BIOALKYLATION CATALYZED BY METHYLTRANSFERASES

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Diese Arbeit ist meinem Opa Viktor gewidmet.

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INTRODUCTION

The methyl group is one of the most widespread functionalities in small molecule drugs occurring in more than 67% of the top-selling drugs of 2011. Upon methylation the chemical, physical and biological properties of a molecule can be altered and optimized in order to achieve adaptation to a certain requirement. A reason for the frequent utilization of the methyl group in drug discovery is the "magic methyl effect". The replacement of C-H by a C-CH_3 group may improve the binding affinity of a small molecule to proteins dramatically. Methylation may energetically favor binding because it reduces the free energy of desolvation required to remove water from the small molecule when it enters the lipophilic active site of the protein. The introduction of a methyl group may improve the binding potency approximately 10-fold if the methyl group fits into a hydrophobic pocket provided that there is space in the active site.[1]

Although significant advances in synthetic chemistry have been achieved allowing the direct methylation of C(sp^2)-H and C(sp^3)-H bonds the call for environmentally benign alternatives has become louder. Since methylation is one of the most common chemical modifications in living cells a variety of enzymes catalyzing the introduction of methyl groups has evolved in nature. These so-called methyltransferases share their requirement of cofactors among which *S*-adenosyl-*L*-methionine (SAM) is by far the most predominant natural source of methyl groups. Since they are involved in many cellular processes their acceptor substrates are diverse ranging from large biopolymers to small metabolites.[1, 2]

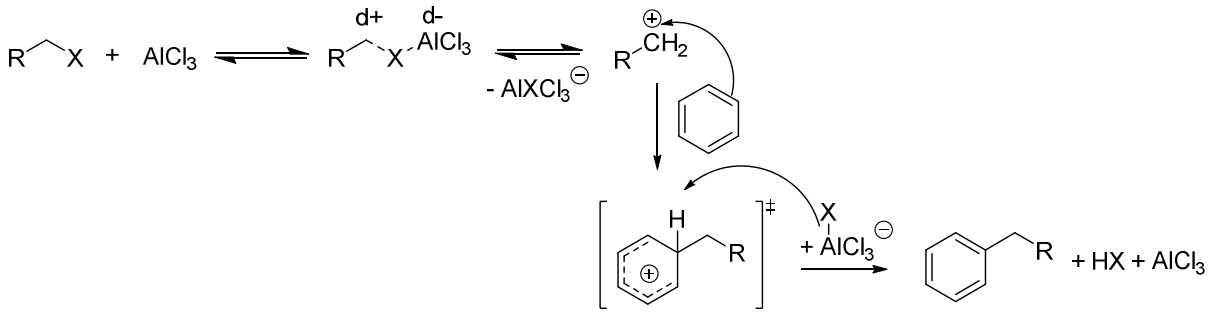
The broad substrate spectrum would allow the utilization of methyltransferases as catalyst for a wide range of methylation reactions. However, to date their technological exploitation is still limited by their cofactor dependence and the lack of a recycling system for SAM. Once this problem has been overcome a toolbox of stable and fully characterized methyltransferases should be provided for biotechnological applications.

THEORETICAL PART

FRIEDEL-CRAFTS alkylation

Carbon-carbon bond formation is one of the most desirable yet challenging reactions in synthetic organic chemistry. The FRIEDEL-CRAFTS alkylation was among the first reactions by which this aim could be accomplished and to date it is the method of choice for the alkylation of aromatic and heteroaromatic compounds.
In1877 Charles FRIEDEL and James Mason CRAFTS published their "*New General Synthetical Method of Producing Hydrocarbons*". In their experiments they converted different alkyl halides like 1-chloropentane, iodoethane and bromomethane with an excess of benzene to the corresponding alkylated benzene derivatives along with some side products utilizing $AlCl_3$ as a LEWIS acid.[3]

The FRIEDEL-CRAFTS alkylation is a LEWIS acid catalyzed electrophilic aromatic substitution reaction which proceeds according to the mechanism shown below:



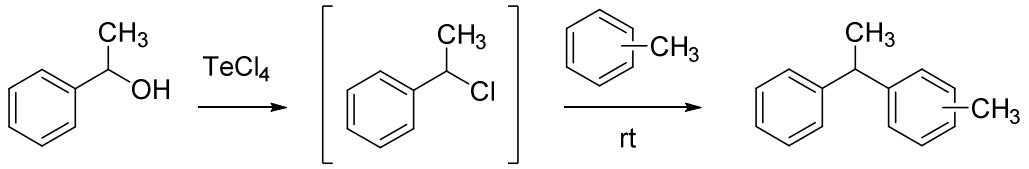
Scheme 1: mechanism of the FRIEDEL-CRAFTS alkylation.

Upon coordination of aluminium chloride to the halogen atom of the alkyl halide the neighboring carbon atom is positively polarized and eventually dehalogenated. The emerging alkyl carbocation is a very reactive electrophile which can be attacked even by weak nucleophiles like benzene. The stable aromatic π system which is temporarily interrupted is restored after deprotonation by the halogen atom yielding the alkylated benzene derivative and the hydrohalogenic acid.

The significance of the FRIEDEL-CRAFTS alkylation for synthetic organic chemistry is unquestioned but the classical reaction has some drawbacks. For the successful alkylation of arenes and heteroarenes alkyl halides and stoichiometric or even super stoichiometric amounts of LEWIS or BRØNSTED acid are required yielding large amounts of salt as a side product.
Therefore the reaction had been further developed and up to now a wide range of new methods is available utilizing only catalytic amounts of LEWIS or BRØNSTED acid and replacing alkyl halides by alcohols and activated alkenes as electrophiles.[4]

Benzylation

One of the first reports of a catalytic FRIEDEL-CRAFTS alkylation with benzyl alcohol was published by UEMURA *et al* in 1986. They performed the chlorination of a benzyl alcohol derivative catalyzed by $SeCl_4$ and $TeCl_4$ in different solvents and obtained the expected chloride in non-aromatic solvents. In aromatic solvents the created chloride was converted to the corresponding 1,1-diarylalkane in a FRIEDEL-CRAFTS alkylation (Scheme 2).[5]

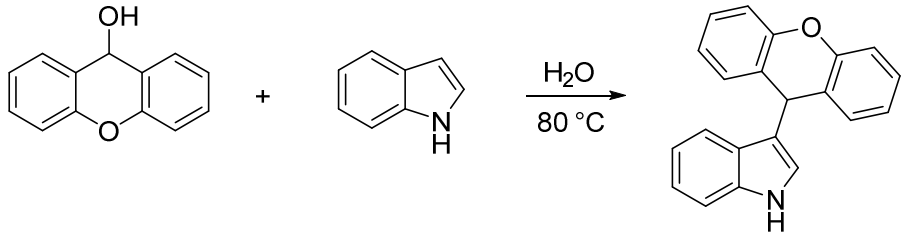


Scheme 2: FRIEDEL-CRAFTS alkylation with 1-phenylethanol and toluene catalyzed by $TeCl_4$.

This discovery was followed by first systematic studies on catalytic FRIEDEL-CRAFTS benzylation by FUKUZAWA and SHIMIZU. SHIMIZU *et al*. utilized a $Mo(CO)_6$ LEWIS acid catalyst which had to be handled under inert conditions.[6] FUKUZAWA *et al*. developed a method utilizing a water and oxygen compatible $Sc(OTf)_3$ catalyst. Among other aromatic compounds benzene and p-xylene were alkylated with benzyl alcohol derivatives to the corresponding 1,1-diarylalkanes.[7,8]

Diverse LEWIS and BRØNSTED acids were tested as catalysts for the FRIEDEL-CRAFTS benzylation. BELLER *et al.* discovered the high activity of late transition metal catalysts and - most worth mentioning -$FeCl_3$ as a readily available and consequently cheap alternative.[9]

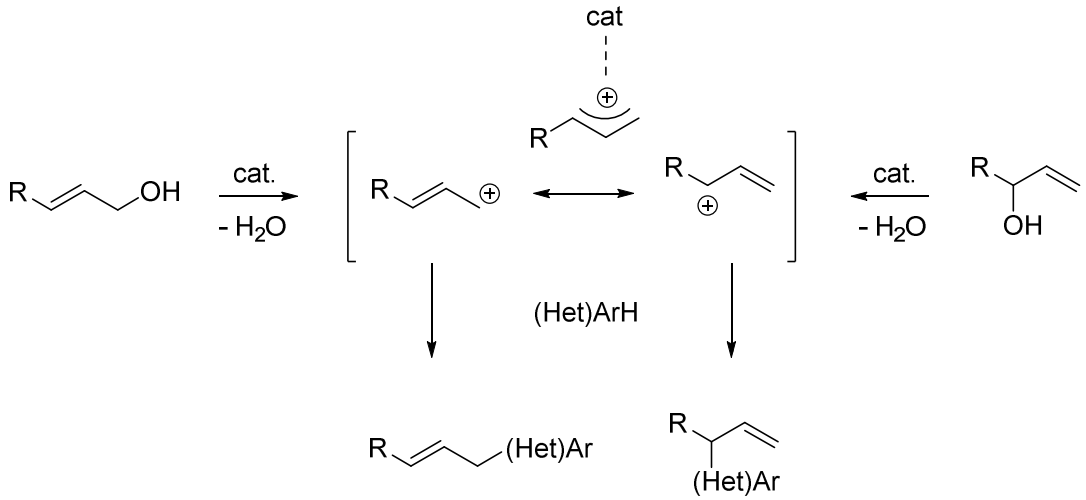
In 2008 COZZI *et al.* published a FRIEDEL-CRAFTS benyzlation protocol which could be performed in water and did not require a LEWIS acid catalyst at all. The reaction is restricted to highly reactive heteroarenes and benzyl alcohol derivatives which form very stable carbocations (Scheme 3).[10]



Scheme 3: FRIEDEL-CRAFTS alkylation without LEWIS acid catalysis.

Allylation

In the transition metal catalyzed FRIEDEL-CRAFTS type allylation the reacting electrophile is a complexed rather than a free allyl cation. Due to the existence of two mesomeric structures linear and branched allylation products can be formed (Scheme 4).[4]



Scheme 4: FRIEDEL-CRAFTS type alkylation with allyl alcohols.

