are not compatible with a suitable environment for biocatalysts.



Scheme 13. Biosynthesis of SAM and its metabolism (MetH methionine synthase, THF tetrahydrofolate, MTHF methyltetrahydrofolate).

5.6 Aldehydes as cofactors for SAM dependent MTases

According to literature some DNA MTases are able to accept formaldehyde and acetaldehyde as a cofactor instead of SAM.[91, 92]

The use of formaldehyde leads to hydroxymethylation, acetaldehyde gives hydroxyethylation at the natural methylation site of the DNA strand. In these experiments the formaldehyde was applied at a concentration of 800 mM. Recently the use of prolonged aldehydes and follow-up chemistry has been described too.[92, 93]

It can be assumed that the enzyme activates the substrate in the same mode as for methylation. But as there is no SAM present in the active site, as a substitute the aldehyde is attacked by the generated nucleophile.

5.7 Other cofactors for MTases

5.7.1 Cobalamin

Cobalamin (vitamin B_{12}) consists of a highly substituted corrin ring system which forms a complex with cobalt as central atom. The four equatorial coordination sites of Co^{II}/Co^{III} are occupied by the corrin nitrogens, the lower axial coordination site is occupied either by the intramolecular dimethylbenzimidazole ligand or a histidine of a protein. Only one axial coordination site is available for ligand binding. Common ligands are methyl, adenosyl, cyano or aqua groups.[94]

Cobalamin acts as a methyl shuttle in methyltransferase reactions. It is involved in the formation of methionine as well as in reactions of methanogenic and acetogenic organisms. Another role of cobalamin is in isomerase catalysed reactions. In methyltransferase reactions the Co-C bond is cleaved heterolytically. In contrast to that the Co-C bond is cleaved homolytically in isomerase reactions.[95]

In methylation reactions cobalamin is always accompanied by another compound which acts as methyl source. As an example cobalamin is able to conduct methyl uptake from methanol[96] or halomethane.[97, 98] In a second step the methyl group is transferred from methyl cobalamin to the substrate which is further metabolised.

(Methyl-)cobalamin is also involved in the Wood-Ljungdahl pathway for CO_2 fixation and formation of acetyl-CoA. In this metabolic pathway two molecules of carbon dioxide are used for the formation of one acetyl unit.[99]

5.7.2 *N*^{5}-methyl-5,6,7,8-tetrahydrofolate

N^5-methyl-5,6,7,8-tetrahydrofolate (MTHF) is the methyl source for the formation of methionine from homocysteine in the classical SAM metabolic cycle.

A special role of tetrahydrofolate is assumed in the methylation of uracil in tRNA in *Bacillus subtilis*.[100] Although the methylating agent could not be clarified, it is assumed to be MTHF.

Accompanied by cobalamin tetrahydrofolate is able to act as a methyl acceptor under formation of MTHF in *O*-demethylase reactions[101-103] and halomethane MTases.[97]

Typical substrates for demethylation are the lignin derived syringate or vanillate.[103] *O*-demethylases are able to accept a great variety of aromatic methyl ethers as substrates.[104]

5.7.3 Betaine

Betaine on one hand describes a substance class where both a cationic functional group as well as an anionic group is situated in one molecule. At the positively charged center no hydrogen is bound. The positively charged center usually is a quarternary ammonium group, the negative center a carboxylate.

On the other hand betaine stands for *N,N,N*-trimethylglycine (Figure 7). This is also the natural substrate for the enzyme betaine:homocysteine MTase (EC [2.1.1.5]).

Investigations on the coenzyme promiscuity of betaine:homocysteine MTase purified from pig liver showed that this enzyme is able to accept a variety of ammonium and sulfonium compounds as methyl donors. Surprisingly sulfonium based compounds showed much higher activity towards methyl transfer compared to betaine. Trimethylsulfonium chloride exhibited about 5-fold activity towards methyl transfer. Dimethylthetin, dimethylpropiothetin (*S,S*-dimethylsulfoniopropanoate) and S-methyl methionine showed even higher methyl transfer activity with about 13-fold, 8.5-fold and 12-fold activity respectively.[105]

5.7.4 Thetin

Thetin is the name of a chemical motif and is a special case of a betaine: Thetin describes a substance that contains an alkylated sulfonium moiety as well as a carboxylate. So it is not a single compound, but a number of similar compounds sharing both moieties. The simplest thetin is dimethylacetothetin (*S,S*-dimethylsulfonioacetate) shown in Figure 7.

Dimethylthetin is meant to be the natural substrate of the enzyme thetin:homocysteine MTase (EC[2.1.1.3]).[106, 107] A study on the distribution of thetin:homocysteine MTase in different species led to the assumption that this enzyme is only found in vertebrates, situated mainly in the liver.[108]

Trimethylsulfonium chloride, dimethylpropiothetin (dimethylsulfoniopropionate) and methyl methionine also serve as a methyl source for this enzyme, but at lower rates.[109]

Dimethylpropiothetin acts as a methyl donor for a special methyltransferase that uses tetrahydrofolate as a substrate. The enzyme is found in the marine sulfate-reducing bacterium strain WN. It was shown, that this enzyme accepts dimethylpropiothetin and the longer thetins dimethylbutyrothetin and dimethylpentanothetin, but does not accept dimethylacetothetin.[110]

Experiments on the influence of betaines and thetins on the homocysteine metabolism in rats showed that dimethylthetin and dimethylpropiothetin also act as methyl source for homocysteine methylation in vivo.[111]

5.7.5 *S*-Methyl-L-methionine

S-Methyl-L-methionine (MeMet), also called vitamin U, is mainly found in plants and acts as a storage form of methionine. MeMet plays an important role in the phloem sulfur transport. It is formed by methylation of methionine catalysed by the SAM dependent enzyme SAM:methionine MTase (EC[2.1.1.12]).[112] The methylation of homocysteine is catalyted by methylmethionine:homocysteine MTase, formerly considered to be a betaine:homocysteine MTase.[113] Also some other enzymes use methyl methionine as their natural methyl source, for instance a group of thiol/selenol MTases.[114]

Methyl methionine was also investigated towards its medical applicability. A variety of medically interesting activities were observed. A preventive and curing effect of MeMet is described for gastric, peptic and duodenal ulcer.[115, 115-121] Cosmetics containing MeMet promote the decomposition of neutral fat accumulations and body fat and show slimming effect. The cosmetics alleviate cellulite and give a smooth, elastic skin.[122] Also an accelerating effect on wound healing is reported.[123, 124]

5.7.6 Trimethylsulfonium

In literature an enzyme from *Pseudomonas* sp. is described that catalyses the transfer of a methyl group from a trimethylsulfonium (TMS) salt to tetrahydrofolate under formation of MTHF and release of dimethyl sulfide.[125] Experiments showed that neither SAM nor dimethylpropiothetin were able to replace TMS as a methyl donor. Mercaptoethanol did not act as methyl acceptor in the reaction catalysed by this enzyme.[126]

6 Aromatic *C*-methyltransferases

6.1 Aminocoumarin MTases

The aminocoumarin motif 3-amino-4,7-dihydroxycoumarin appears in a small group of antibiotics. Coumermycin, novobiocin and chlorobiocin are prominent members of the class of aminocoumarin antibiotics.

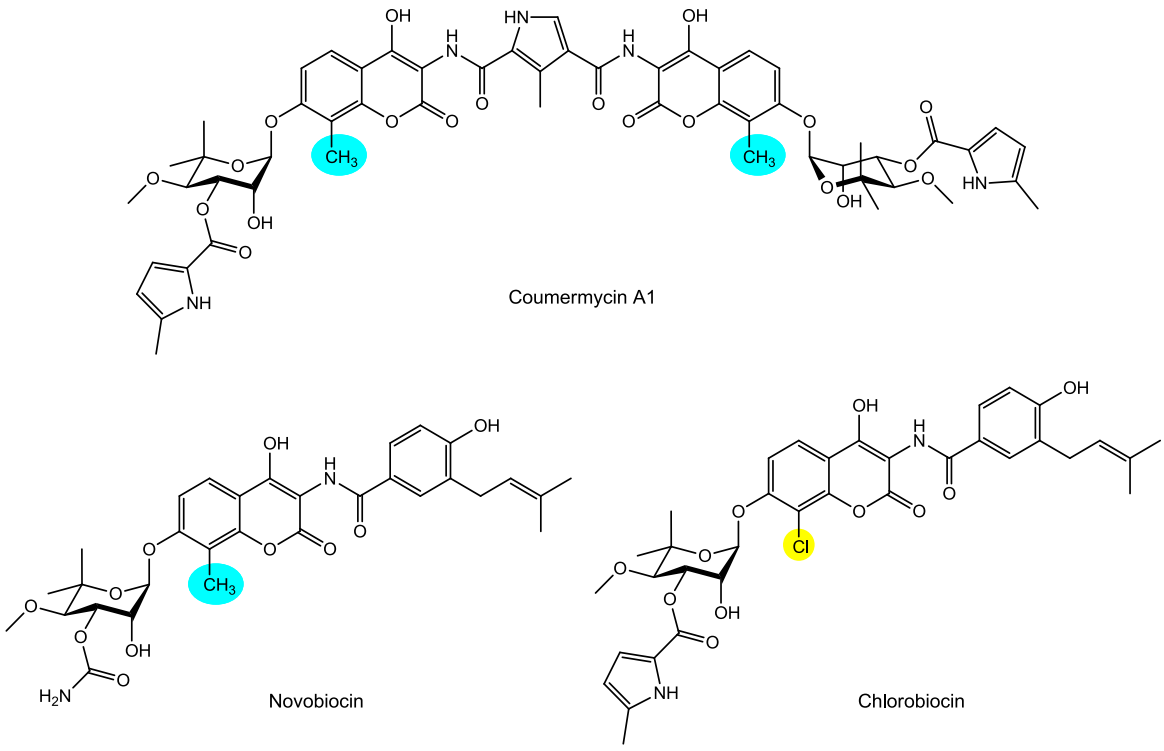


Figure 9. Structures of the antibiotics coumermycin A1, novobiocin and chlorobiocin. Chlorobiocin contains a chlorine atom instead of a methyl group as it is found in coumermycin and novobiocin.

Coumermycin and novobiocin contain a methyl group at the C8-position of the aminocoumarin moiety (shown in turquoise in Figure 9). In chlorobiocin a chlorine is bound to that position instead of the methyl group (shown in yellow in Figure 9).

Coumermycin A1 was first isolated from *Streptomyces rishiriensis* (DSMZ 40489) in 1965 by Kawaguchi *et al*. who also characterized this compound and elucidated the structure.[127, 128] Structurally coumermycin contains two aminocoumarin moieties linked with a 3-methylpyrrol-2,4-dicarboxylic acid by amide formation.

Recently coumermycin was also detected in organisms belonging to the genus *Actinoallomurus*.[129]

The biosynthetic gene cluster for coumermycin A1 production in *Streptomyces rishiriensis* was published in the year 2000.[130]

Novobiocin was first isolated from different strains and with different names nearly simultaneously. Named streptonividin (also albamycin as trading name by Upjohn company) it was isolated from *Streptomyces niveus* (DSMZ 40088) in 1955.[131-136] It was also isolated from *Streptomyces spheroides* (DSMZ 40292) by a second group in the same year and was named cathomycin.[137] A third group reported about the isolation of an antibiotic from *Streptomyces niveus* which they called cardelmycin and found that this compound was identical with streptonividin and cathomycin.[138] As a consequence of the different names the compound was renamed to novobiocin.

The gene cluster for novobiocin production in *Streptomyces spheroides* was published in the year 2000.[139] In 2008 *Streptomyces spheroides* was reclassified as a later synonym of *Streptomyces niveus*.[140]

Two novel novobiocin derivatives, both lacking a methyl group, were isolated from the strain *Streptomyces* TP-A0556. One misses the 8-methyl group on the coumarin, the other one lacks the axial 5-methyl group on the noviose sugar moiety (Figure 10).[141]

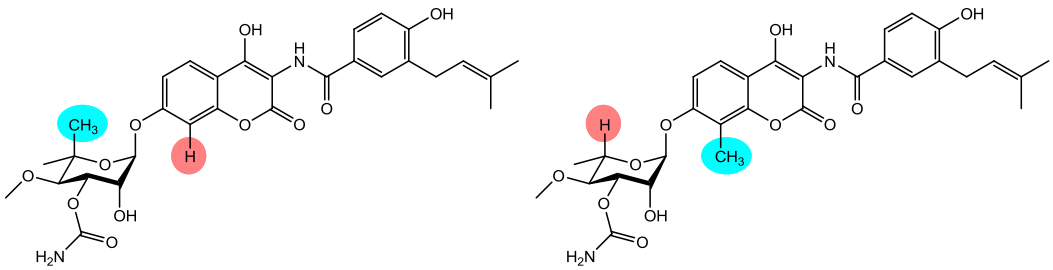


Figure 10. Structures of the two naturally occurring novobiocin derivatives with missing methyl groups. The positions where the methyl groups are absent are highlighted in red.

New aminocoumarin antibiotics were isolated from the strain *Streptomyces* sp. L4-4. The coumabiocins share high structural similarity to novobiocin. The aminocoumarin moiety and the noviose sugar moiety are identical with novobiocin except in coumabiocin F which lacks the noviose moiety. The variation is found in the 4-hydroxy-3-(3-methylbut-2-enyl)benzoyl side chain of novobiovin (Figure 11).[142]

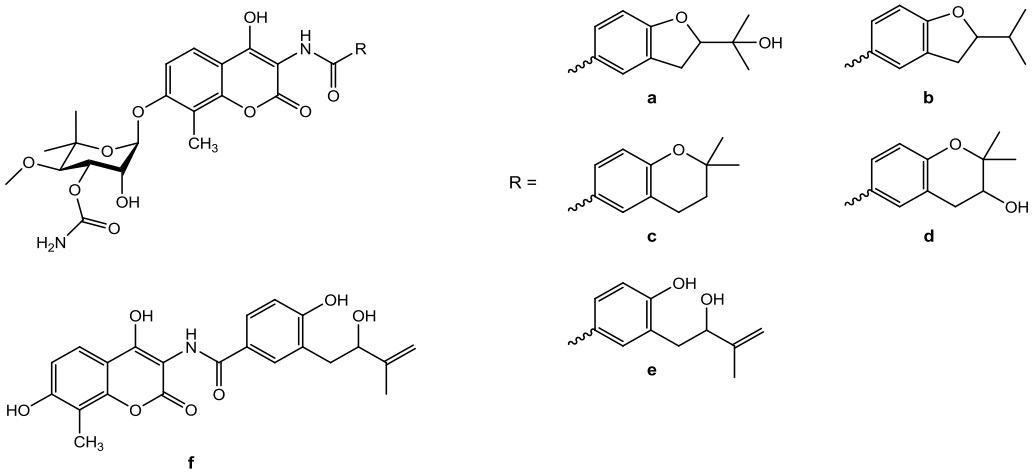


Figure 11. Structures of coumabiocin A-F isolated from *Streptomyces* sp. L4-4. a Coumabiocin A, b Coumabiocin B, c Coumabiocin C, d Coumabiocin D, e Coumabiocin E, f Coumabiocin F.

Chlorobiocin was discovered in *Streptomyces hygroscopicus* (DSMZ 41524) in 1969.[143] Later it was also isolated from *Streptomyces roseochromogenes* var *oscitans* (NCBI 149682) and *Streptomyces albocinerescens* (DSMZ 40794). It was found that the antibiotic contains a chlorine atom.[144] The structure with the exact position of the chlorine was clarified shortly after.[145] The synthetic gene cluster for clorobiocin from *Streptomyces roseochromogenes* was published in 2002, a high similarity with the novobiocin and coumermycin A1 synthetic gene cluster was observed.

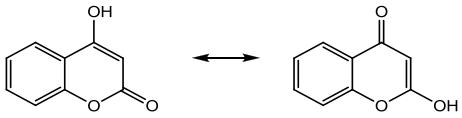
The aminocoumarin antibiotics address DNA gyrase as target.[146] DNA gyrase is a bacterial type II topoisomerase. Topoisomerases are responsible for the topology of DNA with the three main types supercoiling, knotting or catenation. DNA gyrases possess the potential to introduce negative supercoils into DNA using the energy of ATP hydrolysis. DNA gyrase is comprised of two proteins, GyrA and GyrB. The active enzyme is a A_2B_2 complex. The aminocoumarin antibiotics are supposed to interrupt the ATP hydrolase reaction by competitive binding in the ATP binding site which is situated in the *N*-terminal region of GyrB. In this manner they inhibit DNA supercoiling catalysed by DNA gyrase. Both the noviose moiety and the aminocoumarin moiety are necessary for the antibiotic activity. It was observed that one molecule of coumermycin is able to bind and inhibit two molecules of DNA gyrase B because of its dimeric character.[147]

The methyl group in position 8 of the aminocoumarin moiety is found in nearly all aminocoumarin antibiotics except chlorobiocin that contains a chlorine atom instead. The methyl group is introduced into the compound by a SAM dependent MTase. The MTases CouO from *Streptomyces rishiriensis* and NovO from *Streptomyces spheroides* are described in literature.[72, 148]

The methylation of the aminocoumarin moiety follows amide formation and is previous to the connection of noviose sugar moiety to the 7-hydroxyl group. In case of coumermycin biosynthesis

the enzyme is able to introduce the methyl groups into the monomeric and dimeric coumarin amide.[148]

4-Hydroxycoumarins can appear in two tautomeric forms (Scheme 14)



Scheme 14. The two tautomeric forms of 4-hydroxycoumarins: the 4-hydroxy-2-oxo form and the 2-hydroxy-4-oxo form.

The thermodynamically more stable form can either be the 4-hydroxy-2-oxo form (4-hydroxycoumarin derivatives) or the 2-hydroxy-4-oxo form (2-hydroxychrom-4-one derivatives). The predominating tautomeric form is determined by the substitution pattern on the ring system. The two tautomers differ in the absorbance behaviour in IR spectroscopy. This can be used to distinguish between the two tautomers.

According to IR analysis a hydroxyl group at position 5 or 7 causes a preference of the 2-hydroxychromone form (Figure 12). Also the analogous 3-phenyl derivatives appear as the 2-hydroxyisoflavones.

The 7-hydroxy group, also 7-methoxy group, stabilises the chromone form via electron delocalisation. Also a 5-hydroxy group stabilises the chromone form by hydrogen bonding. In contrast a 5-methoxy group as well as a 3-methoxy group favour the coumarin form.[149]

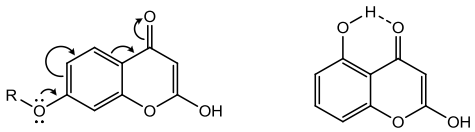


Figure 12. Mode of stabilisation of the chromone form by a 7-hydroxy and 5-hydroxy group respectively.

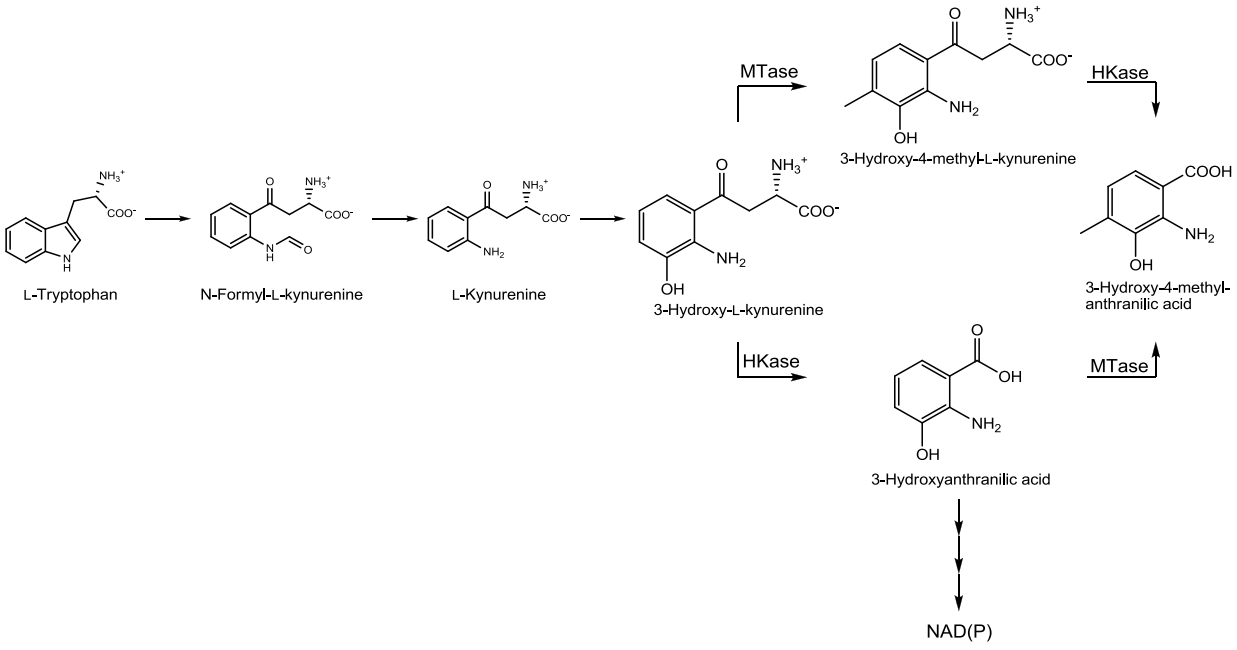
The biosynthesis of the 5-methylpyrrole-2-carboxylic acid motif that is condensed to the noviose moiety of coumermycin and chlorobiocin also employs a MTase. In principle also this MTase would fit into the focus of our project. But the enzyme differs mechanistically from the majority of the aromatic *C*-MTases. In contrast to most other aromatic *C*-MTases there is no hydroxyl group adjacent to the methylation site. In literature a radical mechanism is stated for this MTase.[71]

6.2 3-Hydroxyanthranilate 4-MTase

3-Hydroxy-4-methylanthranilic acid (MeHAA) is an intermediate that occurs in the biosynthesis of diverse antibiotics. Two different pathways for the formation of MeHAA were suggested.
Perlman et al. proposed a methylation of 3-hydroxykynurenine (HKyn) under formation of 3-hydroxy-4-methylhydroxykynurenine (MeHKyn). After the methylation a hydroxykynureninase hydrolytically cleaves off alanine under release of MeHAA.[150]

In contrast to that, an enzyme from *Streptomyces antibioticus* (DSMZ 41481) was mentioned to be able to transfer a methyl group from SAM to 3-hydroxyanthranilic acid (HAA) to position *C*4.[151, 152] A pathway was proposed with cleavage of HKyn under formation of HAA followed by SAM dependent methylation. Experiments with radioactively labeled SAM led to incorporation of the labeled methyl group into MeHAA what was interpreted as an indication for the methylation of HAA.

Both pathways are depicted in Scheme 15.



Scheme 15. Metabolic pathway from tryptophan to 3-hydroxykynurenine (small scale) and the two proposed pathways to 3-hydroxy-4-methylanthranilic acid. Both proposed pathways contain a hydroxykynureninase (HKase) and a MTase in altering order.

As shown above HKyn and HAA occur in the natural metabolic pathway from tryptophan to NAD(P).[153] The enzyme hydroxykynureninase is part of the NAD(P) biosynthesis and hence is a very frequent enzyme. In contrast the MTase is involved in the biosynthesis of secondary metabolites and

is only found in some specific organisms. The intermediate MeHAA does not occur in classical metabolic pathways.

Recently new results were published that favour the proposed pathway of Perlman *et al*. It was shown that HKyn acts as a substrate for the MTases AcmI and AcmL from *Streptomyces chrysomallus* (DSMZ 40128). It also could be demonstrated that these enzymes offer a small substrate promiscuity and are able to methylate L-tyrosine and L-dopa.[154]
It is still not clear if all organisms follow the same synthetic route to MeHAA and use a 3-hydroxykynurenine MTase followed by a hydroxykynureninase or the other proposed pathway is applied as well.

The intermediate MeHAA is part of biosynthetic pathways for the formation of different antibiotics. MeHAA forms the chromophoric part of the actinomycin antibiotics upon oxidative dimerisation (Figure 13).

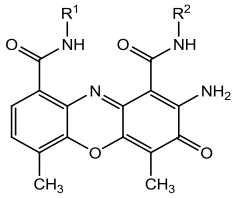


Figure 13. Structure of the chromophoric part of actinomycins formed from MeHAA. R^1 and R^2 are cyclic pentapeptides.

The chromophore is linked to a cyclic pentapeptide on each acid moiety. The peptide is connected through the amide bond of a threonine. D-Valine is part of the peptide ring and also other non canonical amino acids like sarcosine or 4-chlorothreonine can occur in the peptide ring within the different actinomycins.[155]

MeHAA is also integrated in the ring system of some benzodiazepine antibiotics. The characteristic substitution pattern containing the methyl group occurs in the antibiotics anthramycin and sibiromycin (Figure 14).

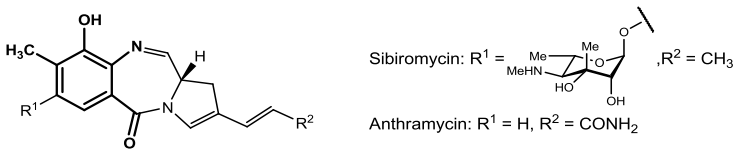


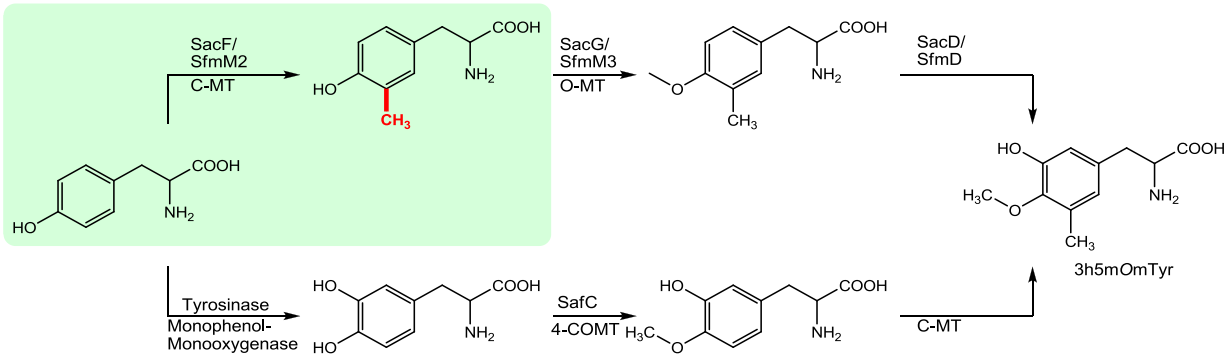
Figure 14. Structure of the antibiotics sibiromycin and anthramycin. The contribution of MeHAA to the benzodiazepine is highlighted.

With the publication of the gene sequences of the anthramycin biosynthetic gene cluster from the organism *Streptomyces refuineus* (NCBI 223296), for the first time the gene sequence of a proposed 3-hydroxyanthranilate 4-MTase was available.[156] In 2009 the sibiromycin biosynthetic gene cluster from *Streptosporangium sibiricum* (DSMZ 44093) was published.[157] The actinomycin biosynthetic gene cluster from *Streptomyces chrysomallus* was published in 2010.[158]

6.3 Tyrosine 3´-MTase

Like most other aromatic *C*-MTases this enzyme class appears in the biosynthesis of antibiotics. The biosynthesis of saframycin and safracin involves a methylation step where a methyl group is introduced to the position *C*3´ of tyrosine (2-amino-3-(4-hydroxyphenyl)propanoic acid). Two different pathways from tyrosine to the intermediate 2-amino-3-(3-hydroxy-4-methoxy-5-methylphenyl)propanoic acid (3h5m*O*mTyr) in the saframycin and safracin biosynthesis are stated in literature.

One biosynthetic pathway starts with *C*3´-methylation step followed by *O*-methylation of the hydroxyl group and introduction of a hydroxyl group to position 5´ to form the intermediate.[159] The other biosynthetic route uses the same reactions but in the reverse order. Both routes are shown in Scheme 16.



Scheme 16. Two pathways leading to the same intermediate. The biosynthesis of the intermediate 2-amino-3-(3-hydroxy-4-methoxy-5-methylphenyl)propanoic acid (3h5m*O*mTyr) via two contrary routes. The reaction which was investigated in this work is highlighted in light green

The upper route is encountered in the organisms *Streptomyces lavendulae* 314 that produces the antibiotic saframycin and in *Pseudomonas fluorescens* A2-2 that produces safracin.

The lower route is part of the saframycin MX1 biosynthesis in *Myxococcus Xanthus*.[37] A very interesting enzyme occurs in this pathway: The *para* hydroxyl group is methylated by a SAM dependent catechol-4-*O*-MTase. The last step in this route is supposed to be an aromatic *C*-methylation. In difference to *C*-MTases investigated in this work the substrate for this *C*-MTase does

not offer a hydroxyl group adjacent to the methylation site. It can be assumed that the proposed MTase uses an unconventional mode of activation or the reaction does not follow an S_N2 reaction mechanism.

Saframycins A, B, C, D and E were first isolated from *Streptomyces lavendulae*.[160] The structure of the saframycins B and C were elucidated two years later.[161]

Safracin A and B were first isolated from *Pseudomonas fluorescens* and characterised regarding structure and bioactivity in 1983.[162-164]

The structures of saframycin A and safracin B are depicted in Figure 15.

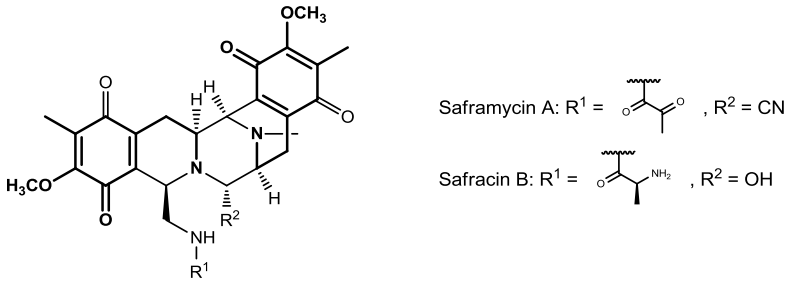


Figure 15. structure of Saframycin A and Safracin B. The parts that originate from 3-hydroxy-5-methyl-*O*-methyltyrosine are marked bold.

Safracin and saframycin are members of the tetrahydroisoquinoline antibiotics. The two tetrahydroisoquinoline core structures, the (hydro-)quinone and the aromatic core, are shown in Figure 16.

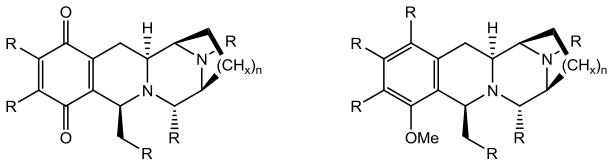


Figure 16. General structure of the tetrahydroquinoline class of antibiotics.

The motif that derives from 3h5m*O*mTyr is also found in other substances of that class. It is part of renieramycins isolated from sponges *Reniera* sp., *Xestospongia caycedoi*, *Haliclona cribricutis* or *Cribrochalia* sp., jorumycin isolated from *Jorunna funebris*, ecteinascidins from *Ecteinascidia turbinata*, naphthyridinomycin from *Streptomyces lusitanus*, cyanocycline from *Streptomyces flavogriseus*, bioxalomycins from *Streptomyces viridostaticus* ssp. *litoralis* and lemonocin from *Streptomyces candidus*.[165] It can be assumed that some of the organisms that produce one of the above secondary metabolites possess a tyrosine 3´-MTase.

Saframycin and safracin are potent antitumor drugs.[166] Synthetic, semisynthetic and biosynthetic methods were introduced for effective derivatisation of the different antibiotics of that class.[167, 168]

6.4 Ubiquinone MTase

Ubiquinone or coenzyme Q (Figure 17) is part of the respiratory system in almost all living cells including mammals. It is used for electron and proton transfer, the primary role is the electron transfer from NADH-quinone oxidoreductase (complex I) or succinate dehydrogenase (complex II) to quinone-cytochrome *c* oxidoreductase (complex III), all part of the electron transport chain. It also serves as lipid soluble antioxidant. Different derivatives are found in nature with variations in the length of the side chain. [169]

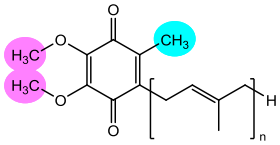
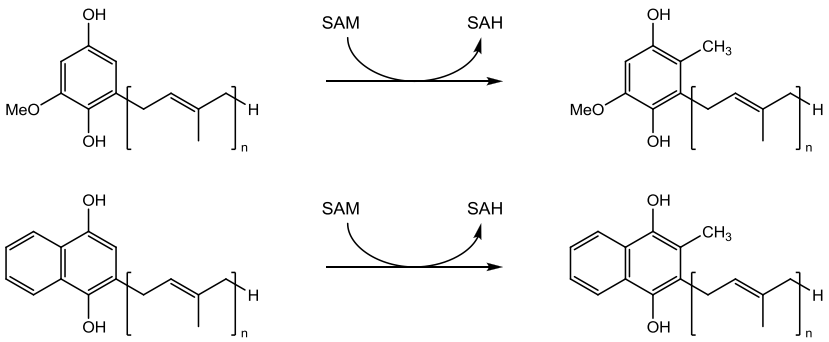


Figure 17. Structure of ubiquinone (n = 6-10) which employs two SAM dependent MTases for biosynthesis. The two methyl groups shown in pink are introduced by the *O*-MTase UbiG / Coq3, the methyl group shown in turquoise is introduced by the *C*-MTase UbiE / Coq5.

Prokaryotic cells and eukaryotic cells follow slightly different biosynthetic pathways, but both biosynthetic strategies employ the *O*-MTase UbiG / Coq3 for two methylation reactions[170] and the *C*-MTase UbiE / Coq5 respectively. The *ubi* genes were sequenced from *E. coli* and are representative for the prokaryotic pathway, the *coq* genes, sequenced from *Saccharomyces cervisiae*, represent the eukaryotic pathway.[169]

The reaction catalysed by the enzyme UbiE (and Coq5 respectively) is the methylation of 2-methoxy-6-polyprenylbenzene-1,4-diol under formation of 3-methoxy-2-methyl-5-polyprenylbenzene-1,4-diol and the methylation of 2-polyprenylnaphthalene-1,4-diol forming 2-polyprenyl-3-methylnaphthalene-1,4-diol (Scheme 17).



Scheme 17. Natural reactions catalysed by the enzyme UbiE from *E. coli*.

This reaction is not only part of the biosynthetic pathway of ubiquinone but also of menaquinone (vitamin K_2). Interestingly the *C*-methylation is the only reaction the two pathways have in common starting from chorismate.[171]

The *O*-MTase involved in ubiquinone biosynthesis uses substrates with two adjacent hydroxyl groups like it is the case for COMT.

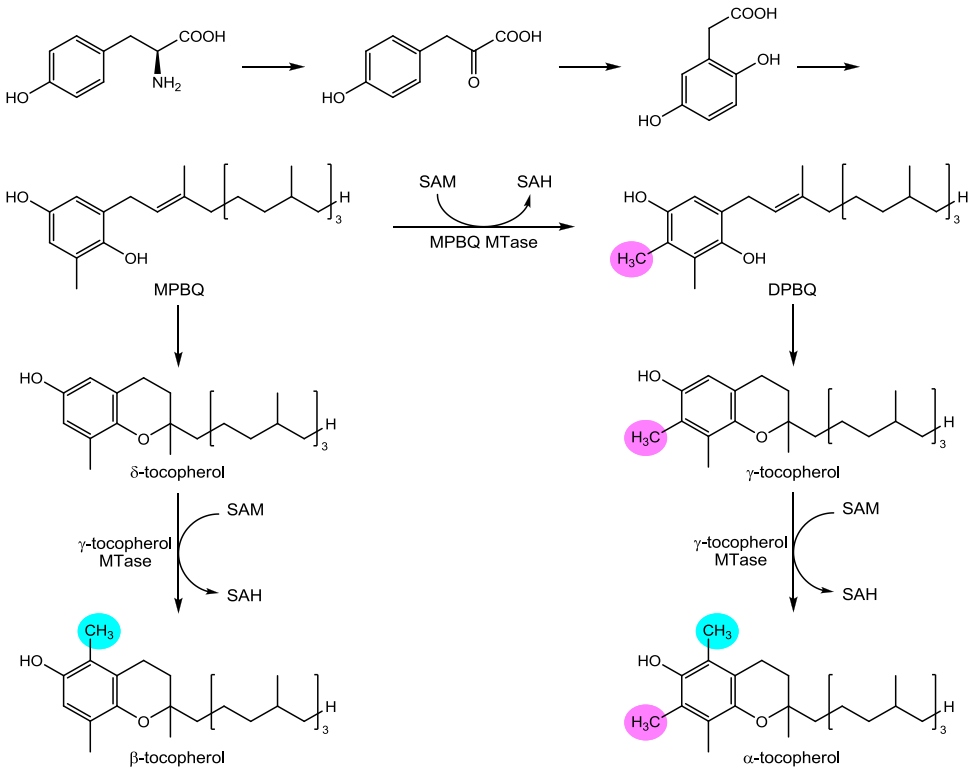
The *C*-MTase introduces the methyl group into position *C*3 next to the hydroxyl group. As in the other aromatic *C*-MTase it can be assumed that the hydroxyl group is involved in the activation of the substrate for the nucleophilic attack at the methyl group of SAM.

6.5 Tocopherol *C*-MTases

Tocopherols are lipid soluble antioxidants which are exclusively found in photosynthetic organisms. Tocopherols and closely related substances with variations in the phytyl side chain are collectively known as vitamin E.[172] For mammals the dietary uptake is essential.

The biosynthesis of tocopherols starts from tyrosine which forms 4-hydroxyphenylpyruvate. This intermediate is converted to homogentisate by a single enzyme that catalyses decarboxylation, dioxygenation and rearrangement of the pyruvate side chain.[173]

Two SAM dependent C-MTases are employed for the biosynthesis of α-tocopherol. One methyltransferase catalyses the methylation of 6-methyl-2-phytyl-1,4-benzoquinol (MPBQ) under formation of 2,3-dimethyl-5-phytyl-1,4-benzoquinol (DPBQ). The second MTase catalyses the methylation of γ-tocopherol to form α-tocopherol and is also able to methylate δ-tocopherol under formation of β-tocopherol (Scheme 18[73]).[174]



Scheme 18. Biosynthetic pathway to the four tocopherols α, β, γ and δ. The methyl group introduced by MPBQ MTase is shown in pink, the methyl group introduced by γ-tocopherol MTase is shown in turquoise.

The existence of SAM dependent γ-tocopherol MTase as the last step in the α-tocopherol biosynthesis was discovered in the early 1980s.[40] The enzyme from *Arabisopsis thaliana* was cloned and overexpressed in *E. coli*. It was characterised in detail together with purified γ-tocopherol MTase from *Capsicum annuum*.[172] The MPBQ MTase was identified and characterised later.[41]

The activities of the two MTases and the enzyme tocopherol cyclase relative to each other determine the content of the different tocopherols α, β, γ and δ in cells.

Both methyl groups are introduced in a position next to a hydroxyl group. It can be assumed that in both cases the hydroxyl group is required for activation of the substrate.

6.6 Mithramycin C-MTase

The antibiotic mithramycin (Figure 18) was isolated from *Streptomyces argillaceus*, *Streptomyces plicatus*, *Streptomyces tanashiensis* and *Streptomyces atroolivaceus*. It was first described in the 1950s and was also known as aureolic acid, plicamycin, LA-7017 and PA-144.[175-177] The different names originated because the compound was isolated by different groups.[178]

The aromatic polyketide drug mithramycin possesses antitumor and other pharmaceutically interesting activities. It contains a tricyclic core that is formed from a polyketide chain via tetracyclic intermediates. One ring is opened oxidatively by a Baeyer-Villiger monooxygenase.[179]

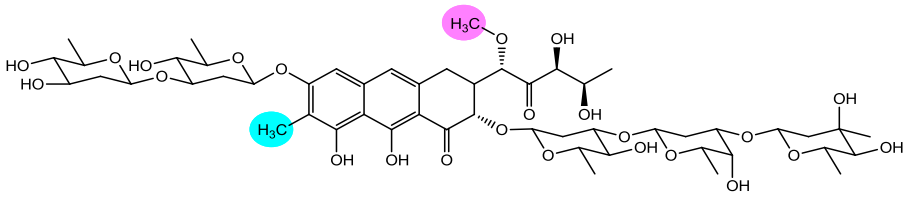


Figure 18. Structure of mithramycin. The methyl group introduced by the MTase MtmMI is highlighted in pink, the aromatic methyl group introduced by the MTase MtmMII is highlighted in turquoise.

Two methyltransferases are employed in the biosynthesis of the tricyclic polyketide core of mithramycin. In the gene cluster for mithramycin biosynthesis two gene sequences were found, MtmMI and MtmMII, which encode SAM dependent MTases. Their role in the biosynthetic pathway was investigated by insertional inactivation of the genes. The knock-out mutants (MtmMI minus mutant and MtmMII minus mutant) were generated and the accumulating mithramycin biosynthetic intermediates were determined.[180]

In case of the MtmMI minus mutant 4-demethylpremithramycinone (4DPMC) was isolated, a compound lacking the methyl groups at positions 4-*O* and 9-*C*. It was deduced that this MTase is responsible for the methylation of the 4-hyxoxyl group in 4DPMC.
The MtmMII minus mutant produced the compounds premithramycin A1 (PMA1), 9-demethylpremithramycin A3 (DPMA3,Figure 19) and 7-demethylmithramycin, which all contain the *O*-methyl group at position 4 and 1´ respectively, but lack the methyl group at the aromatic carbon.[180]

The two enzymes MtmMI and MtmMII were expressed in *Streptomyces lividans*. The extracts of the cultivated strains were used for the determination of the natural substrates. It was confirmed that the natural substrate for the *O*-MTase MtmMI is 4DPMC (Figure 19). In case of MtmMII it was determined that the natural substrate is DPMA3 (Figure 19).[180]

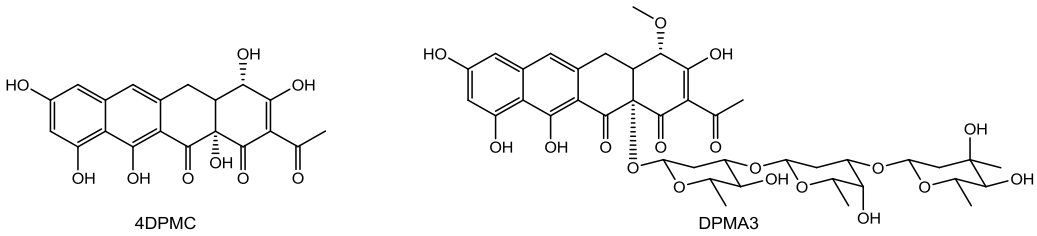


Figure 19. Structures of the natural substrates for the two MTases involved in mithramycin biosynthesis. 4DPMC acts as a substrate for the *O*-MTase MtmMI, DPMA3 is the natural substrate for the aromatic C-MTase MtmMII.

The MTase MtmMII introduces the methyl group to a position that is activated by two neighbouring hydroxyl groups. This is also the case for most of the aromatic *C*-MTases.
The methyl group at aromatic *C*7-position in mithramycin seems to be crucial for its antitumor activity. 7-demethylmithramycin isolated from MtmMII minus mutant showed drastically decreased biological activity.[181]

The methyl group at the aromatic *C*7 is also found in the antibiotic chromomycin which differs only in the carbohydrate chains, and chromocyclomycin, which contains a tetracyclic core structure.[178]
In the gene cluster for chromomycin A_3 biosynthesis from *Streptomyces griseus* subsp. *griseus* a gene encoding the proposed *C*-MTase CmmMII with high similarity to MtmMII was found. It also contains a proposed *O*-MTase CmmMI with high similarity to MtmMI.[182]

Other antibiotics of the aureolic acid family do not carry a methyl group at *C*7-position. They contain hydrogen (olivomycin) or possess an iso-butyl group instead (UCH9, durhamycin).[178]

6.7 Tryptophan 2´-*C*-MTase

A small number of *Actinomycetes* are able to produce the antibiotic thiostrepton (Figure 20). Thiostrepton is a prominent member of the class of thiopeptide antibiotics.[183] It was first isolated from *Streptomyces azureus* (DSMZ 40106, ATCC 14921) in 1954.[184-186] Named bryamycin (or thiacin) it was isolated from *Streptomyces hawaiiensis* (DSMZ 40042, ATCC 12236 and 19771) in 1955.[187] It was ascertained in 1963 that bryamycin and thiostrepton are identical.[188] Later it was also detected in *Streptomyces laurentii* (DSMZ 41684, ATCC 31255).[189]

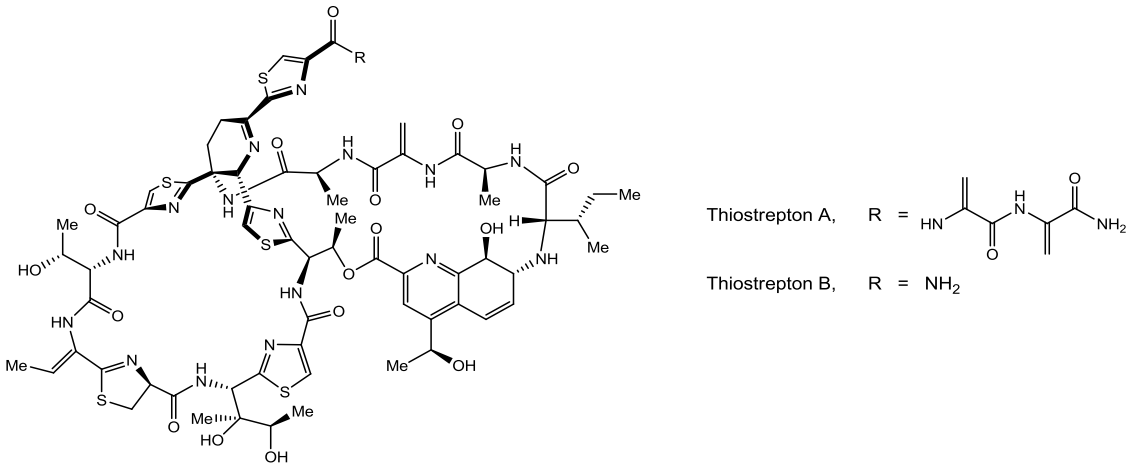
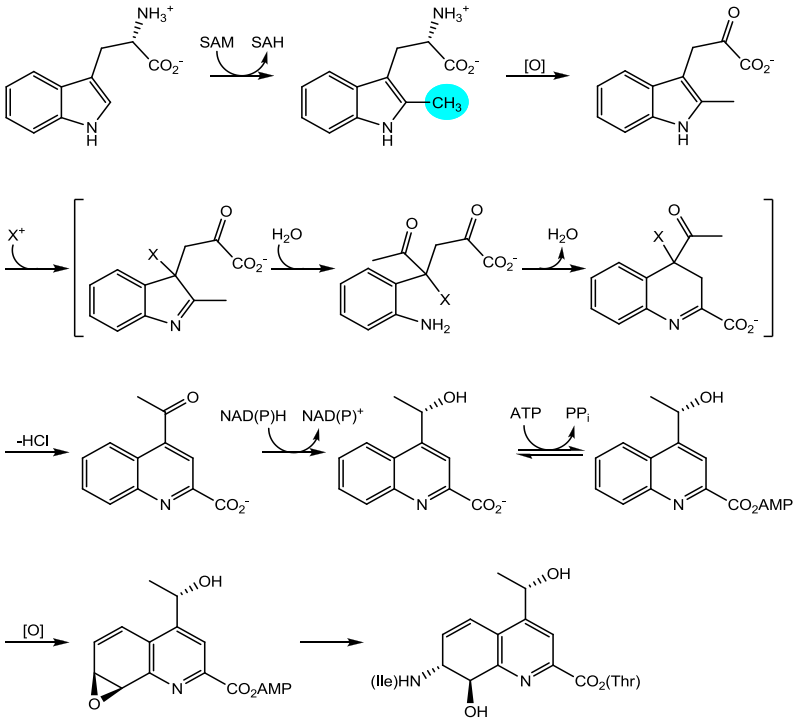


Figure 20. The structure of thiostrepton A and B resp.

Although thiostrepton is already known for more than 50 years, the investigation on its derivatives and the whole class of thiopeptide antibiotics is still of great interest. Numerous interesting lead structures are found within this class of antibiotics and have gained increasing attention in recent science.[190] They inhibit the growth of gram-positive bacteria, especially methicilline resistant *Staphylococcus aureus*. Also some other antibiotic activity could be observed like activities against the malaria parasite *Plasmodium falciparum*.[183, 191] Also its potential in cancer therapy is under investigation.[192]

Tryptophan-2´-*C*-MTase is a SAM dependent enzyme.[193, 194] The methylation of tryptophan at position *C*2 of the indole system is the initial step in the biosynthesis of the quinaldic acid moiety of thiostrepton (Scheme 19).[195, 196]



Scheme 19. Putative biosynthetic route to the quinaldic acid moiety which is part of the antibiotics thiostrepton, siomycin, sporangiomycin and thiopeptin. The first step is the introduction of a methyl group into tryprophan. The methyl group is highlighted in turquoise.

The mechanism of the methyl transfer catalysed by this enzyme seems to differ from the classic S_N2 reaction mechanism. In the S_N2 reaction the methyl group changes its net configuration. In a methyl group containing the isotopes hydrogen, deuterium and tritium inversion of the stereocenter is observed if the transfer reaction follows an S_N2 mechanism. In difference to the expected behaviour net retention of the methyl group is observed with tryptophan-2´-*C*-MTase.[193, 194] Up to now no explanation for the unusual behaviour was found.

Experiments in the early 90s showed that the purification of the enzyme from *Streptomyces laurentii* led to total inactivation. The activity could not be recovered by addition of cobalamin or different metal ions.

The enzyme characteristics were determined in the soluble part of the crude extract. A sharp pH optimum at pH 7.8 and temperature optimum at 45 °C were observed. At the temperature optimum the enzyme was rapidly deactivated, also at 4 °C a fast loss of activity was detected.[194]

As the quinaldic acid moiety is also found in other thiopeptide antibiotics, it was assumed that all microorganisms that are able to produce antibiotics which contain a quinaldic acid moiety should also possess the tryptophan-2´-*C*-MTase.

The strains which were identified as potentially interesting candidates, are *Streptomyces sioyaensis* (DSMZ 40032) producing the antibiotic siomycin,[197] *Planomonospora parontospora* subsp. *antibiotica*

(DSMZ 43869) producing siomycin B (also called sporangiomycin) [198] and *Streptomyces tateyamensis* (DSMZ 41969) producing thiopeptins.[199, 200] Also the strains *Micromonospora arborensis* producing the antibiotic Sch 18640[201] and *Micromonospora carbonecea* var. africana (ATCC 39149), producer of the antibiotic Sch 40832,[202] are able to synthesise the quinaldic acid moiety.

The gene sequences of two putative MTases each involved in the biosynthesis of siomycin and thiostrepton respectively from *S. sioyaensis*[203, 204] and *S. azureus*[204-206] were published recently. One of each is assumed to be a member of the radical SAM superfamily.

7 Aim of the work

Since many years a special interest in biocatalysis has existed in Graz. In particular there was a focus on C-C-bond formation. New reactions were discovered and investigated. Biocatalysts were improved concerning the reaction conditions, conversion and stereoselectivity.
Two highlights: Investigations on hydroxynitrile lyases led to the discovery of a biocatalytic Henry reaction.[207] Also the biocatalytic aldol reaction with threonine aldolases as catalysts was investigated towards its applicability in organic chemistry.[208, 209]

In the tradition of new biocatalytic C-C bond forming reactions effort was made to discover a biocatalytic Friedel-Crafts alkylation reaction. A biocatalytic variant of the Friedel-Crafts alkylation would be a novelty and a real breakthrough.

The application of biocatalytic procedures would be a promising way to overcome the disadvantages of the classic Friedel-Crafts alkylation.
Biocatalysis offers contrary reaction characteristics and conditions to the classic Friedel-Crafts reaction. Biocatalytic reactions usually run at ambient temperature and in aqueous solution. Biocatalysts are environmentally benign and easily degradable. Normally only a single product is formed, unwanted loss of precious substrates via formation of by-products is avoided and simple purification protocols can be applied. Biocatalysts provide overwhelming reaction parameters that no other catalyst class can compete with.

Biocatalysts should be found that are able to catalyse the introduction of strictly just one alkyl group at a defined position. Also the tolerance towards many different functional groups would be a benefit. In the quest for a suitable biocatalyst methyltransferases were identified as potential candidates as catalysts of a biocatalytic Friedel-Crafts alkylation variant.

The methyltransferases should be investigated towards their substrate promiscuity and ability to transfer alkyl chains from artificial cofactors. Also an approach to cofactor recycling should be introduced.

8 Materials and Methods

HPLC analyses were performed on a HP 1100 instrument composed of vacuum degasser G1322A, quaternary pump G1311A, autosampler G1313A, thermostatted column compartment G1316A, refractive index detector G1362A and diode array detector (DAD) G1315A or on an Agilent 1200 instrument composed of vacuum degasser G1379B, binary pump G1312B, autosampler G1367C with autosampler thermostat G1330B, thermostatted column compartment G1316B, and multi wavelength detector (MWD) G1365C connected to an Agilent quadrupol-MS 6120 with electrospray ionisation (ESI) unit.

HPLC colums:

Agilent Poroshell® C_{18} ec 100*3 mm, 2.7 µm
Agilent Zorbax® Eclipse XDB C_{18} ec 50*4.6 mm, 1.8 µm
Macherey Nagel Nucleodur® C_{18} ec 150x4 mm, 5 µm
Macherey Nagel Nucleosil® C_4 150*4mm, 5 µm
Merck Chromolith® C_{18} ec 100*4.6 mm
Merck Chromolith® C_{18} ec 100*3 mm
Merck Chromolith® C_{18} ec 100*2 mm
Merck Purospher® star C_{18} ec 250*4 mm, 5 µm
Merck SeQuant™ ZIC®-HILIC 150*2.1 mm, 3.5 µm

Preparative HPLC purification was performed on a Knauer Smartline composed of Smartline Manager 5000 module, binary pump Smartline 1000, a dynamic mixing chamber, autosampler Smartline 3800, single wavelength detector Smartline 2500 and a Teledyne Isco Foxy Jr. fraction collector.
Preparative HPLC columns:

Macherey Nagel Nucleodur® C_{18} ec 125x21 mm, 5 µm
Macherey Nagel Nucleosil® C_4 125x21 mm, 5 µm

^{1}H- and ^{13}C NMR spectra were recorded on a Bruker Avance 3 (300MHz) or a Varian Inova-500 (500MHz) machine.
Reactions were monitored by TLC using silica gel plates Merck 60 F_{254} plates. Flash chromatography was carried out at a pressure of ca. 1.5 bar, using Merck silica gel 60H.

Chemicals were purchased from Sigma Aldrich, Alfa Aesar, Acros, TCI Europe, ABCR or A1Synth. All commercially available compounds were used as received.

8.1 Tryptophan 2´-*C*-MTase

8.1.1 Cultivation of *Streptomyces laurentii*

Medium 65 was chosen for the cultivation of *Streptomyces laurentii* as recommended by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The composition of the growth medium for agar plate and liquid cultures:

medium 65	glucose	4.0 g l^{-1}
	malt extract	10 g l^{-1}
	yeast extract	4.0 g l^{-1}
	agar	12 g l^{-1}
	$CaCO_3$	2.0 g l^{-1}

Liquid medium did not contain agar and $CaCO_3$.

Streptomyces laurentii was grown on agar plates for 48 h (28 °C). An isolated colony was used for inoculation of a 100 ml preculture in a baffled flask which was incubated for additional 48 h at 28 °C while shaking at 130 rpm. 10 ml were transferred from the preculture to 300 ml main culture (28 °C, 130 rpm). After 34 h incubation, at the transitional period from log-phase to the stationary phase, in accordance to the literature the cells were harvested by centrifugation (30 min, 10.000 rpm, 4 °C). The supernatant was discarded. The cell pellet was resuspended in buffer and centrifuged again. Afterwards the cell pellet was stored at -20 °C.

8.1.2 Assay conditions

The cell pellet from cultivation was resuspended in assay buffer. The suspended cells were used directly, dried by lyophilization or were disrupted by sonication.

Assay conditions:

Substrate	1 mM
SAM	1 mM
Enzyme	disrupted cells, lyophilized cells or whole cells
Buffer	sodium phosphate 50 mM pH 7.8

Stock solutions used:
Substrate 10 mM in assay buffer
SAM 10 mM in assay buffer

In a microcentrifuge tube the substrate and SAM were combined from the stock solutions in assay buffer and the enzyme preparation was added. The mixture was incubated at 45 °C and was shaken in an Eppendorf Thermomixer at 1100 rpm. The reaction was stopped via acidic precipitation of the enzyme by addition of 0.3 times the reaction volume 0.33 M HCl. The precipitate was separated by centrifugation (12 000 rpm for 15 min). The supernatant was directly employed for HPLC analysis.

8.2 CouO and NovO

The CouO and NovO crude lysates were used as received after preparation according to the following procedure:
The genomic DNA was isolated from the strains and the genes were amplified by PCR. The gene was then cloned into the expression vector pET26b(+). The expression vector was transformed into *E.coli* BL21 Gold (DE3) for overexpression.
The transformant cells were grown on LB medium containing kanamycin. The cells were harvested by centrifugation, resuspended in buffer and lysed by sonication. The resultant cell debris was removed via centrifugation and the supernatant was used for the enzyme assays.
For experiments with cells lysed via freeze/thaw method a defined amount of cells was frozen in liquid N_2. The cells were allowed to thaw and were suspended in assay buffer. The resulting suspension was directly used for the activity assays.

8.2.1 Substrate screening assay conditions

The standard assay conditions for CouO and NovO substrate screening experiments were chosen as follows:

Substrate	1 mM
SAM	2 mM (SAM analogs 2.6 mM)
DMSO	10 % v/v
Bovine serum albumin	1 mg ml^{-1}
Enzyme	crude lysate in buffer or disrupted cells
Buffer	sodium phosphate 50 mM pH 7.0 (CouO), pH 6.5 (NovO) resp.

Stock solutions used:
Substrate 10 mM in DMSO
SAM 20 mM and BSA 10mg ml^{-1} in assay buffer

The substrate, SAM and the enzyme preparation were combined in a microcentrifuge tube. The resulting mixture was incubated at 30 °C and shaken in an Eppendorf thermomixer at 1000 rpm. After 24 h the reaction was stopped by thermal denaturation of the enzyme (85 °C for 15 min). To achieve better precipitation of the denaturized enzyme the suspension was cooled to 4 °C for 10 min. The protein was removed via centrifugation (15 min, 12 000 rpm). The supernatant was directly used for HPLC-MS analysis.

8.2.2 Preparative scale experiments

General procedure for the preparation of the SAM analogs for preparative scale experiments

The reaction was monitored by HPLC-MS.
50 mg SAH (0.13 mmol, 1 eq) were dissolved in 5 ml formic acid. 5.2 mmol of the according alkyl bromide and 67mg silver triflate (0.13 mmol, 1 eq) were added. A colourless solid started to precipitate after addition of silver triflate. After 24 h another portion of silver triflate (34 mg, 0.065 mmol, 0.5 eq) was added. After 48 h reaction time 10 ml deion. H_2O was added. The product was washed three times with 10 ml diethyl ether. The aqueous product solution was dried by lyophilisation. The crude product was directly used for the preparative scale experiments.

General procedure for the preparative scale experiments with SAM analogs

Buffer:

CouO	sodium phosphate 50 mM pH 7.0
NovO	sodium phosphate 50 mM pH 6.5

The course of the reaction was monitored by HPLC-MS

To 25 mg of substrate are dissolved in 2.5 ml DMSO (in case of 2,7-dihydroxynaphthalene 1 ml) in a baffled flask, one portion SAM analog (prepared according to the procedure above) in 2.5 ml buffer and 20 ml cells disrupted by freeze/thaw suspended in buffer were added. The mixture was incubated at 30 °C and 150 rpm. After 24 h another portion SAM analog in 2.5 ml assay buffer and 15 ml cells disrupted by freeze/thaw suspended in assay buffer were added. After 48 h reaction time the enzyme was precipitated by addition of 2 ml 3 N HCl. The resulting suspension was extracted with ethyl acetate after vigorous shaking. The phases were separated by centrifugation (45 min, 8000 rpm, 4 °C). The aqueous phase was again extracted two times with ethyl acetate and phases were separated by centrifugation. The combined organic layers were washed with brine and dried over sodium sulfate. After removal of the solvent in vacuo, the crude product was purified by column chromatography or preparative HPLC. The resulting product was characterized by HPLC-MS and NMR.

8.3 SacF and SfmM2

The SacF and SfmM2 crude lysates were used as received after preparation according to the following procedure:

SacF and SfmM2 synthetic genes was purchased and cloned into the expression vector pET26b(+). The expression vector was transformed into *E.coli* BL21 Gold (DE3) for overexpression.

The transformant cells were grown on LB medium containing kanamycin. Induction was carried out with isopropyl-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation, resuspended in buffer and lysed by sonication. The resultant cell debris was removed via centrifugation and the supernatant was used for the enzyme assays.

Standard assay conditions:

Substrate	1 mM
SAM	2 mM (SAM analogs 2.6 mM)
Enzyme	crude lysate in buffer
Buffer	sodium phosphate 50 mM pH 7.0

Stock solutions:
Substrate 10 mM in assay buffer (tyrosine was dissolved and pipetted at elevated temperature)
SAM 20 mM in assay buffer

The substrate, SAM and the enzyme preparation were combined in a microcentrifuge tube. The mixture was incubated at 30 °C and shaken in an Eppendorf thermomixer at 1000 rpm. After 24 h the reaction was stopped by thermal denaturation of the enzyme (85 °C for 15 min). To achieve better precipitation of the denaturized enzyme the suspension was cooled to 4 °C for 10 min. The protein was removed via centrifugation (15 min, 12 000 rpm). The supernatant was directly used for HPLC-MS analysis.

8.4 ORF19 and SibL

The ORF19 and SibL crude lysates were used as received after preparation according to the following procedure:
ORF19 and SibL synthetic genes were purchased and cloned into the expression vector pET26b(+). The expression vector was transformed into *E.coli* BL21 Gold (DE3) for overexpression.
The transformant cells were grown on LB medium containing kanamycin. Induction was carried out with isopropyl-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation, resuspended in buffer and lysed by sonication. The resultant cell debris was removed via centrifugation and the supernatant was used for the enzyme assays.

Standard assay conditions:

Substrate	1 mM
SAM	2 mM (SAM analogs 2.6 mM)
Dithiothreitol (DTT)	10 mM (optional)
Enzyme	crude lysate in buffer
Buffer	sodium phosphate 50 mM pH 7.0

Stock solutions
Substrate 10 mM in assay buffer
SAM 20 mM in assay buffer
DTT 100 mM in assay buffer

The mixture was incubated at 30 °C and shaken in an Eppendorf thermomixer at 1000 rpm. After 24 h the reaction was stopped by thermal denaturation of the enzyme (85 °C for 15 min). To achieve better precipitation of the denaturized enzyme the suspension was cooled to 4 °C for 10 min. The protein was removed via centrifugation (15 min, 12 000 rpm). The supernatant was directly used for HPLC-MS analysis.

The substrate stock solutions of HKyn and HAA were freshly prepared for every assay. Both HKyn and HAA reacted to 2-aminophenoxazinones upon oxidative dimerization which led to fast decrease of the substrate concentration in the stock solution.

8.5 Alternative methyl donors

The standard assay conditions for the experiments with the alternative methyl donors were chosen as follows:

CouBz	1 mM
Methyl donor	20 mM (TMS, TMSO) or 100 mM (MeMet)
DMSO	10 % v/v
Bovine serum albumin	1 mg ml^{-1}
Enzyme	disrupted cells (freeze/thaw)
Buffer	sodium phosphate 50 mM pH 7.0 (CouO), pH 6.5 (NovO) resp.

Stock solutions used:
Substrate 10 mM in DMSO
TMS or TMOS 200 mM or MeMet 1000 mM and BSA 10mg ml^{-1} in assay buffer

CouBz, the methyl donor and the enzyme preparation were combined in a microcentrifuge tube. The resulting mixture was incubated at 30 °C and shaken in an Eppendorf thermomixer at 1000 rpm. After 24 h the reaction was stopped by thermal denaturation of the enzyme (85 °C for 15 min). To achieve better precipitation of the denaturized enzyme the suspension was cooled to 4 °C for 10 min. The protein was removed via centrifugation (15 min, 12 000 rpm). The supernatant was directly used for HPLC-MS analysis.

8.6 Catechol *O*-MTase

A commercially available preparation of porcine liver COMT was purchased from MP Biomedicals. The standard assay conditions were adopted from the conditions described by the supplier.[210]

Assay conditions:

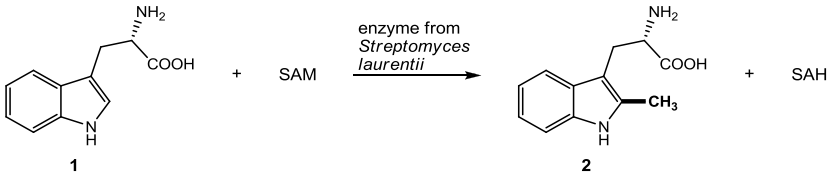
Substrate	1 mM
SAM	1 mM
$MgCl_2$	1 mM
DTT	1 mM
Enzyme	20 U ml^{-1} porcine liver COMT (MP Biomedicals, lyophilized in the presence of Tris buffer and DTT)
Buffer	sodium phosphate 50 mM pH 8.0

The compounds except the substrate were combined and the mixture was preincubated at 37 °C and 1000 rpm for 5 min. After addition of the substrate the mixture was incubated at 37 °C and shaken in an Eppendorf thermomixer at 1000 rpm. After 24 h the reaction was stopped by thermal denaturation of the enzyme (85 °C for 15 min). To achieve better precipitation of the denaturized enzyme the suspension was cooled to 4 °C for 10 min. The protein was removed via centrifugation (15 min, 12 000 rpm). The supernatant was directly used for HPLC-MS analysis.

9 Results and Discussion

9.1 Tryptophan 2´-*C*-MTase

The tryptophan-2´-*C*-MTase from *Streptomyces laurentii* was investigated best. Whole cells, crude lysate or lyophilized cells from *Streptomyces laurentii* were used for the experiments. The reaction catalysed is depicted in Scheme 20.



Scheme 20. Natural reaction catalysed by the tryptophan-2´-*C*-MTase from *Streptomyces laurentii*.

Product formation could be detected with *Streptomyces laurentii* whole cells, lyophilized cells and crude lysate under assay conditions. The product formation was lower with lyophilized cells and disrupted cells in comparison to the conversion with whole cells. A characteristic HPLC chromatogram is depicted in Figure 21.

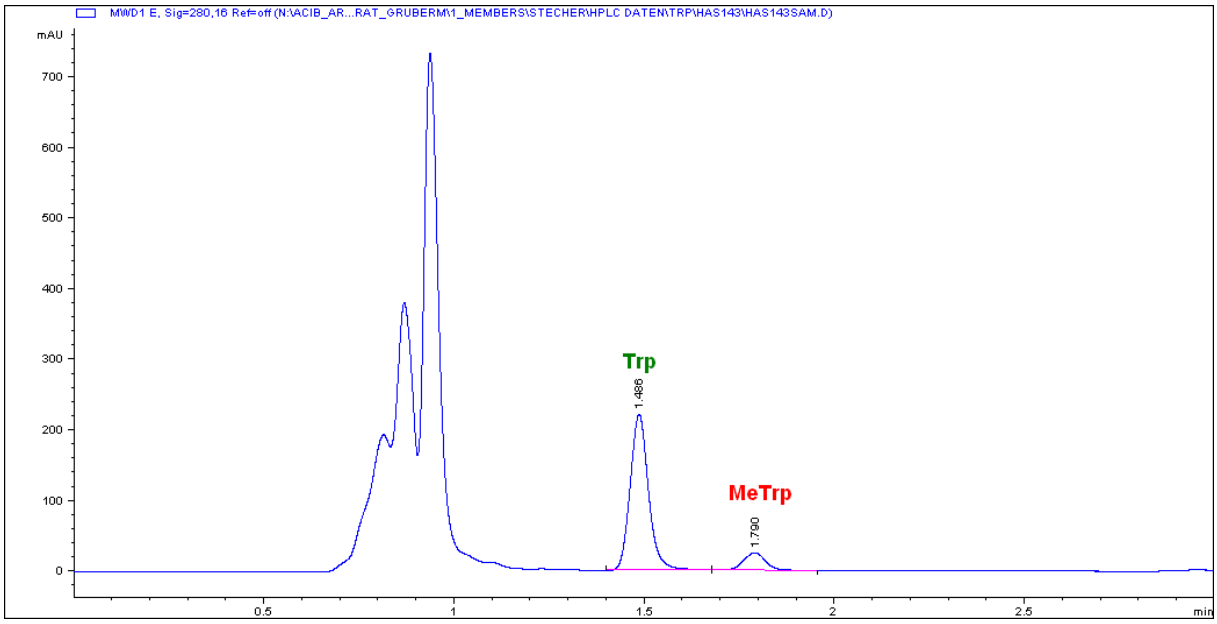


Figure 21. Chromatogram of tryptophan MTase activity assay using whole cells of *Streptomyces laurentii* and tryptophan as a substrate. Method: Agilent Zorbax® XDB C_{18} ec 50*4.6 mm, 1.8 µm; ammonium acetate 10 mM pH 5.0 / MeOH 70:30; 0.6 ml min^{-1}; 40 °C; R_t Trp = 1.49 min, MeTrp = 1.79 min.

Experiments on the methyltransferase activity of the enzyme preparation after a certain growth time showed that after 34-36 h the enzyme preparation was most active.

The substrate promiscuity of this enzyme was explored using whole cells of *Steptomyces laurentii*. A series of substrates closely related to tryptophan were employed to the assay. The compounds which were tested are depicted in Figure 22.

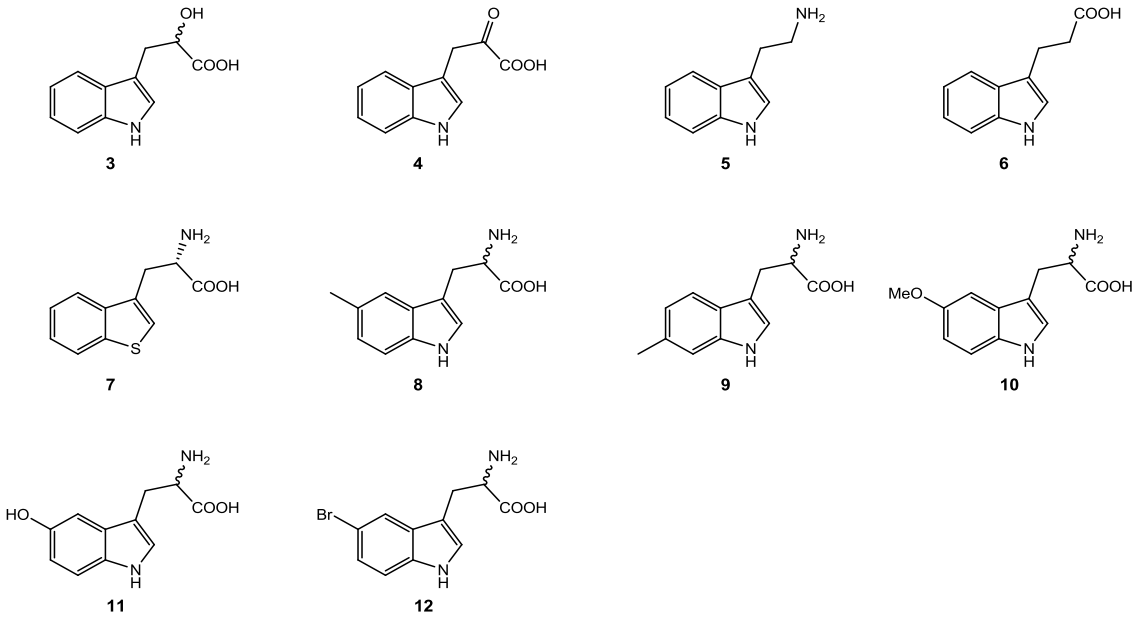


Figure 22. Substrates for tryptophan 2*C*-MTase from *S. laurentii* that were not accepted. 3 DL-3-(3-indolyl)-lactic acid, 4 3-indolpyruvic acid, 5 tryptamine, 6 3-(3-indolyl)-propionic acid, 7 3-(1-benzothiophen-3-yl)-L-alanine, 8 5-methyl-DL-tryptophan, 9 6-methyl-DL-tryptophan, 10 5-methoxy-DL-tryptophan, 11 5-Hydroxy-DL-tryptophan, 12 5-Bromo-DL-tryptophan.

None of the substrates shown in Figure 22 was methylated under assay conditions. In a reference experiment with tryptophan a conversion of 14 % was detected after 24 h. Even with 3-indolylpyruvate as a substrate no product formation was detected although in literature the methylation of 3-indolylpyruvate was described.[194] A possible explanation for the appearance of 3-(2-methylindol-3-yl)pyruvate is that endogeneous tryptophan was methylated and then converted into 3-(2-methylindol-3-yl)pyruvate which is also the next step in the biosynthesis of the quinaldic acid moiety of thiopeptide antibiotics.[193, 195]

Five alternative methyl donors were tested with the whole cells of *Steptomyces laurentii*: SAM-*S*-oxide, trimethylsulfonium iodide, trimethylsulfoxonium iodide, methyl methionine chloride and 5´-deoxy-5´-dimethylsulfoniumadenosine iodide (Figure 23). SAM *S*-oxide was tested in 2mM concentration, the other alternative methyl donors were tested in 1 mM and 10 mM concentration.
In case of 5´deoxy-5´-dimethylsulfoniumadenosine iodide also glycine or alanine was added to the assy. It was assumed that the presence of glycine or alanine is beneficial on the methylation potential

of 5´deoxy-5´-dimethylsulfoniumadenosine iodide by binding to the vacant positions of the SAM binding site.

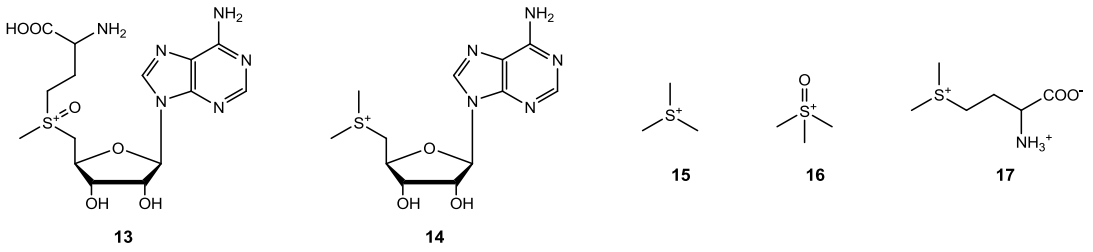


Figure 23. Alternative methyl donors tested with *Streptomyces laurentii* cells for activity. From left to right: 13 SAM-*S*-oxide, 14 5´deoxy-5´-dimethylsulfoniumadenosine iodide, 15 trimethylsulfonium iodide, 16 trimethylsulfoxonium iodide and 17 methyl methionine chloride.

SAM-oxide showed about 10 % of the activity of SAM, none of the other methyl donors led to formation of a detectable amount of the product. Also addition of alanine or glycine to 5´deoxy-5´-dimethylsulfoniumadenosine iodide did not induce product formation.

Also double activated cofactors were tested. Allyl-SAH was applied unpurified and purified by semipreparative HPLC, but no allyl transfer was detected.

Other organisms were tested for their ability to methylate tryptophan. The thiostrepton producers *Streptomyces azureus* and *Streptomyces hawaiiensis* were purchased. Of the organisms which produce thiopeptide antibiotics other than thiostrepton that contain a quinaldic acid moiety *Streptomyces sioyaensis* was the only commercially available organism at that moment, so this was the only organism that does not produce thiostrepton which was investigated.
The different microorganisms were cultivated and used for the reactions.

As proposed we observed MTase activity with all microorganism preparations tested. The thiostrepton producer *Streptomyces azureus* exhibited the highest activity, followed by *Streptomyces laurentii* and *Streptomyces hawaiiensis*. The lowest conversion was detected with *Streptomyces sioyaensis*.

Because of the promising results with the cultivated streptomyces strains, experiments with overexpressed enzyme in a host organism would describe a next step forward.
The gene sequences of two putative methyltransferases each in *Streptomyces azureus* and *Streptomyces sioyaensis* were published in 2009. The MTase genes were cloned into *E. coli* and

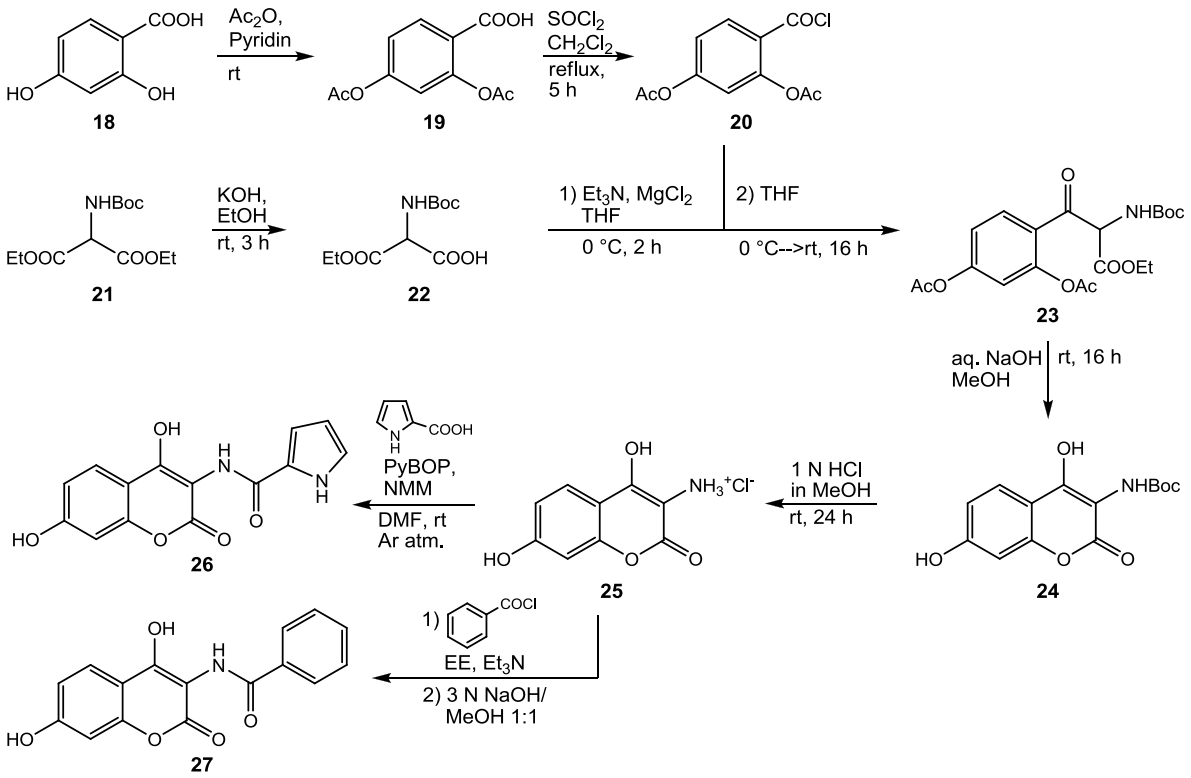
overexpressed. The cells were disrupted by sonication. The lysates prepared by centrifugation were used for an activity assay.