KOCOVSKÝ *et al.* published one of the first catalytic FRIEDEL-CRAFTS alkylation reactions with allyl alcohols. They employed Mo(IV) complexes as LEWIS acid catalysts for the allylation of electron rich aromatic systems such as phenol and anisol. With phenol as a substrate

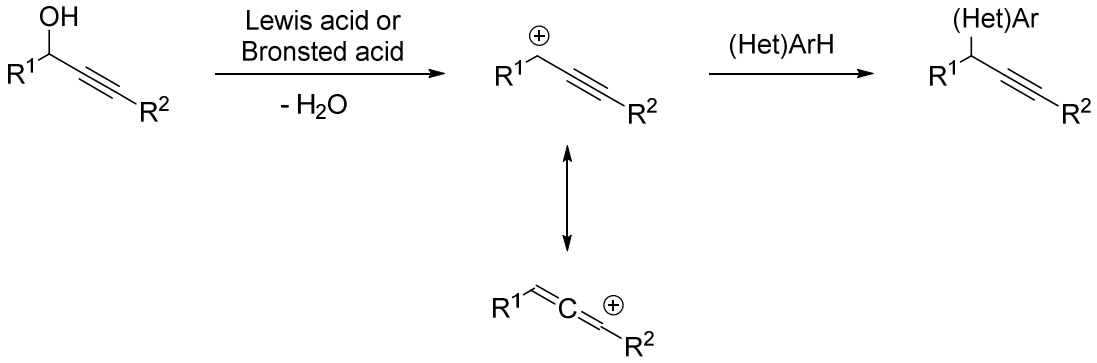
$Mo(acac)_2(SbF_6)_2$ yielded the *para*-*C*-allylated product while with $Mo(acac)_2Cl_2$ as a catalyst *O*-allylation was favored over *C*-allylation.[11]

A method for the catalytic FRIEDEL-CRAFTS alkylation of indol was developed by TAMARU *et al*. utilizing allyl alcohols as alkylating reagents and $Pd(PPh_3)_4$ and triethylborane as the catalytic system. Indoles with electron donating and electron withdrawing groups were tested but the substituents seem not to exert any effect. The reaction selectively yielded the C3-allylated products and no N-allylation was detected.[12]

CHAN *et al.* discovered a diverse collection of BRØNSTED acids and transition metal salts that can act as catalysts for the FRIEDEL-CRAFTS allylation. Electron rich arenes and heteroarenes could be alkylated with allyl alcohols including cinnamyl alcohols and 1-arylated allyl alcohols.[13]

Propargylation

The synthesis of (prop-2-ynyl)arenes by direct propargylation of aromatic systems with propargyl alcohols would be most efficient but it is difficult to accomplish. The propargyl cation is in equilibrium with the allenylium cation which is prone to γ-substitution (Scheme 5).[4]



Scheme 5: FRIEDEL-CRAFTS type alkylation with propargyl alcohols.

FRIEDEL-CRAFTS propargylation was reported with α-arylated propargyl alcohols and different arenes and heteroarenes such as furan, pyrrol and thiophene catalyzed by diruthenium complexes.[14]

For another method for aromatic propargylation the water and oxygen compatible rhenium oxo complex (dppm)Re(O)Cl_3 was utilized as a catalyst. TOSTE *et al.* were able to establish a regioselective reaction yielding no allenylic side products but only the propargylic arenes.[15]

Methylation

FRIEDEL-CRAFTS alkylation is not the method of choice for the methylation of aromatic systems.
The first alkyl group that was attached to the arene exerts a positive inductive effect which increases the nucleophilicity of the aromatic system and activates it for further electrophilic aromatic substitution reactions. Therefore the already alkylated arene has a higher reactivity than the non-alkylated arene and the formation of polyalkylated products is favored.

Methylation of arenes can be accomplished by alternative methods such as formylation followed by conversion of the aldehyde to the corresponding alkane by CLEMMENSEN or WOLFF-KISHNER reductions. For acid or base sensitive substrates milder methods (e.g. MOZINGO reduction) must be applied.

Thiol alkylation

One of the first methods for thioether synthesis was established by MCALLAN*et al.*[16] The production of dialkyl sulfides was accomplished by a nucleophilic substitution reaction in which the alkyl or aryl thiol was activated as a nucleophile upon deprotonation by an inorganic base in aqueous solution. Due to the application of strong electrophiles like alkyl halide which could also react with other nucleophiles like alcohols the chemoselectivity of the method is unsatisfying.

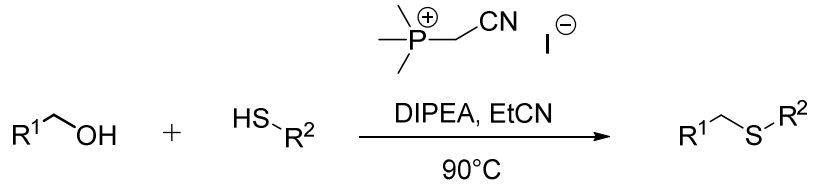
To date a variety of methods is available for the synthesis of thioethers. Some of them are shortly summed up in this section.

S-alkylation of thiols with alcohols

The conversion of benzyl alcohol with 4-methylthiophenol in the presence of $ZrCl_4$ dispersed on molecular sieves, alumina, or silica gel was studied as a model reaction for thiol alkylation. By this method the synthesis of different thioethers from aliphatic and aromatic thiols and benzyl alcohol derivatives and cinnamyl alcohol could be accomplished. Aliphatic primary and secondary alcohols could not be utilized for alkylation.[17]

MITSUNOBU-type reactions

Primary aliphatic alcohols could be employed for the *S*-alkylation of thiols in the presence of (cyanomethyl)trimethylphosphonium iodide yielding unsymmetric thioethers (Scheme 6).[18]

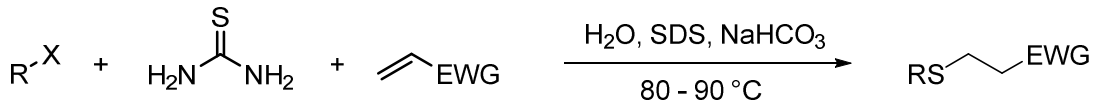


Scheme 6: alkylation of thiols with alcohols mediated by a phosphonium iodide.

These two methods are alternatives for which the conversion of the alcohol to an alkylating agent is not required.

Thia-MICHAEL addition reaction

Thia-Michael products could be obtained from primary, allyl and benzyl bromides and iodides, thiourea and electron deficient alkanes in a sodiumdodecyl sulfate (SDS) catalyzed reaction. Hydrolysis of the intermediate *S*-alkylisothiouronium salts yields the corresponding thiols which are added to the alkanes to give the thia-Michael products (Scheme 7).[19]

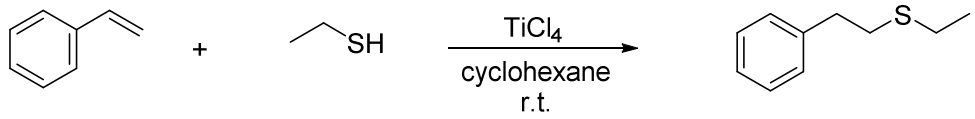


Scheme 7: thia-MICHAEL addition catalyzed by SDS micellar solution.

Addition of thiols to alkenes

The synthesis of MARKOVNIKOV thioethers can be accomplished be the BRØNSTED or LEWIS acid catalyzed addition of thiols to alkenes.[20]

One of the few methods for the synthesis of anti-MARKOVNIKOV thioethers was developed by MUKAIYAMA *et al* in 1973. $TiCl_4$ was employed as a promoter for the addition of ethanethiol to styrene and α-methylstyrene yielding the corresponding anti-MARKOVNIKOV products (Scheme 8).[21]



Scheme 8: synthesis of anti-MARKOVNIKOV thioether.

Addition of thiols to alkynes

The addition of thiols to alkynes is a method for the synthesis of vinyl sulfides. Thiols are known to add to alkynes according to a radical mechanism to yield anti-MARKOVNIKOV vinylic sulfides with excellent regioselectivity. The synthesis of the MARKOVNIKOV products can be accomplished by palladium catalyzed hydrothiolation of alkynes with $PdCl_2(PhCN)_2$. Anti-MARKOVNIKOV addition of thiols to alkynes can be obtained by utilizing rhodium catalysts such as $RhCl(PPh_3)_3$.[22]

Biocatalytic Alkylation

Impact of methylation on biological and chemical properties

As implied by the name, methyltransferases are enzymes that catalyze the transfer reaction of a methyl group from a donor to an acceptor molecule. Methylation may equip a molecule with the chemical properties which are essential for its biological function. Addition or subtraction of a methyl group may affect the biological activity of a molecule dramatically.[23]

An example of this effect is X-inactivation by which most of the genes on one of the two X chromosomes of female mammalian cells are silenced. Upon enzymatic methylation of the 5' position of cytosine nucleobases[24] the DNA is tightly packaged into the more compact type of chromatin called heterochromatin which is inaccessible for transcription. X-inactivation is essential to prevent female cells from having a twice as high expression level of genes on the X chromosome as male cells.

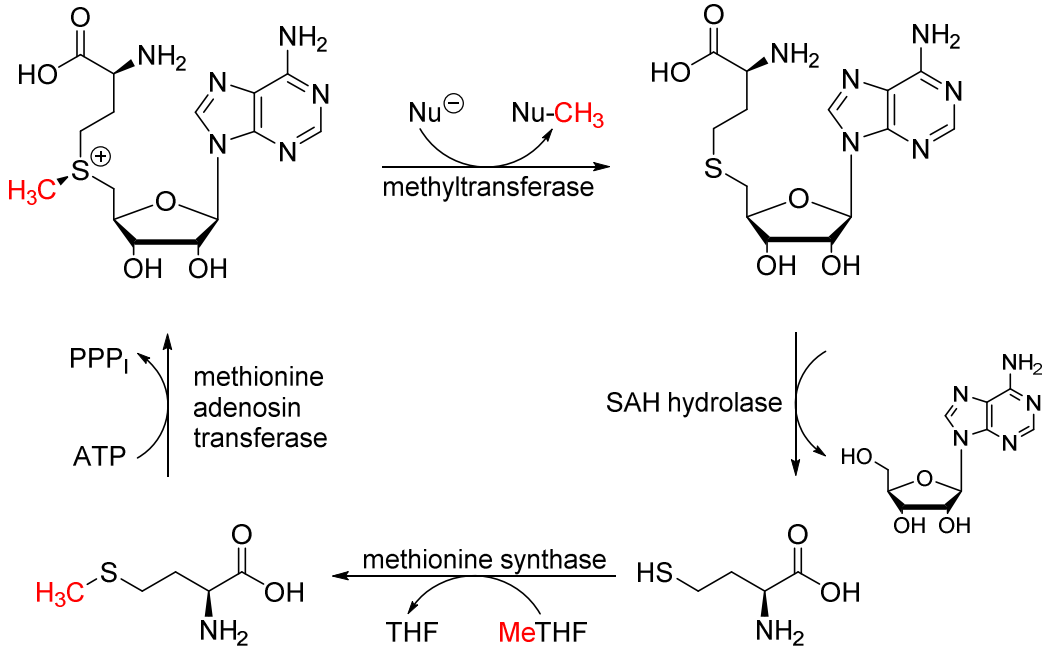
Regarding the chemical properties of a molecule, methylation may lead to an Umpolung and lipophilization of alcohols, thiols and amines or to a change of its electronic and steric properties.[23]

Methyltransferases

Methyltransferases are ubiquitous enzymes that occur throughout all kingdoms of life which indicates the importance of methylation in nature.
The substrates of methyltransferases are diverse ranging from large biomolecules like DNA, RNA, proteins, lipids and carbohydrates to small molecules. The enzymes are highly selective regarding the type of substrate and the atom they transfer the methyl group to. Therefore methyltransferases are classified according to their substrate as DNA, RNA, protein and small molecule methyltransferases or according to their acceptor atom as C-, N-, O- and S-methyltransferases.[23]

What all methyltransferases have in common is their requirement of a coenzyme. In nature *S*-adenosyl-*L*-methionine is the most frequently employed methyl group donor and was first described by CANTONI.[25] Alternative coenzymes accepted by specific methyltransferases are among others *S*-methyl-*L*-methionine, methyltetrahydrofolate, methylcobalamin and betaine.