Unfortunately none of the four tested enzyme lysates showed any activity towards methyl transfer.

9.2 CouO and NovO

9.2.1 Model substrate synthesis

Model substrates for CouO and NovO were designed and synthesized. The synthetic routes to the model substrates *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-1H-pyrrole-2-carboxamide (CouPy) for CouO and *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide (CouBz) for NovO is depicted in Scheme 21.



Scheme 21. Synthetic route to the model substrates *N*-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)-1H-pyrrole-2-carboxamide for CouO and *N*-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)benzamide for NovO.

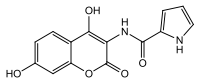
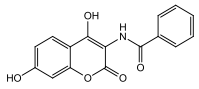
2,4-Dihydroxybenzioc acid **18** is protected with acetyl groups in pyridine.[211, 212] The according acid chloride **20** is generated with thionyl chloride.[212] Also oxalyl chloride can be applied for the formation of **20**.[213] Ethyl *N*-Boc-malonate **22** is synthesized by cleavage of one ethyl ester using a solution of

potassium hydroxide in ethanol.[214, 215] Cleavage of the second ester is prevented by precipitation of the desired product as a potassium salt. Alternatively KOH in THF/H_2O can be applied to get **22**.[216] **22** is deprotonated with Et_3N and is coupled with **20** in the presence of $MgCl_2$.[217] Cyclisation of **23** under basic conditions and removal of the Boc protecting group gave **25** which was an important intermediate for the synthesis of the model substrates and related compounds.[217] CouBz **27** was produced with benzoyl chloride and following basic cleavage of the esters which were formed as side products in the reaction. CouPy **26** was synthesized from **25** with PyBOP (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate),[218] an agent that activates acids for selective reaction with amines frequently used in solid phase peptide synthesis.[219]

9.2.2 CouO and NovO preliminary experiments

In the presence of SAM CouO was able to transfer a methyl group to its model substrate CouPy, NovO was able to methylate CouBz. Both enzymes also were able to methylate the model substrate of the other enzyme.

Table 2. Conversions of the two model substrates CouPy and CouBz with the enzymes CouO and NovO

n°	substrate		CouO	NovO
26	*N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-pyrrole-2-carboxamide CouPy		>99	>99
27	*N*-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)benzamide CouBz		>99	>99

As an example a chromatogram of the enzymatic formation of *N*-(4,7-dihydroxy-8-methyl-2-oxo-2H-chromen-3-yl)benzamide (MeCouBz) is displayed in Figure 24. A chromatogram was chosen where both the substrate CouBz and the product MeCouBz appear. The peaks were identified with a characterized reference substance both for the substrate and the product and the MS data as well.

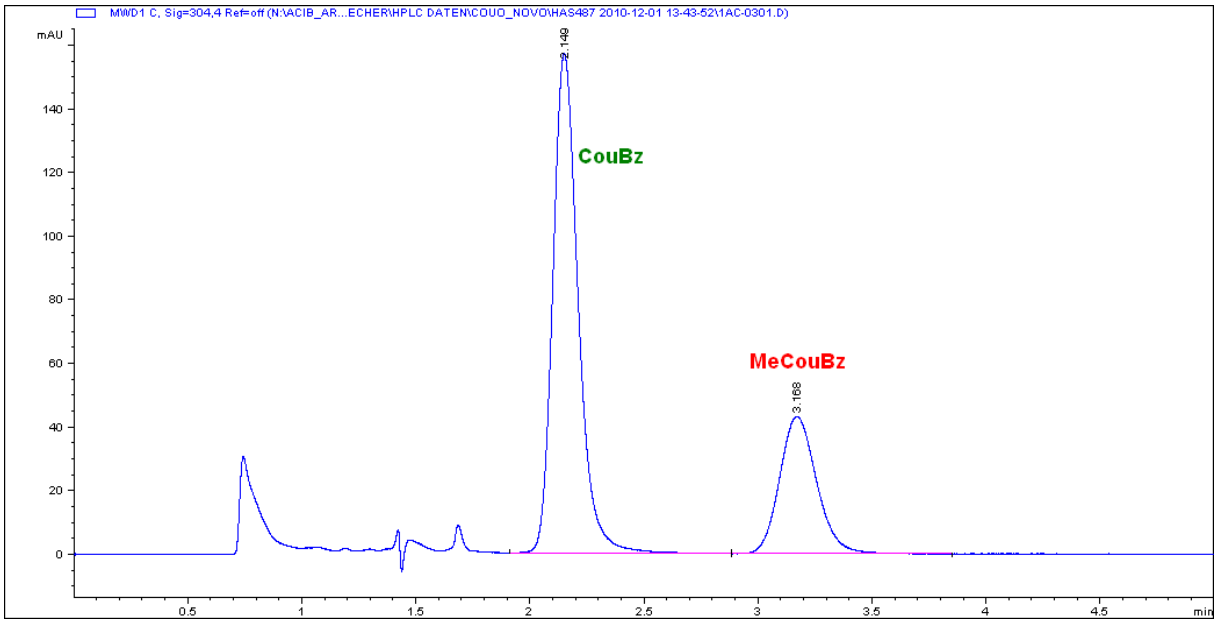


Figure 24. Chromatogram of the enzymatic reaction of CouBz. The UV-trace at 304 nm is shown. Method: Macherey Nagel Nucleodur® C_{18} ec 150x4 mm, 5 µm; ammonium acetate 10 mM pH 6.8 / acetonitrile 90:10; 1.0 ml min^{-1}; 25 °C; R_t CouBz = 2.15 min, MeCouBz = 3.17 min.

From the data of HPLC analysis collected with a DAD detector the UV traces were chosen. 220 nm was picked as a general trace, 260 nm because the adenine ring and compounds containing it show good UV absorbance at that wavelength, 304 nm because the substrate and the product exhibit an absorption maximum at that wavelength and 330 nm as a trace where both the substrate and the product appear, but nearly no interfering peaks occur.

In the SIM trace a big peak appeared in the chromatogram at the CouBz [M+H] mass beside the substrate. The peak correlated to a peak present at 220 nm and 260 nm. It was observed that the peak occurred in all samples that contain SAM, but not in blank reactions without SAM. It was assumed that the compound derives from SAM.

Considering the mass of the observed by-product it was suggested that 5´-deoxy-5´-methylthioadenosine is the detected by-product. In analyses with purchased 5´-deoxy-5´-methylthioadenosine reference material our hypothesis could be verified.

Our observation made obvious that SAM is partly removed from the methyl transfer reaction by hydrolytic cleavage under formation of 5´-deoxy-5´-methylthioadenosine and homoserine as products.

To overcome the hydrolytic loss of SAM and to enable complete conversion an excess of SAM was applied to the assays.

The two enzymes were characterized according to their pH and temperature optimum. The temperature optimum was determined to be 34 °C for CouO with CouPy as substrate and 35 °C for NovO with CouBz as a substrate (Figure 25).

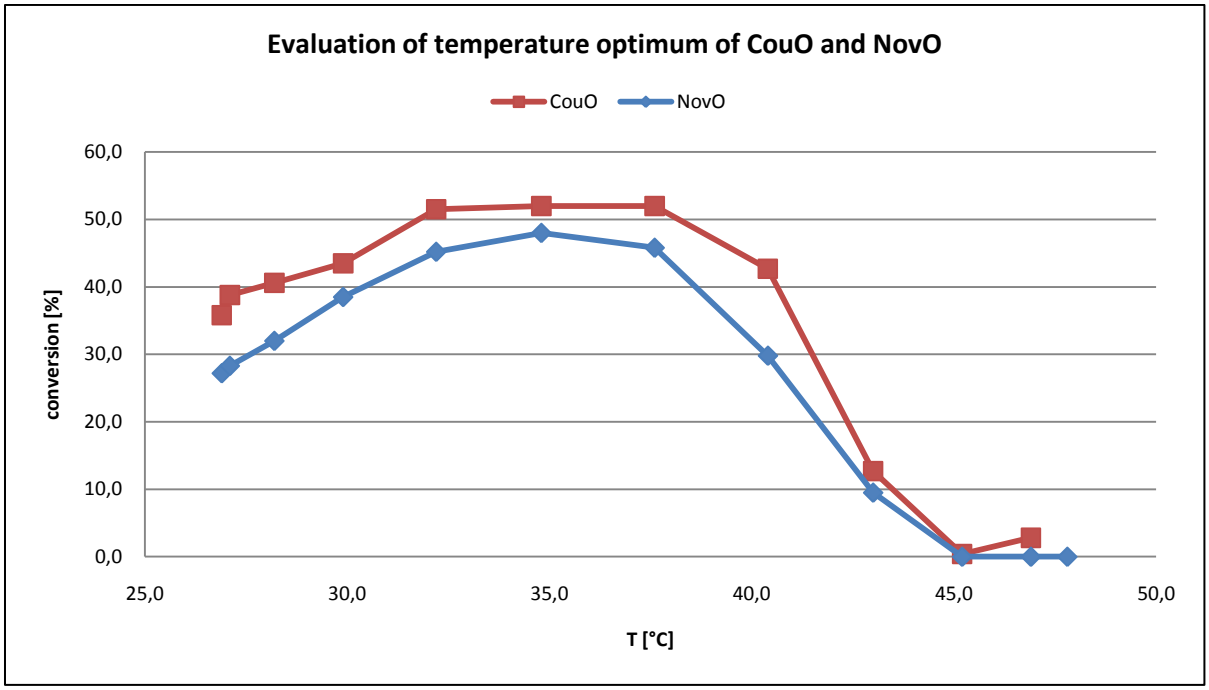


Figure 25. Determination of the temperature optimum of CouO with CouPy as a substrate and NovO with CouBz as a substrate.

The substrate activity assays were carried out at 30 °C because the long term stability of the enzymes was increased at lower temperature and higher conversion could be achieved at 30 °C in overnight experiments.

The pH optimum was determined in sodium phosphate buffer and Tris HCl buffer. The pH optimum was at pH 7.0 for CouO (Figure 26) and pH 6.5 for NovO (Figure 27) in sodium phosphate buffer. At the same time it was found that Tris HCl buffer has an inhibitory effect on CouO and NovO.

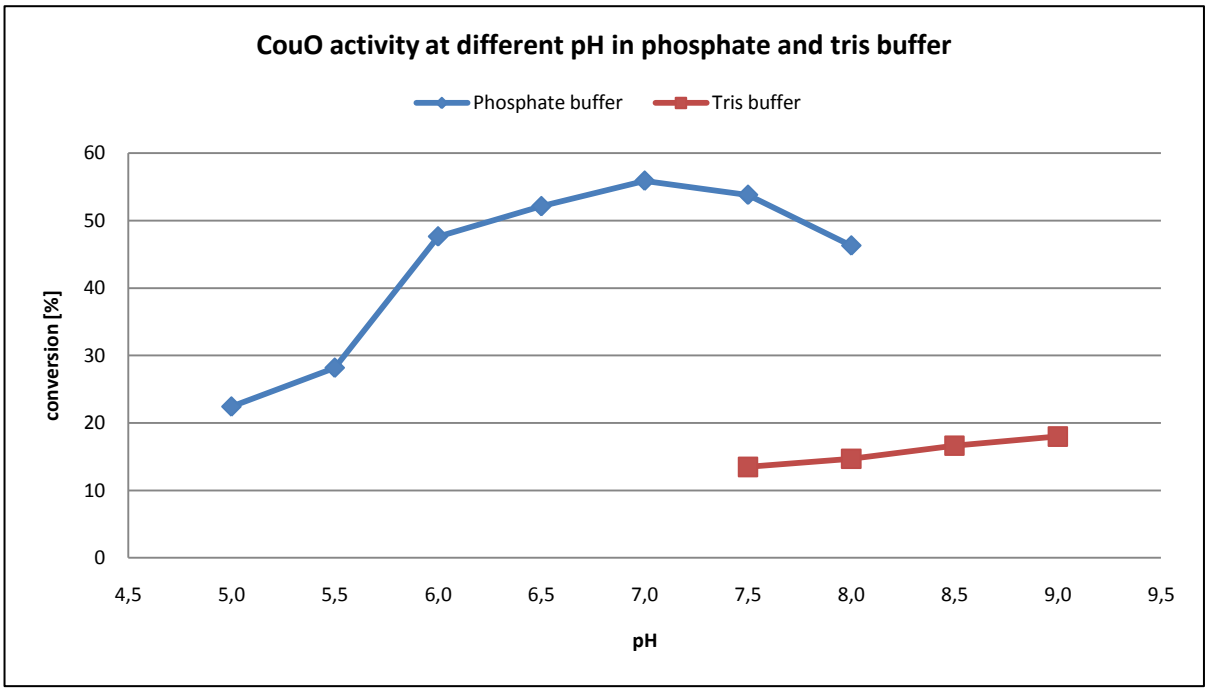


Figure 26. Data curve for the determination of the pH optimum of CouO with CouPy as substrate in phosphate buffer and Tris buffer.

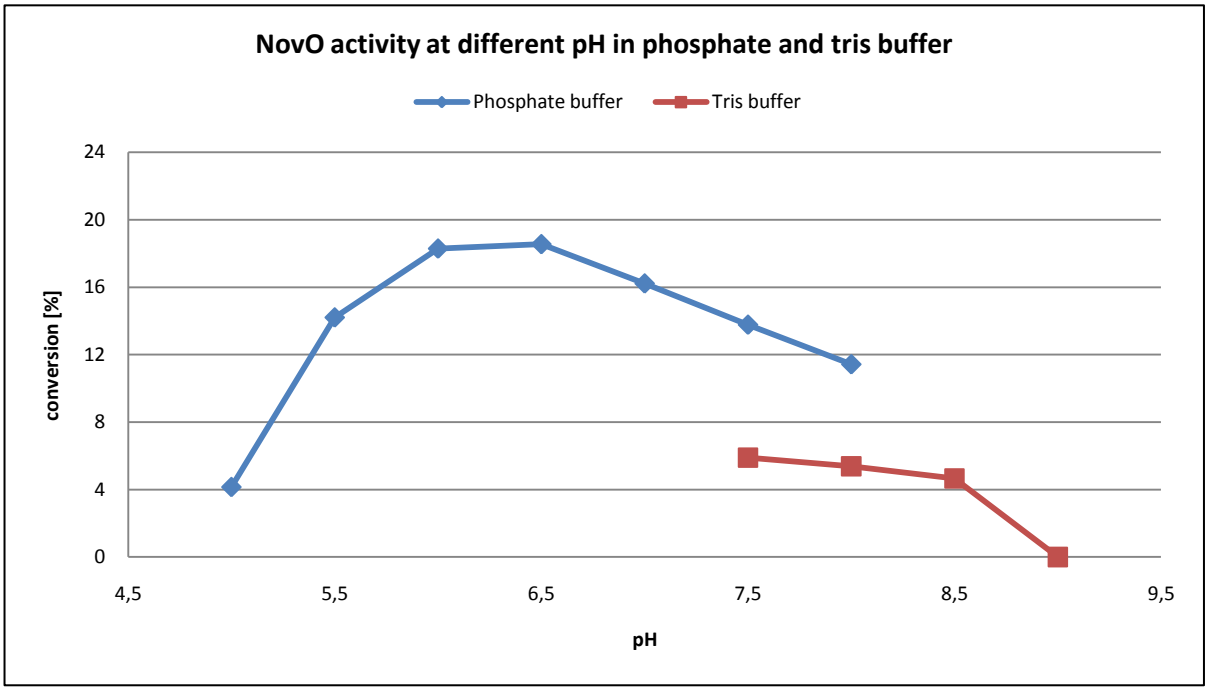


Figure 27. Data curve for the determination of the pH optimum of NovO with CouBz as substrate in phosphate buffer and Tris buffer.

It was observed that both enzymes are active over a broad pH range. With CouO high conversion is observed from pH 6.0 up to pH 8.0, with NovO high conversion is observed from pH 5.5 up to pH 8.0.

Further experiments were carried out in ammonia buffer (NH_3/NH_4^+) pH 8.0 to pH 10.0 with an enzyme preparation showing much higher activity than used for the experiments for the

determination of the pH optima. Even at pH 10 complete conversion was observed with CouO and NovO.

DMSO was present in the enzymatic reactions to guarantee full solubilization of the model substrates under assay conditions.

It was observed that the solubility of CouPy and CouBz strongly depends on the pH of the solvent. At acidic conditions CouPy and CouBz dissolve in organic solvents like ethyl acetate and can be extracted from aqueous environment into the organic phase quantitatively. In contrast to that CouPy and CouBz fully dissolve in alkaline aqueous solvents and can be quantitatively extracted to the aqueous phase. This behaviour can be useful for workup and purification of the model substrates and the alkylation products derived from them.

Around neutral pH only small amounts of the model substrates dissolve in sodium phosphate buffer. The presence of DMSO enhanced the solubility of the model substrates.

The tolerance of CouO and NovO for acetonitrile, DMF and DMSO as co-solvents were tested at different co-solvent levels. The results are shown in Figure 28 for CouO and Figure 29 for NovO.

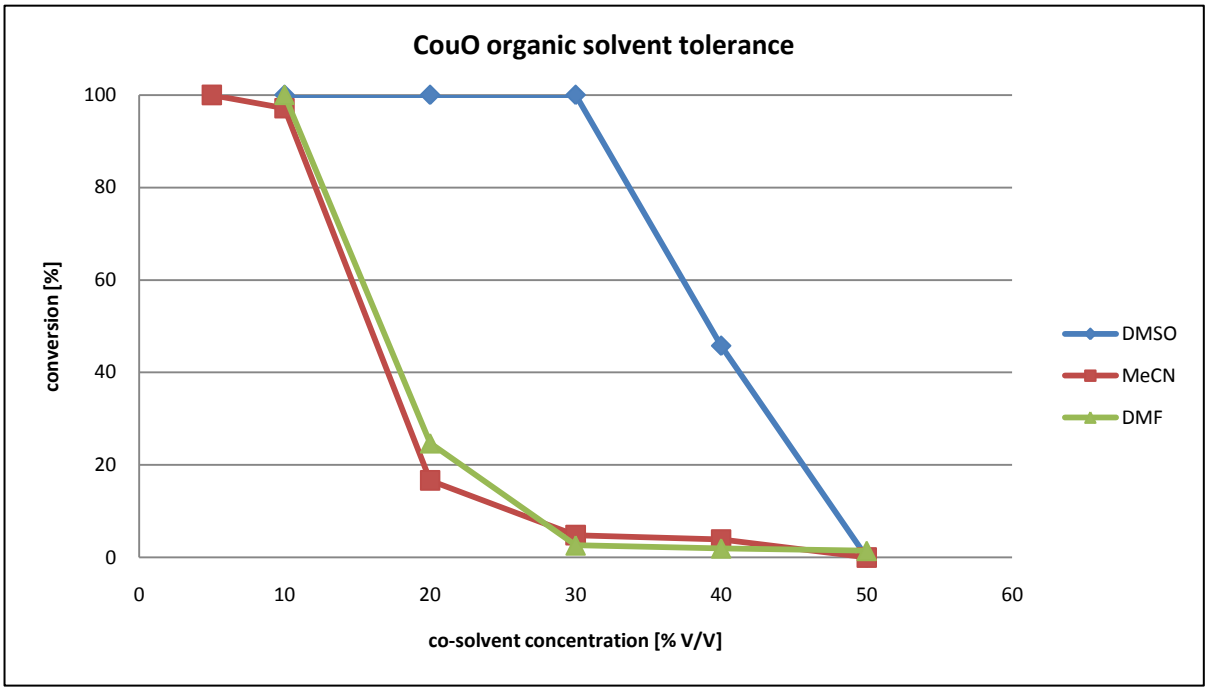


Figure 28. Influence of the presence of acetonitrile, DMF and DMSO at different concentrations on the activity of CouO after a reaction time of 24 h.

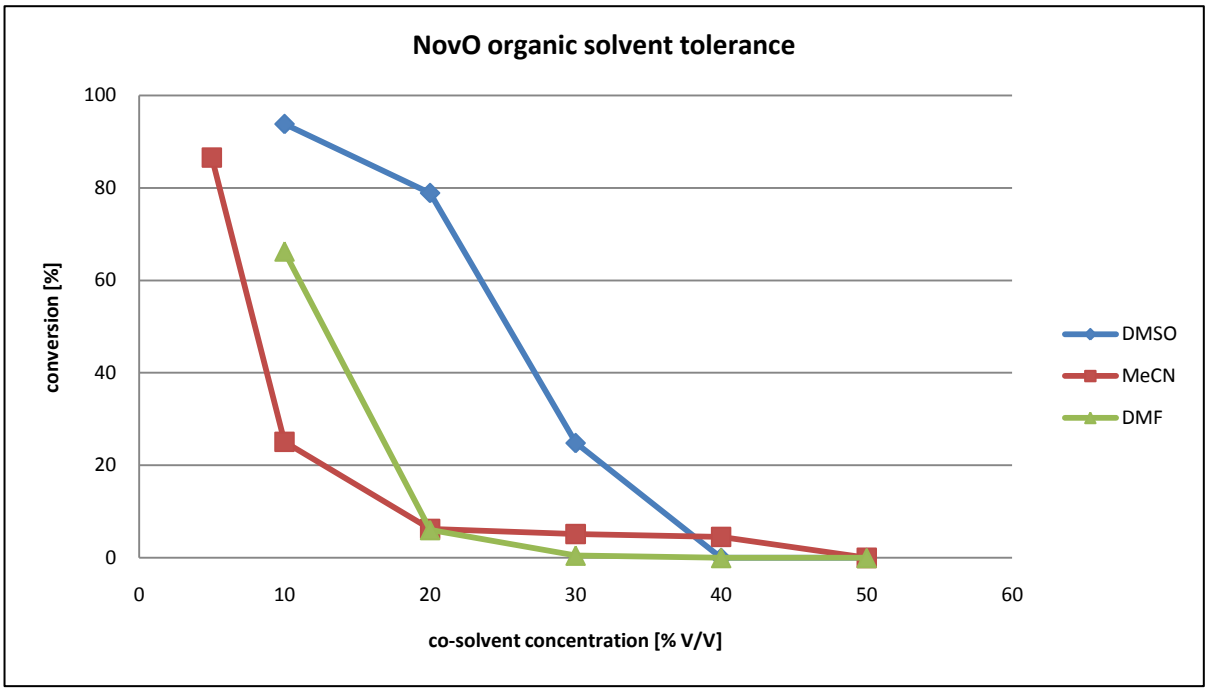


Figure 29. Influence of the presence of acetonitrile, DMF and DMSO at different concentrations on the activity of NovO after a reaction time of 24 h.

Both CouO and NovO are able to tolerate DMSO quite good, acetonitrile and DMF are tolerated much worse than DMSO and the conversion dramatically drops down at 20 % V/V organic solvent.

9.2.3 Substrate screening for CouO and NovO

The substrate promiscuity of CouO and NovO was tested with a series of substrates which contain different variations. The variations tested are highlighted in Figure 30, but also substrates with combinations two and more of these variations were tested.

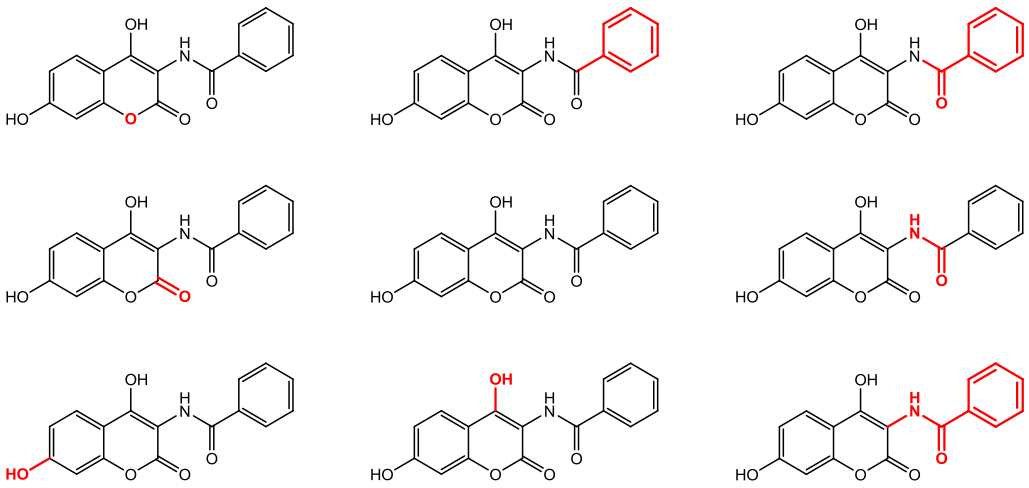


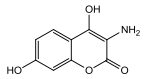
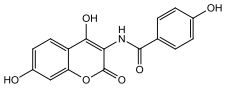
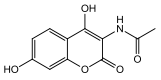
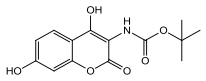
Figure 30. Suitable variations of the model substrate CouBz to test with CouO and NovO. The groups which were changed or left out are highlighted in red.

9.2.3.1 Variations in the amide side chain

The acceptance of substrates with different amide side chains and free amine was tested.

The substrates with an acetamide side chain and 4-hydroxybenzamide (CouOHBz) were prepared using the corresponding acid chlorides according to the method for the CouBz synthesis. The 4-hydroxy moiety in 4-hydroxybenzoic acid was protected with an acetyl group prior to acid chloride formation. The acetyl group was removed in the basic reaction step for cleavage of the esters formed with the acid chloride.

Table 3. List of tested substrates for CouO and NovO which vary in the amide side chain (- no product formation detected).

n^{o}	substrate		CouO	NovO
25	3-amino-4,7-dihydroxycoumarin		-	-
28	*N*-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)-4-hydroxybenzamide CouOHBz		>99	>99
29	*N*-(4,7-dihydroxy-2-oxo-2H-chromen-3-yl)acetamide		-	-
24	tert-butyl 4,7-dihydroxy-2-oxo-2H-chromen-3-ylcarbamate		-	-

With the free amine, the Boc-protected amine and the acetyl amide no conversion was observed. The 4-hydroxybenzoylamide was fully converted under standard assay conditions. The results suggest that an aromatic amide is necessary to get an active substrate.

To investigate the activity of CouOHBz in comparison to CouBz a series of experiments with diluted enzyme was carried out. The results are summarized in Figure 31 for CouO and Figure 32 for NovO.

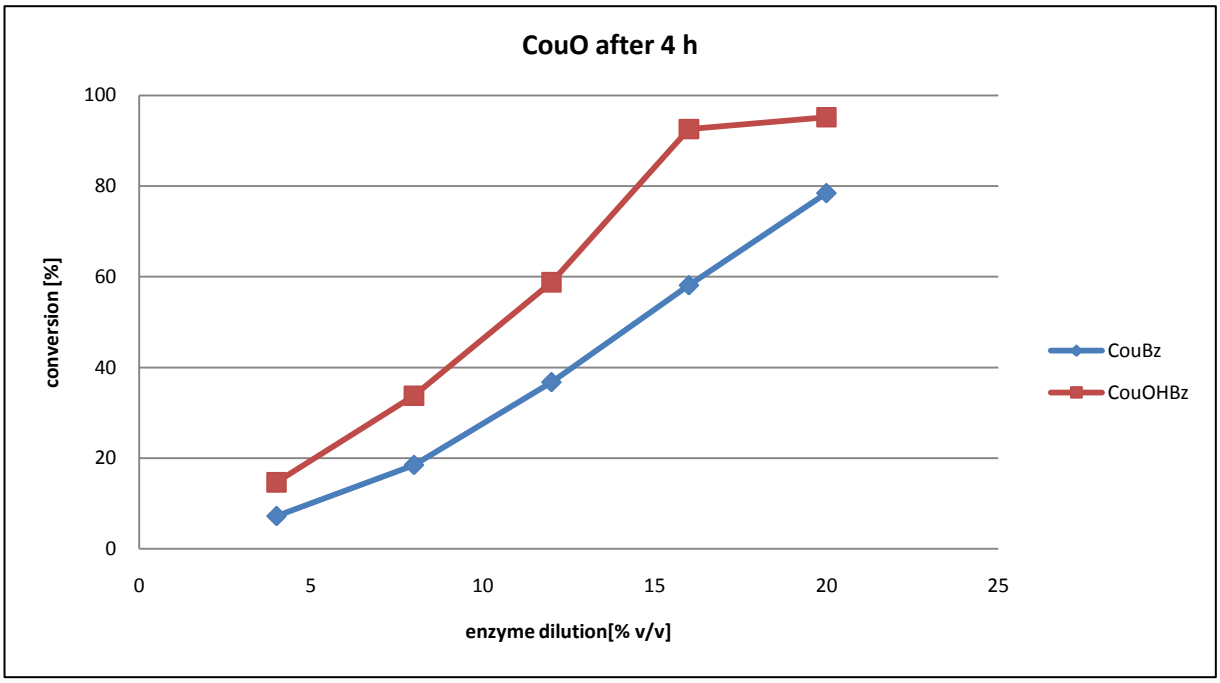


Figure 31. Comparison of the substrates CouBz and CouOHBz after 4 h at different CouO crude lysate dilution levels.

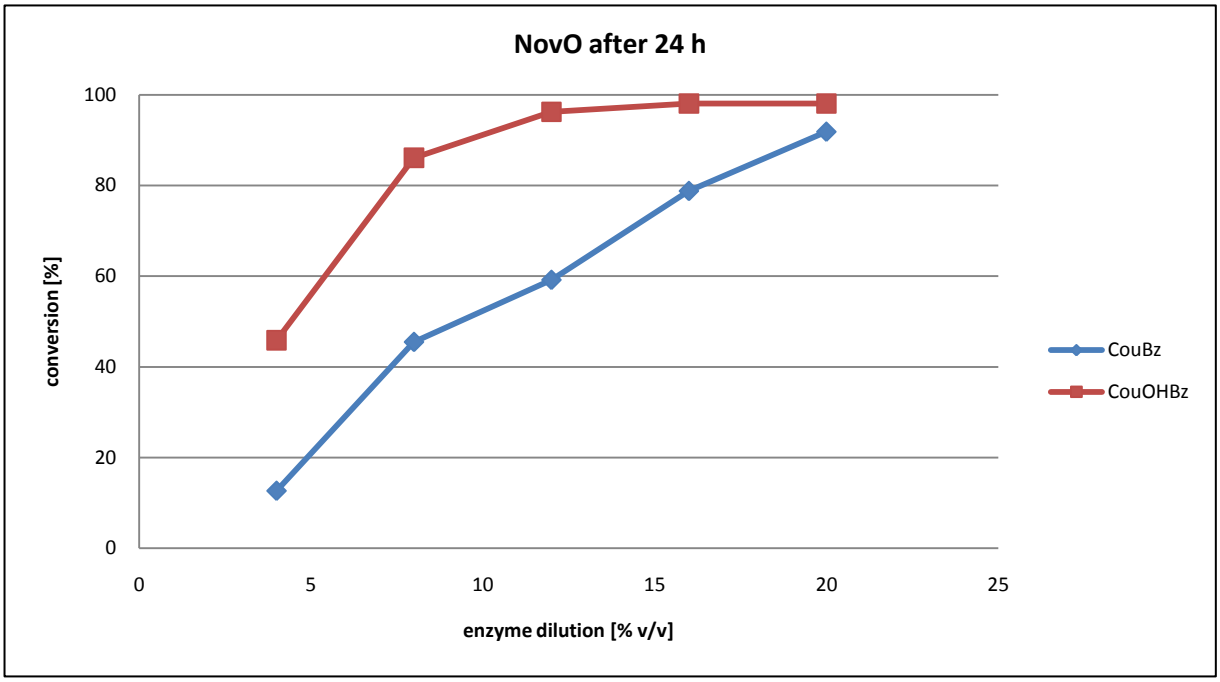


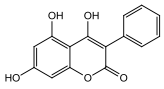
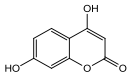
Figure 32 Comparison of the substrates CouBz and CouOHBz after 24 h at different NovO crude lysate dilution levels.

The experiments with diluted enzyme were carried out using the crude lysates in concentrations from 4 to 20 % of the overall volume. In case of CouO after 24 h the conversion of both substrates was very high. The results of these experiments were not representing the difference in the activity of the two substrates. To overcome the high conversions after 24 h data was collected after 4 h. Also for NovO the conversion after 4 h was determined, but the comparison of the substrates is more descriptive using the data after 24 h.

9.2.3.2 Substrates without 3-aminofunction

Substrates were tested which did not contain the amide function. The substrates are shown in Table 4.

Table 4. List of substrates without an amino function at position 3.

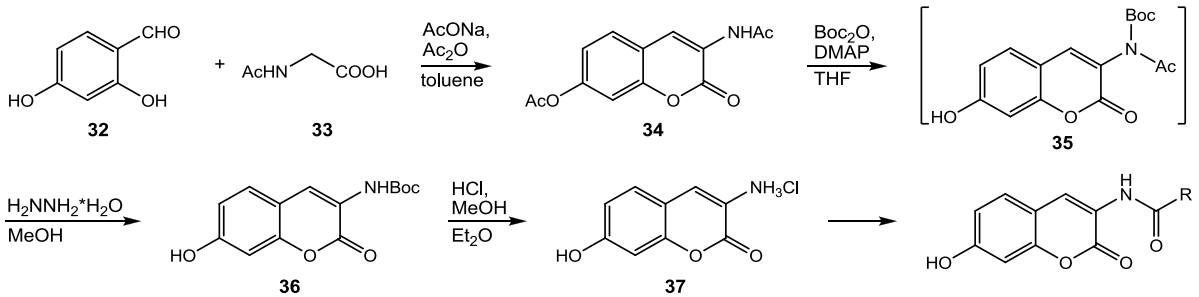
n°	substrate	structure	CouO	NovO
30	4,5,7-trihydroxy-3-phenylcoumarin		>99	>99
31	4,7-dihydroxycoumarin		>99	13

4,5,7-Trihydroxy-3-phenylcoumarin was readily accepted as a substrate. Also 4,7-dihydroxycoumarin was able to act as a substrate, but was less active with NovO.
It can be assumed that the amide at position 3 has minor relevance for substrate recognition.

9.2.3.3 Substrates without 4-hydroxyl function

The influence of the 4-hyroxyl group on the coumarin ring was tested with substrates that miss the hydroxyl group. Also one substrate was tested where the hydroxyl group is derivatized as ether.

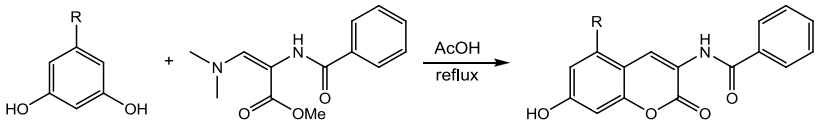
For the synthesis of the substrate *N*-(7-hydroxy-2-oxo-2*H*-chromen-3-yl)benzamide **41** two strategies were worked out. The first strategy uses 3-amino-7-hydroxycoumarin as important intermediate and is shown in Scheme 22.



Scheme 22. Synthesic route to *N*-(7-hydroxy-2-oxo-2*H*-chromen-3-yl)amides via 3-amino-7-hydroxycoumarin as intermediate.

The cyclisation reaction of 2,4-dihydroxybenzaldehyde **32** and *N*-acetyl glycine **33** under formation of **34** is described in literature.[220] Direct removal of the N-acetyl group was not possible at that reaction stage. It was necessary to exchange the *N*-acetyl group with a Boc group in a two step reaction.[220, 221] The idea of using *N*-Boc-glycine instead of **33** to form **36** directly did not work. The only product formed in the reaction was **34**. Cleavage of the Boc group of **36** was achieved in HCl in methanol / Et_2O. The free amine **37** can be used for the formation of various amides with standard methods.

The second synthetic strategy is depicted in Scheme 23.



38 R = H
39 R = OH

40

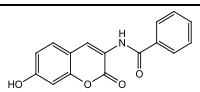
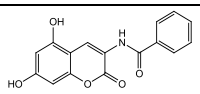
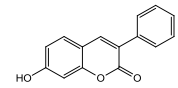
41 R = H
42 R = OH

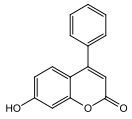
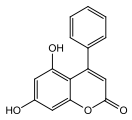
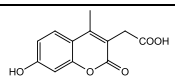
Scheme 23. Alternative synthesis of *N*-(7-hydroxy-2-oxo-2*H*-chromen-3-yl)benzamide and its derivative *N*-(5,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide.

This synthesis was much shorter and turned out to be very simple. The two reagents resorcinol **38** or phloroglucinol **39** and (E)-methyl 2-benzamido-3-(dimethylamino)acrylate **40** were stirred in acetic acid and heated to reflux. The product precipitated upon cooling to room temperature and was collected by filtration in pure form without further purification.[222, 223]

A variety of substrates was tested that do not contain a 4-hydroxyl group also paired with other variations already mentioned. The substrates are listed in Table 5.
Many substrates were complicated to test for their activity under standard assay conditions because they were scarcely soluble. As seen for CouBz and CouPy the solubility was much higher in more alkaline conditions. These substrates were tested at increased pH either in TrisHCl or in NH_3/NH_4^+ buffer.

Table 5. List of substrates that miss the hydroxyl group at position 4 ([1] the substrates were also tested at higher pH to increase the solubility, [2] product formation assumed from SIM at product mass).

n°	substrate	structure	CouO	NovO
41	*N*-(7-hydroxy-2-oxo-2*H*-chromen-3-yl)benzamide		2[1]	1[1]
42	*N*-(5,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide		traces[1]	traces[1]
43	7-hydroxy-3-phenylcoumarin 3-phenylumbelliferone		traces[1,2]	traces[1,2]

44	7-hydroxy-4-phenylcoumarin 4-phenylumbelliferone		traces[1,2]	traces[1,2]
45	5,7-dihydroxy-4-phenylcoumarin		traces[1,2]	traces[1,2]
46	2-(7-Hydroxy-4-methyl-2-oxo-2H-chromen-3-yl)acetic acid		30	1

Besides the substrates shown in Table 5 also other substrates were tested which did not lead to detectable product formation. These substrates are summarized in Figure 33.

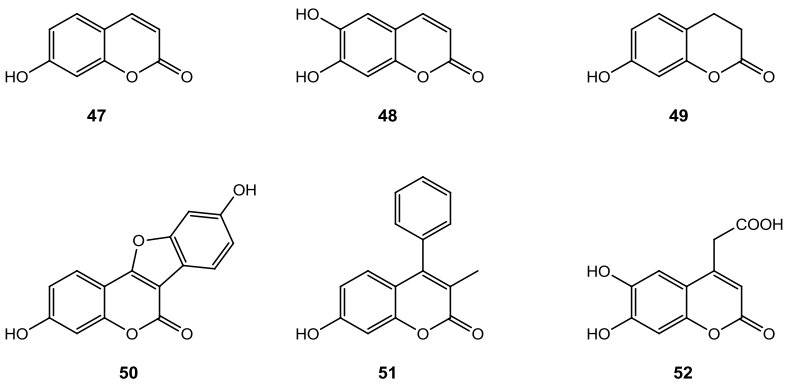


Figure 33. Substrates that were not accepted as substrates by CouO and NovO. 47 7-hydroxycoumarin (umbelliferone), 48 6,7-dihydroxycoumarin (esculetin, cichorigenin), 49 7-hydroxy-3,4-dihydrocoumarin, 50 3,9-dihydroxy-6H-benzofuro[3,2-c]chromen-6-one (coumestrol), 51 7-hydroxy-3-methyl-4-phenylcoumarin, 52 2-(6,7-Dihydroxy-2-oxo-2H-chromen-4-yl)acetic acid.

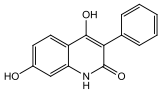
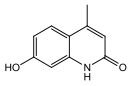
It was found that the hydroxyl group at position 4 has a major impact on the acceptance of the substrates. The substrates which do not contain the hydroxyl group show drastically decreased or no activity for methyl transfer reaction.

Coumestrol **50** was an interesting substrate as it contains an ether bridge to the phenyl ring instead of the hydroxyl group. Coumestrol shows high structural similarity to 4,5,7-trihydroxy-3-phenylcoumarin **30** which was readily accepted as substrate In contrast to **30** with a hydroxyl group at position 4 the ether oxygen is not able to participate in the tautomerization of the coumarin to a 2-hydroxy-4-oxo form. Coumestrol was found to be not accepted as substrate.
Because of the results derived from these experiments it can be assumed that the ability of tautomerization to a 2-hydroxy-4-oxo form improves the acceptance of the substrates.

9.2.3.4 Quinolone, naphthalene and benzofuran substrates

The importance of the coumarin ring system was investigated via exchange of the ring oxygen by carbon or nitrogen. Also benzofuran derivatives were applied as substrates.
The quinoline substrates are listed in Table 6.

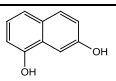
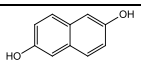
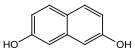
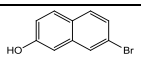
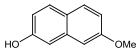
Table 6. Quinolones as substrates for CouO and NovO (2) product formation assumed from SIM at product mass; - no product formation detected).

n°	substrate	structure	CouO	NovO
53	4,7-diydroxy-3-phenylquinolin-2(1*H*)-one		33	17
54	7-Hydroxy-4-methylquinolin-2(1*H*)-one		traces[2]	-

Although with diminished activity in comparison to the according coumarins, both CouO and NovO were able to accept quinolines as substrates.

2-Naphthol, 7-substituted 2-naphthols and a series of dihydroxynaphthalenes were tested as substrates for CouO and NovO. The results of the accepted substrates are summarized in Table 7.

Table 7. List of naphthalenes which were accepted by CouO and NovO (2) product formation assumed from SIM at product mass; - no product formation detected).

n°	substrate	structure	CouO	NovO
55	1,7-dihydroxynaphthalene		15	-
56	2,6-dihydroxynaphthalene		21	-
57	2,7-dihydroxynaphthalene		93	58
58	7-bromo-2-hydroxynaphthalene		traces[2]	-
59	2-hydroxy-7-methoxynaphthalene		traces[2]	-

The best results were achieved with 2,7-dihydroxynaphthalene, also 2,6-dihydroxynaphthalene and 1,7-dihydroxynaphthalene were accepted as substrates. Also with 7-bromo-2-hydroxynaphthalene and 2-hydroxy-7-methoxynaphthalene a minimal conversion was detected in the SIM signal at the product mass [M+H] with CouO as a catalyst, but the substitution of the hydroxyl group with other functional groups strongly affects the substrate acceptance.

No product formation was detected with the naphthalene based substrates depicted in Figure 34.

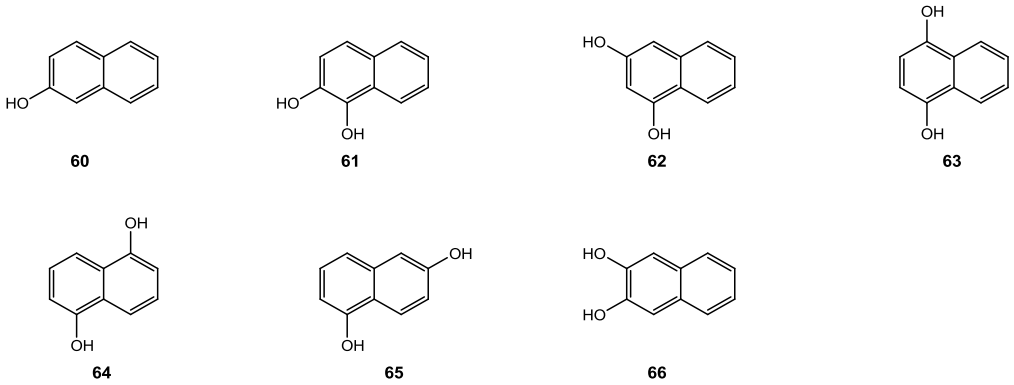


Figure 34. Naphthalene based substrates for CouO and NovO which exhibited no product formation. 60 2-naphthol, 61 1,2-dihydroxynaphthalene, 62 1,3-dihydroxynaphthalene, 63 1,4-dihydroxynaphthalene, 64 1,5-dihydroxynaphthalene, 65 1,6-dihydroxynaphthalene, 66 2,3-dihydroxynaphthalene

From the results it was assumed that a hydroxyl group in position 2 is crucial and a second hydroxyl group at position 7 or one of the adjacent positions 6 or 8 has to be present for substrate acceptance (Figure 35).

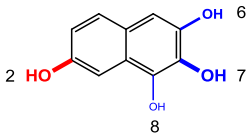
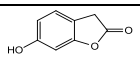
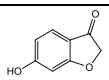


Figure 35. Substitution pattern that allows for substrate acceptance. The hydroxyl group at position 2 (red) is crucial, the second hydroxyl group (blue)can be placed at position 6, 7 (highest conversion) or 8 (lowest conversion).

Generally CouO showed higher conversion than NovO with the quinolines and naphthalenes as substrates.

Two 6-hydroxybenzofuranons were tested with CouO and NovO (Table 8).

Table 8: results of tests with 6-hydroxybenzofuranons (2) product formation assumed from SIM at product mass 3) compound not stable under assay conditions; - no product formation detected).

n°	substrate		CouO	NovO
67	6-hydroxybenzofuran-2(3*H*)-one		-[3]	-[3]
68	6-hydroxybenzofuran-3(2*H*)-one		traces[2]	-

While 6-hydroxybenzofuran-3(2*H*)-one **68** was a poor substrate for CouO and showed no conversion with NovO, no results were obtained with 6-hydroxybenzofuran-2(3*H*)-one **67** because it was not stable under assay conditions and neither the substrate nor the product could be found in HPLC analysis.

9.2.3.5 Substrates without 2-oxo group

The influence of the 2-oxo group was tested with substrates that do not contain the functionality. The investigated substrates were 4-oxochromenes or flavonoids (Figure 36).

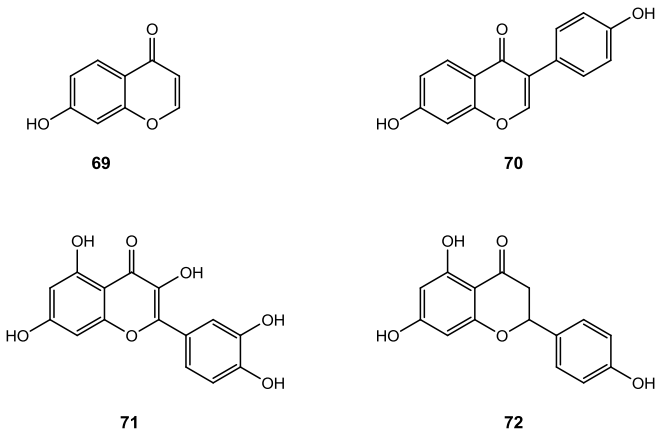


Figure 36. Chromene substrates that do not contain a 2-oxo group. 68 7-hydroxy-4*H*-chromen-4-one, 69 7-hydroxy-3-(4-hydroxyphenyl)-4*H*-chromen-4-one (daidzein), 70 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one (quercetin), 71 5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one (naringenin)

The results of these experiments indicate a great importance of the 2-oxo group in chromene substrates. With substrates that do not contain a 2-oxo group no product formation was observed. Only in case of naringenin as a substrate and CouO as catalyst a minimal product formation was assumed from the SIM at the methylation product mass [M+H].

9.2.3.6 Influence of the 7-hydroxyl group

It is supposed that the 7-hydroxyl group, which is adjacent to the methylation site, participates in the activation of the substrate. This goes in line with observations of other MTases. The effect of substitution or deletion of the 7 hydroxyl group was checked with the substrates depicted in Figure 37.

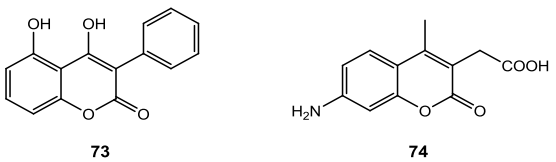


Figure 37. Substrates that do not contain a hydroxyl group at position 7. 73 4,5-dihydroxy-3-phenylcoumarin, 74 2-(7-amino-4-methyl-2-oxo-2H-chromen-3-yl)acetic acid

Consistent with our expectations no conversion could be detected with substrates that do not contain the 7-hydroxyl group. It is assumed that this hydroxyl group is crucial for substrate activation.

9.2.3.7 Monocyclic substrates

The selective methylation of a specific position of a phenyl ring represents a very useful reaction. It was tested if CouO and NovO are able to catalyse this reaction type. A variety of monocyclic substrates was tested (Figure 38).

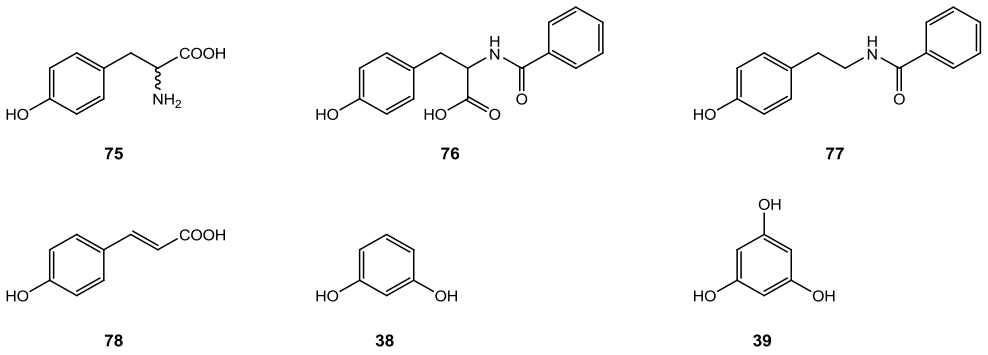


Figure 38. Monocyclic substrates for CouO and NovO. 75 DL-tyrosine, 76 *N*-benzoyl-L-tyrosine, 77 *N*-benzoyltyramine, 78 3-(4-hydroxyphenyl)propenoic acid (para-coumaric acid), 38 1,3-dihydroxybenzol (resorcinol), 39 1,3,5-trihydroxybenzol (phloroglucinol).

With none of the monocyclic substrates product formation by CouO or NovO was detected.

9.2.4 CouO and NovO substrate model

Based on the results a basic substitution pattern with features that have to be present for substrate acceptance can be designed.

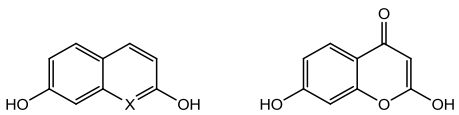


Figure 39. Basic substitution pattern for a substrate that is accepted by CouO and NovO (X = C,N).

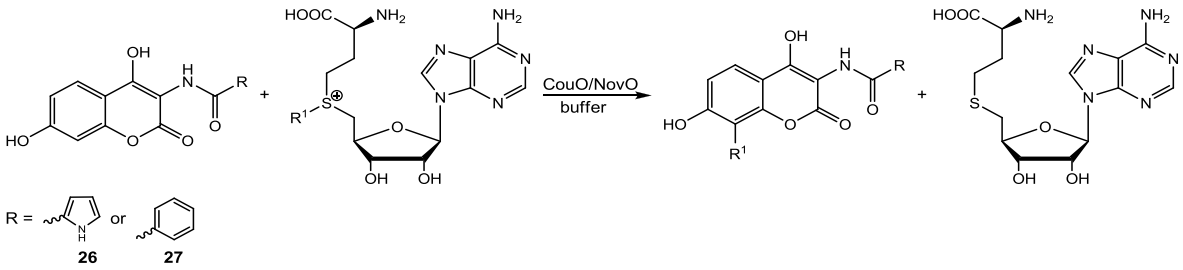
The results indicate that a substrate for CouO and NovO has to consist of a bicyclic aromatic system. The bicyclic aromatic system can be naphthalene, quinoline or coumarin. The hydroxyl group next to the methylation site is crucial for activity. A hydroxyl or oxo group has to be situated at position 2. It is assumed that in coumarin substrates the 4-hydroxy group is important to allow tautomerization to

a 2-hydroxy-4-oxo form. The substitution pattern on a 4-hydroxycoumarin substrate is assumed to influence the acceptance in correlation to the favored tautomer.

9.2.5 SAM analogs as co-substrates

The ability of CouO and NovO to accept SAM analogs as co-substrates instead of SAM was tested (Scheme 24). The SAM analogs were synthesized as described in this work and were directly applied without purification.

The results are summarized in Table 9.



Scheme 24. Reaction of SAM analogs with the model substrates XX and XX

Table 9. Summarized results of the reaction of the model substrates 1 and 2 with SAM analogs as alkyl donors. The conversion [%] after 24 h is listed.

$R^{1=}$	~CH3											
substrate	**26**	**27**	**26**	**27**	**26**	**27**	**26**	**27**	**26**	**27**	**26**	**27**
CouO	>99	>99	42	>99	11	38	11	>99	-	77	21	45
NovO	>99	>99	96	>99	30	42	35	>99	28	41	24	40

It was found that both CouO and NovO were able to accept the SAM analogs for alkyl transfer. The reaction rate was influenced by the group that was transferred.

Also with 2,7-dihydroxynaphthalene as a substrate allyl transfer and butynyl transfer cound be detected using CouO and NovO as catalysts.

9.2.6 Preparative scale experiments

For the characterization of methylated and alkylated compounds a series of preparative scale experiments were carried out. A sufficient amount for NMR analysis was converted and purified.

Methylated and alkylated CouPy and CouBz derivatives were produced in preparative scale experiments applying CouO and NovO as catalysts. For the workup the pH dependent solubility in aqueous and organic solvents could be utilized. Upon acidification of the reaction mixture the product could be extracted with organic solvent. At the same time acidification was used for the removal of the majority of the enzyme preparation components by precipitation and subsequent centrifugation. The products formed were purified by flash chromatography or preparative HPLC. The purified products were characterized by NMR analyses (except **90**).

The products which were isolated and characterized are depicted in Figure 40.

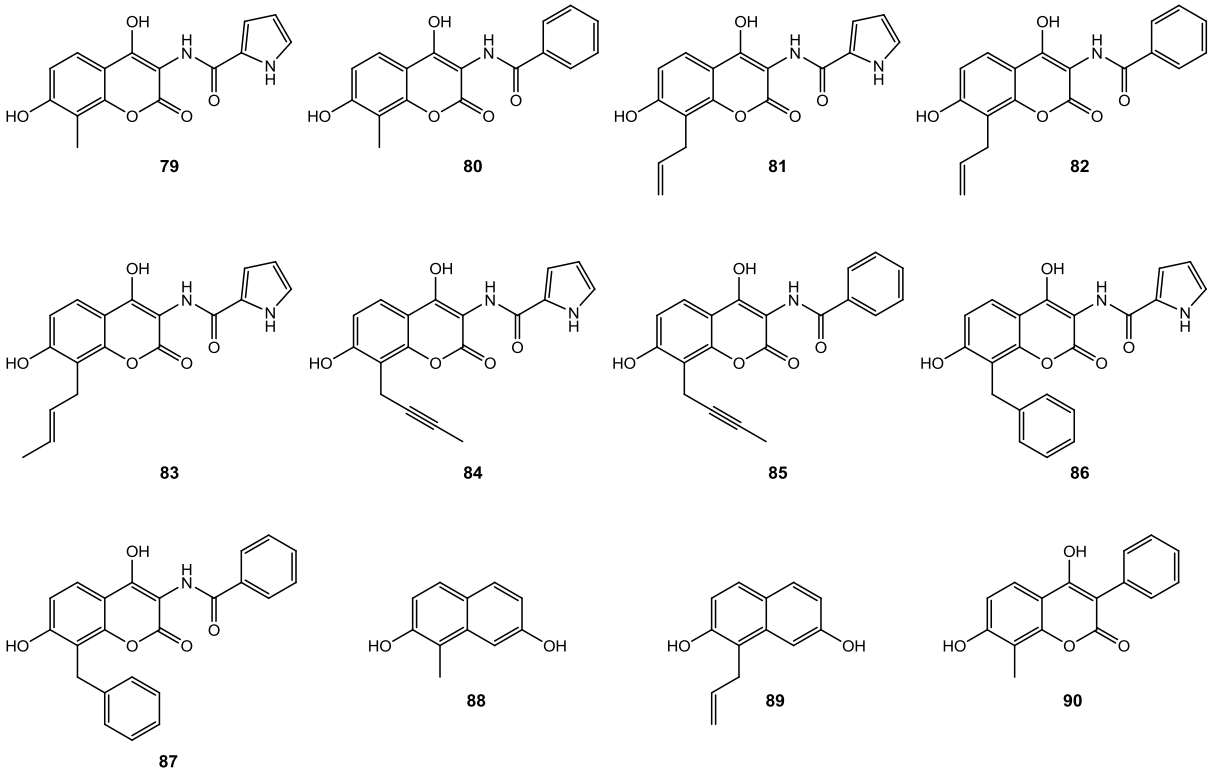


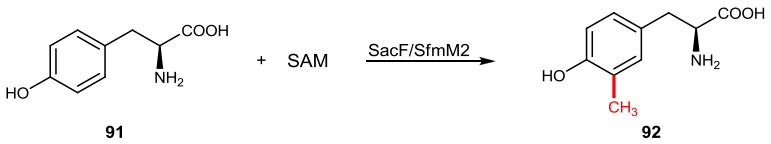
Figure 40. Compounds which were synthesized in preparative scale and characterized by NMR.

Also for 2,7-dihydroxynaphthalene as substrate preparative scale reactions were carried out with SAM and allyl-SAH as cofactors. The crude products were purified either by repeated flash chromatography in case of **88** or by preparative HPLC in case of **89**. The products were characterized by NMR. From the NMR data the exact position of the introduced methyl or alkyl group could be determined.

90 was prepared from 4,5,7-trihydroxy-3-phenylcoumarin as a substrate and SAM as cofactor. The product was characterized only by HPLC-MS. No NMR-data was recorded because a great amonut of the product was lost due to problems during purification with preparative HPLC.

9.3 SacF and SfmM2 results

The enzymes SacF and SfmM2 were investigated with their natural substrate L-tyrosine. The natural reaction is depicted in Scheme 25.



91 92

Scheme 25. Natural reaction of the enzymes SacF and SfmM2.

Both SacF and SfmM2 were active for methyl transfer with L-tyrosine under the chosen standard assay conditions.

Figure 41 shows an exemplary chromatogram (UV at 220 nm) of the enzymatic conversion of L-tyrosine **91** to 3-methyl-L-tyrosine **92**.

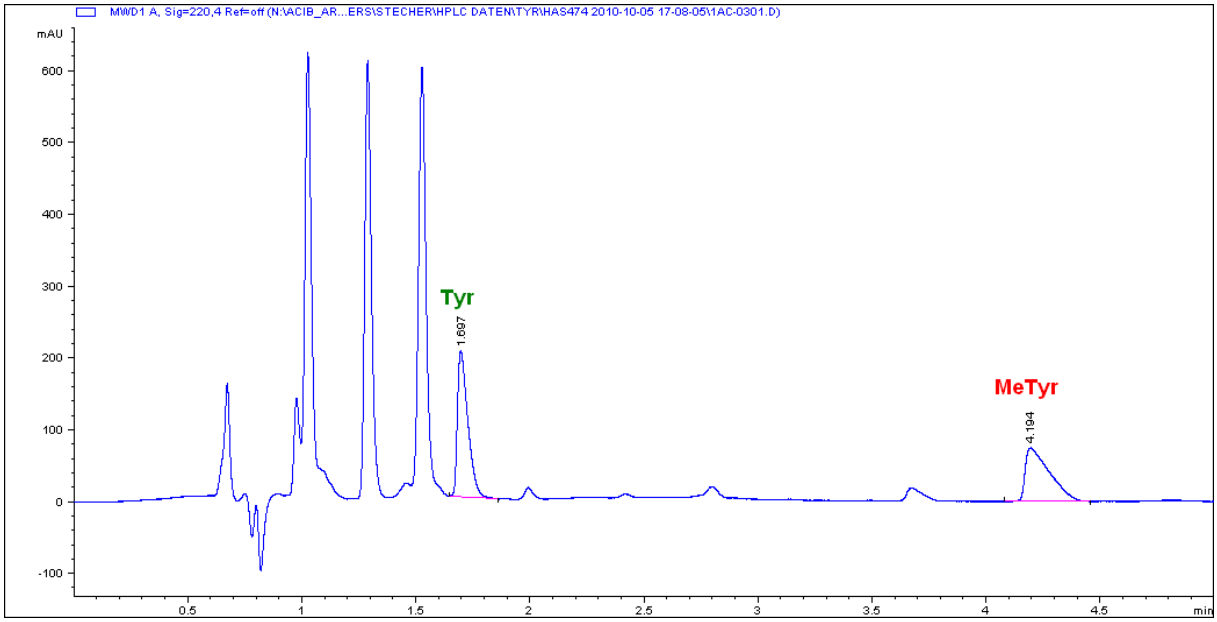


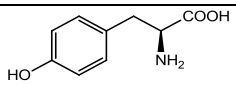
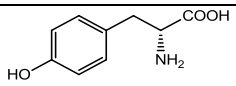
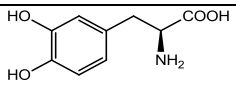
Figure 41. Chromatogram at 220 nm of SacF MTase activity assay with tyrosine as a substrate. Method: Agilent Poroshell® C_{18} ec 100*3 mm, 2.7µm; ammonium acetate 10 mM pH 6.8 / MeOH 95:5; 0.7 ml min^{-1}; 25 °C; R_t Tyr = 1.70 min, MeTyr = 4.19 min.

For analysis of SacF and SfmM2 catalysed methyl transfer to tyrosine the wavelengths 220 nm, 260 nm and 276 nm were chosen.

9.3.1 Substrate screening for SacF and SfmM2

The substrate promiscuity of SacF and SfmM2 was tested with various substrates that are closely related to the natural substrate L-tyrosine. The accepted substrates are summarized in Table 10.

Table 10. List of accepted substrates for SacF and SfmM2.

n°	substrate		SacF	SfmM2
91	L-tyrosine		87	65
93	D-tyrosine		64	24
94	L-dopa		2	1

Also further substrates were tested. The substrates are depicted in Figure 42. None of the substrates led to formation of a detectable amount of product.

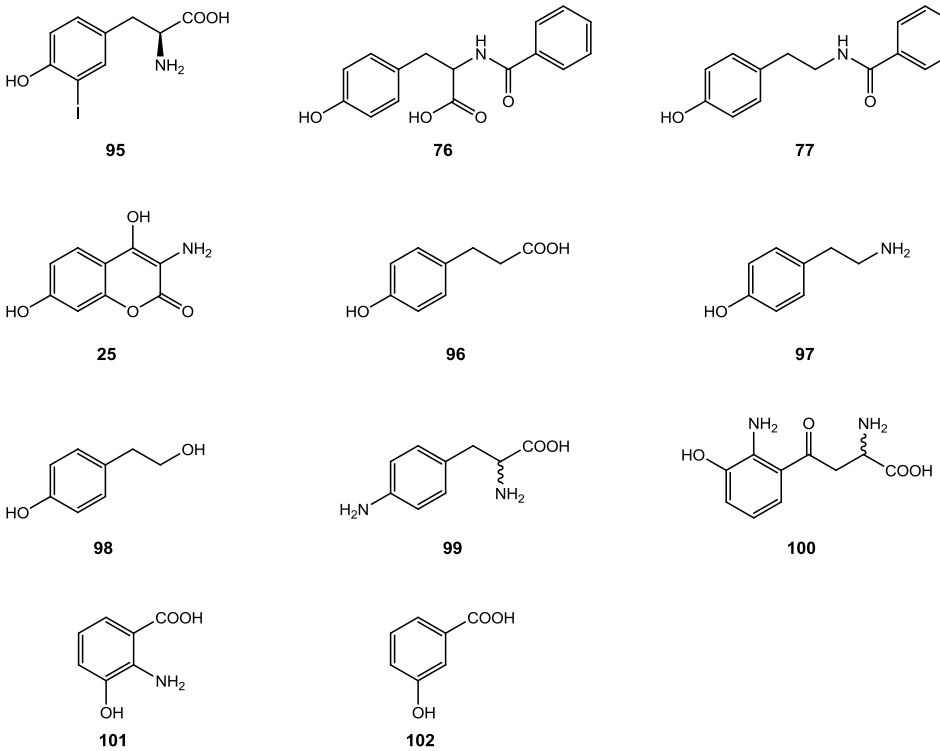


Figure 42. Substrates that were not accepted by SacF and SfmM2. 95 3-iodo-L-tyrosine, 76 *N*-benzoyltyrosine, 77 *N*-benzoyltyramine, 25 3-amino-4,7-dihydroxycoumarin, 96 3-(4-hydroxyphenyl)propionic acid, 97 2-(4-hydroxyphenyl)ethanamine, 98 2-(4-hydroxyphenyl)ethanol, 99 4-aminophenyl-DL-alanine and 100 DL-3-hydroxykynurenine, 101 3-hydroxyanthranilic acid, 102 3-hydroxybenzoic acid.

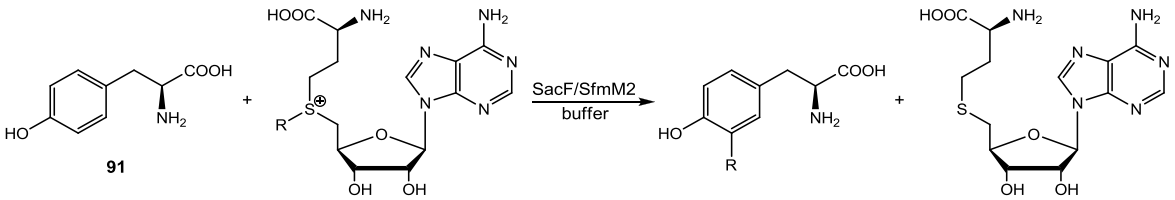
The experiments exhibited that SacF and SfmM2 offer a narrow substrate spectrum. Only L-dopa was accepted as non-natural substrate at very low rates, no other substrate was accepted. Interestingly

both enantiomers of tyrosine were accepted by SacF and SfmM2. L-tyrosine, the naturally occurring enantiomer, gave higher conversion.

The 4-amino analog to tyrosine was not accepted as a substrate. This promotes the idea of mechanistic importance of the hydroxyl group next to the methylation site.

9.3.2 SAM analogs as co-substrates

The ability of SacF and SfmM2 to accept SAM analogs as co-substrates was tested. The SAM analogs were synthesized as described in this work and were directly applied without purification.



Scheme 26. Reaction of SAM analogs with L-tyrosine catalysed by SacF and SfmM2.

Table 11. Summarized results of the reaction of SAM analogs as alkyl donors with tyrosine as substrate. The conversion [%] after 24 h is listed

R =	~CH3	~	~	~
SacF	87	73	31	19
SfmM2	65	56	31	14

It was observed that both SacF and SfmM2 were able to accept the SAM analogs for alkyl transfer. In comparison to SAM the application of SAM analogs as cofactors led to lower conversion. The reaction rate seemed to be determined by the steric demand of the chain that was transferred.

In case of but-2-enyl and propynyl analogs of SAM no product formation was detected. It is assumed that the formation of the according SAM analogs did not proceed satisfactory.

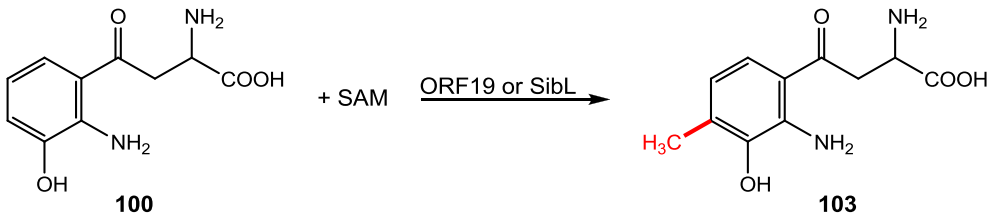
9.4 ORF19 and SibL results

9.4.1 3-Hydroxyanthranilate 4-methyltransferase from *Streptomyces antibioticus*

In former investigations the actinomycin producing strain *Streptomyces antibioticus* was cultivated. The grown cells were harvested by filtration and disrupted by sonication. Both the lysate and the pellet got from centrifugation as well as the unseparated cell disruption were tested for their activity with 3-hydroxyanthranilic acid (HAA) as substrate, but no product formation could be observed. Also different growth conditions were applied, but the resulting enzyme preparations showed no activity towards methyl transfer to 3-hydroxyanthranilic acid.

9.4.2 ORF19 and SibL

In accordance to recently published results[154] with MTases from *Streptomyces chrysomallus* the natural substrate for the enzymes ORF19 and SibL was identified to be 3-hydroxykynurenine (HKyn) and not 3-hydroxyanthranilic acid. The reaction catalysed by ORF19 and SibL is depicted in Scheme 27.



Scheme 27. Natural reaction of the enzymes ORF19 and SibL.

An exemplary chromatogram of the conversion of HKyn with ORF19 is depicted in Figure 43.

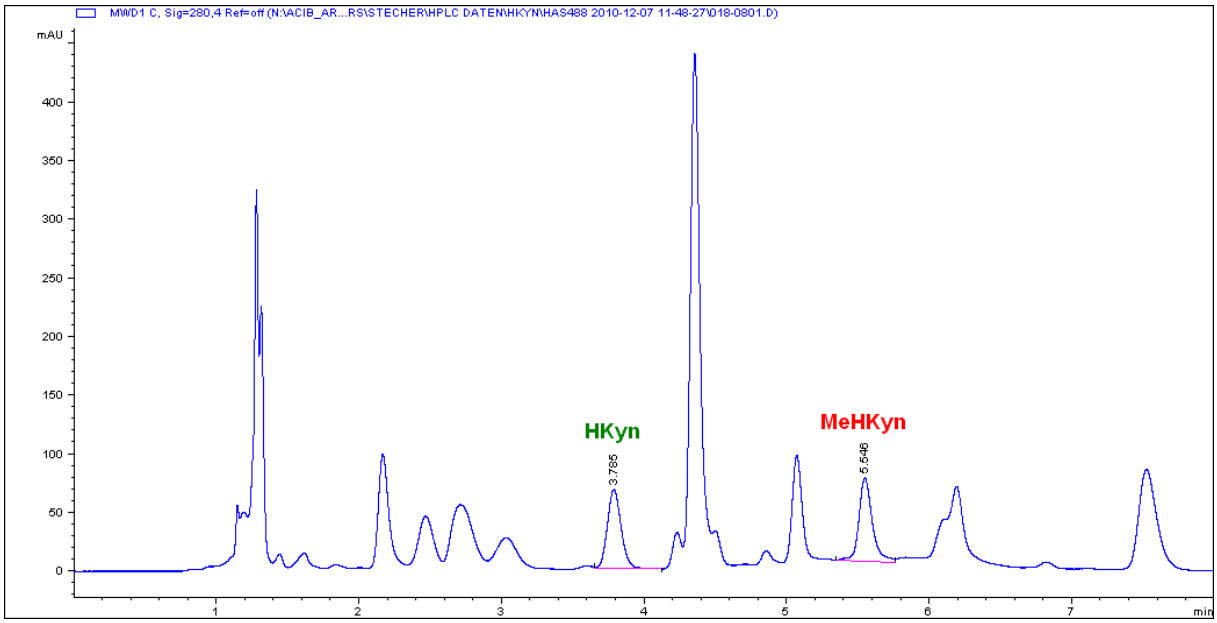


Figure 43: Chromatogram at 280 nm of ORF19 MTase activity assay with HKyn as a substrate. Method: Macherey Nagel Nucleodur® C_{18} ec 150x4 mm, 5 µm; ammonium acetate 10 mM pH 6.8 / MeOH 98:2 for 1.5 min, in 3.5 min to 75:25, hold for 0.9 min, in 0.1 min to 98:2, hold for 2 min; 1.0 ml min^{-1}; 25 °C; R_t HKyn = 3.79 min, MeHKyn = 5.55 min.

The data was collected at the wavelengths 220 nm, 260 nm and 280nm. The separation of HKyn from peaks that arise out of the enzyme matrix required a gradient based method.

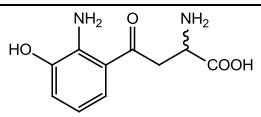
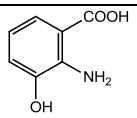
In case of SibL the overexpressed enzyme was nearly exclusively found in the pellet. The addition of mercaptoethanol had no effect on the activity of SibL, the addition of dithiothreitol showed a beneficial effect on the product formation.

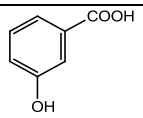
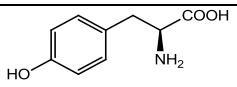
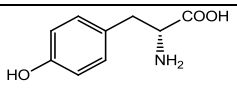
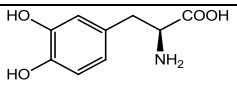
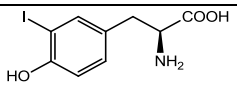
It was found that the addition of the reducing agent DTT increases the amount of both substrate and product recovered from the enzymatic reaction. It is assumed that DTT suppresses the oxidative dimerisation as a side reaction that consumes both the substrate and the product.

9.4.3 Substrate screening for ORF19 and SibL

The substrate promiscuity of ORF19 and SibL was tested. The results are summarized in Table 12.

Table 12. List of substrates tested with ORF19 and SibL (- not detected, n/a not tested)

n°	substrate	structure	Orf19	SibL
100	3-hydroxy-DL-kynurenine		51	10
101	3-hydroxyanthranilic acid		-	-

102	3-hydroxybenzoic acid		-	-
91	L-tyrosine		3	n/a
93	D-tyrosine		1	n/a
94	L-dopa		1	n/a
95	L-3-iodotyrosine		-	n/a

In line with the results reported by Crnovcic *et al* with a related MTase from *Streptomyces chrysomallus* also tyrosine and dopa were accepted as substrates, but at low reaction rates. In difference to the results of Crnovcic *et al* product formation was observed with both enantiomers of tyrosine.

Experiments with HAA as a substrate and SibL or ORF19 as catalyst showed no product formation. Also the addition of mercaptoethanol or dithiothreitol as stabilizing agents for the enzyme did not lead to product formation.

9.4.4 Preparative scale preparation of MeHKyn

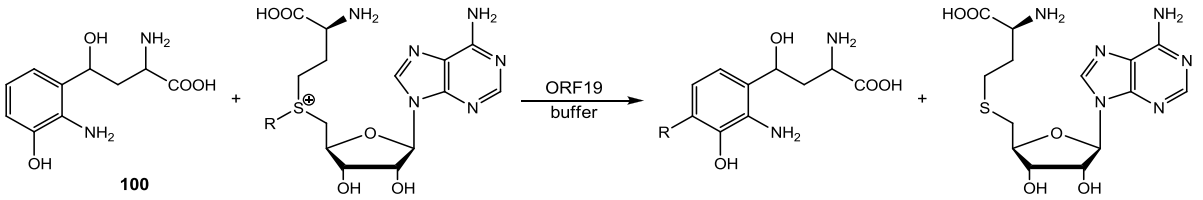
To exactly determine the position of methylation a preparative scale experiment with HKyn as substrate and ORF19 as catalyst was carried out. The reaction was monitored by HPLC-MS. After work-up the crude product was purified by HPLC-MS.

The purified product was subjected to NMR analysis. To clear the structure and exact position of the methyl group NMR data was collected from ^{1}H, COSY, HMBC and HSQC experiments. With the data all H and C atoms (except the carboxylic C which did not appear) could be allocated.

It could be ascertained that as expected the methyl group is introduced adjacent to the hydroxyl group.

9.4.5 SAM analogs as co-substrates

The ability of ORF19 to accept SAM analogs as co-substrates was tested. The SAM analogs were synthesized as described in this work and were directly applied without purification.



Scheme 28. Reaction of SAM analogs with 3-hydroxykynurenine catalysed by ORF19.

Table 13 Summarized results of the reaction of SAM analogs as alkyl donors with HKyn as substrate. The conversion [%] after 24 h is listed.

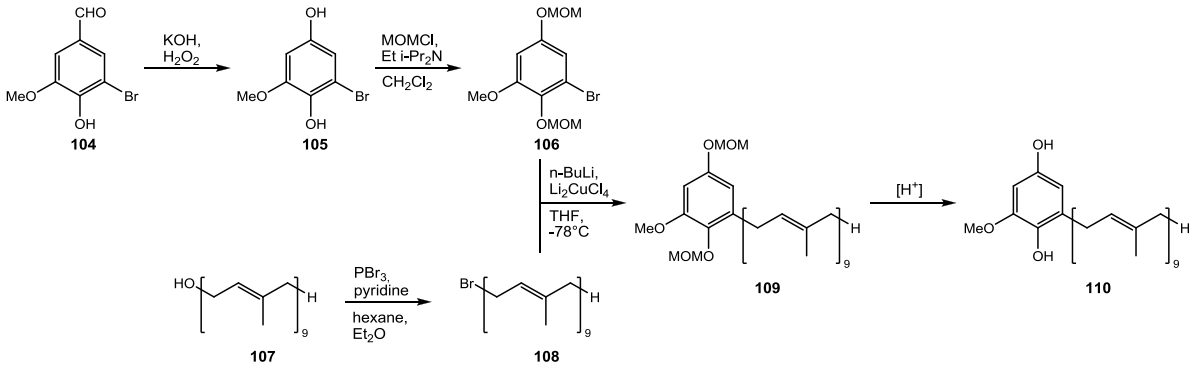
R =	~CH3			
ORF19	51	17	13	3

The investigations showed that the enzyme ORF19 is able to accept SAM analogs as cofactors. In comparison to SAM the application of SAM analogs as cofactors led to lower conversion. The reaction rate seemed to be determined by the steric demand of the chain that was transferred.

In case of but-2-enyl and propynyl transfer no product formation was detected. It is assumed that the formation of the according SAM analogs did not proceed satisfactory.

9.5 UbiE substrate synthesis

A concept for the synthesis of a model substrate for UbiE was developed. The synthetic route is shown in Scheme 29.



Scheme 29. Proposed synthetic route to the model substrate for UbiE.

The synthesis was carried out until the last step, the deprotection of **109**. It could be demonstrated, that the proposed route was successful for the synthesis of the (protected) model substrate.

The aldehyde moiety of 5-bromovanillin **104** was turned into the alcohol **105**. Both hydroxyl groups were protected with MOM chloride. Bromine was introduced into solanesol **107**. The next step is the copper catalysed coupling of solanesyl bromide **108** and protected 2-bromo-5-methoxy-1,4-dihydroxybenzene **106**.

UbiE could be cloned and overexpressed. Characterization of this enzyme regarding substrate and cofactor scope has not been carried out yet.