The methyl transfer reaction proceeds according to the mechanism of a nucleophilic substitution reaction. In the active site of the methyltransferase the substrate is activated upon deprotonation by an acidic amino acid. The resulting nucleophile attacks the carbon atom of the methyl group and *S*-adenosyl-*L*-homocysteine (SAH) acts as a leaving group. SAH is subsequently hydrolyzed to adenine and homocysteine by SAH hydrolase. The next step of the so called "activated methyl cycle" is the methylation of homocysteine by methionine synthase and methyltetrahydrofolate as a methyl donor yielding methionine. Ultimately the adenosine moiety of ATP and methionine are attached to each other by the methionine adenosine transferase yielding *S*-adenosyl-*L*-methionine (Scheme 9).
The methyl group of *S*-adenosyl-*L*-methionine was described as "natural methyl iodide" in the literature.[26]

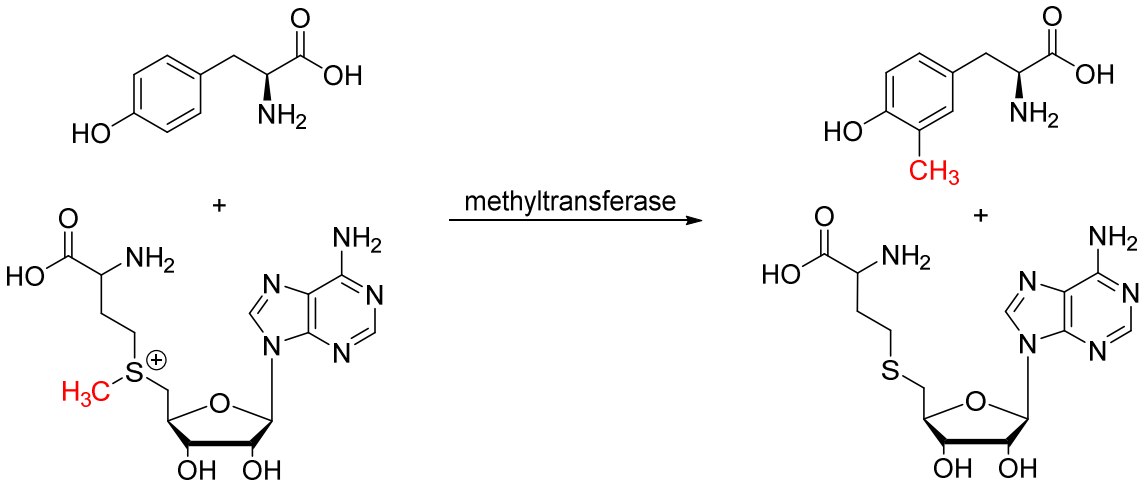


Scheme 9: activated methyl cycle.

The enzymes investigated in the course of this thesis belong to the class of small molecule *C*- and *S*-methyltransferases. The respective enzymes are described in the following section.

Tyrosine *C*-Methyltransferases

The *S*-adenosyl-*L*-methionine dependent methyltransferases SacF and SfmM2 catalyze the methylation of *L*-tyrosine yielding 3-methyltyrosine (Scheme 10) which is a precursor of the tetrahydroisoquinoline moiety in the antibiotics Safracin and Saframycin.



Scheme 10: methylation of *L*-tyrosine catalyzed by a methyltransferase.

SacF is involved in the biosynthesis of Safracin which is produced by *Pseudomonas fluorescence*[27] and SfmM2 is the tyrosine *C*-methyltransferase in *Streptomyces lavendulae* producing Saframycin.[28] Both compounds are isoquinoline alkaloids with antibiotic and antitumor activity.[29, 30]

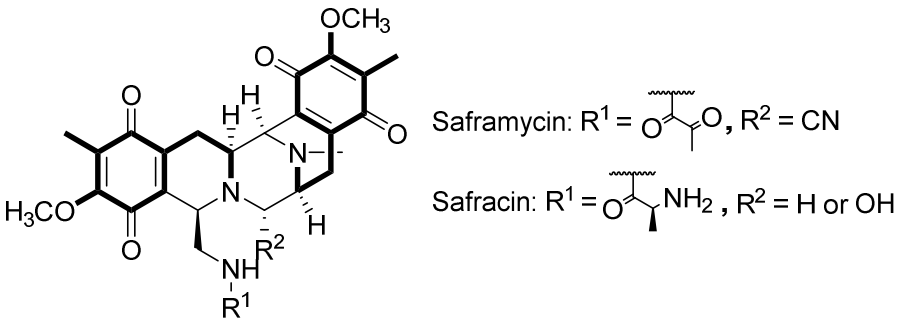


Figure 1: structures of Saframycin A and Safracin B. The moieties of the molecule deriving from 3'-methyltyrosine are drawn in bold.[31]

Aminocoumarin *C*-Methyltransferases

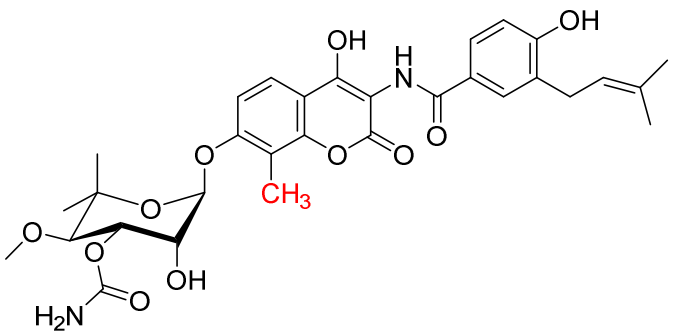
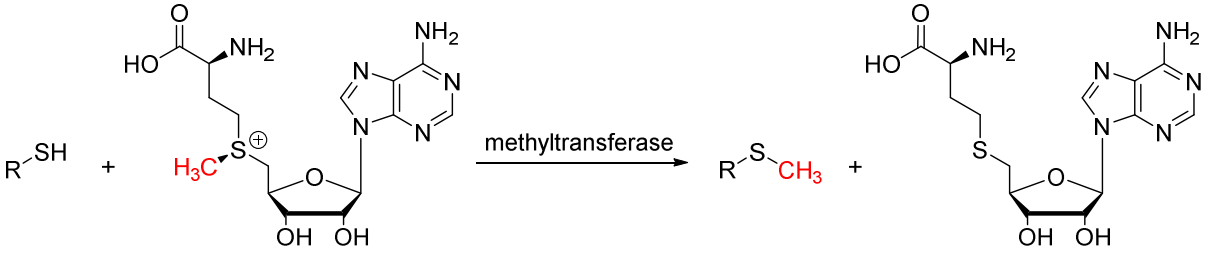


Figure 2: structure of novobiocin. The methyl group that is transferred by NovO is drawn in red. [31]

The enzyme NovO catalyzes the transfer of a methyl group from *S*-adenosyl-*L*-methionine to the C-8 atom of the aminocoumarin moiety in the antibiotic novobiocin. The biosynthesis of novobiocin was first discovered by SAVAGE *et al.* in *Streptomyces niveus*[32] and by FOLKERS *et al.* in *Streptomyces spheroides*[33] simultaneously and the compound was published as streptonividin[23] and cathomycin[24] respectively. *Streptomyces niveus* and *Streptomyces spheroids* were later classified as the same species[34] and the compound was named novobiocin.

S-Methyltransferases

*Bo*tmt is an *S*-adenosyl-*L*-methionine dependent methyltransferase that was purified from the leaves of *Brassica oleracea*. The plant enzyme is involved in sulfur metabolism and the detoxification of reactive thiols which are methylated to yield the more volatile thioethers. The enzyme was reported to catalyze the methyl transfer to the halides iodide, bromide and chloride and to thiols (e.g. thiocyanate and thiophenol). Since *O*- and *N*-methylation was not observed *Bo*tmt was classified as *S*-methyltransferase.[35, 36, 37]



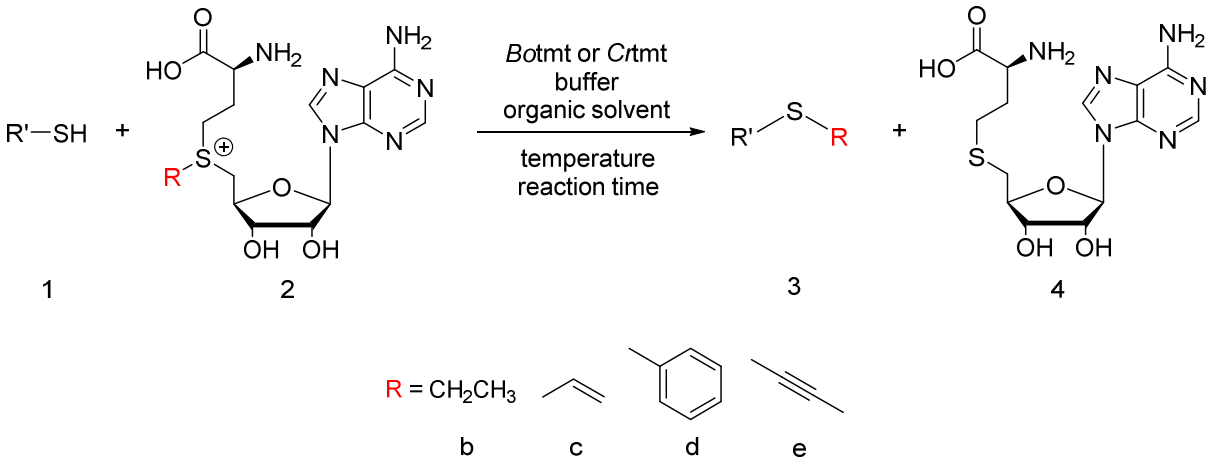
Scheme 11: methylation of a thiol catalyzed by a *S*-methyltransferase.