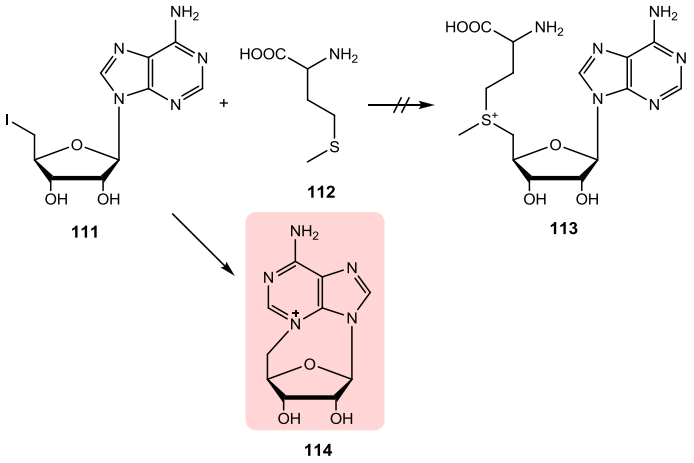
9.6 Synthesis of SAM and SAM analogs

9.6.1 Synthesis of SAM

Because SAM is very expensive also for the use in our enzyme assays, effort was made to find a synthesis for SAM that uses cheaper starting materials and still gives SAM in a moderate to good yield. In literature different attempts for the formation of sulfonium salts are described. Some of these methodologies are potentially capable for SAM synthesis starting from adenosine, 2´,3´-isopropylidene adenosine[224, 225], 5´deoxy-5´-iodoadenosine[226] or 5´deoxy-5´-chloroadenosine.[227] Adenosine and 2´,3´-isopropylidene adenosine were purchased, 5´-iodoadenosine and 5´-chloroadenosine were synthesized from adenosine.

The coupling reaction of adenosine and methionine in trifluoroacetic acid did not lead to product formation. Also the reaction of 2´,3´-isopropylidene adenosine and methionine in DMF acidified with trifluoroacetic acid did not give product formation.[228-230]

Different conditions for the reaction of 5´deoxy-5´-iodoadenosine as starting material with methionine were tested.[230] The starting material was partly consumed during the reaction, but no SAM formation was observed. The product which had formed was assumed to be 3-5´-cycloadenosine by HPLC-MS data.



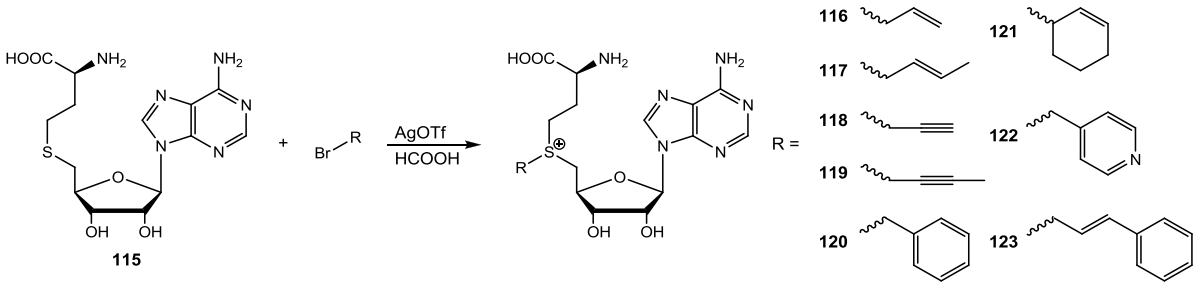
Scheme 30. 5´deoxy-5´-iodoadenosine did not react as desired to form SAM as a product. Instead 3,5´-cycloadenosine was supposed to form under the applied conditions according to HPLC-MS data.

9.6.2 Double activated cofactors

The synthesis of the double activated cofactors turned out to be rather simple. An excess of the alkyl bromide and SAH is stirred in formic acid.[77] It was found, that the addition of silver triflate significantly accelerates the reaction.[79] Independent from the employed bromide the reaction has reached completion not later than 48 h.

According to this method a series of cofactors (Scheme 31) was synthesized and tested with our enzymes.

Preparative HPLC was applicable for purification of the double activated cofactors.



Scheme 31. The SAM analogs which were synthesized and tested as cofactors.

Allyl, propynyl, butenyl, butynyl and benzyl analogs of SAM were accepted as alkyl donors by CouO and NovO. In contrast with 4-pyridinyl, benzylpropenyl and cyclohexenyl analogs no product formation could be detected with CouO and NovO as catalysts. Allyl, butynyl and benzyl were also transferred by SacF, SfmM2 and Orf19.

The addition of silver triflate has a beneficial effect on the formation of the double activated cofactors from the alkyl bromides. It is assumed that the formation of silver bromide which is

removed from the reaction by precipitation is a driving force to reach complete conversion. Also the back reaction is suppressed. In comparison to the reaction without addition of silver triflate the reaction is faster and higher conversion is achieved.

As proposed in literature it was found that also silver perchlorate and sodium perchlorate are promoting the formation of the SAM analogs.[231] But the application of perchlorates diminishes the activity of the cofactors. SAM that is synthesized with methyl iodide in the presence of perchlorate exhibits less methyl transfer activity than SAM with other counter ions.

It seems that also the species of the counterion of SAM and analogs influences the alkyl transfer reaction. In case of perchlorate as counter ion it can be assumed that the oxidative potential of the perchlorate ion has a deactivating effect on the enzyme.

9.6.3 Immobilisation of SAM

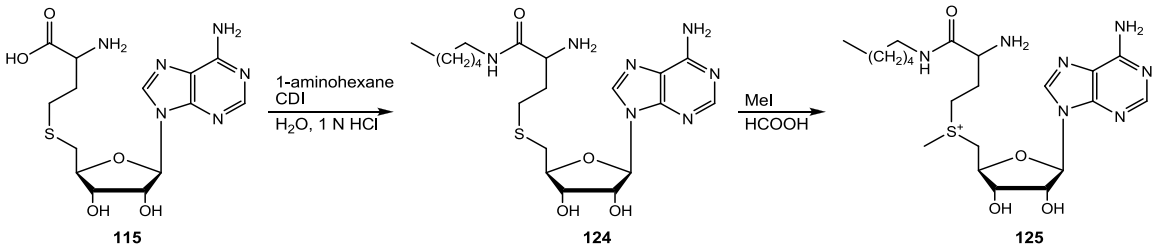
Also the immobilization of SAM was investigated. In comparison to the synthesis of SAM the immobilization has to conquer some additional problems. Immobilization of SAH is described in literature for affinity chromatography of methyltransferases.[232] In difference to the immobilization of SAH, we have to deal with the much lower stability of SAM. The influence of binding a linker to a functional group of SAM on the methyl transfer activity has not been fully cleared yet.

Another difficulty with the immobilization is the fact that since the starting material is linked to the bead, no direct reaction control is possible anymore. Also the solvents used in the reactions must be chosen with an eye on the stability of the bead and the swelling behaviour.

The acid function of the amino acid chain was chosen as target for the immobilization. SAM is attached to the bead by amide formation with an amine linked to the bead.

To try if the concept works, a reference compound was synthesized. Different synthetic strategies were worked out.

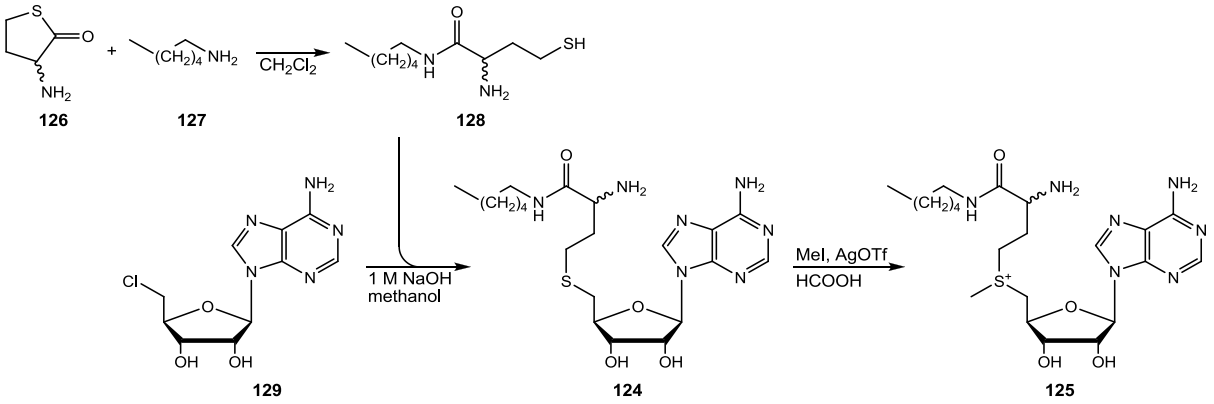
In literature carbonyl diimidazole (CDI) is described as amide coupling agent for unprotected α-amino acids and various aliphatic, aromatic and heteroaromatic amines.[233] The direct connection of 1-aminohexane to SAM or SAH using CDI as a coupling agent (Scheme 32) was investigated.



Scheme 32. Proposed synthesis of the hexylamide of SAM with SAH as a starting material.

Both in the reaction with SAM and SAH no product formation could be detected.
Also synthetic attempts to form *N*-hexylhomocysteine from homocysteine were not successful.

A new synthetic strategy was formulated (Scheme 33). Homocysteine thiolactone was used as starting material. For amide formation aminolytic ring opening with 1-aminohexane gave 2-amino-*N*-hexyl-4-mercaptobutanamide **128** as sole product.[234] This compound was then coupled with 5´-deoxy-5´-chloroadenosine **129** under basic conditions to give *N*-hexyl SAH amide **124**.[227] The methyl group was introduced with methyl iodide in formic acid promoted by silver triflate to give *N*-hexyl SAM amide **125**.[235]



Scheme 33. Synthetic route to *N*-hexyl SAM amide via ring opening substitution reaction for the formation of 2-amino-*N*-hexyl-4-mercaptobutanamide which was coupled with 5´-deoxy-5´-chloroadenosine followed by methylation.

The product was tested wether it is still active as a cofactor for the MTases CouO and NovO. A minimal residual methylation activity was assumed.

For immobilization a bead had to be applied that is able to swell both in organic and aqueous solvents. The bead also had to be stable upon drying as well to allow a change of the solvent. As potentially interesting beads α,o-diaminopolyethyleneglykols and Rapp TentaGel were identified.
With TentaGel the synthetic route was followed to the immobilized SAM as product.

9.7 Alternative Cofactors

The potential of aldehydes to act as a cosubstrate for CouO and NovO was investigated together with some proposed methyl donors. First experiments were performed under similar assay conditions regarding concentration of alkyl donor as applied by Klimasauskas and coworkers for the DNA MTases.[91]

Trimethylsulfonium (TMS) iodide, trimethylsulfoxonium (TMSO) iodide and *S*-methyl-DL-methionine (MeMet) chloride (Figure 44) were tested as methyl donors. Formaldehyde was tested for the hydroxymethylation.
The co-products of methyl transfer would be dimethyl sulfide, dimethyl sulfoxide (DMSO) and methionine respectively.

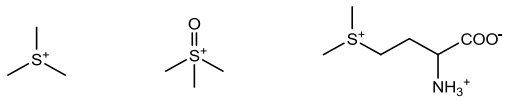


Figure 44. Proposed alternative methyl donors for SAM dependent MTases

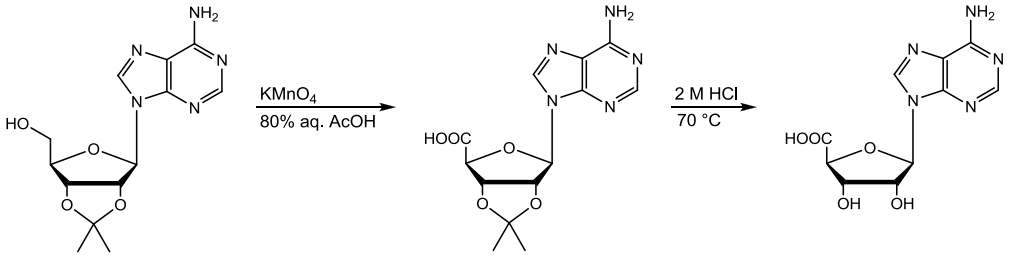
The methyl transfer activity assays were carried out either with the enzyme CouO or NovO. CouBz was the standard substrate for the assay. The activity assay was performed under the standard assay conditions described in the chapter materials and methods except that the alternative methyl donors were added instead of SAM and at higher concentrations. CouO and NovO were applied as cell preparations lysed via freeze/thaw method: The cell pellet was frozen in liquid N_2, after thawing the lysed cells were suspended in assay buffer.

In first experiments methylation of the substrates CouBz and CouPy was detected at a methyl donor concentration of 1 M. The experiments exhibited that formaldehyde was not accepted as a cofactor by the enzymes.
A solubility problem occurred in case of TMSO. The solubility of TMSO is limited in aqueous buffers to about 200 mM.

Different to TMS, TMSO and MeMet SAM is a highly functionalized substance which is bound to the enzyme in a very rigid and defined way. The methyl group is directed to the ideal position for nucleophilic attack of the substrate. In contrast TMS and TMSO do not offer moieties for recognition and binding in the SAM binding site. MeMet contains the amino acid functionalities, but it is dubious

if it is bound in the SAM binding site. Even if it is bound via the amino acid moieties, the methyl group on the sulfonium center is not fixed in the ideal position for methyl transfer.

It was aspired to find a substance that is able to bind in the SAM binding site and is able to expose a directing effect on the sulfonium center of the alternative methyl donors by Coulomb attraction. Adenosine-5´-carboxylic acid was found to be an interesting substance that possibly exhibits the desired behaviour.



130 131 132

Scheme 34. Synthesis of adenosine-5´-carboxylic acid.

The compound was synthesized and tested as additive to the alternative cofactors. The presence of adenosine-5´-carboxylic acid showed no effect on the methyl transfer.

Experiments were made to investigate the influence of the concentration of the methyl donors in a concentration range from 50 mM to 1 M. MeMet showed the highest conversion at a concentration of 100 mM while TMS and TMSO showed the highest conversion at 50mM, the lowest concentration that was tested in that series of experiments. Because of these results further experiments were carried out to elucidate the concentration dependence at low concentrations of the methyl donor in more detail. The combined results are depicted in Figure 45, Figure 46 and Figure 47. The different behaviour of TMS and TMSO on one hand and MeMet on the other hand could be demonstrated again.

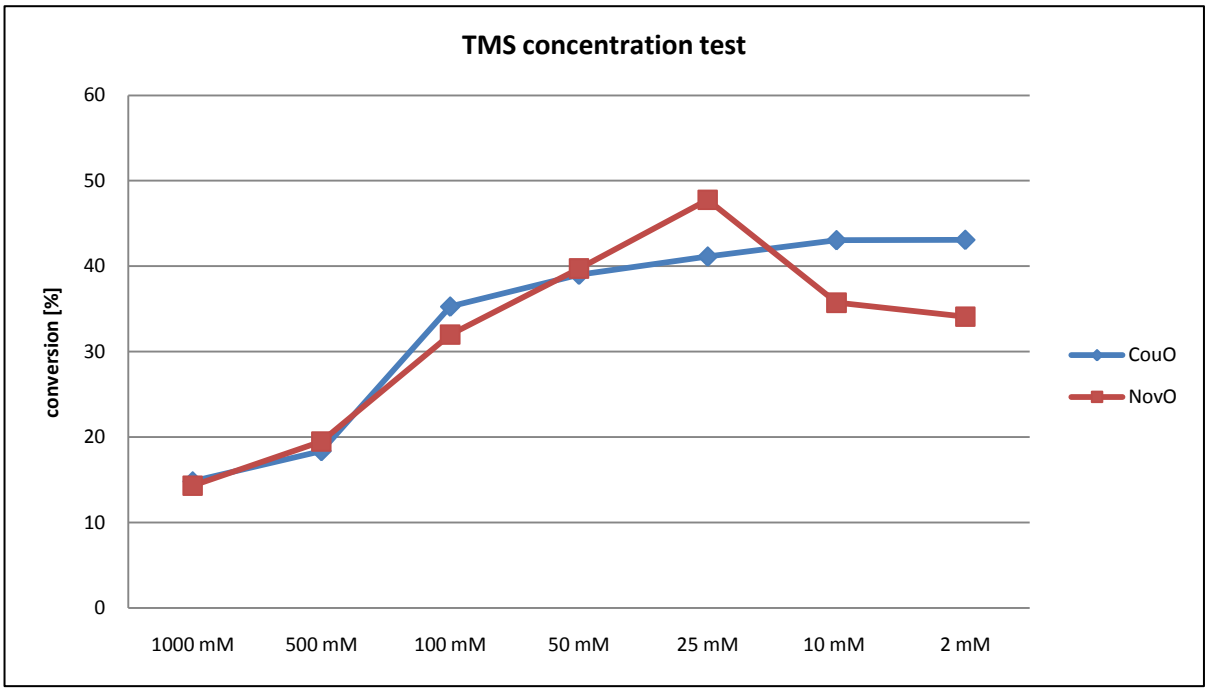


Figure 45. Concentration test with TMS as methyl donor. CouO and NovO are applied as cell lysates (freeze/thaw).

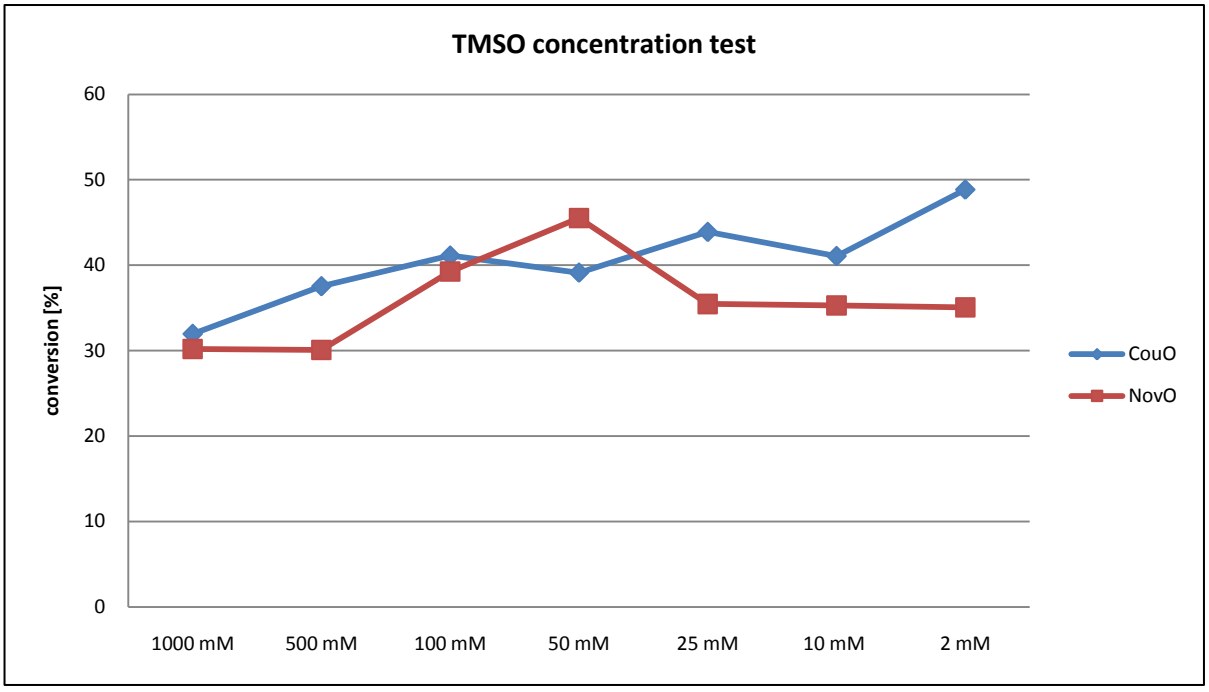


Figure 46. Concentration test with TMSO as methyl donor. CouO and NovO are applied as cell lysates (freeze/thaw).

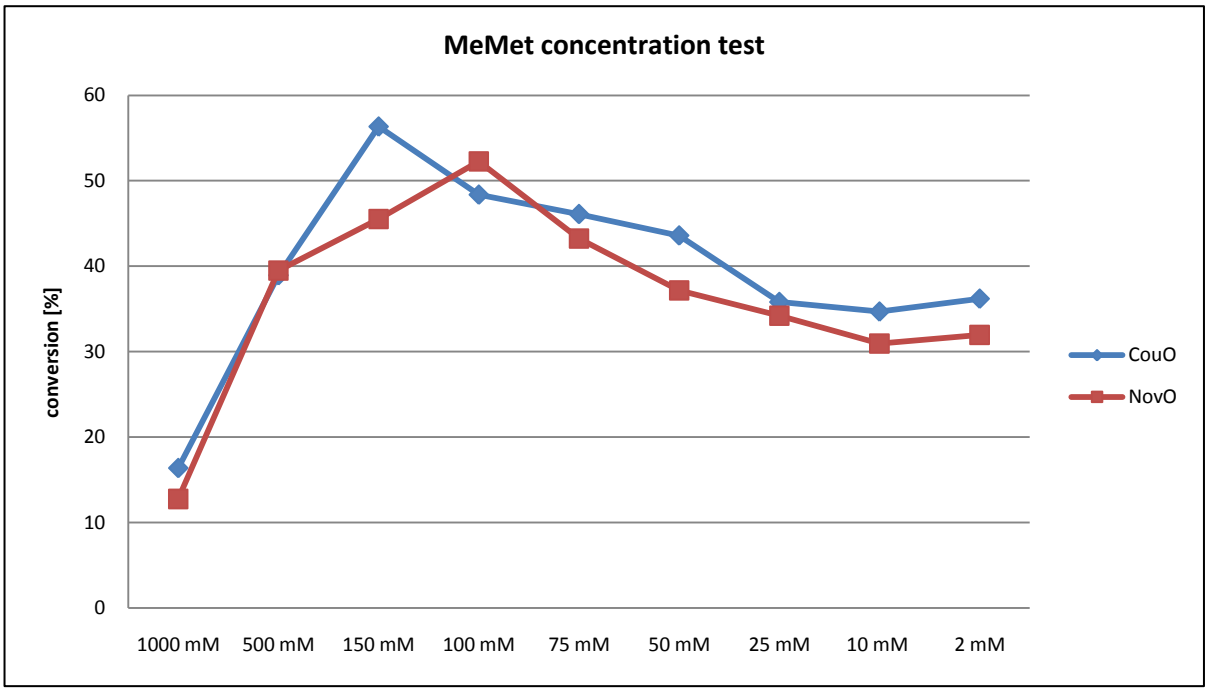


Figure 47. Concentration test with MeMet as methyl donor. CouO and NovO are applied as cell lysates (freeze/thaw).

CouO showed an increase of conversion with decreasing concentration of TMS and TMSO. With MeMet a concentration optimum at 100 mM concentration was observed.
NovO exposed a concentration optimum at 25 mM with TMS, 50 mM with TMSO and 150 mM with MeMet.

Based on these results the standard assay concentrations were chosen to be 20 mM for TMS and TMSO, 100 mM for MeMet both for CouO and NovO. 20 mM TMSO assay concentration was chosen because of the limited solubility of TMSO under assay conditions to ensure a good intercomparability between the experiments.

The background reaction was found to be stronger than expected, even without addition of a methyl donor (SAM or alternatives) product formation was observed. Nevertheless a higher conversion was found in the samples with methyl donor added.
In general MeMet gave better results than TMS and TMSO.

As already mentioned before MeMet is the natural substrate of the enzyme methyl methionine:homocysteine MTase which shows high similarity to betaine:homocysteine MTase.[113] Because of that betaine was considered to be a potential methyl donor for CouO and NovO. In an assay different concentrations of betaine were checked for their methylation activity with CouO and NovO (Figure 48).

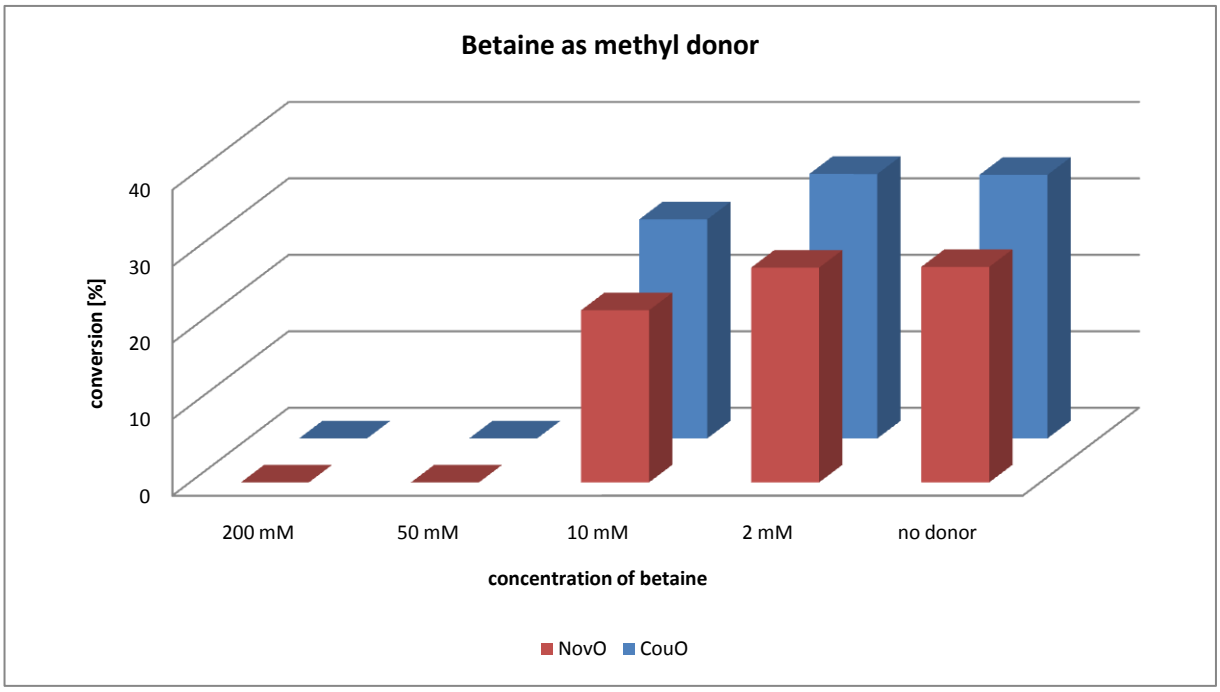


Figure 48. Conversion of CouBz to MeCouBz with CouO and NovO at different concentrations of betaine.

It was observed that betaine is poorly tolerated at higher concentrations by both CouO and NovO. At low concentrations the conversion was nearly the same as without methyl donor, but it seems that betaine does not act as a cofactor for CouO and NovO. No further experiments were carried out with betaine.

All above mentioned experiments were carried out with cell extracts lysed via freeze / thaw method. CouO and NovO crude lysates were also tested with the alternative methyl donors. The crude lysates were prepared via centrifugation after disruption of the cells by sonication.
Unfortunately no product formation could be observed with the alternative methyl donors when the enzymes were applied as crude lysates.

To check the influence of the presence of *E.coli* cell matrix towards the methyl transfer activity a special enzyme preparation was composed. *E.coli* cell pellet that contains an empty vector plasmid was disrupted by freeze/thaw method and suspended in CouO or NovO crude lysate.
CouO and NovO were tested under these conditions. The results are depicted in Figure 49 and Figure 50.

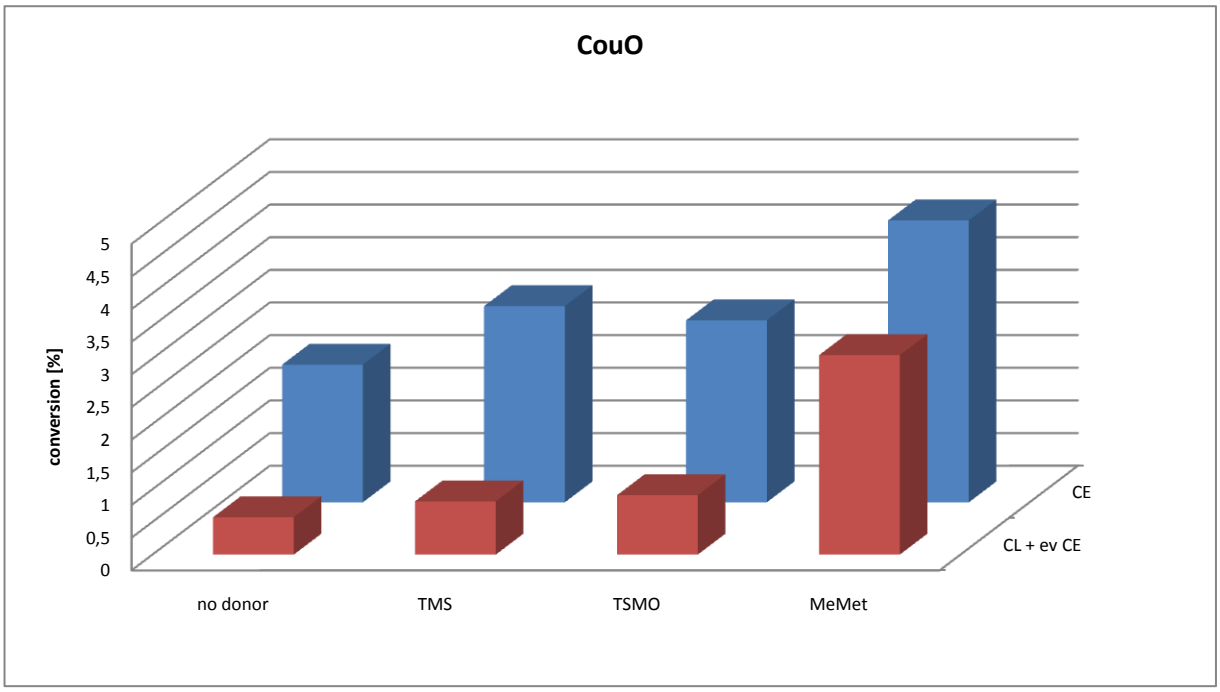


Figure 49. The conversion applying two enzyme preparation compositions of CouO in presence of the alternative methyl donors were compared. In blue the conversion with CouO cells disrupted by freeze/thaw method suspended in buffer, in red the conversion with *E.coli* cells containing an empty vector plasmid disrupted by freeze/thaw method suspended in CouO crude lysate is shown.

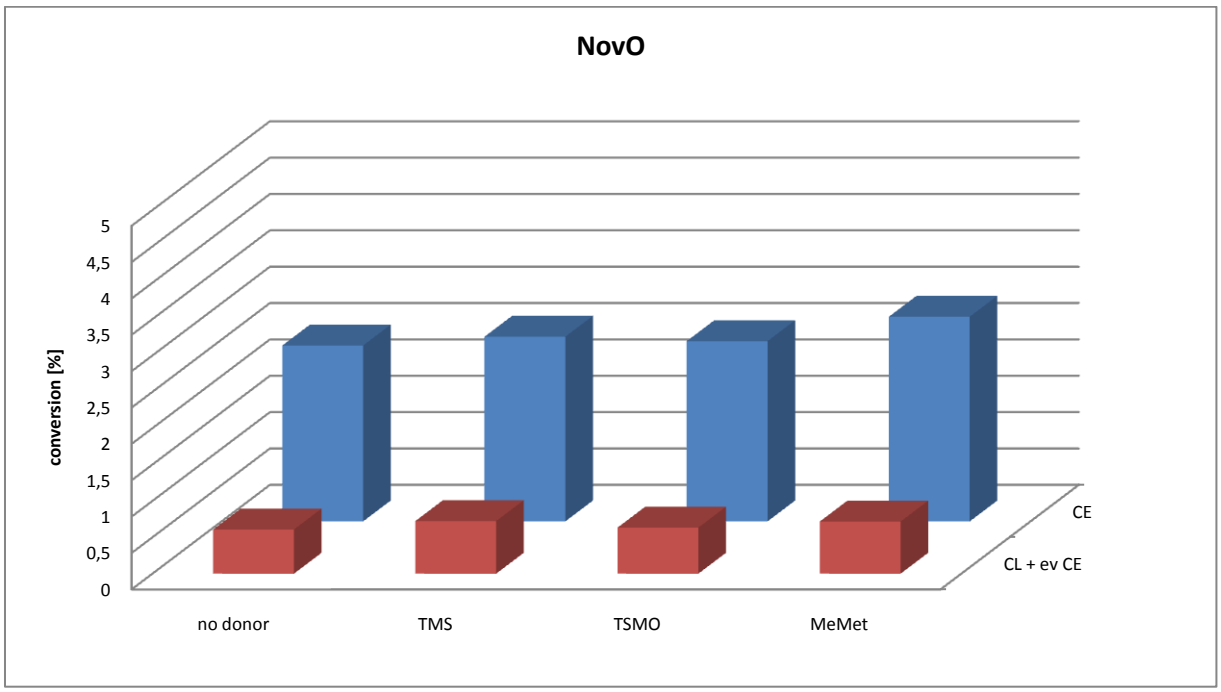


Figure 50. The conversion applying two enzyme preparation compositions of NovO in presence of the alternative methyl donors were compared. In blue the conversion with NovO cells disrupted by freeze/thaw method suspended in buffer, in red the conversion *E.coli* cells containing an empty vector plasmid disrupted by freeze/thaw method suspended in NovO crude lysate is shown.

It was found that the methyl transfer activity of the CouO/NovO crude lysate in presence of the alternative cofactors could be partly restored by addition of smoothly disrupted *E.coli* cells.

The applicability of the alternative methyl donors for the methylation of 2,7-dihydroxynaphthalene and 4,5,7-trihydroxy-3-phenylcoumarin was investigated. The results are shown in Figure 51 and Figure 52.

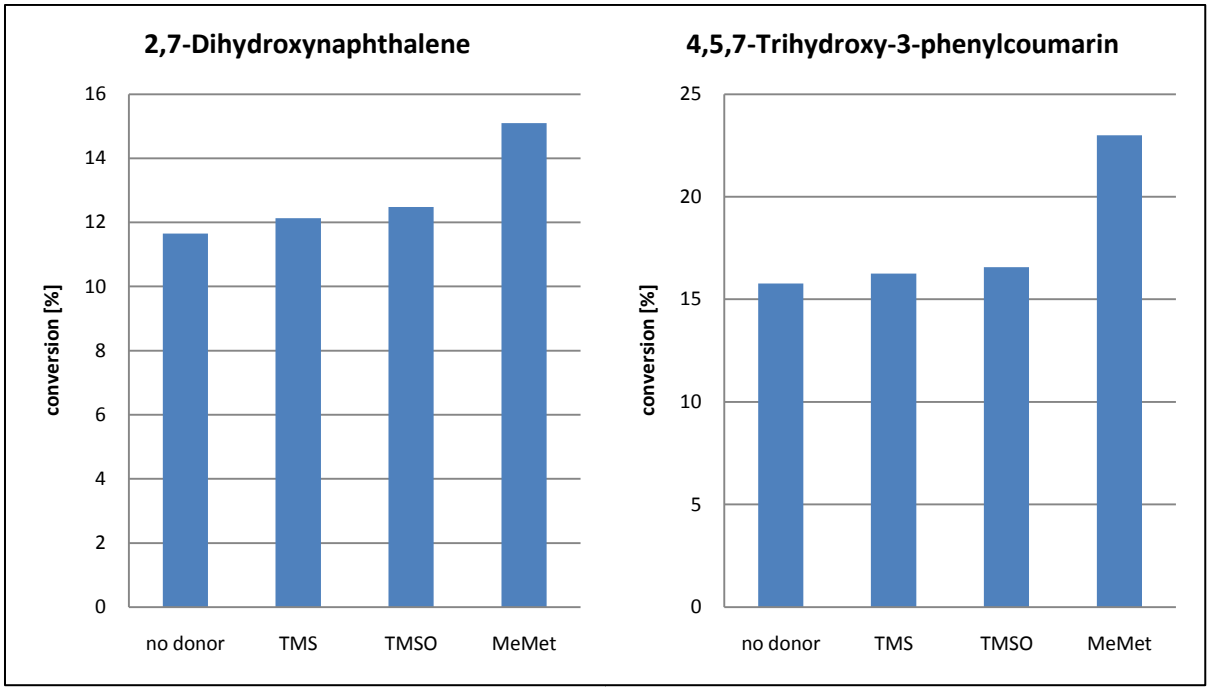


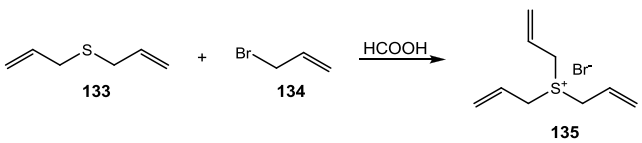
Figure 51: Activity of the alternative methyl donors with CouO as catalyst and 2,7-dihydroxynaphthalene as substrate.

Figure 52 Activity of the alternative methyl donors with CouO as catalyst and 4,5,7-Trihydroxy-3-phenyl-coumarin as substrate.

With both substrates a higher conversion was observed in presence of the alternative methyl donors compared to the background reaction without methyl donor added.

In order to extend the alkyl transfer reaction from methyl to other alkyl groups other sulfonium salts than methylated ones were tested. Due to this triallylsulfonium bromide was subjected to an alkyl transfer reaction.

Triallylsulfonium bromide **135** was synthesized from diallylsulfide **133** and allyl bromide **134** (Scheme 35).



Scheme 35. Synthesis of triallylsulfonium bromide

To explain the observations with triallylsulfonium bromide a short excursion to the possible mode of the methyl transfer is necessary.

The mode of methyl transfer with the alternative methyl donors is not clear. The methyl group can either be directly transferred or the methyl donor is used for the formation of SAM which then is used as methylating agent.

In a series of experiments it was tested if allyl bromide and triallylsulfonium bromide were able to introduce an allyl group into SAH under assay conditions, sodium phosphate buffer pH 7.0. If no SAH is alkylated under assay conditions, it is a hint against a transfer reaction via SAM or a SAM analog formation.

It was found that both allyl bromide and triallylsulfonium bromide were able to form allyl SAH under assay conditions, but at different levels.

With allyl bromide as an alkylating agent nearly the whole SAH was consumed and beside the desired product (at least) two byproducts appeared (Figure 53). These byproducts were not formed in the reaction in formic acid, the standard solvent for the formation of SAM analogs.

In contrast triallylsulfonium bromide led to the formation of only a small amount of product with a conversion of approximately 1 % after 18 h. In this case no byproduct formation was detected.

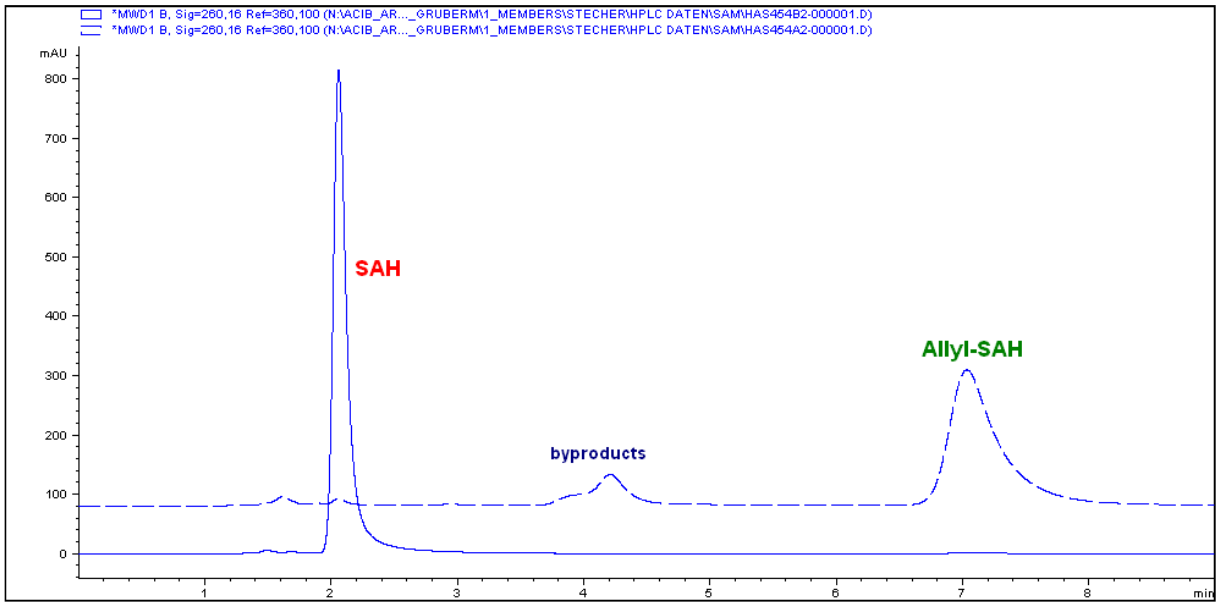


Figure 53. Chromatograms of the reaction of triallylsulfonium bromide (full line) and allyl bromide (dashed line) with SAH in 50 mM sodium phosphate buffer pH 7.0 after 18 h (Merck SeQuant™ ZIC®-HILIC column 150x2.1 mm, conditions: eluent 10 mM ammonium acetate pH 5.5 / MeCN 1:1, flow 0.3 ml min^{-1}, 30 °C. Peak at R_t 2.05 min is SAH, R_t 7.03 min peak shows the product allyl-SAH)

The chromatogram (Figure 53) shows the UV trace recorded at 260 nm. 260 nm was chosen because the adenine bicycle exhibits an absorption maximum at that wavelength. The shouldered peak at R_t

4.21 min comes from two different compounds with detected masses of 364.1 m/z and 465.2 m/z. The supposed byproducts with corresponding masses are shown in Figure 54.

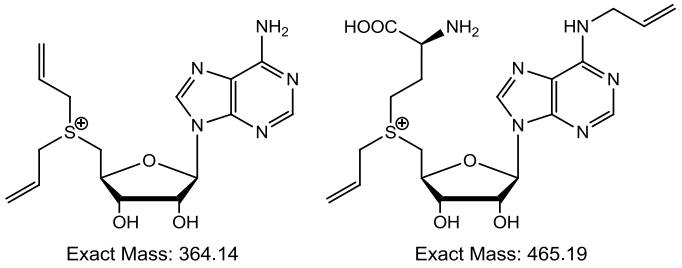


Figure 54. Proposed structures of the byproducts which occured in the reaction of allyl bromide with SAH in aqueous sodium phosphate buffer pH 7.0.

If triallylsulfonium bromide is added as alkyl donor, according to the prior results a small amount of allyl SAH should be formed under assay conditions. In spite of this no product formation could be detected. It was assumed that under assay conditions the alkylation power of triallylsulfonium bromide is sufficient for the allylation of the enzyme. This would cause deactivation of the enzyme.

The methylation of SAH under formation of SAM in formic acid was described in literature using TMS as methylating agent.[236] Nevertheless with TMS, TMSO and MeMet no formation of SAM from SAH was observed under assay conditions.

A comparing study between MeMet and methionine showed that whereas MeMet increased the overall conversion methionine in the same concentration exhibited an inhibitory effect.

In order to check the influence of SAH present in the reaction mixture on the methylation level with the alternative methyl donors as cofactors, experiments at different SAH concentrations were carried out with CouO as catalyst. SAH was subjected to the reaction mixture in 0.1 mM and 1 mM concentration, also reference data without addition of SAH were collected. The results are depicted in Figure 55.

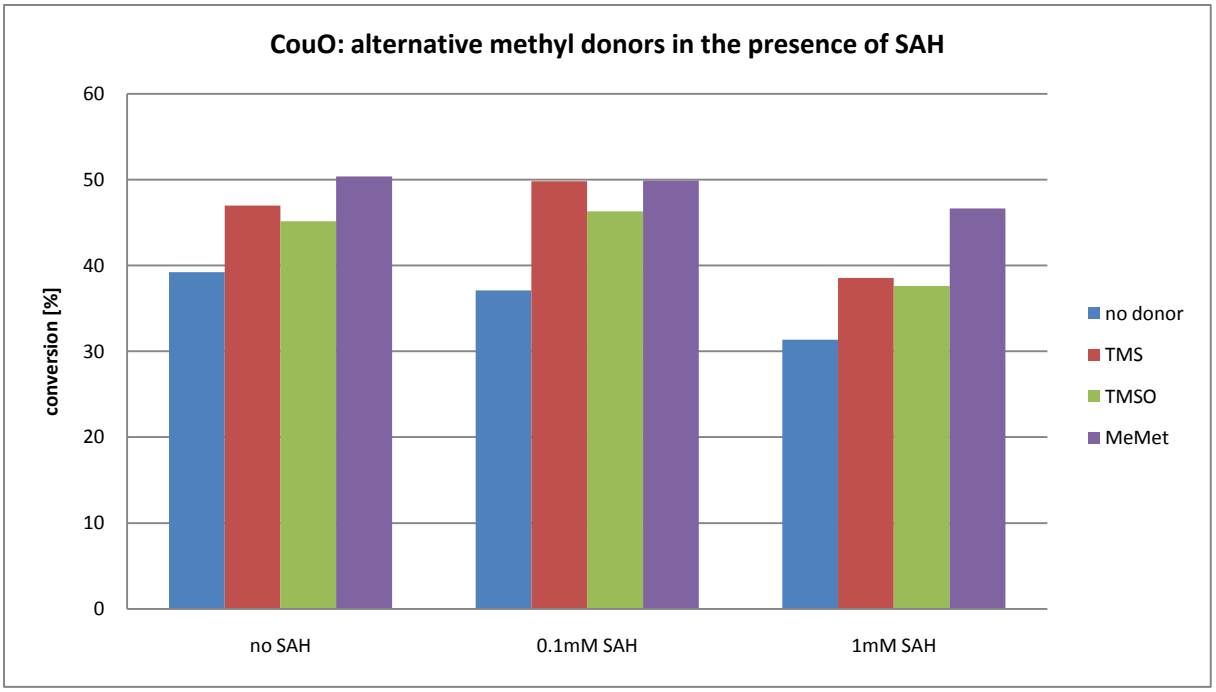


Figure 55. Influence of the addition of different concentrations of SAH to the methylation reaction with the alternative methyl donors as cofactors and CouO as catalyst.

SAH seems to have little influence on the product formation at 0.1 mM concentration, at a concentration of 1 mM an inhibitory effect occurs. This observation promotes the assumption of a methylation mechanism of the alternative methyl donors without participation of SAH.

The influence of the presence of SAM in the methylation reaction with the alternative methyl donors was investigated as well. SAM was added to the reaction mixture in 0.1 mM and 0.5 mM concentration and compared to the reactions without addition of SAM. The results of this assay are summarized in Figure 56.

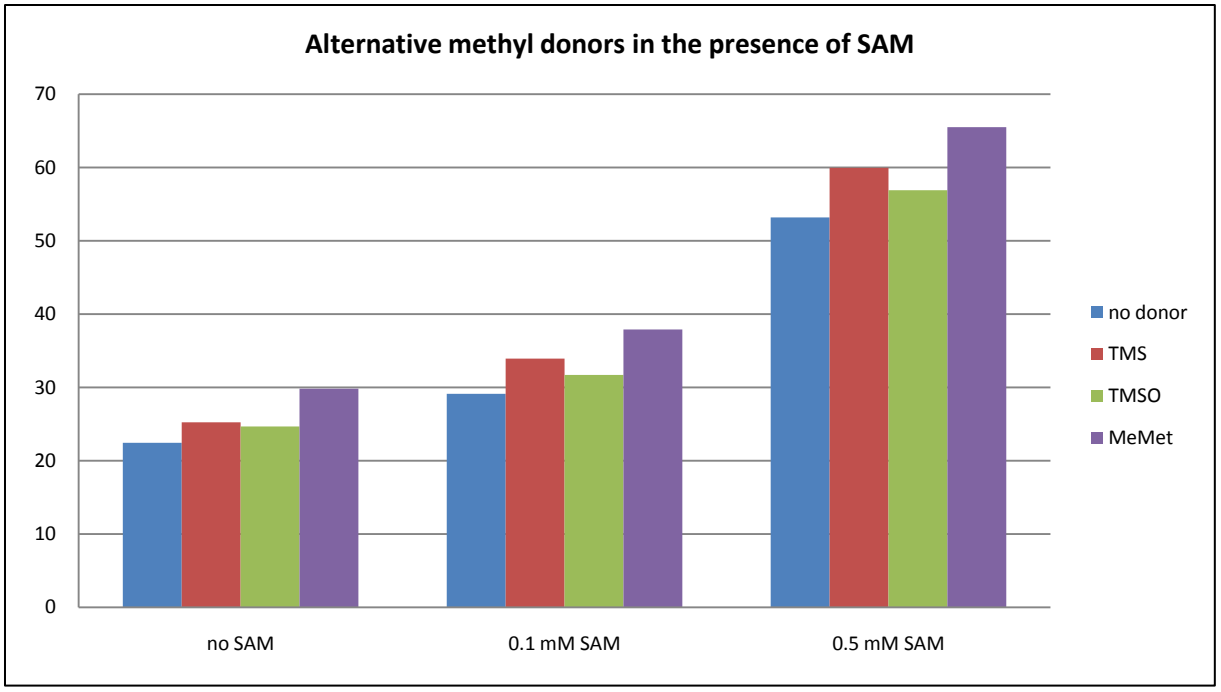


Figure 56. In the diagram the conversion with CouO applying the alternative methyl donors and different SAM concentrations is shown.

In Figure 56 the contribution of the alternative methyl donors to the overall conversion at different SAM concentrations present is depicted more clearly. The conversion detected in the reference reaction without addition of a methyl donor was substracted from the conversion observed in the reaction with alternative methyl donors present.

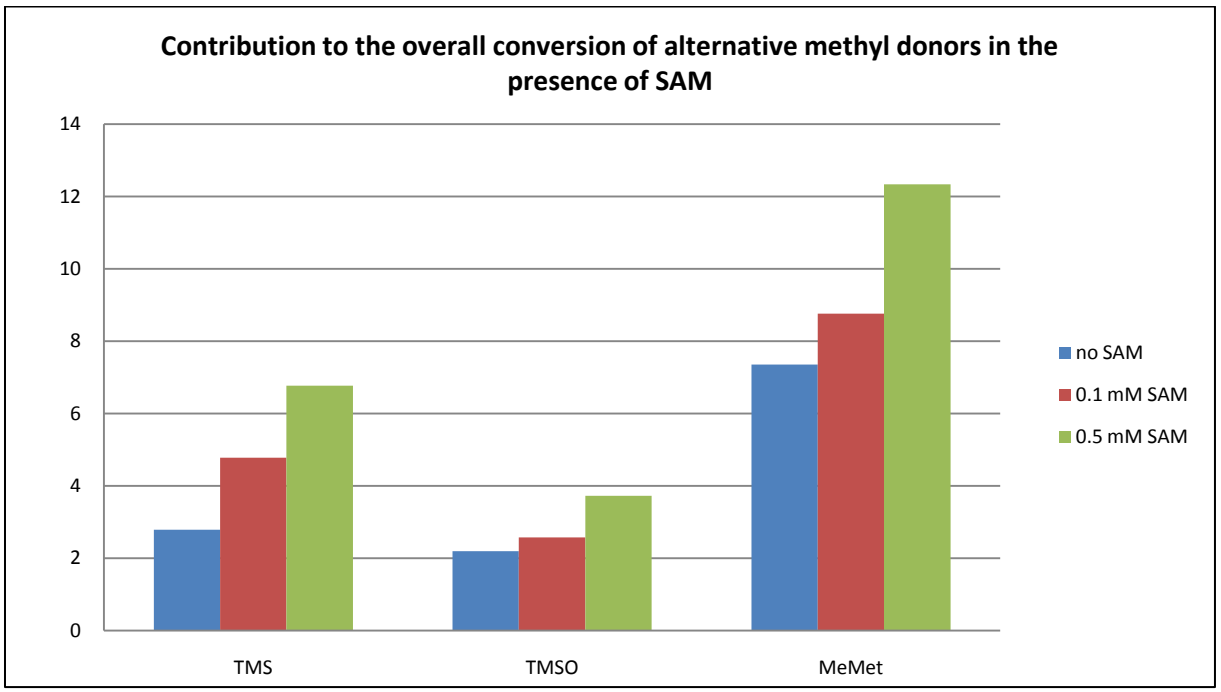


Figure 57. The contribution of the alternative methyl donors to the overall product formation with CouO as a catalyst is shown. The conversion detected without addition of an alternative methyl donor is substracted from the conversion detected in the presence of an alternative methyl donor.

Apparently the presence of SAM is beneficial for the methylation reaction with the alternative methyl donors. With increasing SAM concentration also an increase of product formation is observed in the reactions with one of the three alternative methyl donors in comparison to the reactions without addition of an alternative methyl donor. MeMet led to the best results with a contribution of up to 14 % to the overall conversion with 0.5 mM SAM present.

Motivated by these results, the conversion with CouO crude lysate in the presence of 0.5 mM SAM was investigated. The results are shown in Figure 58.

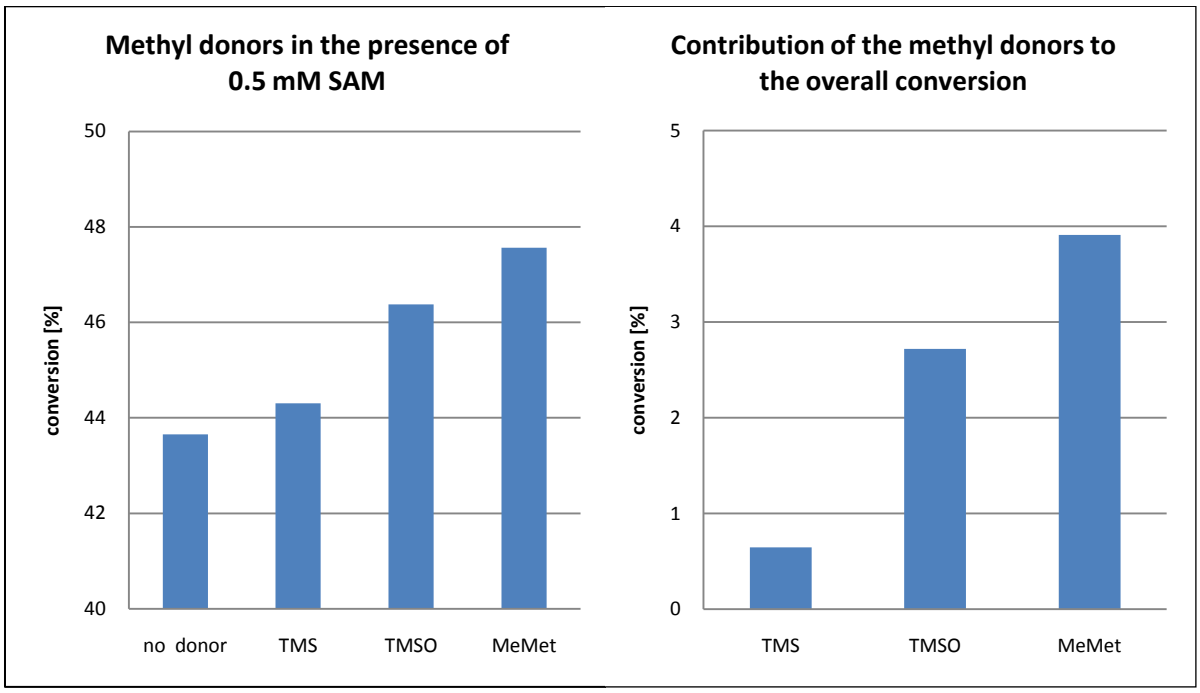


Figure 58. Conversion of CouBz with CouO crude lysate using the alternative methyl donors at 0.5 mM SAM present.

Figure 59. The contribution of the alternative methyl donors to the overall conversion in the reaction of CouBz with CouO crude lysate in the presence of 0.5 mM SAM.

In line with the former results no conversion could be detected in the absence of SAM with CouO crude lysate also when alternative methyl donors were added. When SAM was present in the reaction together with the alternative methyl donors, a slight increase of the conversion could be observed. To highlight the contribution of the alternative methyl donors to the overall conversion in presence of 0.5 mM SAM the difference between the background reaction in the absence of alternative methyl donors and the conversion in the presence of them is depicted in Figure 59.

The enhancement in product formation with CouO crude lysate by addition of alternative methyl donors in the presence of 0.5 mM SAM is between 1 and 4 percent. As observed in all other experiments MeMet gave the best results.

It is assumed that the presence of SAM promotes the correct folding for substrate activation. The activated substrate then attacks a methyl group of the alternative methyl donor.

9.8 Catechol-*O*-MTase

Because of its availability COMT was chosen as a representative enzyme of the *O*-MTases to test its ability to accept alternative methyl donors for methyl transfer.

As substrate L-3,4-dihydroxyphenylalanine (L-dopa) was chosen for activity experiments, but unfortunately L-dopa only gave low conversion around 2 %. Furthermore dopa was not stable under assay conditions and a huge quantity of the L-dopa was lost and could not be detected in the analyzed samples. Nevertheless first tests for finding suitable assay conditions concerning pH and buffer were carried out with L-dopa as a substrate (Figure 60, Figure 61).

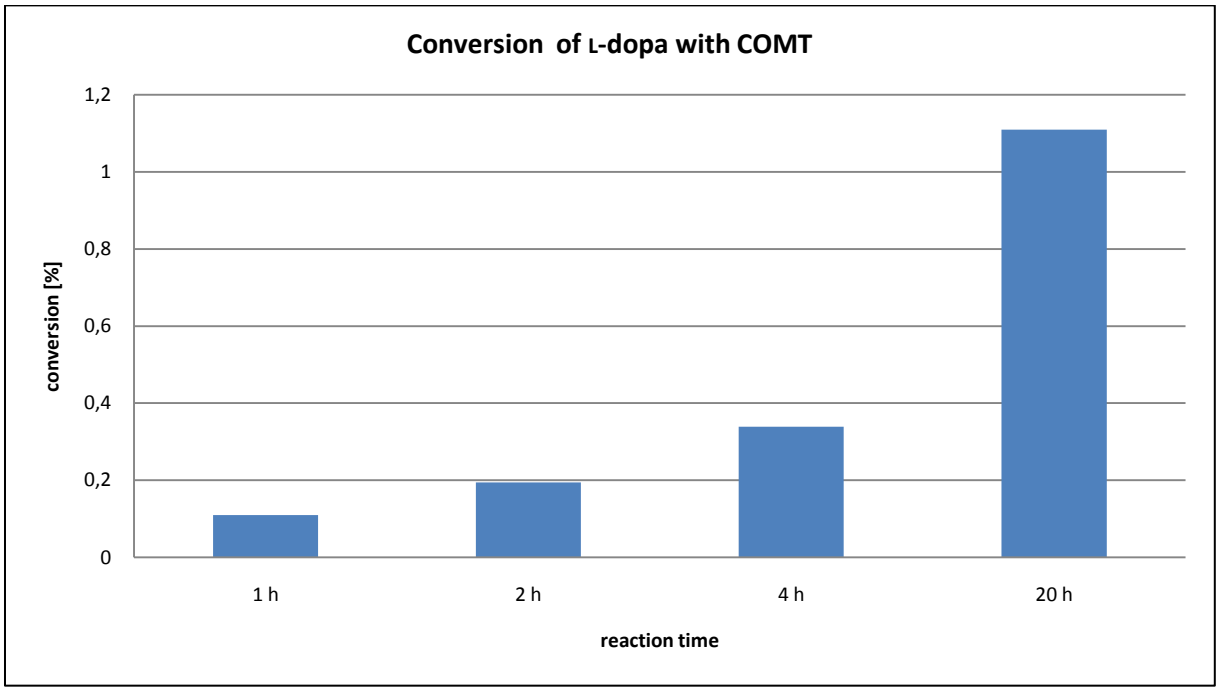


Figure 60. Conversion of L-dopa after 1 h, 2 h, 4 h and 20 h, assay conditions: TrisHCl buffer pH 8.0.

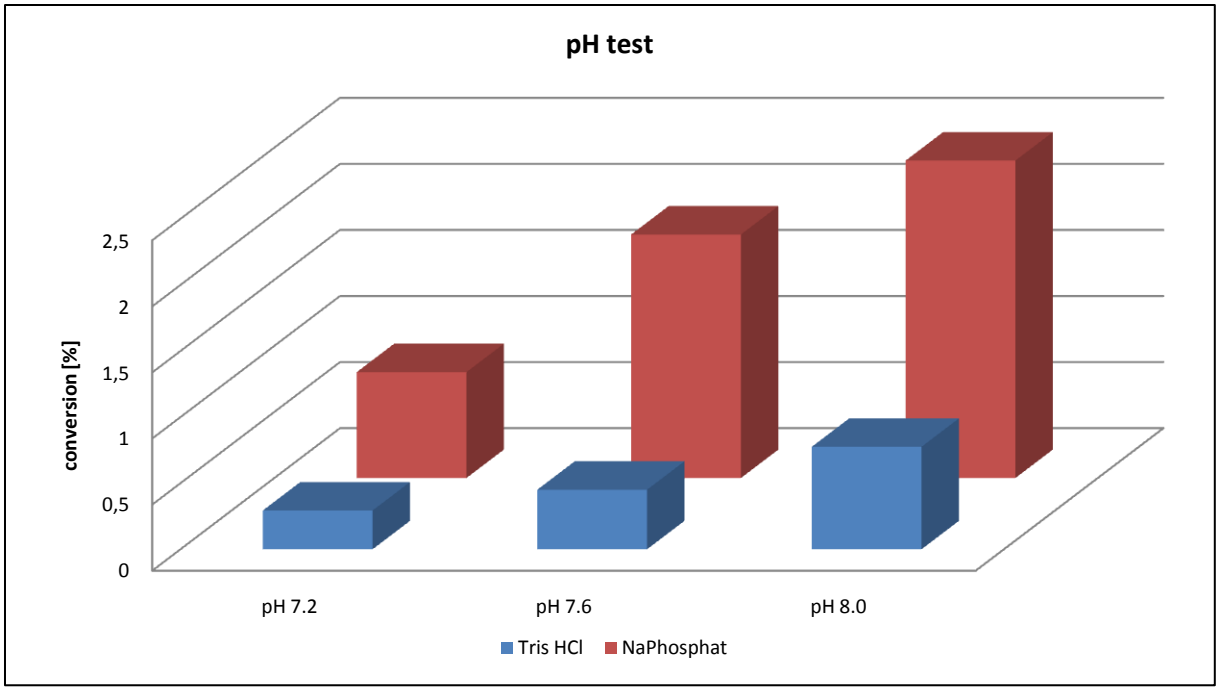


Figure 61. Rough pH and buffer test with L-dopa as a substrate.

The conversion was determined with HPLC-MS using SIM mode of MS at the mass [M+H] of the product. Commercially available reference material was used for calibration.

Because dopa turned out to be unstable under assay conditions some other probable substrates for COMT were tested. The best conditions determined in the preliminary experiments were applied for the following assays. From a list of already characterized substrates [35, 237] four compounds were chosen which were available in-house. The substrates investigated are depicted in Figure 62.

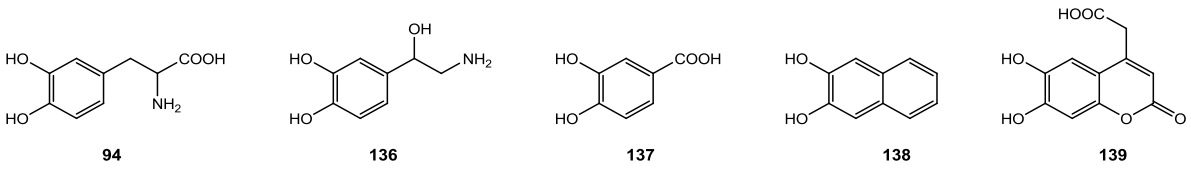


Figure 62. Structure of the compounds tested with COMT (from left to right: L-dopa, adrenaline, 3,4-dihydroxybenzoic acid (DHBA), 2,3-dihydroxynaphthaline, 6,7-dihydroxycoumarin-4-ylacetic acid (DHCAA))

Adrenaline and 2,3-dihydroxynaphthalene showed no activity, poor conversion was again observed with L-dopa. With DHBA and DHCAA about 10 % conversion was observed. DHCAA was picked for the following experiment because of the characteristic UV-absorption maximum at 352 nm.

In the reaction with the alternative methyl donors TMS, TMSO and MeMet the HPLC analysis showed the evolution of a new peak with TMS and TMSO that did not correspond to the desired product. The unknown product had a shorter retention time than the substrate. Also the HPLC trace of the mass scan did not indicate formation of the desired product. Interestingly the unidentified product was

formed in the blank reaction to an even larger extend as in the enzymatic reactions. This is an indication for an uncatalysed chemical reaction.

To avoid the formation of unwanted side products, the assay to test the alternative methyl donors was repeated with DHBA as a substrate. As an example the chromatogram of the enzymatic formation of 4-hydroxy-3-methoxybenzoic acid (HMBA) from DHBA with SAM as cofactor is depicted in Figure 63.

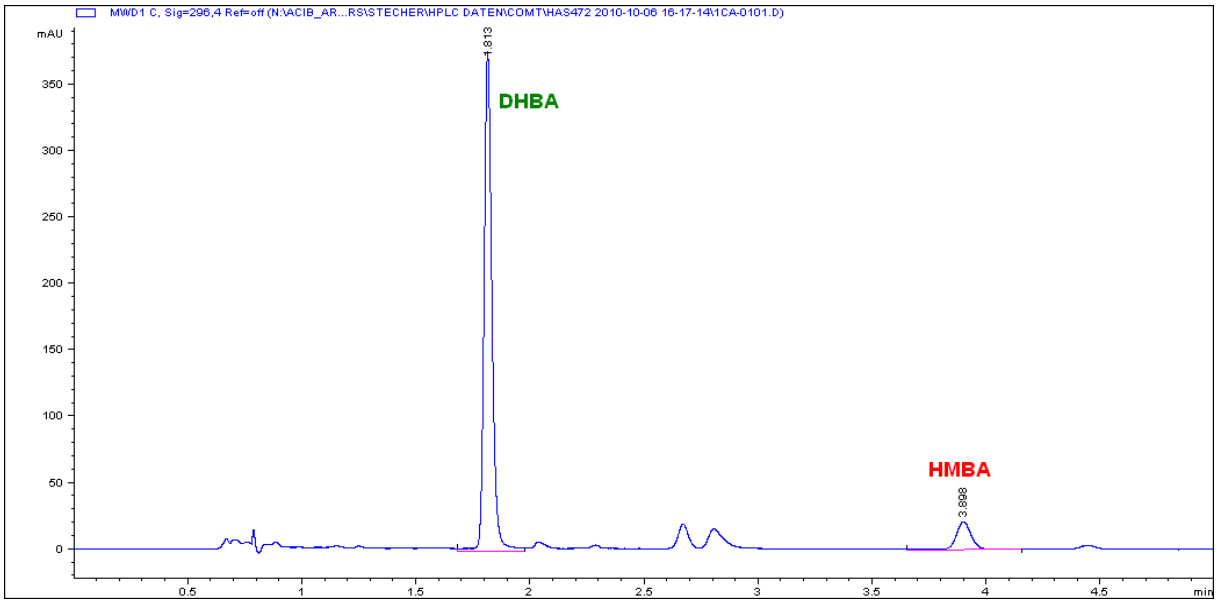


Figure 63. Chromatogram at 296 nm of COMT activity assay with DHBA as a substrate. Method: Agilent Poroshell® C_{18} ec 100*3 mm, 2.7 µm; 0.1% formic acid / MeOH 80:20; 0.7 ml min^{-1}; 25 °C; R_t DHBA = 1.81 min, HMBA = 3.90 min.

In this series of reactions no product formation was detected with any of the alternative methyl donors and no side products evolved. Also the addition of *E. coli* crude lysate or *E. coli* cells lysed by freeze / thaw method did not provoke product formation with the alternative methyl donors as methyl source.

10 Conclusion

A biocatalytic variant of the Friedel-Crafts reaction could be introduced. Methyltransferases were identified as suitable catalysts. Besides methyl transfer the enzymes were able to transfer various alkyl chains from synthetically prepared double activated cofactors.

A new methodology for an absolutely regioselectie alkylation of bicyclic aromatic systems was discovered. With CouO and NovO two enzymes were found that offer a broad substrate acceptance. Diverse substances are alkylated regioselectively. Even with symmetrical substrates no dimethylation is observed. Experiments in a preparative scale always led to single product formation.
SacF, SfmM2 and ORF19 only were able to accept a narrow substrate scope in comparison to CouO and NovO. The cofactor specificity of the three enzymes is lower, the enzymes were able to transfer larger groups than methyl from double activated cofactors. The natural substrates of SacF, SfmM2, ORF19 and SibL, L-tyrosine and DL-hydroxykynurenine respectively, participate in classic metabolic pathways. It can be assumed that a precise substrate recognition is necessary to avoid damage to the organism.
In contrast the natural substrates for CouO and NovO do not occur in common metabolism. Exact recognition of the natural substrate is not important, the substrate and the product are only employed for aminocoumarin antibiotics biosynthesis.

New reactions for the alkylation of tyrosine and 3-hydroxykynurenine were presented. Double activated cofactors are accepted and can be applied for alkyl transfer.

Tryptophan methylation is observed with all microorganisms that produce antibiotics containing a quinaldic acid moiety. No other substrate than the natural substrate is accepted by the enzyme from *Streptomyces lautentii*. Also no cofactor analogs are accepted for alkyl transfer.

Recently the transfer of an acetonyl group by application of the acetonyl analog of SAM as a cofactor was described with COMT and three *S*-MTases.[81] In near future this cofactor will be synthesized and tested with the enzymes covered in this work. Also other cofactors that contain a *C*-hetero atom multiple bond will be synthesized and tested.

A first step in substitution of SAM by cheaper methyl donors could be accomplished.
The alternative methyl donors TMS, TMSO and MeMet contribute to the overall conversion. MeMet gives better results than TMS and TMSO. Addition of SAH to the reaction mixture shows no beneficial

effect, in contrast the presence of SAM increases the amout of product that can be dedicated to the alternative methyl donors.

An easy way to modulate the alkylation potential of alkyl sulfonium salts is to change the substitution pattern as the results already indicate. It is not possible to detect formation of SAM from SAH with TMS, TMSO or MeMet under assay conditions, in contrast to this finding triallylsulfonium bromide is able to allylate SAH under formation of allyl-SAH, but triallylbromide seems to be not compatible with our biocatalysts.

It would be of great interest to test the alternative methyl donors in presence of a potent inhibitor of SAM dependent MTases. Many MTases are known to undergo a conformational change when SAM is bound into the SAM binding site. The idea is that the inhibitor binds into the SAM binding site in such a way as SAM that the conformational change occurs and the conformation is fixed by the inhibitor. Sinefungin is an interesting candidate to be tested as inhibitor.[238-240] It shows great structural similarity to SAM.

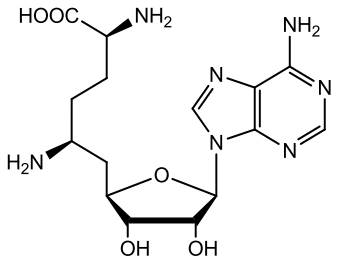


Figure 64. Structure of sinefungin

In literature in spite of the similarity to SAM, the binding mode of Sinefungin in the SAM binding site is mentioned to be similar with SAH, but not SAM, in the DNA MTases on which these results base.[241]

11 Chromatographic methods:

HPLC columns:	referred to in the following tables as
Agilent Poroshell® C_{18} ec 100*3mm, 2.7µm	Poroshell
Agilent Zorbax® XDB C_{18} ec 50*4.6 mm, 1.8 µm	Zorbax
Macherey Nagel Nucleodur® C_{18} ec 150x4 mm, 5 µm	Nucleodur
Macherey Nagel Nucleosil® C_4 150*4mm, 5 µm	Nucleosil
Merck Chromolith® C_{18} ec 100*4.6 mm	Chrom4.6
Merck Chromolith® C_{18} ec 100*3 mm	Chrom3
Merck Chromolith® C_{18} ec 100*2 mm	Chrom2
Merck Purospher® star C_{18} ec 250*4 mm, 5 µm	Purospher
Merck SeQuant™ ZIC®-HILIC 150*2.1 mm, 3.5 µm	HILIC

Buffer A: ammonium acetate 10 mM

Buffer B: sodium acetate 20mM pH7.0 (not compatible with HPLC-MS)

Buffer C: 0.1 % formic acid

Buffer D: 0.01 % trifluoroacetic acid

Buffer E: NH_3/NH_4^+ pH 8.5

Table 14. Chromatographic methods for the substrates of CouO and NovO; Rt = retention time; S = substrate; P = methylated product.

substrate	Column	Method			Rt [min]	
		eluent	Flow [ml min^{-1}]	T [°C]	S	P
24	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	19.8	
25	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	5.1	
26	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	30	7.3	11.9
	Chrom3	Buffer A pH 5.5/MeCN 96:4	1.5	40	1.8	3.9
	Chrom2	Buffer A pH 5.5/MeCN 97:3	0.7	40	1.7	4.0
	Nucleodur	Buffer E /MeOH 90:10	1.0	25	3.72	
27	Purospher	Buffer A pH 6.0/MeCN 80:20	0.6	40	4.2	4.8
	Chrom4.6	Buffer A pH 5.5/MeCN 95:5	2.0	40	2.6	
	Chrom3	Buffer A pH 5.5/MeCN 95:5	1.2	40	2.4	4.7
	Chrom2	Buffer A pH 5.5 /MeCN 95:5	0.7	40	2.3	4.3

	Nucleodur	Buffer A pH 6.8/MeCN 90:10	1.0	25	2.1	3.1
	Poroshell	Buffer A pH 6.8/MeOH 90:10	0.5	40	1.4	1.8
28	Chrom3	Buffer A pH 5.5/MeCN 96:4	1.5	40	1.8	3.8
29	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	4.8	
30	Purospher	Buffer A pH 5.5/MeCN 80:20	0.6	40	8.2	9.5
	Chrom2	Buffer A pH 5.5/MeCN 90:10	0.7	40	2.0	3.0
31	Chrom3	Buffer A pH 5.5/MeCN 99:1	1.5	40	1.5	2.2
38	Chrom3	Buffer A pH 5.5/MeCN 70:30	1.5	40	0.6	
39	Chrom3	Buffer A pH 5.5/MeCN 96:4	1.5	40	0.7	
41	Chrom2	Buffer A pH 5.5/MeCN 70:30	0.7	40	2.8	4.3
42	Chrom3	Buffer A pH 5.5/MeCN 93:7	1.5	40	1.9	
43	Chrom3	Buffer A pH 5.5/MeCN 70:30	1.5	40	3.3	4.7
44	Chrom3	Buffer A pH 5.5/MeCN 70:30	1.5	40	2.8	
45	Chrom3	Buffer A pH 5.5/MeCN 80:20	1.5	40	4.5	7.4
46	Chrom3	Buffer A pH 5.5/MeCN 93:7	1.5	40	1.7	3.0
47	Purospher	Buffer A pH 6.0/MeCN 70:30	0.6	40	7.3	
	Chrom3	Buffer A pH 5.5/MeCN 93:7	1.5	40	3.9	
48	Chrom3	Buffer A pH 5.5/MeCN 93:7	1.5	40	1.9	
49	Chrom3	Buffer A pH 5.5/MeCN 93:7	1.5	40	2.2	
50	Chrom3	Buffer A pH 5.5/MeCN 70:30	1.2	40	1.6	
51	Chrom3	Buffer A pH 5.5/MeCN 70:30	1.5	40	5.2	
52	Chrom3	Buffer A pH 5.5/MeCN 98:2	1.5	40	2.2	
53	Chrom2	Buffer A pH 5.5/MeCN 88:12	0.7	40	2.9	3.3
54	Chrom3	Buffer A pH 5.5/MeCN 90:10	1.5	40	2.8	3.0
55	Chrom3	Buffer A pH 5.5/MeCN 90:10	1.5	40	2.3	5.4
56	Chrom3	Buffer A pH 5.5/MeCN 90:10	1.5	40	7.3	9.1
57	Chrom3	Buffer A pH 5.5/MeCN 90:10	1.5	40	4.3	5.4
	Chrom2	Buffer A pH 5.5/MeCN 90:10	0.7	40	2.5	3.1
58	Chrom3	Buffer A pH 5.5/MeCN 65:35	1.5	40	3.3	6.0
59	Chrom3	Buffer A pH 5.5/MeCN 75:25	1.5	40	3.2	5.6
60	Chrom3	Buffer A pH 5.5/MeCN 90:10	1.5	40	1.2	
68	Chrom3	Buffer A pH 5.5/MeCN 98:2	1.5	40	1.8	4.4
69	Chrom2	Buffer A pH 5.5/MeCN 93:7	0.7	40	2.7	
70	Chrom2	Buffer A pH 5.5/MeCN 80:20	0.7	40	2.2	

71	Purospher	Buffer A pH 5.0/MeOH 70:30	0.6	40	3.0	
72	Chrom3	Buffer A pH 5.5/MeCN 75:25	1.5	40	2.1	5.2
73	Chrom2	Buffer A pH 5.5/MeCN 85:15	0.7	40	2.0	
74	Chrom3	Buffer A pH 5.5/MeCN 96:4	1.5	40	2.8	
75	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	4.3	5.2
	Poroshell	Buffer A pH 6.8/MeOH 95:5	0.7	25	1.7	4.2
	Nucleodur	Buffer A pH 6.8/MeOH 90:10	1.0	25	2.1	3.8
76	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	13.8	
77	Purospher	Buffer A pH 6.0/MeCN 60:40	0.6	40	7.7	
78	Chrom3	Buffer A pH 5.5/MeCN 98:2	1.5	40	1.2	

Table 15. Chromatographic methods for the substrates of SacF and SfmM2; Rt = retention time; S = substrate; P = methylated product.

substrate	Column	Method			Rt [min]	
		eluent	Flow [ml min^{-1}]	T [°C]	S	P
75, 91, 93	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	4.3	5.2
	Poroshell	Buffer A pH 6.8/MeOH 95:5	0.7	25	1.7	4.2
	Nucleodur	Buffer A pH 6.8/MeOH 90:10	1.0	25	2.1	3.8
25	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	5.1	
77	Purospher	Buffer A pH 6.0/MeCN 60:40	0.6	40	7.7	
94	Poroshell	Buffer A pH 6.8/MeOH 90:10	0.3	50	1.9	2.5
95	Poroshell	Buffer A pH 6.8/MeOH 90:10	0.3	50	4.8	
96	Purospher	Buffer A pH 5.0/MeOH 85:15	0.6	40	4.2	
97	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	4.6	
98	Purospher	Buffer A pH 5.0/MeOH 85:15	0.6	40	3.9	
99	Purospher	Buffer A pH 5.0/MeOH 85:15	0.6	40	3.2	
100	Nucleodur	NH4OAc pH6.8 / MeOH 98:2 for 1.5 min, in 3.5 min to 75:25, hold for 0.9 min, in 0.1 min to 98:2, hold for 2 min	1.0	40	3.7	5.5
	Poroshell	Buffer A pH 6.8/MeOH 90:10	0.3	50	2.0	3.1
101	Chrom3	Buffer A pH 5.5/MeCN 99.9:0.1	1.5	40	0.7	
102	Chrom3	Buffer A pH 5.5/MeCN 99.9:0.1	1.5	40	0.7	

Table 16. Chromatographic methods for the substrates of ORF19 and -SibL; Rt = retention time; S = substrate; P = methylated product.