Type I and II *S*-adenosyl-*L*-methionine dependent *O*-methyltransferases catalyze the majority of the methylations of hydroxyl group of small molecules in plants. SCHRÖDER *et al.* identified new type I OMT family members in *Catharanthus roseus*. Unexpectedly, one of the enzymes catalyzed the methylation of thiols instead of hydroxyl groups. *Cr*tmt is the only known enzyme in the family of small molecule plant *O*-methyltransferases acting as a *S*-methyltransferase.[38]

AIMS OF THE MASTER THESIS

It is estimated that the (bio)synthesis of one half of the medicinally applied natural products involves a methylation step.[23] Therefore there is a great demand on chemo- and regioselective methods by which a methyl or alkyl group can be introduced into a molecule.
However, for many chemical alkylation reactions rough conditions and / or toxic alkyl halides need to be employed. The presence of other functional groups is often not tolerated resulting in the requirement of protecting groups.
Up to now a range of metal catalyzed alkylation methods has been developed utilizing metals like Ru, Rh and Pd. These precious metals are not only expensive but sometimes also difficult to remove completely from the reaction mixture which is a problem in the pharmaceutical industry in particular.
Therefore a biocatalytic equivalent would be a mild and selective alternative by which precious metals and rough reaction conditions could be avoided.

This work should contribute to the establishment of methyltransferases as valuable catalysts for alkylation reactions in academic research and subsequently in industrial applications.
The focus is on selected small molecule *C*- and *S*-methyltransferases. To date small molecule methyltransferases are rather unexplored enzymes and research is still at the beginning.
In order to determine basic characteristics such as substrate scope of the two plant *S*-methyltransferases *Bo*tmt and *Cr*tmt a reliable and reproducible assay should be developed. This involves the investigation upon the temperature optimum, the organic solvent tolerance and the preferred aqueous system for each enzyme. Furthermore the ability of both *S*-methyltransferases to act as alkyltransferases with artificial cofactors (**2 b-e**) should be tested (Scheme 12). Analogs of the natural cofactor *S*-adenosyl-*L*-methionine (**2 b-e**) should be synthesized which may act as alkyl donors in the alkyl transfer reaction.



Scheme 12: alkylation of a thiol catalyzed by a *S*-methyltransferase.

For the *C*-methyltransferases SacF, SfmM2 and NovO the assay development and the characterization regarding their substrate and cofactor scope were already done.[31] The most promising substrates should be alkylated applying the synthesized cofactors (**2 c-e**) and the corresponding products should be isolated and characterized.

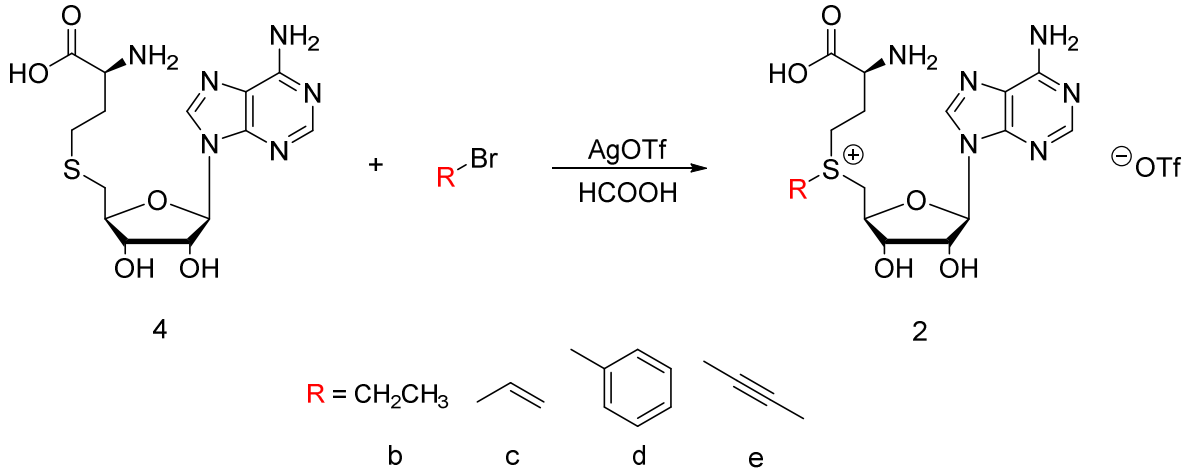
RESULTS & DISCUSSION

Syntheses of artificial cofactors

S-adenosyl-*L*-homocysteine (**4**) was alkylated with allyl, benzyl and 2-butynyl bromide utilizing formic acid as a solvent and silver trifluoromethanesulfonate as a LEWIS acid activator and catalyst.[39]

The addition of silver triflate leads to the formation and precipitation of silver bromide which is a driving force of the reaction. Since silver bromide is removed from the reaction mixture the back reaction is suppressed and the equilibrium is shifted to the product side.

Studies on the stability of *S*-adenosyl-*L*-methionine (**2a**) in solution showed that the counterion of the sulfonium should be a large, non-nucleophilic anion such as trifluormethanesulfonate.[40]



Scheme13: syntheses of SAM analogs.

According to HPLC-MS allyl, benzyl, 2-butynyl and ethyl SAH (**2 b-e**) were successfully synthesized. However, complete conversion could not be achieved with any of the alkyl moieties with 40 eq. of the corresponding alkyl bromide. The reactions were run for 48 hours but the conversion of SAH (**4**) to SAM analogs (**2 b-e**) stopped after the first 24 hours. Subsequently the remaining SAH (**4**) was converted to its degradation product adenine.

All artificial cofactors (**2 b-e**) were applied as crude products without purification for the cofactor scope screening with the S-methyltransferase *Bo*tmt. The allyl, benzyl and 2-butynyl analogs of *S*-adensoyl-*L*-methionine (**2 c-e**) were employed as alkyl donors for the

experiments with the *C*-methyltransferase NovO and 4,5,7-trihydroxy-3-phenylcoumarin (**8**) as a substrate.

C-Methyltransferases

SacF and SfmM2

Aqueous phase screening and pH tests with SacF

The substrate scope screening showed the narrow substrate spectrum of the enzymes SacF and SfmM2. Only their natural substrate *L*-tyrosine (**5**), *D*-tyrosine and *L*-dopa were accepted by the enzymes.[31]

Due to the low solubility of *L*-tyrosine (**5**) in water aqueous NaOH solution has been used to dissolve the substrate. This experiment should clarify if the buffer capacity of the salts contained in the lyophilized cells is sufficient for pH maintenance. Therefore SacF (lyophilized crude enzyme preparation) has been suspended in either water or sodium phosphate buffer (50 mM pH 7).

Table 1: pH tests for tyrosine *C*-methyltransferases assay.

	components	pH
1	5.5 mg/ml SacF in water	7.3
2	10 mg/ml SacF in water	7.3
3	5.5 mg/ml SacF in buffer	7.2
4	10 mg/ml SacF in buffer	7.1
5	5.5 mg/ml SacF in water + 1eq *L*-tyrosine in $NaOH_{aq}$	8.3
6	10 mg/ml SacF in water + 1eq *L*-tyrosine in $NaOH_{aq}$	7.7
7	5.5 mg/ml SacF in buffer + 1eq *L*-tyrosine in $NaOH_{aq}$	7.3
8	10 mg/ml SacF in buffer + 1eq *L*-tyrosine in $NaOH_{aq}$	7.2
9	5.5 mg/ml SacF in water + 1eq *L*-tyrosine in $NaOH_{aq}$ + 1 eq SAM	6.1*
10	10 mg/ml SacF in water + 1eq *L*-tyrosine in $NaOH_{aq}$ + 2 eq SAM	5.6*
11	5.5 mg/ml SacF in buffer + 1eq *L*-tyrosine in $NaOH_{aq}$ + 1 eq SAM	7.0
12	10 mg/ml SacF in buffer + 1eq *L*-tyrosine in $NaOH_{aq}$ + 2 eq SAM	6.9

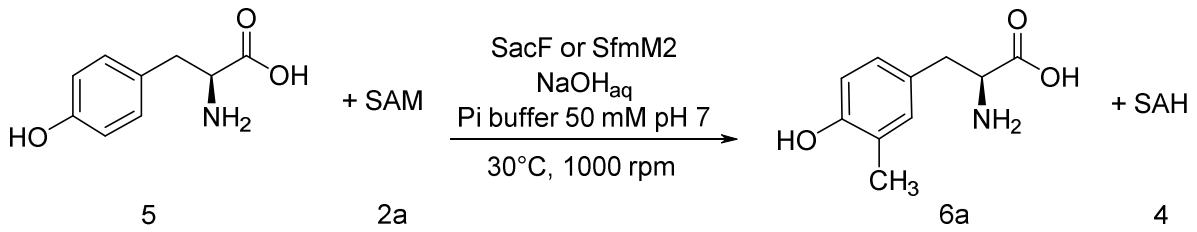
*precipitation occurred

There are differences between 5.5 mg/ml and 10 mg/ml in water and in buffer because the lyophilized cells contain buffer salts. Since there is more phosphate in the 10 mg/ml samples the pH is closer to pH 7 than that of the samples with 5.5 mg/ml (Table 1 entries 3-8).

The phosphate concentration in the lyophilisate is not sufficient to keep the pH constant. To buffer the basicity of NaOH and the acidity of *S*-adenosyl-*L*-methionine chloride dihydrochloride (**2a**) the lyophilized crude enzyme preparation must be suspended in sodium phosphate buffer (50 mM pH 7) instead of pure water.

Activity assay of SacF and SfmM2

The assay that was applied for all screenings with SacF and SfmM2 was already established.[31]



Scheme 14: methylation of *L*-tyrosine (5) catalyzed by SacF or SfmM2.

The activities of the *C*-methyltransferases SacF as crude lysate and SfmM2 as purified enzyme have been compared (Scheme 14).