substrate	Column	Method			Rt [min]	
		eluent	Flow [ml min^{-1}]	T [°C]	S	P
100	Nucleodur	NH4OAc pH6.8 / MeOH 98:2 for 1.5 min, in 3.5 min to 75:25, hold for 0.9 min, in 0.1 min to 98:2, hold for 2 min	1.0	40	3.7	5.5
	Poroshell	Buffer A pH 6.8/MeOH 90:10	0.3	50	2.0	3.1
	Chrom3	Buffer A pH 5.5/MeCN 99.9:0.1	1.5	40	0.7	1.4
101	Chrom3	Buffer A pH 5.5/MeCN 99.9:0.1	1.5	40	0.7	
102	Chrom3	Buffer A pH 5.5/MeCN 99.9:0.1	1.5	40	0.7	
91, 93	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	40	4.3	5.2
	Poroshell	Buffer A pH 6.8/MeOH 95:5	0.7	25	1.7	4.2
	Nucleodur	Buffer A pH 6.8/MeOH 90:10	1.0	25	2.1	3.8
94	Poroshell	Buffer A pH 6.8/MeOH 90:10	0.3	50	1.9	2.5
95	Poroshell	Buffer A pH 6.8/MeOH 90:10	0.3	50	4.8	

Table 17. Chromatographic methods for the substrates of COMT; Rt = retention time; S = substrate; P = methylated product.

substrate	Column	Method			Rt [min]	
		eluent	Flow [ml min^{-1}]	T [°C]	S	P
94	Nucleodur	Buffer C/MeOH 95:5	1.0	25	2.1	4.9
136	Nucleodur	Buffer A pH6.8/MeOH 95:5	1.0	25	2.0	
137	Nucleodur	Buffer C/MeOH 70:30	1.0	25	2.7	5.0
	Poroshell	Buffer C/MeOH 80:20	0.7	25	1.8	3.9
138	Nucleodur	Buffer C/MeOH 40:60	1.0	25	2.8	
139	Nucleodur	Buffer C/MeOH 65:35	1.0	25	3.1	4.8

Table 18. Chromatographic methods for the substrates of tryptophan 2´-*C*-MTase; Rt = retention time; S = substrate; P = methylated product.

substrate	Column	Method			Rt [min]	
		eluent	Flow	T [°C]	S	P

			[ml min^{-1}]			
1	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	1.5	1.8
3	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	1.6	
4	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	2.6	
5	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	1.7	
6	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	5.3	
7	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	4.0	
8	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	2.6	
9	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	2.5	
10	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	1.6	
11	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	1.0	
12	Zorbax	Buffer A pH 5.0/MeOH 70:30	0.6	40	4.5	

Table 19. Chromatographic methods for the alkylation transfer reactions; Rt = retention time; S = substrate; P = alkylated product.

substrate	Column	Method			Rt [min]	
		eluent	Flow [ml min^{-1}]	T [°C]	S	P
81	Chrom3	Buffer A pH 5.5/MeCN 100:0 for 1 min, in 0.5 min to 95:5, hold for 2.5 min, in 2 min to 0:100, hold for 2 min	1.5	40	2.8	5.5
82	Chrom3	Buffer A pH 5.5/MeCN 100:0 for 1 min, in 0.5 min to 95:5, hold for 2.5 min, in 2 min to 0:100, hold for 2 min	1.5	40	3.7	5.6
83	Purospher	Buffer A pH 5.0/MeOH 70:30	0.5	35	5.9	18.5

84	Chrom2	Buffer A pH 5.5/MeCN 97:3 for 3 min, in 2 min to 100:0, hold for 5 min	0.7	40	1.6	5.3
85	Chrom2	Buffer A pH 5.5/MeCN 95:5 for 3 min, in 2 min to 100:0, hold for 5 min	0.7	40	2.1	5.4
86	Chrom2	Buffer A pH 5.5/MeCN 97:3 for 3 min, in 2 min to 100:0, hold for 5 min	0.7	40	1.6	5.6
87	Chrom2	Buffer A pH 5.5/MeCN 95:5 for 3 min, in 2 min to 100:0, hold for 5 min	0.7	40	2.1	5.6
89	Chrom2	Buffer A pH 5.5/MeCN 90:10 for 3 min, in 2 min to 100:0, hold for 5 min	0.7	40	4.8	5.6
allylTyr	Nucleodur	Buffer A pH 6.8/MeCN 95:5	1.0	25	1.9	9.6
butynyl-Tyr	Nucleodur	Buffer A pH 6.8/MeOH 90:10 for 2 min, in 2 min to 30:70, hold for 2 min	1.0	25	2.1	5.1
benzylTyr	Nucleodur	Buffer A pH 6.8/MeOH 90:10 for 2 min, in 2 min to 30:70, hold for 2 min	1.0	25	2.1	5.5
allylHKyn	Nucleodur	Buffer A pH 6.8/MeCN 95:5	1.0	25	2.1	13.7
butynyl-HKyn	poroshell	Buffer A pH 6.8/MeOH 90:10 for 2 min, in 1 min to 30:70, hold for 2 min	0.4	40	1.5	4.5
benzyl-HKyn	poroshell	Buffer A pH 6.8/MeOH 90:10 for 2 min, in 1 min to 20:80, hold for 3 min	0.4	40	1.5	4.6

Table 20. Chromatographic methods for SAH, SAM, SAM analogs and substances in the formation of *N*-hexyl SAM; Rt = retention time.

analyte	Column	Method			Rt [min]
		eluent	Flow [ml min^{-1}]	T [°C]	

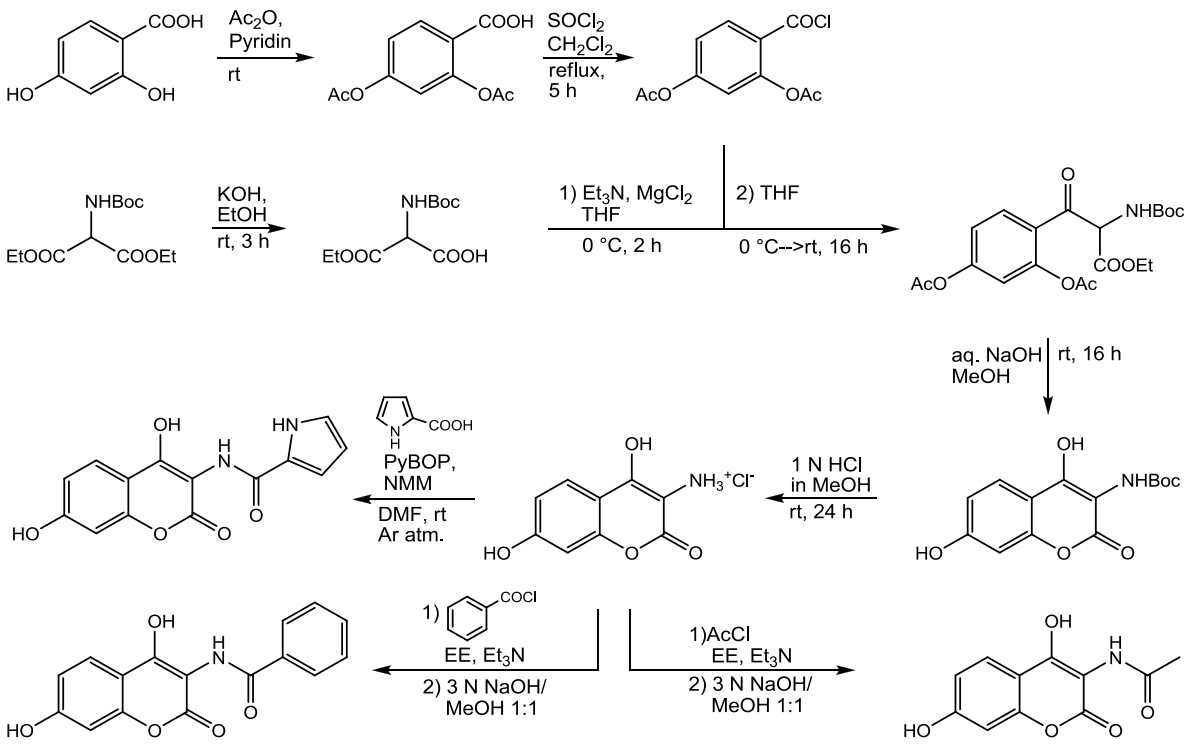
115	Chrom3	Buffer A pH5.5/MeCN 100:0 for 2 min, to 95:5 in 0.5 min, hold	1.2	40	3.9
	Chrom2	Buffer A pH5.5/MeCN 100:0 for 1 min, to 95:5 in 0.5 min, hold	0.7	40	3.4
	Nucleodur	Buffer C/MeOH 95:5	1.0	25	2.1
	HILIC	Buffer A pH 5.5/MeCN 50:50	0.3	25	2.1
113	Purospher	Buffer A pH 6.0/MeOH 70:30	0.5	30	3.8
116	Chrom2	Buffer A pH5.5/MeCN 100:0 for 1 min, to 95:5 in 0.5 min, hold	0.7	40	1.1
	Nucleodur	Buffer D	0.5	25	4.7; 5.2
	HILIC	Buffer A pH 5.5/MeCN 50:50	0.3	25	7.0
117	Chrom3	Buffer A pH5.5/MeCN 100:0 for 2 min, to 95:5 in 0.5 min, hold	1.2	40	1.1
118	Chrom3	Buffer A pH5.5/MeCN 100:0 for 2 min, to 95:5 in 0.5 min, hold	1.2	40	2.2
119	Chrom2	Buffer A pH5.5/MeCN 100:0 for 1 min, to 95:5 in 0.5 min, hold	0.7	40	3.9
128	Chrom3	Buffer A pH 5.5/MeCN 95:5	1.5	40	2.4
129	Nucleodur	Buffer C/MeOH 80:20	1.5	40	2.9
	Chrom3	Buffer A pH5.5/MeCN 100:0 for 1.5 min, to 95:5 in 0.5 min, hold for 1.5 min, to 90:10 in 0.5 min, hold for 1.5 min, to 80:20 in 2 min, hold	1.5	40	4.9
124	Nucleosil	H_2O deion./MeOH 95:5	1.0	40	3.7
	Chrom3	Buffer A pH5.5/MeCN 100:0 for 1.5 min, to 95:5 in 0.5 min, hold for 1.5 min, to 90:10 in 0.5 min, hold for 1.5 min, to 80:20 in 2 min, hold	1.5	40	8.3
125	Chrom3	Buffer A pH5.5/MeCN 100:0 for 1.5 min, to 95:5 in 0.5 min, hold for 1.5 min, to 90:10 in 0.5 min, hold for 1.5 min, to 80:20 in 2	1.5	40	7.3

		min, hold			

12 Experimentals

NMR data are reported in ppm using the solvent as an internal standard (s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, br = broad). All coupling constants are given as *J* in Hz.

Synthesis of *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide, *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)acetamide and *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-pyrrol-2-carboxamide



2,4-diacetoxybenzoic acid[211, 212]

To a stirred and light protected solution of 5.0 g 2,4-dihydroxybenzoic acid (32 mmol, 1 eq) in 30 ml pyridine 15 ml acetic anhydride (16 g, 160 mmol, 5 eq) and 0.40 g DMAP (3.2 mmol, 0.1 eq) were added. After 24 h ice was added to the reaction mixture and it was acidified with aqueous 3 N HCl. The resulting suspension was extracted three times with ethyl acetate. The organic layers were

pooled and washed once with brine. After drying over Na_2SO_4 the solvent was removed until about 15 ml remained, which were allowed to crystallise. The resulting crystals were collected and dried, further product was crystallized from the mother lye. In total 7.2 g (93 %) off-white crystals were collected. ^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 13.12 (br, 1H, -COOH), 7.98 (d, *J* = 8.6 Hz, 1H, -CH_{ar}-), 7.18 (dd, J_1 = 8.6, J_2 = 2.3 Hz, 1H, -CH_{ar}-), 7.09 (d, *J* = 2.2 Hz, 1H, -CH_{ar}-), 2.29 (s, 3H, -CH_3), 2.25 (s, 3H, -CH_3). ^{13}C (75 MHz) δ ppm: 168.97, 168.61, 164.99, 154.01, 151.05, 132.47, 121.63, 119.65, 117.62, 20.84, 20.80.

2,4-diacetoxybenzoyl chloride[212]

2.0 g 2,4-diacetoxybenzoic acid (8.4 mmol, 1 eq) was solved in 20 ml CH_2Cl_2. 12 ml $SOCl_2$ (20 g, 165 mmol, 20 eq) was added to the stirred solution. The mixture was heated to reflux for 5 h, then CH_2Cl_2 and $SOCl_2$ were distilled off. The resulting brown solid was used directly for the next step and not further characterized.

2-(*tert*-butoxycarbonylamino)-3-ethoxy-3-oxopropanoic acid[215]

To a suspension of 4.5 g KOH (80 mmol, 1 eq) in 100 ml EtOH 20 ml diethyl 2-(*tert*-butoxycarbonylamino)propandioate (22 g, 80 mmol, 1 eq) was added. The mixture was stirred for 3 h, then about ninety percent of the solvent was removed. 150 ml of aqueous 1 M $NaHCO_3$ were added and washed twice with ethyl acetate to remove the unreacted educt. The solution was cooled to 0 °C, acidified with $KHSO_4$ and extracted three times with ethyl acetate. The combined organic layers were washed once with brine and dried over Na_2SO_4. The solvent was evaporated in vacuo (bath temperature 20 °C). The resulting oily residue was dried in vacuo to give 14 g (73 %) of a white powder. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 7.73 (d, *J* = 4.4 Hz, 0.6H, -NH-), 5.65 (d, *J* = 6.8 Hz, 0.4 H, -NH-), 4.98 (d, *J* = 7.3 Hz, 0.4H, -CH-), 4.77 (d, *J* = 4.9 Hz, 0.6H, -CH-), 4.23-4.31 (m, 2H, -CH_2-), 1.42 (s, 9H, 3 –CH_3), 1.31 (t, *J* = 7.3 Hz, 3H, -CH_3). ^{13}C (125 MHz) δ ppm:168.51, 166.86, 156.35, 82.95, 62.53, 58.90, 28.33, 14.31.

4-(2-(*tert*-butoxycarbonylamino)-3-ethoxy-3-oxopropanoyl)-1,3-phenylene diacetate[217]

To a ice-cooled solution of 2-(*tert*-butoxycarbonylamino)-3-ethoxy-3-oxopropanoic acid (2.9 g, 12 mmol, 1.4 eq) in dry THF (30 ml) 7.4 ml triethylamine (5.4 g, 53 mmol, 6.3 eq) and 2.7 g $MgCl_2$ (28 mmol, 3.4 eq) were added and the resulting slurry was stirred vigorously for 2 h. Then crude 2,4-diacetoxybenzoyl chloride (theor. 8.4 mmol, 1 eq) in 50 ml dry THF was added and the resulting

suspension was stirred overnight at room temperature. The reaction mixture was quenched with saturated aqueous NH_4Cl until the suspension cleared. The solution was extracted three times with ethyl acetate. The combined organic layers were dried over Na_2SO_4. Removal of the solvent yielded 4.3 g (121 %) of dark-brown oil, which was used for the next step without further purification.

tert-butyl 4,7-dihydroxy-2-oxo-2*H*-chromen-3-ylcarbamate[217]

The crude 4-(2-(*tert*-butoxycarbonylamino)-3-ethoxy-3-oxopropanoyl)-1,3-phenylene diacetate (4.3 g) was dissolved in 24 ml MeOH and 30 ml 1.5 N NaOH was added. The solution was stirred for 3 h at room temperature. Then the solution was acidified with 1 N HCl to pH 3 and extracted three times with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent was removed under vacuum. The crude product was purified with column chromatography to give 1.2 g (50 % over three steps) of an orange solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 7.78 (s, 1H, -NH-), 7.64 (d, J = 8.8 Hz, 1H, -CH_{ar}-), 6.77 (dd, J_1 = 8.8, J_2 = 2.0 Hz, 1H, -CH_{ar}-), 6.66 (d, J = 2 Hz, 1H, -CH_{ar}-), 1.40 (s, 9H, -CH_3). ^{13}C NMR (125 MHz) δ ppm: 166.38, 161.94, 161.63, 155.40, 154.11, 125.68, 113.48, 108.71, 102.51, 100.80, 79.25, 28.86.

3-amino-4,7-dihydroxy-2*H*-chromen-2-one hydrochloride[217]

To a cooled mixture of 25 ml Et_2O and 5 ml MeOH 4.3 ml acetyl chloride was carefully added. This acidic mixture was poured onto a stirred and cooled solution of 1.2 g *tert*-butyl 4,7-dihydroxy-2-oxo-2*H*-chromen-3-ylcarbamate in 25 ml Et_2O and 5 ml MeOH (1 N HCl in the reaction mixture). The solution was allowed to stir for 24 h at room temperature. The precipitate formed was collected and dried to give 0.52 g (53 %) light brown solid. ^{1}H NMR (500MHz, DMSO-*d6*) δ ppm: 7.96 (d, J = 8.8 Hz, 1H, -CH_{ar}-), 6.84 (dd, J_1 = 8.8 Hz, J_2 = 2.0 Hz, 1H, -CH_{ar}-), 6.75 (d, J = 2.0 Hz, 1H, -CH_{ar}-). ^{13}C NMR (125 MHz) δ ppm:162.71, 161.45, 160.37, 154.19, 126.44, 113.86, 108.73, 102.95, 95.49.

N-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide

To a stirred solution of 200 mg 3-amino-4,7-dihydroxy-2*H*-chromen-2-on hydrochloride (0.87 mmol, 1 eq) in 10 ml ethyl acetate 0.51 ml benzoyl chloride (0.61 g, 4.4 mmol, 5 eq) and 1.2 ml triethylamine (0.88 g, 8.7 mmol, 10 eq) were added. The suspension was stirred overnight at room temperature. After removal of the solid by filtration the solvent was removed under reduced pressure. The resulting residue was dissolved in 10 ml MeOH and 10 ml 1 N NaOH was added. The reaction mixture was stirred for 24 h, subsequently the mixture was acidified with 1 N HCl to pH 3

and extracted three times with ethyl acetate. The combined organic layers were washed once with brine and dried over Na_2SO_4. Celite was added and the solvent was removed *in vacuo*. The resulting fine brown powder was used for purification with column chromatography to give 243 mg (94%) of an orange solid. ^{1}H NMR (500 MHz, DSMO-*d6*) δ ppm: 11.82 (s, 1H, -OH), 10.56 (s, 1H, -OH), 9.42 (s, 1H, -NH-), 8.00 (d, J = 7.3 Hz, 2H, -CH_{ar}-), 7.72 (d, J = 8.8 Hz, 1H, -CH_{ar}-), 7.58 (t, J = 7.3 Hz, 1H, -CH_{ar}-), 7.51 (t, J = 7.6 Hz, 2H, -CH_{ar}-), 6.83 (dd, J_1 = 8.8 Hz, J_2 = 2.0 Hz, 1H, -CH_{ar}-), 6.74 (d, J = 2.4 Hz, 1H, -CH_{ar}-). ^{13}C (125 MHz) δ ppm: 166.57, 161.46, 160.83, 160.40, 153.54, 133.93, 131.61, 128.19, 128.02, 125.07, 112.99, 107.97, 101.94, 100.10.

N-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)acetamide

To a stirred solution of 100 mg 3-amino-4,7-dihydroxy-2*H*-chromen-2-on hydrochloride (0.44 mmol, 1.0 eq) in 1 ml DMF 0.038 ml pyridine (38 mg. 0.48 mmol, 1.1 eq) and 0.041 ml acetic anhydride (45 mg, 0.44 mmol, 1.0 eq) were added. After 19 h further 0.082 ml acetic anhydride (89 mg, 0.88 mmol, 2 eq) and 0.12 ml pyridine (11 mg, 1.5 mmol, 3.3 eq) were added and the reaction mixture was stirred for 5 h. 1.0 ml MeOH and 0.44 ml NaOMe were added and the mixture was stirred overnight. Then the solution was diluted with ethyl acetate and acidified with 1 N HCl. After separation of the phases the aqueous phase was extracted twice with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and the solvent was removed. The resulting residue was dried *in vacuo* to give 68 mg (66 %) of an off-white solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 12.06 (s, 1H, -OH), 10.51 (s, 1H, -OH), 9.30 (s, 1H, -NH-), 7.66 (d, J = 8.8 Hz, 1H, -CH_{ar}-), 6.79 (dd, J_1 = 8.8, J_2 = 2.0 Hz, 1H, -CH_{ar}-), 6.68 (d, J = 2 Hz, 1H, -CH_{ar}-), 2.05 (s, 3H, -CH_3). ^{13}C NMR (125 MHz) δ ppm: 171.70, 161.98, 161.22, 158.89, 153.71, 125.68, 113.74, 108.72, 102.47, 101.42, 23.32.

N-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-pyrrol-2-carboxamide[218]

A mixture of 200 mg 3-amino-4,7-dihydroxy-2*H*-chromen-2-on hydrochloride (0.87 mmol, 1 eq), 97 mg *1H*-pyrrole-2-carboxylic acid (0.87 mmol, 1 eq), 450 mg PyBOP (0.87 mmol, 1eq) and 0.77 ml *N*-methylmorpholin (700 mg, 7.0 mmol, 8 eq) in 12 ml DMF was stirred for 27 h at room temperature under an argon atmosphere. After addition of further 230 mg PyBOP (0.44 mmol, 0.5 eq) the solution was again stirred for 66 h. The reaction mixture was acidified with 1 N HCl and extracted three times with ethyl acetate. The combined organic layers were washed once with brine. After drying over Na_2SO_4 the solvent was removed under reduced pressure. The resulting brown oil was dissolved in ethyl acetate what gave a suspension. The solid was collected to give the desired product. Further product was precipitated from the mother lye by addition of cyclohexane. Both fractions were dried

in vacuo to yield overall 180 mg (72 %) of a brown solid. ^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 12.01 (br, 1H, -OH), 11.65 (s, 1H, -NH-) 10.57 (br, 1H, -OH), 9.30 (s, 1H, -NH-), 7.69 (d, J = 8.3 Hz, 1H, -CH_{ar}-) 7.01 (s, 1H, -CH_{ar}-), 6.94 (s, 1H, -CH_{ar}-), 6.81 (dd, J_1 = 8.8, J_2 = 2.0 Hz, 1H, -CH_{ar}-), 6.71 (d, J = 2 Hz, 1H, -CH_{ar}-), 6.15 (s, 1H, -CH_{ar}-). ^{13}C NMR (125 MHz) δ ppm: 162.00, 161.55, 161.49, 160.34, 153.97, 126.21, 125.70, 123.07, 113.68, 112.88, 109.67, 108.77, 102.56, 100.92.

General procedure for the preparation of the SAM analogs for activity assays and preparative scale experiments

50 mg SAH (0.13 mmol, 1 eq) are dissolved in 5.0 ml formic acid. 5.2 mmol of the according alkyl bromide and 67mg silver triflate (0.13 mmol, 1 eq) were added. A colourless solid started to precipitate after addition of the silver triflate. After 24 h another portion of silver triflate (34 mg, 0.065 mmol, 0.5 eq) was added. The reaction was monitored by HPLC-MS. After 48 h reaction time 10 ml deion. H_2O was added. The product was washed three times with 10 ml diethyl ether. The aqueous product solution was dried by lyophilisation. The crude product was directly used for the assay or preparative scale experiments.

General procedure for the preparative scale experiments with SAM analogs and CouO or NovO

To 25 mg of substrate are dissolved in 2.5 ml DMSO (in case of 2,7-dihydroxynaphthalene 1.0 ml) in a baffled flask, one portion SAM analog (prepared according to the procedure above) in 2.5 ml buffer (CouO: sodium phosphate 50 mM pH 7.0; NovO: sodium phosphate 50 mM pH 6.5) and 20 ml cells disrupted by freeze/thaw suspended in buffer were added. The mixture was incubated at 30 °C and 150 rpm. The reaction was monitored by HPLC-MS After 24h another portion SAM analog in 2.5 ml assay buffer and 15 ml cells disrupted by freeze/thaw suspended in assay buffer were added. After 48 h reaction time the enzyme was precipitated by addition of 2.0 ml 3N HCl. The resulting suspension was extracted with ethyl acetate after vigorous shaking. The phases were separated by centrifugation (45 min, 8000 rpm, 4 °C). The aqueous phase was again extracted two times with ethyl acetate and phases were separated by centrifugation. The combined organic layers were washed with brine and dried over sodium sulfate. After removal of the solvent in vacuo, the crude product was purified by column chromatography or preparative HPLC. The resulting product was characterized by HPLC-MS and NMR.

N-(8-methyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-benzamide

Purified by column chromatography (CH_2Cl_2/MeOH 20:1)

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11,68 (s, 1H, -OH), 10.40 (s, 1H, -OH), 9.39 (s, 1H, -NH-) 7.98 (d, *J* = 7.0 Hz, 2H, -CH_{ar}-), 7.57-7.45 (m, 4H, -CH_{ar}-), 6.86 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 2.14 (s, 3H, -CH_3). ^{13}C NMR (75 MHz) δ ppm: 166.53, 160.83, 160.47, 159.10, 151.46, 133.90, 131.56, 129.24, 128.15, 127.99, 121.55, 111.84, 110.43 107.91, 99.85, 8.08.

N-(8-methyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-*1H*-pyrrol-2-carboxamide

Purified by column chromatography (CH_2Cl_2/MeOH 20:1)

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.89 (br, 1H, -OH), 11,68 (s, 1H, -NH_{ar}-), 10.44 (s, 1H, -OH), 9.04 (s, 1H, -NH-), 7.59 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 7.06 (s, 1H, -CH_{ar}-), 6.97 (s, 1H, -CH_{ar}-), 6.90 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 6.18 (d, *J* = 3.2 Hz, 1H, -CH_{ar}-), 2.19 (s, 3H, -CH_3). ^{13}C NMR (75 MHz) δ ppm: 160.86, 160.76, 159.68, 158.96, 151.23, 125.48, 122.39, 121.53, 112.25, 111.84, 110.36, 108.97, 108.15, 100.04, 8.07.

N-(8-allyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide

Purified by column chromatography (CH_2Cl_2/MeOH 20:1)

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.75 (br, 1H, -OH), 10.47 (s, 1H, -OH), 9.39 (s, 1H, -NH-), 8.00 (d, *J* = 4.2 Hz, 2H, -CH_{ar}-), 7.63 (d, *J* = 5.2 Hz, 1H, -CH_{ar}-), 7.59 (t, *J* = 4.2 Hz, 1H, -CH_{ar}-), 7.52 (t, *J* = 4.3 Hz, 2H, -CH_{ar}-), 6.91 (d, *J* = 5.2 Hz, 1H, -CH_{ar}-), 5.93 (ddd, J_1 = 13.5 Hz, J_2 = 6.7 Hz, J_3 = 3.7 Hz, 1H, -CH=), 4,96 (dd, J_1 = 11.3 Hz, J_2 = 4.8 Hz, 2H, -CH_2=), 3.46 (d, *J* = 2.9 Hz, 2H, -CH_2-).

N-(8-allyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-*1H*-pyrrol-2-carboxamide

Purified by column chromatography (CH_2Cl_2/MeOH 20:1)

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.98 (br, 1H, -OH), 11,66 (s, 1H, -NH_{ar}-), 10.50 (s, 1H, -OH), 9.03 (s, 1H, -NH-) 7.63 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 7.05 (s, 1H, -CH_{ar}-), 6.96 (s, 1H, -CH_{ar}-), 6.92 (d, *J* = 5.2 Hz, 1H, -CH_{ar}-), 6.18 (s, 1H, -CH_{ar}-), 5.95 (m, 1H, -CH=), 4,94 (m, 2H, -CH_2=), 3.48 (d, 2H, -CH_2-).

N-(8-but-2-en-1-yl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-*1H*-pyrrol-2-carboxamide

Purified by column chromatography (CH_2Cl_2/MeOH 20:1)

^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 11.87 (br, 1H, -OH-), 11,63 (s, 1H, -NH_{ar}-), 10.38 (s, 1H, -OH), 8.95 (s, 1H, -NH-) 7.63 (d, *J* = 8.6 Hz, 1H, -CH_{ar}-), 7.05 (s, 1H, -CH_{ar}-), 6.96 (s, 1H, -CH_{ar}-), 6.92 (d, *J* = 8.6 Hz, 1H, -CH_{ar}-), 6.18 (s, 1H, -CH_{ar}-), 5.52 (m, 1H, -CH=), 5.41 (m, 1H, -CH=), 3.38 (d,, *J* = 7.1 Hz, 2H, -CH_2-), 1.56 (d, *J* = 5.9 Hz, 3H, -CH_3).

N-(8-(but-2-ynyl)-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide

Purified by preparative HPLC (Macherey Nagel Nucleodur® C_{18} ec 125x21 mm, 5 µm; buffer NH_3/NH_4^+ pH 8.2 / MeOH 85:15; 15 ml min^{-1}, R_t 9.2 min

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 10.69 (s, 1H, -OH), 9.42 (s, 1H, -NH-) 8.02 (d, *J* = 7.2 Hz, 1H, -CH_{ar}-), 7.66 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 7.60-7.50 (m, 3H, -CH_{ar}-), 6.92 (d, *J* = 8.3 Hz, 1H, -CH_{ar}-), 3.94 (s, 2H, -CH_2-), 1.71 (s, 3H, -CH_3). ^{13}C NMR (75 MHz) δ ppm: 166.50, 160.72, 158.50, 151.06, 133.97, 131.51, 128.33, 128.13, 127.96, 127.21, 122.81, 112.12, 110.77, 99.78, 76.89, 74.50, 12.18, 3.14.

N-(8-(but-2-ynyl)-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-pyrrol-2-carboxamide

Purified by preparative HPLC (Macherey Nagel Nucleodur® C_{18} ec 125x21 mm, 5 µm; buffer NH_3/NH_4^+ pH 8.2 / MeOH 85:15; 15 ml min^{-1}, R_t 7.8 min

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.90 (br, 1H, -OH), 11.62 (s, 1H, -NH_{ar}-) 10.66 (s, 1H, -OH), 9.00 (s, 1H, -NH-), 7.60 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 7.00 (s, 1H, -CH_{ar}-), 6.92 (s, 1H, -CH_{ar}-), 6.87 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 6.13 (s, 1H, -CH_{ar}-), 3.49 (s, 2H, -CH_2-), 1.65 (s, 3H, -CH_3).

N-(8-benzyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide

Purified by preparative HPLC (Macherey Nagel Nucleodur® C_{18} ec 125x21 mm, 5 µm; buffer NH_3/NH_4^+ pH 8.5 / MeOH 60:40; 15 ml min^{-1}, R_t 10.4 min

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.80 (br, 1H, -OH), 10.61 (s, 1H, -OH), 9.41 (s, 1H, -NH-), 8.00 (d, *J* = 7.3 Hz, 2H, -CH_{ar}-), 7.65 (d, *J* = 8.6 Hz, 1H, -CH_{ar}-), 7.61-7.49 (m, 3H, -CH_{ar}-), 7.25 (m, 4H, -CH_{ar}-), 7.16 (m, 1H, -CH_{ar}-), 6.94 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 4.06 (s, 2H, -CH_2-). ^{13}C NMR (75 MHz) δ ppm: 166.65, 160.70, 158.96, 151.33, 140.15, 133.92, 131.57, 128.23, 128.22, 128.21, 128.17, 127.99, 125.82, 122.58, 113.92, 112.27, 108.20, 99.93, 28.02.

N-(8-benzyl-4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-1*H*-pyrrol-2-carboxamide

Purified by preparative HPLC (Macherey Nagel Nucleodur® C_{18} ec 125x21 mm, 5 µm; buffer NH_3/NH_4^+ pH 8.5 / MeOH 60:40; 15 ml min^{-1}, R_t 8.0 min

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.67 (br, 1H, -NH_{ar}-), 10.62 (s, 1H, -OH), 9.04 (s, 1H, -NH-), 7.66 (m, 2H, -CH_{ar}-), 7.27-6.97 (m, 7H, -CH_{ar}-), 6.19 (s, 1H, -CH_{ar}-), 4.08 (s, 2H, -CH_2-).

1-methyl-2,7-dihydroxynaphthalene

Purified by column chromatography (CH_2Cl_2/MeOH 20:1)

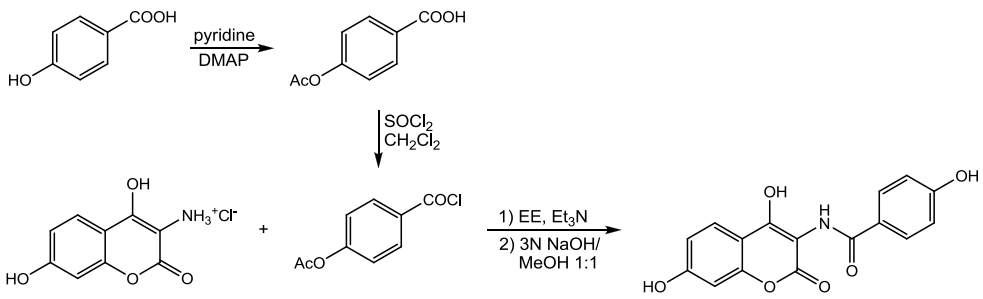
^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 9.62 (s, 1H, -OH), 9.40 (s, 1H, -OH), 7.67 (d, *J* = 8.6 Hz, 1H, -CH_{ar}-), 7.54 (d, *J* = 8.8 Hz, 1H, -CH_{ar}-), 7.12 (d, *J* = 2.1 Hz, 1H,-CH_{ar}-), 6.99 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 6.91 (dd, J_1 =8.7 Hz, J_2 =2.2 Hz, 1H, -CH_{ar}-), 2.27 (s, 3H, -CH_3). ^{13}C NMR (75 MHz) δ ppm: 156.25, 153.07, 136.15, 130.44, 127.17, 123.30, 115.35, 115.25, 113.23, 105.22, 11.21.

1-allyl-2,7-dihydroxynaphthalene

Purified by preparative HPLC (Macherey Nagel Nucleodur® C_{18} ec 125x21 mm, 5 µm; buffer NH_3/NH_4^+ pH 8.5 / MeOH 50:50; 15 ml min^{-1}, R_t 7.1 min

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 8.46 (s, 1H, -OH-), 7.58 (d, *J* = 8.8 Hz, 1H, -CH_{ar}-), 7.48 (d, 1H, *J* = 8.8 Hz-CH_{ar}-), 7.07 (s, 1H, -CH_{ar}-), 6.96 (d, *J* = 8.7 Hz, 1H, -CH_{ar}-), 6.83 (dd, J_1 =8.7 Hz, J_2 =1.9 Hz, 1H, -CH_{ar}-), 5.92 (m, 1H, -CH=), 4.92 (m, 2H, =CH_2), 3.58 (d, 2H, *J* =5.7 Hz, -CH_2-).

Synthesis of *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-4-hydroxybenzamide



4-acetoxybenzoic acid[211, 212]

To a stirred and light protected solution of 1.0 g 4-hydroxybenzoic acid (7.2 mmol, 1 eq) in 6.0 ml pyridine 1.7 ml acetic anhydride (1.8 g, 18 mmol, 2.5 eq) and 0.088 g DMAP (0.72 mmol, 0.1 eq) were added. After 20 h ice was added to the reaction mixture and it was acidified with aqueous 3 N HCl. The resulting suspension was extracted two times with ethyl acetate. The organic layers were pooled and washed once with brine. After drying over Na_2SO_4 the solvent was removed. The resulting solid was recrystallized from EtOH to give 0.85 g (64 %) white crystals.
^{1}H NMR (300 MHz, $CDCl_3$ + 1 drop DMSO-d_6) δ ppm: 10.5 (br, 1H, -COOH), 8.08 (d, J = 8.7 Hz, 2H, -CH_{ar}-), 7.14 (dd, J_1 = 8.7, J_2 = 2.3 Hz, 2H, -CH_{ar}-), 2.29 (s, 3H, -CH_3). ^{13}C (75 MHz) δ ppm: 169.01, 168.96, 154.45, 131.59, 127.97, 121.60, 21.19.

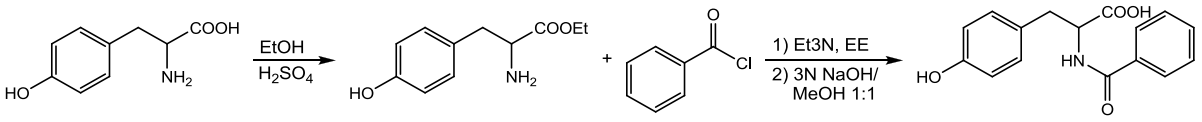
4-acetoxybenzoyl chloride[242]

0.25 g 4-acetoxybenzoic acid (1.4 mmol) was suspended in 5.0 ml CH_2Cl_2. 2.5 mL $SOCl_2$ was added to the stirred suspension and the solid dissolved. The mixture was heated to reflux for 18 h, then CH_2Cl_2 and $SOCl_2$ were distilled off. The crude product was purified by Kugelrohr distillation to give 0.10 g (36 %) of a clear liquid.

N-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)-4-hydroxybenzamide

To a stirred solution of 0.050 g 3-amino-4,7-dihydroxy-2*H*-chromen-2-on hydrochloride (0.22 mmol, 1 eq) in 5.0 ml ethyl acetate 0.10 g 4-hydroxybenzoyl chloride (0.50 mmol, 2.3 eq) and 0.31 ml triethylamine (0.22 g, 2.2 mmol, 10 eq) were added. The suspension was stirred overnight at room temperature. After removal of the solid by filtration the solvent was removed by rotary evaporation. The resulting residue was dissolved in 4.0 ml MeOH and 4.0 ml 3 N NaOH was added. The reaction mixture was stirred for 7 d. The mixture was acidified with 1 N HCl to pH 4 and extracted four times with ethyl acetate. The combined organic layers were washed once with brine and dried over Na_2SO_4. Celite was added and the solvent was removed in vacuo. The resulting fine brown powder was used for purification with column chromatography to give 50 mg (73%) of an off white solid.
^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 10.72 (s, 1H, -OH), 10.23 (s, 1H, -OH), 9.22 (s, 1H, -NH-), 7.87 (d, J = 8.6 Hz, 2H, -CH_{ar}-), 7.71 (d, J = 8.7 Hz, 1H, -CH_{ar}-), 6.86 (d, J = 8.5 Hz, 2H, -CH_{ar}-), 6,84 (dd, J_1 = 8.7 Hz, J_2 = 2.1 Hz, 1H, -CH_{ar}-) 6.75 (d, J = 2.1 Hz, 1H, -CH_{ar}-). ^{13}C NMR (75 MHz) δ ppm: 166.38, 161.46, 160.88, 160.74, 159.92, 153.40, 130.07, 125.01, 124.32, 114.81, 113.01, 108.13, 101.92, 100.59.

Synthesis of 2-benzamido-3-(4-hydroxyphenyl)propanoic acid (*N*-Benzoyltyrosine)



Ethyl 2-amino-3-(4-hydroxyphenyl)propanoate

25 ml ethanol was stirred and cooled in an ice-bath. Slowly 3.9 ml acetyl chloride (4.3 g, 55 mmol, 20 eq) was added to give an acidic solution. 0.50 g L-tyrosine (2.8 mmol, 1 eq) was added. The resulting suspension was heated to reflux. Because the reaction has not run to completion after 2 d 2.0 ml H_2SO_4 was added and the suspension was heated to reflux again. After 18 h the reaction mixture was neutralized with $NaHCO_3$ and extracted two times with ethyl acetate. The organic phase was dried over Na_2SO_4. The solvent was removed under reduced pressure to yield 0.52 g of a yellow oil (90 %) which was directly used for the next step without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 6.98 (d, *J* = 8.3 Hz, 2H, -CH_{ar}-), 6.66 (d, *J* = 8.3 Hz, 2H, -CH_{ar}-), 4.18 (q, *J* = 7.2 Hz, 2H, -CH_2-), 3.71 (dd, J_1 = 7.8 Hz, J_2 = 4.9 Hz, 1H, -CH-), 3.03 (dd, J_1 = 13.7 Hz, J_2 = 4.9 Hz, 1H, -C*H*H-), 2.81 (dd, J_1 = 13.7, J_2 = 7.8 Hz, 1H, -CH*H*-), 1.26 (t, *J* = 7.1 Hz, 3H, -CH_3). ^{13}C NMR (125 MHz) δ ppm: 174.85, 155.69, 130.61, 127.90, 116.00, 61.54, 55.71, 39.76, 14.43.