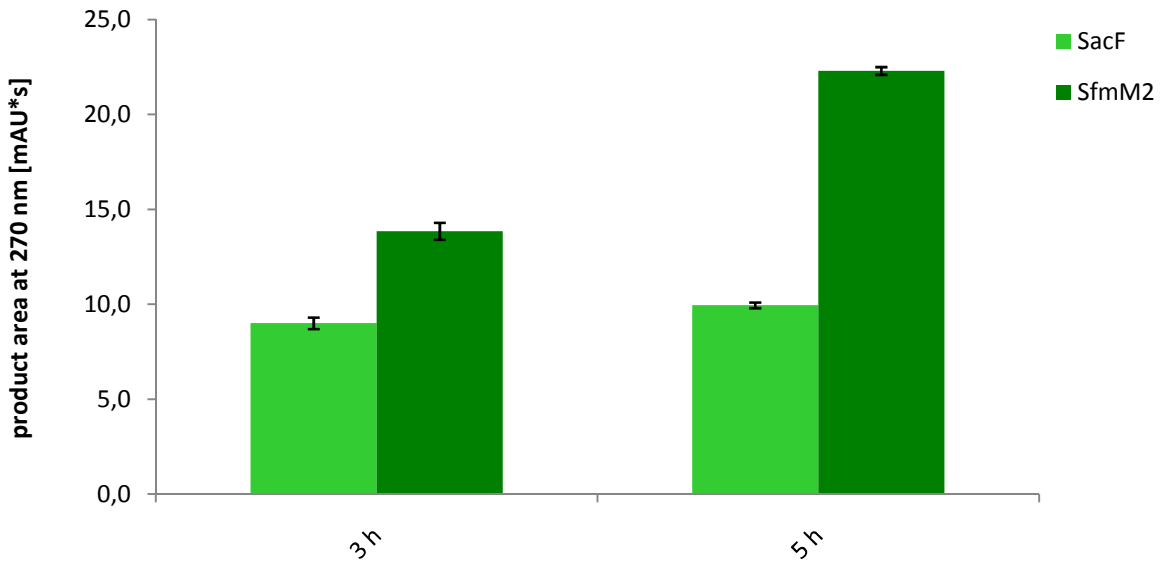
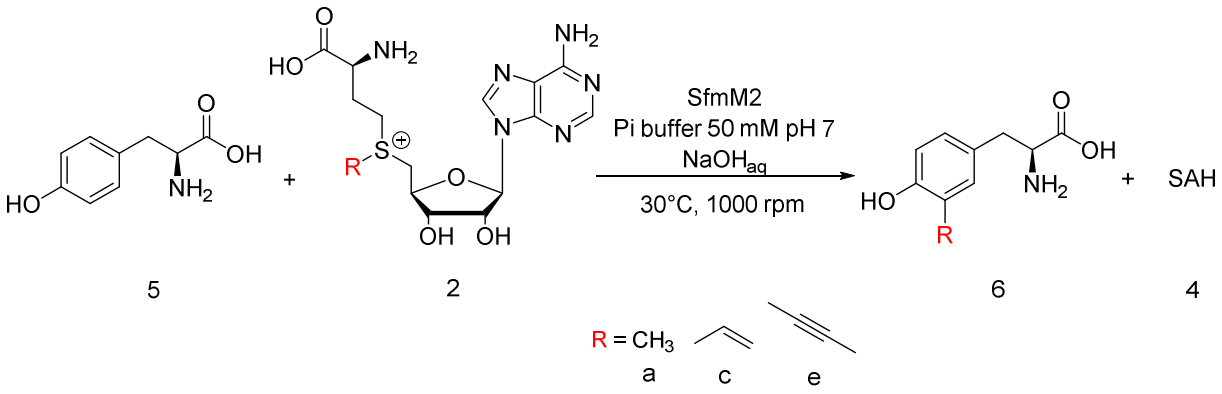


Figure 3: formation of 3-methyltyrosine catalyzed by SacF and SfmM2.

In the case of SfmM2 the substrate *L*-tyrosine (**5**) is not metabolized by the other enzymes that are contained in the crude lysate of SacF. Therefore the purified enzyme is provided with a higher substrate concentration compared to the lysate and consequently more product can be formed (Figure 3). Furthermore the formation of 3-methyltyrosine (**6a**) is exclusively due to the *C*-methyltransferase SfmM2.

Cofactor scope screening



Scheme 15: alkylation of *L*-tyrosine catalyzed by SfmM2.

The *C*-methyltransferase SfmM2 has been screened as alkyltransferase. Therefore *S*-adenosyl-*L*-methionine analogs with allyl and 2-butynyl moieties (**2c, 2e**) have been synthesized and utilized for conversion to the corresponding 3-alkyl-*L*-tyrosines (**6c, 6e**) (Scheme 15).

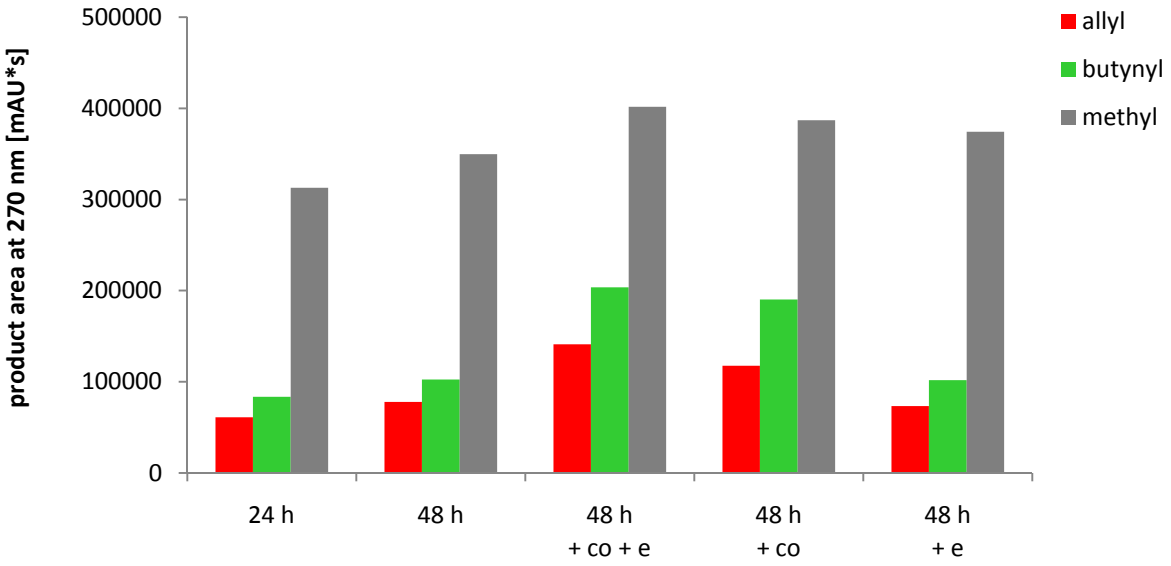
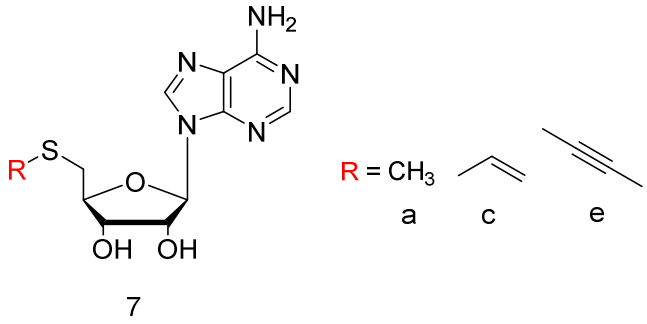


Figure 4: effect of addition of cofactor and / or enzyme on the product concentration. 24 h: the reaction was stopped after 24 hours. **48 h**: the reaction was stopped after 48 hours. **48 h + co + e**: after 24 hours another portion of cofactor and enzyme was added and the reaction was stopped after 48 hours. **48 h + co**: after 24 hours another portion of cofactor was added and the reaction was stopped after 48 hours. **48 h + e**: after 24 hours another portion of enzyme was added and the reaction was stopped after 48 hours.

Comparing the product areas after 24 and 48 hours shows that the concentration of 3-methyl-*L*-tyrosine (**6a**) increases by 28 % when cofactor and enzyme are added and by 12 % without addition. The concentrations of 3-allyl-*L*-tyrosine (**6c**) and 3-(2-butynyl)-*L*-tyrosine (**6e**) have more than doubled upon addition of cofactor and enzyme (Figure 4).

100 % conversion of *L*-tyrosine (**5**) can be achieved with neither of the alkyl moieties. Cofactor instability might be a possible explanation for the incomplete conversion because after 24 h and 48 h none of the cofactors can be detected in the intact form. Only degradation products such as adenine and (**7a, 7c, 7e**) are found.



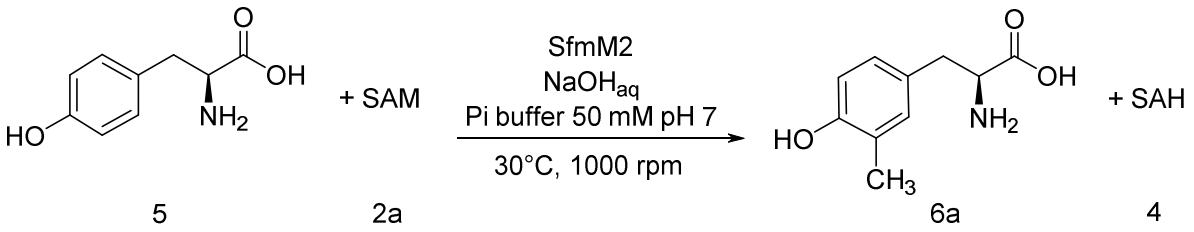
7

Figure 5: degradation products of the cofactors.

In reactions catalyzed by *S*-adenosyl-*L*-methionine (**2a**) dependent methyltransferases *S*-adenosylhomocysteine (**4**) is formed as side product. In literature SAH (**4**) is reported repeatedly to be an inhibitor of most SAM (**2a**) dependent methyltransferases[41] which may be another argumentation for incomplete conversion of L-tyrosine (**5**).

A possibility to enhance product formation might be adding of *S*-adenosylhomocysteine (**4**) hydrolase which selectively degrades *S*-adenosylhomocysteine (**4**) to adenosine and homocysteine and consequently prevents inhibition.

Cofactor concentration screening



5 2a 6a 4

Scheme 16: methylation of *L*-tyrosine (5) catalyzed by SfmM2.

The cofactor concentration screening with the purified enzyme SfmM2 should show whether there is a significant increase in product concentration with 2 eq. of *S*-adenosyl-*L*-methionine (**2a**) compared to 1 eq (Scheme 16).

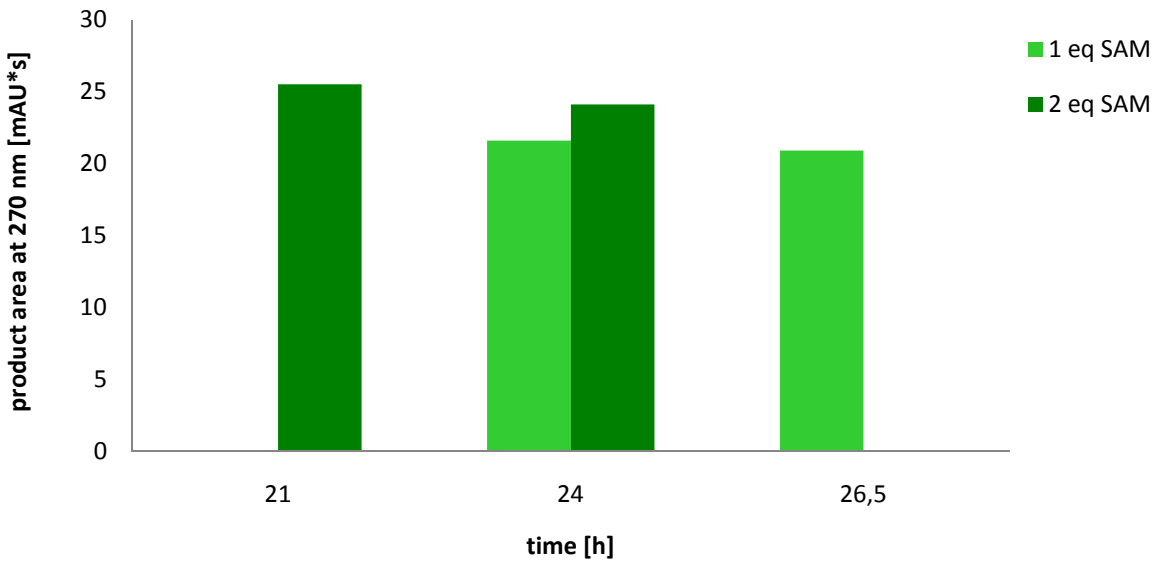


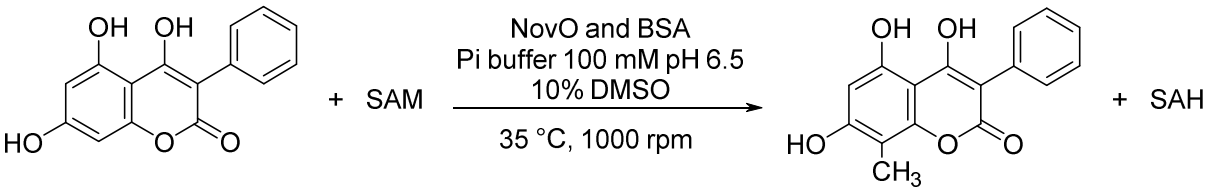
Figure 6: formation of 3-methyltyrosine (6a) with 1 eq. and 2 eq. of SAM (2a) after different reaction times.

After 24 hours more than 10 % less product is formed with 1 eq. of SAM (**2a**). Therefore 2 eq. of SAM (**2a**) are used for further experiments as it was done in the previous ones.
Comparison of the product areas in Figure 3 and Figure 6 shows that after 5 hours almost the same amount of product is formed as after 21 hours. This is possibly due to the instability of SfmM2 and SAM (**2a**) or to inhibition of SfmM2 by SAH (**4**).

NovO

Activity assay

From the previously performed substrate scope screening 4,5,7-trihydroxy-3-phenylcoumarin (**8**) is known to be one of the substrates accepted by NovO (Scheme 17). The *C*-methyltransferase showed high activity with the substrate and complete conversion was achieved within 24 hours.[31]



8 2a 9a 4

Scheme 17: methylation of 4,5,7-trihydroxy-3-phenylcoumarin (8) catalyzed by NovO.

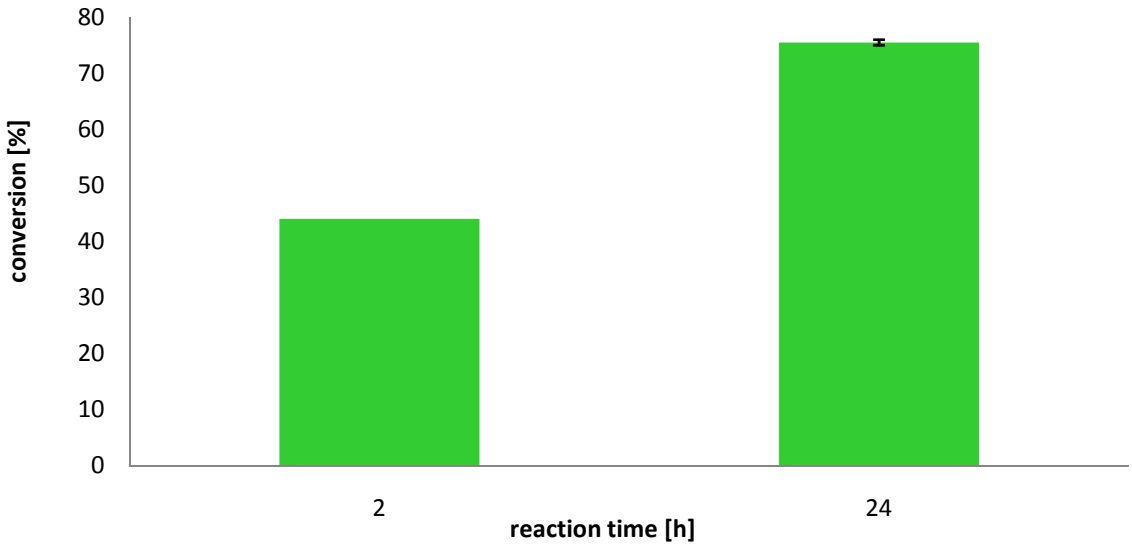
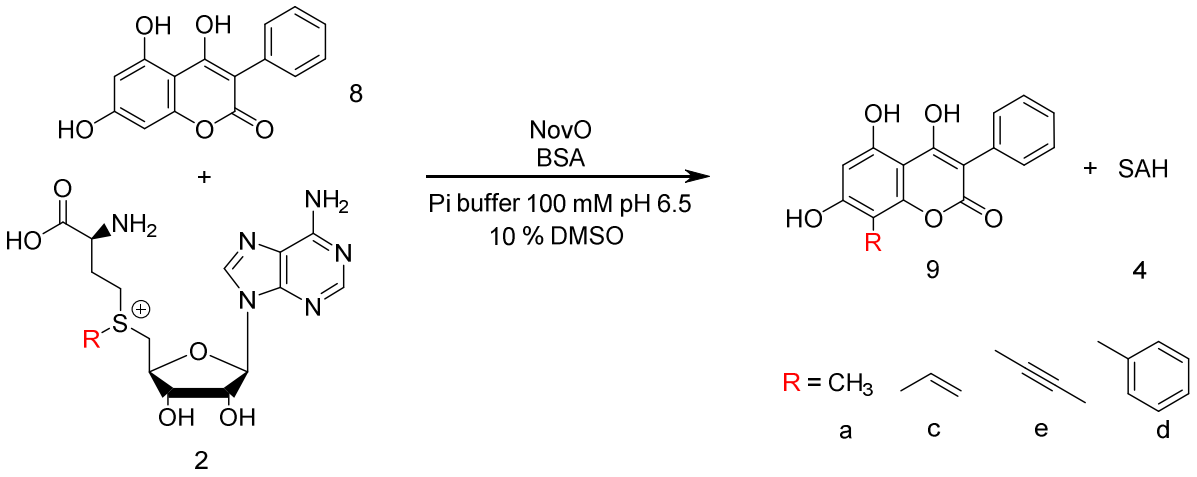


Figure 7: activity and stability of NovO.

The crude lysate has been frozen since 2009 and therefore its activity has to be tested before using it for preparative experiments. Apart from the satisfying activity, the enzyme also shows good stability. Comparison of the conversions after 2 and 24 hours showed that there was an increase of approximately 30 % (Figure 7).

Syntheses of alkylated 4,5,7-trihydroxy-3-phenylcoumarins



Scheme 18: alkylation of 4,5,7-trihydroxy-3-phenylcoumarin (8) catalyzed by NovO.

In previous experiments methyl, allyl, benzyl and 2-butynyl groups could be transferred from the corresponding cofactor (**2a, 2c-e**) to the substrate by the *C*-methyltransferase NovO.[31] These experiments have been up-scaled in order to isolate and characterize the alkylation products.

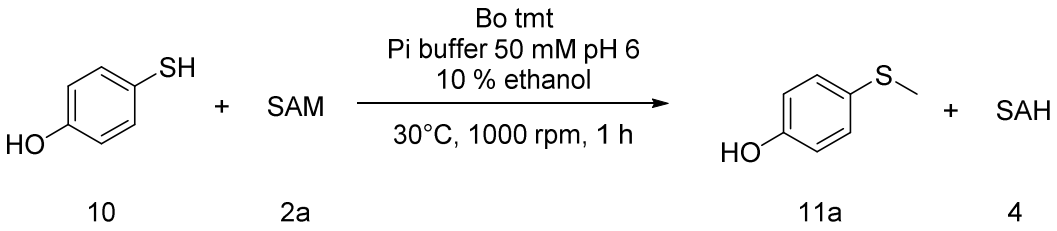
According to the HPLC-MS analysis allylated (**9c**), benzylated (**9d**) and 2-butynylated 4,5,7-trihydroxy-3-phenylcoumarin (**9e**) and 4,5,7-trihydroxy-8-methyl-3-phenylcoumarin (**9a**) have been successfully synthesized. However, only the methylated product can be purified by silica filtration and characterized. The other alkylation products cannot be obtained in high purity by preparative silica gel TLC according to NMR. It is likely that the higher alkylated products are instable and subject to decomposition on the acidic silica gel.

S-Methyltransferases

*Bo*tmt

For the chemical characterization of the enzyme regarding its substrate and cofactor scope a reliable and reproducible assay needed to be set up. By variation of parameters such as buffer,

solvent and temperature the ideal conditions for the enzyme and the reaction should be found. For the assay development 4-hydroxythiophenol (**10**) was chosen as a substrate. At the start the conditions shown in Scheme 19 were applied.



Scheme 19: first assay set up for the methylation of 4-hydroxythiophenol catalyzed by the *S*-methyltransferase *Bo*tmt.

Expression condition screening

In this screening the activity of the *S*-methyltransferases *Bo*tmt cultivated under different conditions has been compared. The enzyme is expressed in two different *E.coli* strains (BL 21 Gold (DE 3) and C43) and two different media (LB or 2xTY). Expression is induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) in one half of the cultures.