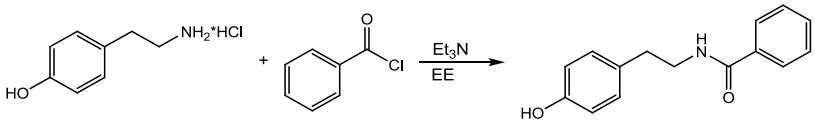
2-benzamido-3-(4-hydroxyphenyl)propanoic acid

0.50 g Ethyl 2-amino-3-(4-hydroxyphenyl)propanoate (2.4 mmol, 1 eq) was dissolved in 20 ml ethyl acetate. To the stirred solution 0.83 ml Et_3N (1.0 g, 7.2 mmol, 3 eq) and 2.0 ml benzoyl chloride (1.5 g, 14 mmol, 6 eq) were added and a white solid started to precipitate. After 16 h the precipitate was removed by filtration The solvent was removed to give an orange solid. The intermediate was suspended in 10 ml methanol. 10 ml NaOH were added and the reaction mixture was stirred for 18h. The resulting clear orange solution was washed two times with CH_2Cl_2 to remove the benzoic acid formed. After acidification with 3 N HCl the mixture was extracted three times with ethyl acetate. The organic phase was dried over Na_2SO_4. Removal of the solvent under reduced pressure gave the crude product as an orange solid which was purified by recrystallisation from ethyl acetate / cyclohexane. In the first fraction 0.41 g orange crystals (60 %) were collected, no further fractions were collected.

^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm:12.69 (broad, 1H, -COOH), 9.17 (s, 1H, -OH), 8.62 (d, 1H, *J* = 8.3 -NH-), 7.77 (d, *J* = 8.3 Hz, 2H, -CH_{ar}-), 7.50 (t, *J* = 7.3 Hz, 1H, -CH_{ar}-), 7.43 (t, *J* = 7.6 Hz, 2H, -CH_{ar}-), 7,08

(d, *J* = 8.3 Hz, 2H, -CH_{ar}-), 6.62 (d, *J* = 8.3 Hz, 2H, -CH_{ar}-), 4.50 (ddd, J_1 = 10.8 Hz, J_2 = 8.3 Hz, J_3 = 4.2 Hz, 1H, -CH-), 3.04 (dd, J_1 = 13.9, J_2 = 4.2 Hz, 1H, -C*H*H-), 2.93 (dd, J_1 = 13.5, J_2 = 10.9 Hz, 1H, -C*H*H-). ^{13}C NMR (125 MHz) δ ppm: 174.05, 167.03, 156.49, 134.64, 132.03, 130.67, 128.93, 128.87, 128.03, 115.65, 55.26, 36.17

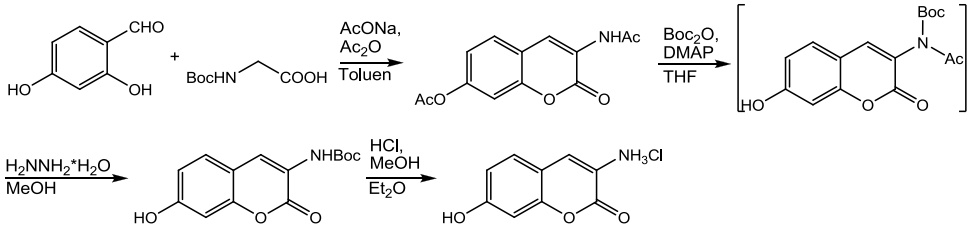
N-(4-hydroxyphenethyl)benzamide



0.50 g tyramine hydrochloride (2.9 mmol, 1 eq) was dissolved in 30 ml ethyl acetate. To the stirred solution 3.6 ml Et_3N (2.6 g, 26 mmol, 9 eq) and 1.0 ml benzoyl chloride (1.2 g, 8.6 mmol, 3 eq) were added to form a colourless suspension. After 18 h a yellow suspension had formed. The white solid was filtered off and the solvent was removed from the filtrate. The resulting solid was suspended in 24 ml methanol, 12 ml 3 N NaOH was added and the suspension was stirred for 18 h at rt. The reaction resulted in a yellow solution which was acidified with 3 N HCl and extracted three times with ethyl acetate. The organic phase was dried over Na_2SO_4. The solvent was removed under reduced pressure to give 0.7 g of an off-white solid (101 %). The crude product was purified by recrystallisation from acetonitrile to yield 203 mg of an off-white solid (29 %).

^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 8.48 (s, 1H, -NH-), 7.79 (d, *J* = 6.8 Hz, 2H, -CH_{ar}-), 7.47 (t, *J* = 7.3 Hz, 1H, -CH_{ar}-), 7.43 (t, *J* = 7.3 Hz, 2H, -CH_{ar}-), 7,01 (d, *J* = 8.3 Hz, 2H, -CH_{ar}-), 6.66 (d, *J* = 8.3 Hz, 2H, -CH_{ar}-), 3.40 (dd, J_1 = 13.2 Hz, J_2 = 7.3 Hz, 1H, -CH-), 2.71 (t, *J* = 7.6, 2H, -CH_2-). ^{13}C NMR (125 MHz) δ ppm: 166.78, 156.31, 135.37, 131.69, 130.25, 130.18, 128.92, 127.81, 115.81, 41.92, 35.02.

Synthesis of 3-amino-7-hydroxy-2*H*-chromen-2-one



3-acetamido-2-oxo-2*H*-chromen-7-yl acetate

1.0 g 2,4-Dihydroxybenzaldehyde (7.2 mmol, 1 eq), 1.3 g *N*-Boc-glycine (7.2 mmol, 1 eq) and 2.4 g sodium acetate (29 mmol, 4 eq) were dissolved in 3.6 ml acetic anhydride. The stirred mixture was heated to 125 °C for 4 h. The mixture was then allowed to cool to rt. The resulting solid was suspended in ice water, the resulting solid was collected by filtration. The solid was suspended in ethyl acetate and filtrated. The mother liquor was evaporated to dryness. The resulting solid was resuspended in ethyl acetate and another product fraction was collected by filtration. The product was dried in vacuo to yield 0.80 g (43 %) yellow crystals.

^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 9.74 (s, 1H, -OH), 8.59 (s, 1H, -NH-), 7.72 (d, J = 8.3 Hz, 1H, -CH_{ar}-), 7.24 (d, J = 2.0 Hz, 1H, -CH_{ar}-), 7.11 (dd, J_1 = 8.3 Hz, J_2 = 2.0 Hz, 1H, -CH_{ar}-), 2.28 (s, 3H, -CH_3), 2.14 (s, 3H, -CH_3). ^{13}C NMR (125 MHz) δ ppm: 170.92, 169.64, 157.99, 151.64, 150.58, 129.18, 124.81, 123.82, 119.71, 118.13, 110.40, 24.61, 21.55.

tert-butyl 7-hydroxy-2-oxo-2*H*-chromen-3-ylcarbamate

0.54 g 3-acetamido-2-oxo-2*H*-chromen-7-yl acetate (2.1 mmol, 1 eq) and 50 mg 4-dimethylaminopyridine (0.41 mmol, 0.2 eq) were stirred in 13 ml THF. To the resulting suspension 1.9 ml (1.9 g, 8.7 mmol, 4.2 eq) Boc anhydride were added. After 3 h a brown solution formed. To the solution 1.0 ml hydrazine hydrate (1.0 g, 21 mmol, 10 eq) and 10 ml methanol were added. After 3 h the solvent was removed in vacuo. The residue was suspended in dichloromethane. The suspension was washed with 1 M HCl, 0.5 M $Cu(OAc)_2$ and saturated $NaHCO_3$. The organic phase was dried over sodium sulfate and evaporated to dryness. The crude product was purified by column chromatography (ethyl acetate/cyclohexane 1:4) to give 0.36 g of the product (63 %) as a yellow solid.

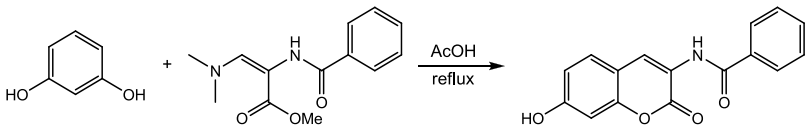
^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 10.30 (s, 1H, -OH), 8.26 (s, 1H, -NH-), 8.07 (s, 1H, -CH_{ar}-), 7.49 (d, J = 8.8 Hz, 1H, -CH_{ar}-), 6.76 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1H, -CH_{ar}-), 6.70 (d, J = 2.0 Hz, 1H, -CH_{ar}-), 1.45 (s, 9H, -CH_3). ^{13}C NMR (125 MHz) δ ppm: 160.07, 158.64, 153.43, 152.09, 129.47, 121.67, 114.22, 112.13, 102.60, 80.73, 28.62.

3-amino-7-hydroxy-2*H*-chromen-2-one

0.36 g *tert*-butyl 7-hydroxy-2-oxo-2*H*-chromen-3-ylcarbamate (1.3 mmol) was dissolved in 2 ml diethyl ether. 0.37 ml methanol in 5.0 ml diethyl ether was cooled in an ice bath and 0.64 ml acetyl chloride was slowly added. The two solutions were combined and stirred. After 16 h the precipitate which was formed was collected to give 0.085 g product as yellow solid. The mother liquor was

evaporated to dryness. The residue was dissolved in 7.0 ml diethyl ether and 2.0 ml trifluoroacetic acid was added. The solution was stirred for 5 d. The reaction mixture was made basic with triethylamine and the product was extracted to H_2O. After acidification with acetic acid the product was extracted with ethyl acetate and dried over sodium sulfate. The solvent was removed by evaporation. The product was isolated by crystallization from H_2O to give additional 0.10 g product as a light brown solid. The overall yield was 0.19 g (82 %). The product was identified by HPLC-MS.

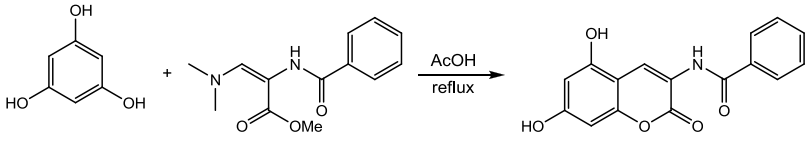
Synthesis of *N*-(7-hydroxy-2-oxo-2*H*-chromen-3-yl)benzamide[222]



0.55 g 1,3-Dihydroxybenzene (5.0 mmol, 1 eq) and 1.2 g (*E*)-methyl 2-benzamido-3-dimethylaminopropenoate (5.0 mmol, 1 eq) were suspended in 15 ml acetic acid and heated to reflux. At elevated temperature the starting materials were fully dissolved. After 7 h the solution was allowed to cool to rt slowly. The product precipitated while cooling down and was collected by filtration to give 0.20 g pure product (14 %) as an orange solid.

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 9.53 (s, 1H, -NH-), 8.42 (s, 1H, -CH_{ar}-), 7.93 (d, J = 7.2 Hz, 2H, -CH_{ar}-), 7.60-7.48 (m, 4H, -CH_{ar}-), 6.80 (dd, J_1 = 8.3 Hz, J_2 = 2.0 Hz, 1H, -CH_{ar}-), 6.75 (s, 1H, -CH_{ar}-). ^{13}C NMR (75 MHz) δ ppm: 165.63, 160.20, 158.25, 152.26, 133.62, 132.04, 129.68, 129.30, 128.57, 127.51, 120.39, 113.70, 111.19, 102.06.

Synthesis of *N*-(5,7-dihydroxy-2-oxo-2*H*-chromen-3-yl)benzamide[222]

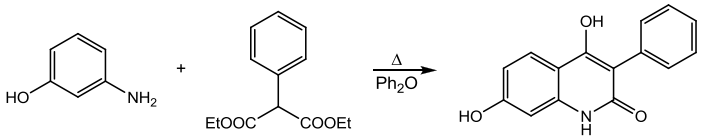


0.63 g 1,3,5-trihydroxybenzene (5.0 mmol, 1 eq) and 1.2 g (*E*)-methyl 2-benzamido-3-dimethylaminopropenoate (5.0 mmol, 1 eq) were suspended in 15 ml acetic acid and heated to reflux. After 16 h the solution was allowed to cool to rt. The precipitate formed was collected by

filtration. Another product fraction was collected from the mother liquor after removal of most of the solvent. Over all 0.91 g product (61%) were isolated as a brown solid.

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 9.46 (s, 1H, -NH-), 8.49 (s, 1H, -CH_{ar}-), 7.90 (d, *J* = 7.1 Hz, 2H, -CH_{ar}-), 7.58-7.46 (m, 3H, -CH_{ar}-), 6.29 (s, 1H, -CH_{ar}-), 6.22 (s, 1H, -CH_{ar}-). ^{13}C NMR (75 MHz) δ ppm: 165.56, 160.88, 158.53, 155.51, 153.27, 133.79, 132.01, 128.61, 127.53, 125.34, 118.38, 101.31, 98.76, 93.91.

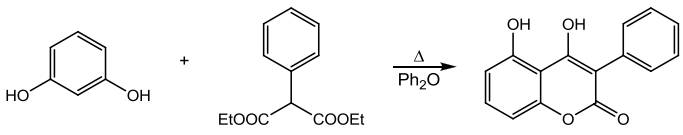
Synthesis of 4,7-dihydroxy-2-oxoquinoline[243]



1.1 g 3-hydroxyaniline (10 mmol, 1 eq) and 2.2 ml diethyl phenylmalonate (2.4 g, 10 mmol, 1 eq) were suspended in 20 ml diphenyl ether (bp. 256 °C). The stirred suspension was heated to reflux for 16h. Upon cooling a solid precipitated which was filtered off. The crude product was purified by flash chromatography (dichloromethane/methanol 20:1) to give 0.27 g pure product (11 %) and furthermore 0.22 g (9 %) 4,5-dihydroxy-2-oxoquinolin.

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.26 (s, 1H, -OH), 10.10 (s, 1H, -OH), 9.85 (s, 1H, -NH-), 7.78 (d, *J* = 8.8 Hz, 2H, -CH_{ar}-), 7.36 (m, 4H, -CH_{ar}-), 7.27 (m, 1H, -CH_{ar}-), 6.70 (d, *J* = 2.1 Hz, 1H, -CH_{ar}-), 6.64 (dd, J_1 = 8.7 Hz, J_2 = 2.0 Hz, 1H, -CH_{ar}-). ^{13}C NMR (75 MHz) δ ppm: 163.11, 159.77, 157.78, 139.94, 133.77, 131.36, 127.59, 126.51, 124.77, 110.95, 109.58, 108.20, 99.63.

Synthesis of 4,5-dihydroxy-3-phenylcoumarin[243, 244]



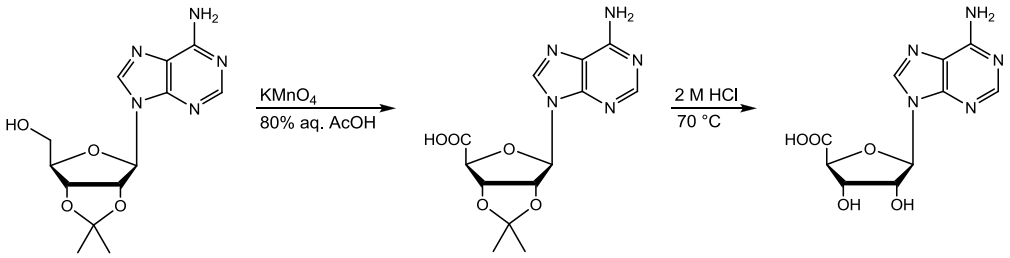
1.1 g resorcinol (10 mmol, 1 eq) and 2.2 ml diethyl phenylmalonate (2.4 g, 10 mmol, 1 eq) were suspended in 20 ml diphenyl ether (bp. 256 °C). The stirred suspension was heated to reflux for 3h. Upon cooling a solid precipitated which was separated and dried to give 1.2 g pure product (45 %) as an off-white solid.

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 11.34 (br, 2H, -OH), 7.47-7.34 (m, 5H, -CH_{ar}-), 7.27 (t, *J* = 7.1 Hz, 1H, -CH_{ar}-), 6.86 (d, *J* = 8.3 Hz, 1H, -CH_{ar}-), 6.76 (d, *J* = 8.2 Hz, 1H, -CH_{ar}-). ^{13}C NMR (75 MHz) δ ppm: 163.36, 161.72, 155.55, 153.27, 132.60, 132.32, 130.76, 127.49, 126.79, 110.22, 107.59, 104.11, 103.02.

Preparative scale formation of 3-hydroxy-4-methylkynurenine

25 mg 3-hydroxykynurenine (0.11 mmol), 48 mg SAM (0.11 mmol), 25 ml ORF19 crude lysate in sodium phosphate buffer pH 7.0, 1 ml SAH hydrolase and 86 mg DTT were combined in a baffled flask and incubated at 30 °C and 150 rpm. After 48 h 20 ml crude lysate and 24 mg SAM (0.056 mmol) were added. After another 24 h the conversion was determined to be 27 %. 15 ml crude lysate and 24 mg SAM (0.056 mmol) were added and the mixture was incubated for further 72 h. The protein was precipitated by addition of 1 M HCl and removed by centrifugation (10000 rpm, 4 °C, 45 min). The pellet was washed two times with 50 mM HCl. The combined aqueous layers were combined and dried by lyophilisation. The resulting crude product was purified by preparative HPLC (Macherey Nagel Nucleodur® C_{18} ec 125x21 mm, 5 µm; buffer 0.01% TFA / MeOH 100:0 for 3 min, to 75:25 in 10 min, to 60:40 in 3 min, hold for 4 min; 10 ml min^{-1}, R_t 13.0 min) and 12 mg yellow product (45 %) were isolated.
^{1}H NMR (300 MHz, D_2O) δ ppm: 7.45 (d, *J* = 8.3 Hz, 1H, -CH_{ar}-), 7.01 (d, 1H, *J* = 8.2 Hz-CH_{ar}-), 4.29 (t, *J* = 5.8 Hz, 1H, -CH-), 3.73 (d, *J* = 5.8 Hz, 2H, -CH_2-), 2.20 (s, 3H, -CH_3).

Synthesis of adenosine-5´-carboxylic acid



2´,3´-*O*-isopropylideneadenosine-5´-carboxylic acid[245]

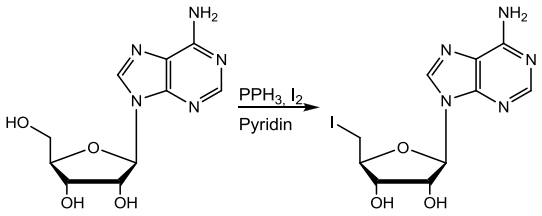
1.5 g 2´,3´-*O*-isopropylideneadenosine (5 mmol, 1 eq) was dissolved in 25 ml aqueous acetic acid (80 % v/v). 2.0 g. The solution was stirred and cooled to 0 °C. 2.0 g potassium permanganate was added

in small portions. After 2 h the reaction mixture was allowed to warm to rt. After 18 h 12.5 ml aqueous acetic acid (80 % v/v) were added and the reaction was quenched by addition of aqueous H_2O_2 (30 % v/v) till the reaction mixture cleared. The product was precipitated by addition of ice water and collected by filtration. The wet product was dried to give 0.98g (61%) of a white solid. The product was directly used for the next step.

adenosine-5´-carboxylic acid

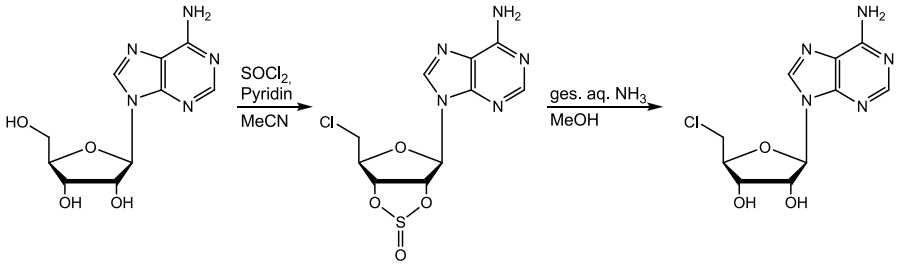
0,97 g 2´,3´-*O*-isopropylideneadenosine-5´-carboxylic acid (3.0 mmol) was stirred in 12 ml 2 M hydrochloric acid. The mixture was heated to 70 °C and all the starting material dissolved. After 45 min the reaction mixture was allowed to cool to rt. A colourless solid precipitated. 10 ml 2M hydrochloric acid was added and the mixture was again heated to 70 °C for 45 min. The colourless precipitate was separated by filtration and dried to give 0.30 g (35 %) pure product.
^{1}H NMR (500 MHz, D_2O + NaOD) δ ppm: 8.22 (s, 1H, -CH_{ar}-), 7.84 (s, 1H, -CH_{ar}-), 5.63 (d, *J* = 6.8 Hz, 1H, -CH-), 4.33 (t, *J* = 5.1 Hz, 1H, -CH-), 4.12 (s, 1H, -CH-), 3.98 (s, 1H, -CH-).

Synthesis of 5´-Deoxy-5´-iodoadenosine[246]



2.5 g adenosine (9.4 mmol, 1 eq) was suspended in 20 ml pyridine and 3.6 g Iodine (14 mmol, 1.5 eq) and 3.7 g triphenylphosphane (14 mmol, 1 eq) were added. After 30 min a clear orange solution formed. After 20 h the reaction was stopped by addition of a saturated sodium thiosulfate solution. After removal of the solvent the residue was crystallized from methanol. The crude product (6.54 g) was purified by column chromatography (dichloromethane / methanol 9:1) to give 2.1 g (59 %) of an off-white solid.
^{1}H NMR (500 MHz, DMSO-*d6*) δ ppm: 8.37 (s, 1H, -CH_{ar}-), 8.14 (s, 1H, -CH_{ar}-), 7.30 (br, 2H, -NH_2), 5.91 (d, *J* = 5.9 Hz, 1H, -CH-), 5.58 (d, *J* = 5.8 Hz, 1H, -OH), 5.47 (d, *J* = 5.8 Hz, 1H, -OH) 4.80 (q, *J* = 5.4 Hz, 1H, -CH-), 4.16 (q, *J* = 4.9 Hz, 1H, -CH-), 3.98 (m, 1H, -CH-), 3.60 (dd, J_1 = 10.3 Hz, J_2 = 5.9 Hz, 1H, -C*H*H-), 3.45 (dd, J_1 = 10.5 Hz, J_2 = 7.1 Hz, 1H, -CH*H*-). ^{13}C NMR (125 MHz) δ ppm: 156.74, 153.39, 150.13, 140.61, 119.76, 88.07, 84.56, 73.80, 73.39, 8.60.

Synthesis of 5´-deoxy-5´-chloroadenosine[227]



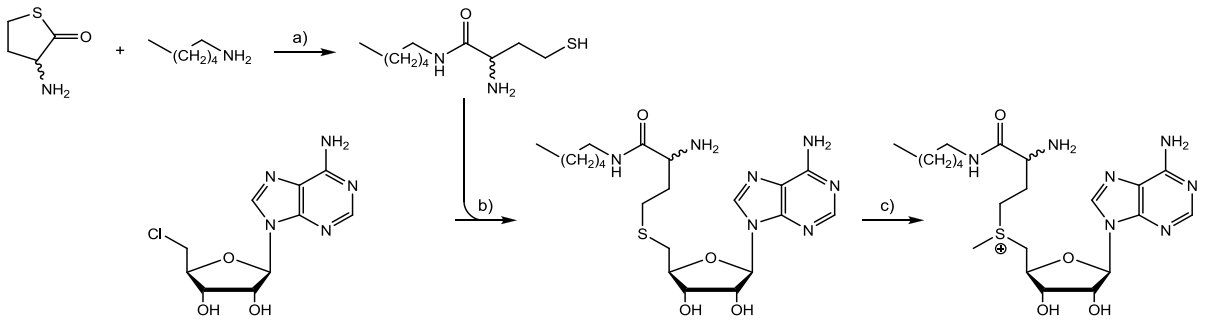
0.5 g adenosine (1.9 mmol, 1 eq) was dissolved in 2 ml acetonitrile and cooled to 0 °C. Then 0.41 ml thionyl chloride (0.67 g, 5.6 mmol, 3 eq) and 0.30ml pyridine (0.30 g, 3.7 mmol, 2 eq) were added and the reaction mixture was allowed to slowly warm to rt. A colourless precipitate formed. After 42 h the suspension was cooled to 0 °C again and 15 ml H_2O was slowly added. After the precipitate fully dissolved, the solution was extracted two times with ethyl acetate. The combined organic phases were washed with saturated $NaHCO_3$ and H_2O and dried over sodium sulfate. 4 ml toluene was added and most of the ethyl acetate was removed in vacuo. The remaining solution was put in the refrigerator to crystallize. After 24 h the intermediate was collected by filtration, another fraction was isolated from the mother liquor to give an overall yield of 0.63 g (99 %) of an off white solid.

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 8.40 (s, 1H, -CH_{ar}-), 8.23 (s, 1H, -CH_{ar}-), 7.48 (br, 2H, -NH_2), 6.45 (d, J = 2.8 Hz, 1H, -CH-), 6.37 (dd, J_1 = 6.3 Hz, J_2 = 2.9 Hz, 1H, -CH-), 5.94 (dd, J_1 = 6.4 Hz, J_2 = 3.6 Hz, 1H, -CH-) 4.56 (dd, J_1 = 9.8 Hz, J_2 = 6.1 Hz, 1H, -CH-), 4.51 (m, 2H, -CH_2-). ^{13}C NMR (75 MHz) δ ppm: 156.22, 152.92, 148.77, 139.74, 118.97, 87.28, 85.89, 85.39, 83.68, 43.44.

0.59 g intermediate (1.8mmol) was suspended in methanol. To the stirred mixture 1 ml H_2O and 0.50 ml concentrated NH_3 were added and the suspension clarified. After 16 h a white had precipitated. After removal of the methanol the solid was recrystallized from 25 ml H_2O. The product was collected by filtration to yield 0.37 mg (72 %) of a white solid.

^{1}H NMR (300 MHz, DMSO-*d6*) δ ppm: 8.35 (s, 1H, -CH_{ar}-), 8.17 (s, 1H, -CH_{ar}-), 7.33 (br, 2H, -NH_2), 5.94 (d, J = 5.0 Hz, 1H, -CH-), 5.62 (d, J = 5.4 Hz, 1H, -OH), 5.48 (d, J = 4.3 Hz, 1H, -OH), 4.76 (d, J = 4.9 Hz, 1H, -CH-), 4.24 (d, J = 3.2 Hz, 1H, -CH-), 4.10 (d, J = 3.2 Hz, 1H, -CH-), 3.92 (dd, J_1 = 10.4 Hz, J_2 = 1.8 Hz, 1H, -C*H*H-), 3.88 (dd, J_1 = 10.6 Hz, J_2 = 1.8 Hz, 1H, -CH*H*-). ^{13}C NMR (75 MHz) δ ppm: 156.08, 152.71, 149.43, 139.74, 119.12, 87.44, 83.63, 72.65, 71.24, 44.80.

Synthesis of *S*-adenosyl-*N*-hexyl-DL-methionine



N-Hexyl-DL-homocysteine[234]

1.0 g DL-Homocysteine thiolactone hydrochloride (6.5 mmol, 1 eq) was suspended in 45 ml dichloromethane and stirred under an argon atmosphere. After addition of 3.44 ml 1-hexanamine (2.63 g, 26 mmol, 4 eq) a clear colourless solution emerged and was protected from light. After 28 h the reaction was stopped with aqueous 1 M NaOH. The phases were separated and the aqueous phase was washed twice with dichloromethane. The aqueous phase was acidified with 1 M HCl to pH around 1 followed by neutralization with saturated $NaHCO_3$. The resulting liquid was extracted three times with dichloromethane. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to give 0.87 g of the pure product (62 %) as a colourless liquid.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 3.49 (dd, J_1 = 7.8, J_2 = 5.0 Hz, 1H, -CH-), 3.20 (q, J = 7.0 Hz, 2H, -CH_2-), 2.61 (m, 2H, -CH_2-), 1.82 (m, 2H, -CH_2-) 1.47 (m, 2H, -CH_2-), 1.26 (m, 6H, -CH_2-), 0.85 (t, J = 6.6 Hz, 3H, -CH_3). ^{13}C (75 MHz) δ ppm: 174.17, 54.03, 39.29, 39.21, 31.53, 29.64, 26.68, 22.61, 21.45, 14.09.

N-hexyl-*S*-adenosyl-DL-homocysteine[227]

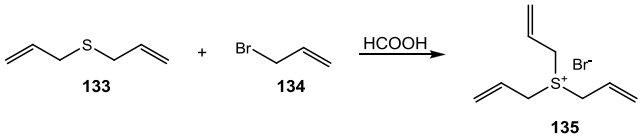
44 mg *N*-hexyl-DL-homocysteine (0.20 mmol, 1.0 eq) was dissolved in 4ml 1 M NaOH in methanol. After addition of 62 mg 5´-chloro-5´-deoxyadenosine (0.22 mmol, 1.1 eq) the solution was stirred and heated to 50 °C. After 3 d the reaction mixture was filtered and purified by preparative HPLC (Macherey Nagel Nucleosil® C_4 150*4mm, 5 µm; H_2O deion./MeOH 60:40; 20 ml min^{-1}; R_t 3.5 min) to give 21 mg of the product (23 %) of a white solid. The product identity was determined by HPLC-MS.

N-hexyl-*S*-adenosyl-DL-methionine

13 mg *N*-hexyl-*S*-adenosyl-DL-homocysteine (0.028 mmol, 1 eq) was dissolved in 2.5 ml formic acid and the resulting yellow solution was cooled to 0 °C. 34.6 µl iodomethane (78.9 mg, 0.56 mmol, 20 eq) and 43 mg silver triflate (0.17 mmol, 6 eq) were added and a solid started to precipitate from the solution. After 24 h water was added and the mixture was washed twice with diethyl ether. The

remaining solid was separated from the aqueous layer by filtration. The aqueous layer was dried by lyophilisation to give 13 mg of the product (77 %). The product identity was determined by HPLC-MS.

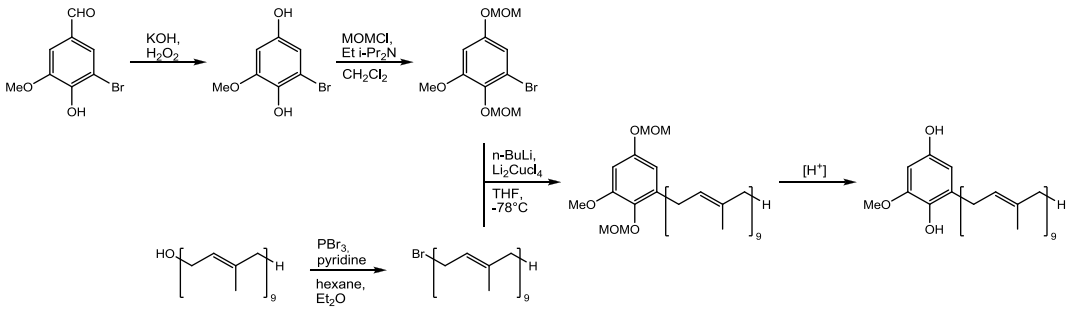
Synthesis of triallylsulfonium bromide



133 134 135

0.64 g diallylsulfide (5.0 mmol, 1 eq) diallylsulfide was dissolved in 10 ml CH_2Cl_2 and 1.7 ml (2.4 g, 20 mmol, 4 eq). The resulting clear and light yellow solution was stirred for 3 d. Because no conversion was detected 0.068 g $ZnCl_2$ (0.50 mmol, 0.1 eq) was added. After another day 0.067 g $AlCl_3$ (0.50 mmol, 0.1 eq) was added and the mixture was stirred for 3d. The reaction was stopped by addition of deion. H_2O and the phases were separated. The aqueous phase was washed once with CH_2Cl_2 and dried by lyophilisation to give 0.38 g (32 %) of a white solid. The product was characterized by HPLC-MS.

Synthesis of 2-methoxy-6-solanesylbenzene-1,4-diol



2-Bromo-6-methoxy-1,4-dihydroxybenzene[247]

5.0 g 5-bromovanillin (22 mmol) were stirred in 25 ml 1 M KOH. 50 ml 3 % H_2O_2 were added to the greenish reaction mixture. A red solution formed. After 16 h pH was determined (pH 6), 5 ml 1 M KOH were added and the solution was heated to 50 °C. After 4 h pH was 7, 10 ml 1 M KOH and 1 ml H_2O_2 were added. After additional 4 h the solution was acidified with 1 N HCl and extracted three times with diethyl ether. The combined organic layers were washed with brine and dried over

sodium sulfate. Purification of the crude product by column chromatography (cyclohexane/ethyl acetate 2:1) yielded a 3:1 mixture of product and starting material. Recrystallisation from toluene gave 1.0g product (22%, according to NMR about 10:1 product/starting material) as light pink solid.
^{1}H NMR (300 MHz, acetone-*d6*) δ ppm: 6.58 (d, *J* = 2.6 Hz, 1H, -CH_{ar}-), 6.49 (d, *J* = 2.6 Hz, 1H, -CH_{ar}-), 3.81 (s, 3H, -CH_3). ^{13}C NMR (75 MHz) δ ppm: 151.55, 149.53, 138.19, 110.91, 109.00, 100.57, 56.51.

Alternative reaction conditions according to literature:[248] Na_2CO_3x1.5 H_2O_2, THF, H_2O; not tested yet.

2-Bromo-6-methoxy-1,4-bis(methoxymethoxy)benzene[248]

0.5 g 2-Bromo-6-methoxy-1,4-dihydroxybenzene (2.3 mmol, 1 eq) were suspended in 15 ml CH_2Cl_2. After addition of of 3.2 ml ethyl diisopropylamine (2.4 g, 18 mmol, 8 eq) all the starting material dissolved under formation of a dark red solution. The addition of 1.1 ml chloromethoxymethane (1.1 g, 13.7 mmol, 6 eq) resulted in gas evolution and the formation of a greenish solution. After 2.5 h the solution turned blue. The reaction was stirred overnight. 20 ml aqueous 2.5 M NH_3 were added and the phases were separated. The aqueous phase was extracted once with diethyl ether. The combined organic layers were washed with water and brine and dried over sodium sulfate. The crude product was purified by column chromatography (cyclohexane/ethyl acetate 10:1) to give 0.24 g pure product (34 %) and 0.48 g impure mixture (68 %).
^{1}H NMR (300 MHz, $CDCL_3$) δ ppm: 6.86 (d, *J* = 2.7 Hz, 1H, -CH_{ar}-), 6.57 (d, *J* = 2.7 Hz, 1H, -CH_{ar}-), 5.11 (s, 2H, -CH_2-), 5.08 (s, 2H, -CH_2-), 3.82 (s, 3H, -CH_3), 3.65 (s, 3H, -CH_3), 3.47 (s, 3H, -CH_3). ^{13}C NMR (75 MHz) δ ppm: 154.36, 153.96, 138.50, 117.84, 111.86, 101.66, 98.84, 95.05, 58.05, 56.21, 56.17.

Solanesyl bromide[249]

0.63 g solanesol (1.0 mmol, 1 eq) was dissolved in 1 ml hexane. 0.030 ml pyridine (0.029 g, 0.37 mmol, 0.37 eq) and 0.5 ml diethyl ether were added and the stirred solution was cooled to 0 °C. 0.06 ml PBr_3 (0.17 g, 0.64 mmol, 1.9 eq) in 0.3 ml hexane was added dropwise over a period of 1 h. After the mixture was stirred for 20 h ice and water was added and the mixture was extracted once with cyclohexane and twice with hexane. The organic layers were combined, washed with brine and dried over sodium sulfate. After the solvent was removed by evaporation, the crude product was purified by column chromatography (cyclohexane/ethyl acetate 10:1) to give 0.62 g of a colourless liquid (89 %).
^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 5.54 (t, *J* = 8 Hz, 1H, =C*H*-CH_2-Br), 5.14-5.10 (m, 8H, -CH=), 4.02 (d, *J* = 8.4 Hz, 2H, -CH_2-Br), 2.15-1.98 (m, 32H, -CH_2-), 1.73 (s, 3H, -CH_3), 1.69 (s, 3H, -CH_3), 1.61 (m, 24H, -

CH_3). ^{13}C NMR (75 MHz) δ ppm: 143.72, 135.81, 135.15, 135.09, 135.07, 135.05, 135.02, 131.36, 124.58, 124.43, 124.33, 123.53, 120.72, 39.90, 39.85, 39.69, 29.73, 26.93, 26.87, 26.84, 26.79, 26.27, 25.83, 17.82, 16.21, 16.18, 16.12.

2-methoxy-6-solanesyl-1,4-bis(methoxymethoxy)benzene[248]

In a schlenk reaction vessel 92 mg 2-Bromo-6-methoxy-1,4-bis(methoxymethoxy)benzene (0.30 mmol, 1.2 eq) was dissolved in 2 ml THF and was stirred under argon. After the solution was cooled to -78 °C 0.16 ml n-butyl lithium 2 M in hexane (1.25 eq) diluted in 1.5 ml THF was added dropwise over a period of 15 min. The solution was stirred for 15 min before 0.10 ml Li_2CuCl_4 0.1 M in THF (0.04 eq) and 0.75 ml THF were added. After additional 30 min 170 mg solanesyl bromide (0.25 mmol, 1.0 eq) in 2.3 ml THF were added dropwise over a period of 20 min. The solution was stirred for 2 h at -78 °C before it was allowed to slowly warm to rt. After 40 h the reaction was quenched with 5 ml saturated aqueous NH_4Cl, made basic with 10 ml aqueous 2N NH_3 and stirred for 20 min. The resulting mixture was extracted with diethyl ether three times. The combined organic layers were washed with water and brine and dried over sodium sulfate. Removal of the solvent gave 0.24 g crude product which was purified by column chromatography (cyclohexane/ethyl acetate 6:1) to yield 0.039mg pure product (19%) as a yellow liquid.

^1H NMR (300 MHz, $CDCL_3$) δ ppm: 6.51 (d, *J* = 2.6 Hz, 1H, -CH_{ar}-), 6.46 (d, *J* = 2.6 Hz, 1H, -CH_{ar}-), 5.33 (m, 1H, -CH_2-), 5.12-5.09 (m, 12H, -CH_2-), 3.85 (s, 3H, -CH_3), 3.65 (s, 3H, -CH_3), 3.34 (d, *J* = 7.3 Hz, 2H, -CH_2-), 2.08-2.00 (m, 32H, -CH_2-), 1.72 (s, 3H, -CH_3), 1.68 (s, 3H, -CH_3), 1.60 (m, 24H, -CH_3). ^{13}C NMR (75 MHz) δ ppm: 150.56, 146.69, 138.62, 136.73, 135.21, 135.06, 131.36, 127.84, 124.57, 124.43, 124.33, 122.01, 109.43, 98.89, 95.67, 56.19, 56.00, 39.89, 39.88, 28.63, 28.24, 27.07, 27.04, 26.93, 26.87, 25.83, 17.82, 16.33, 16.16.

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