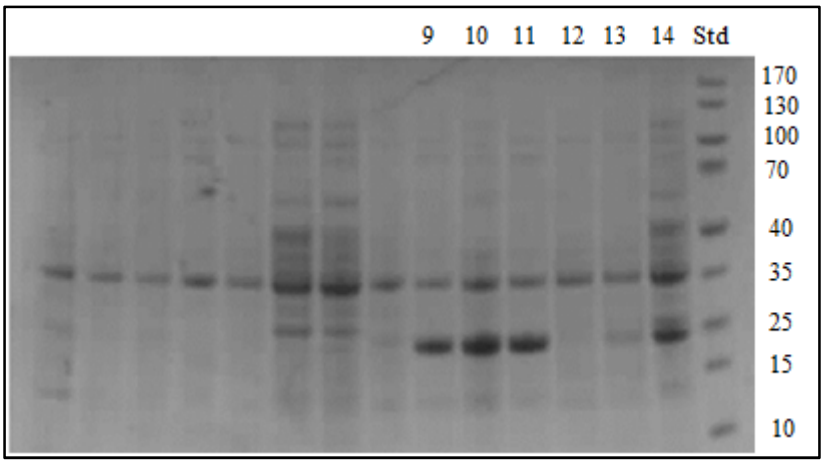
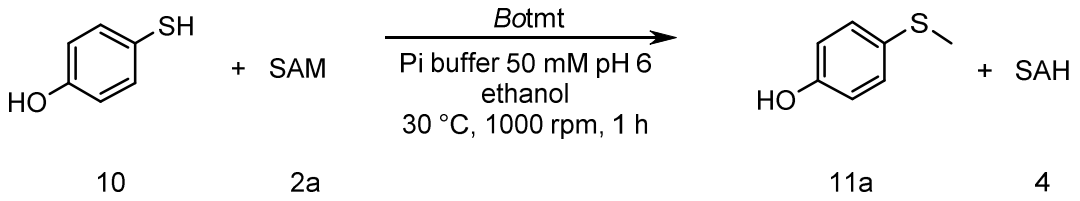


Figure 8: SDS-PAGE of different *E.coli* cultures expressing *Bo*tmt (26 kDa). Lysates with protein concentrations from 20 to 30 mg/ml were applied. 9 *Bo*tmt BL 21 Gold (DE 3) LB with IPTG, 10 *Bo*tmt BL 21 Gold (DE 3) 2xTY without IPTG, 11 *Bo*tmt BL 21 Gold (DE 3) 2xTY with IPTG, 12 *Bo*tmt C43 LB without IPTG, 13 *Bo*tmt C43 LB with IPTG, 14 *Bo*tmt C432xTY without IPTG, Standard PageRuler Prestained Protein Ladder.

According to the SDS-PAGE non-induced expression in 2xTY medium shows the highest levels of *Bo*tmt for both, *E.coli* BL 21 Gold (DE 3) and *E.coli* C43 respectively (Figure 8).



Scheme 20: methylation of 4-hydroxythiophenol catalyzed by *Bo*tmt.

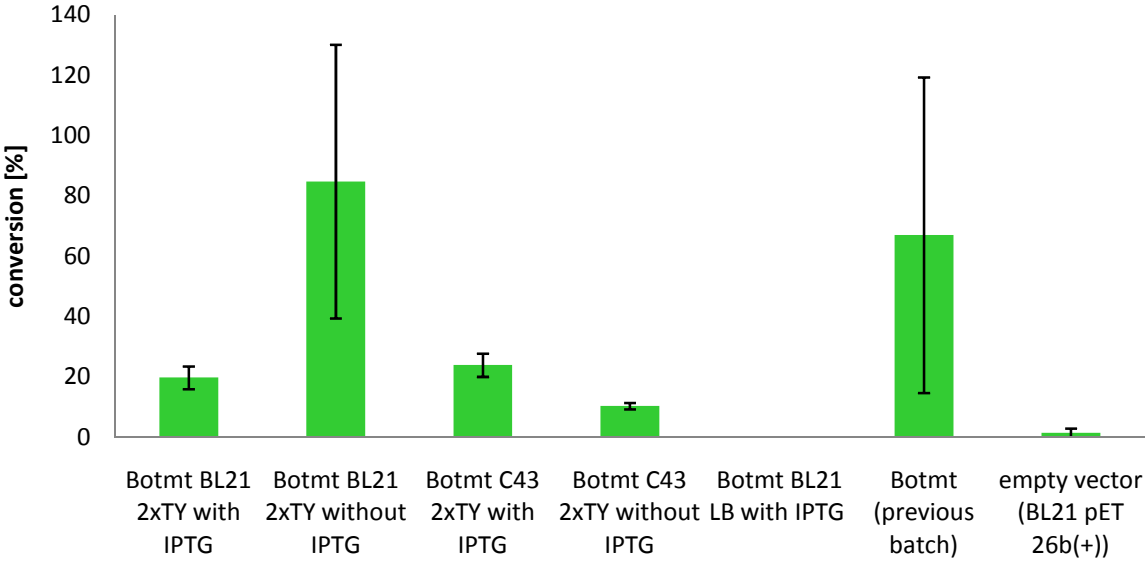


Figure 9: activity of *Bo*tmt cultivated under different conditions.

Corresponding to its high level expression in *E.coli* BL21 Gold (DE 3) and 2xTY medium (without IPTG) *Bo*tmt expressed under these conditions shows the highest activity. Despite the good expression of soluble enzyme BL 21 Gold (DE 3) cells cultivated in LB medium did not catalyze the methyl transfer reaction. An older batch of *Bo*tmt was used as a positive control. BL 21 Gold (DE 3) cells with the empty vector pET26b(+) were used as a negative control to determine the *E.coli* background product formation (Figure 9).

Solubility screening

Since the substrate 4-hydroxythiophenol (**10**) and the corresponding S-methylated product 4-(methylthio)phenol (**11a**) are not soluble in water a set of water miscible and immiscible solvents is tested.

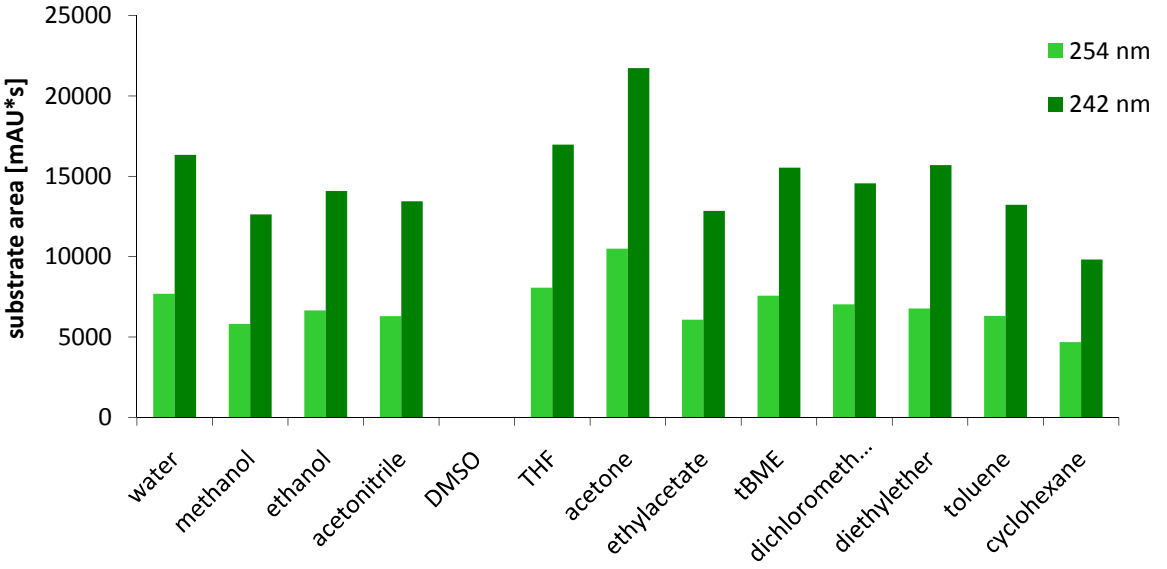


Figure 10: substrate (10) solubility in different solvents.

Similar solubility of the substrate is found for ethanol and acetonitrile as water miscible solvents and for *tert*-butyl methyl ether (tBME) and ethyl acetate as water immiscible solvents (Figure 10) which are used for further experiments.

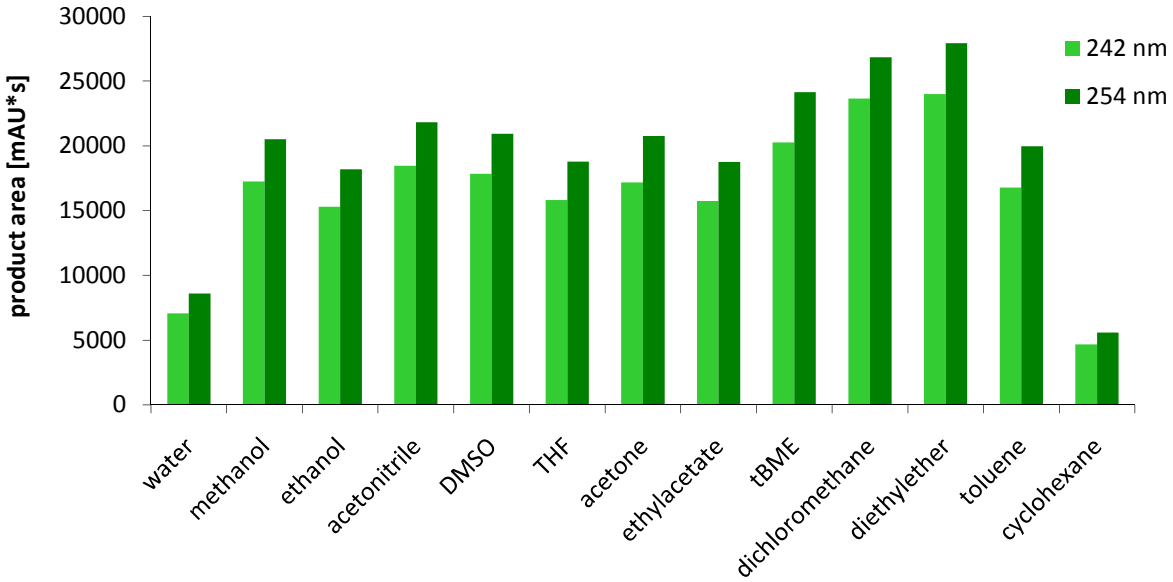


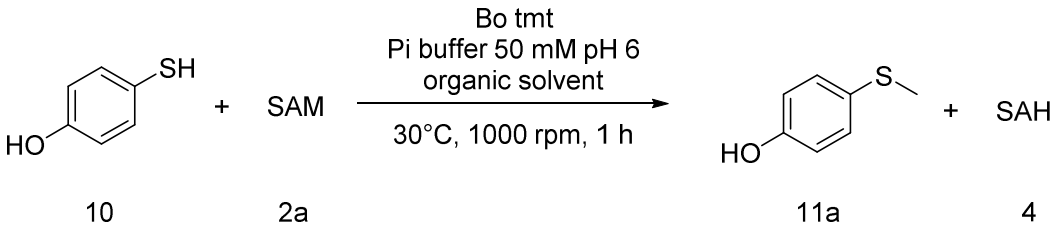
Figure 11: product (11a) solubility in different solvents.

There are no significant differences in the solubility of the product in water miscible solvents. The product is best soluble in dichloromethane, diethylether and tBME (Figure 11). Dichloromethane and diethylether are not selected as water immiscible solvents for the

following experiments because they have very low boiling points. Due to their high volatility the samples may appear more concentrated than they actually were.

In the following experiments the substrate is detected at a wavelength of 242 nm and the product at 254 nm.

Solvent screening



Scheme 21: methylation of 4-hydroxythiophenol (10) catalyzed by *Bo*tmt in different organic solvents and solvent contents.

In the solvent screening the compatibility of the solvent and the enzyme has been tested. Therefore the water miscible solvents ethanol and acetonitrile and the water immiscible solvents ethyl acetate and tBME are selected (Scheme 21).

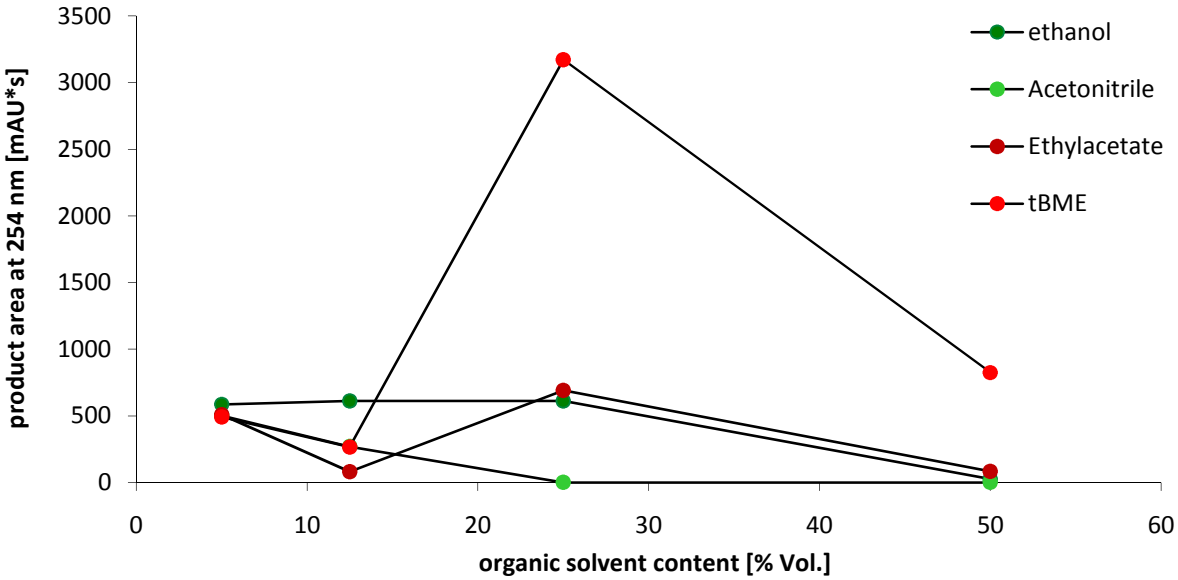
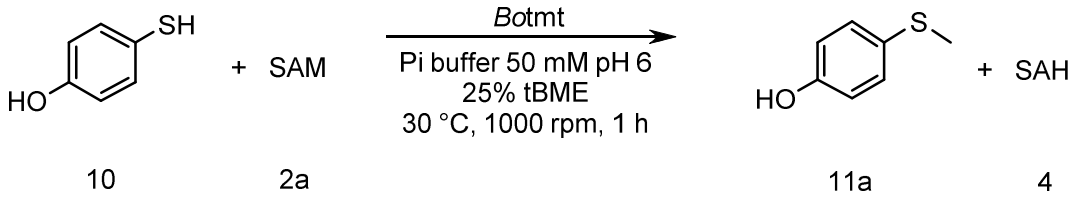


Figure 12: activity of *Bo*tmt in different organic solvents and solvent contents.

Two assays are set up. A one phase assay with a water miscible solvent is set up for HPLC analysis. A two phase assay with a water immiscible solvent is set up for GC analysis (Figure 12).

The following experiments are performed with either 5 % Vol. or 10 % Vol. ethanol or 25 % Vol. tBME as organic solvents.

Dry cell concentration screening



Scheme 22: methylation of 4-hydroxythiophenol (10) catalyzed by *E.coli* cells expressing *Bo*tmt.

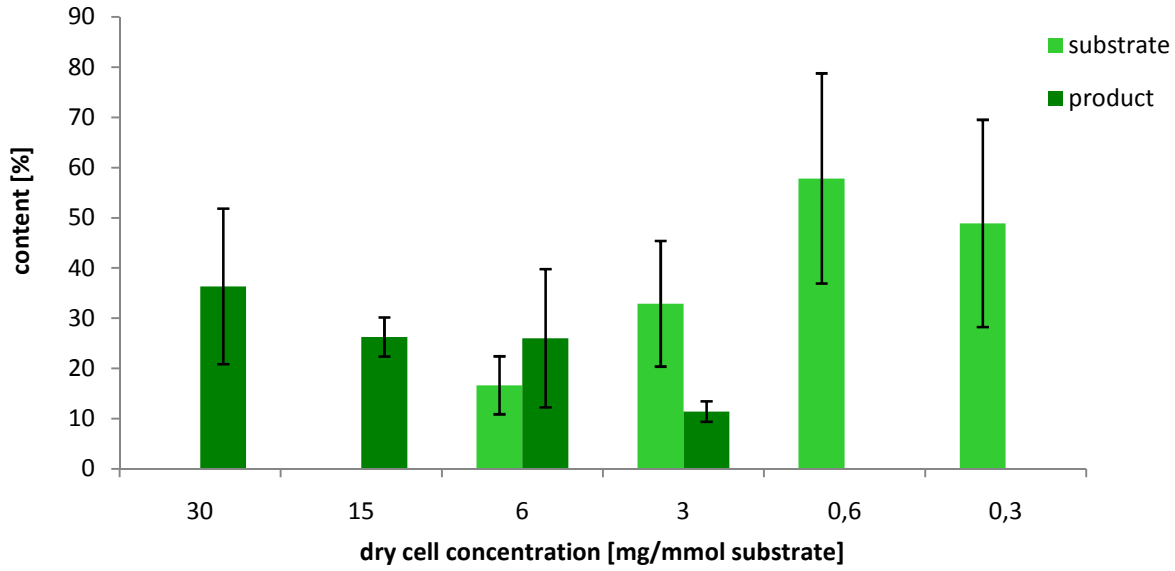
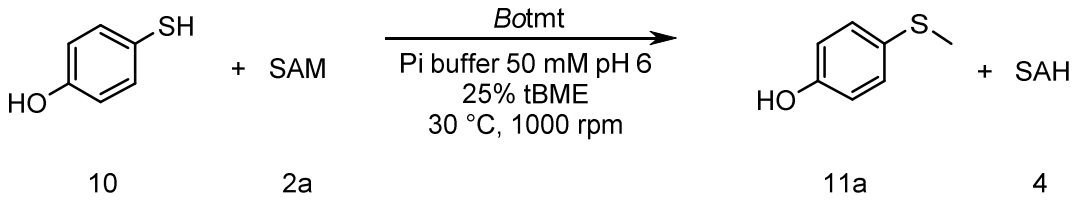


Figure 13: substrate and product contents at different dry cell concentrations.

For the studies of the enzyme kinetics it is important that the substrate is not converted too quickly. With a dry cell concentration of 6 mg lyophilized cells per mmol substrate a satisfying product concentration was reached and there was still substrate left for conversion after an hour (Figure 13). Therefore the kinetic studies were performed with a dry cell concentration of 6 mg/mmol substrate.

Kinetics



Scheme 23: methylation of 4-hydroxythiophenol (10) catalyzed by *Bot*mt.

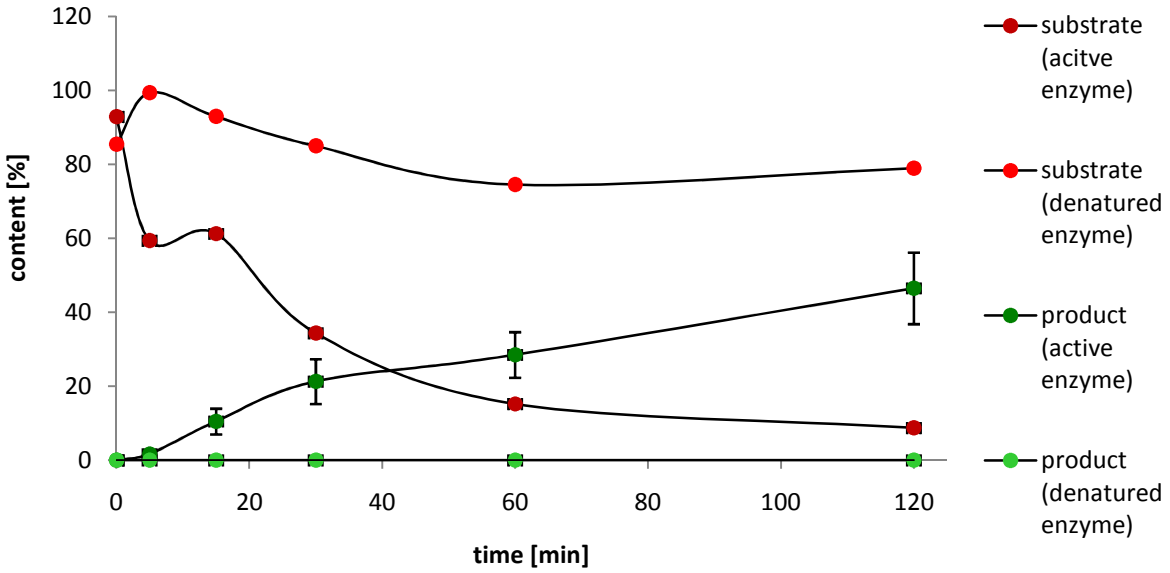
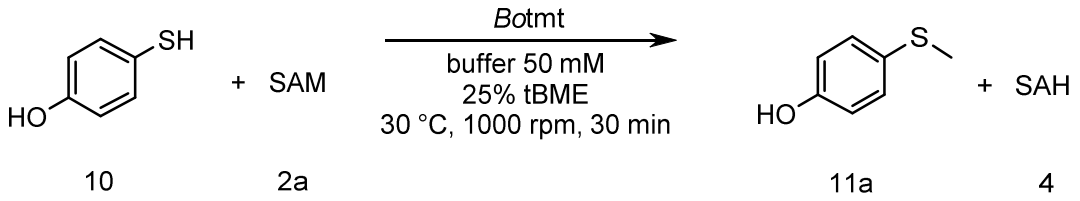


Figure 14: changes of substrate and product contents by applying functional and heat-deactivated enzyme.

As expected the substrate and product concentrations are constant throughout the reaction in the samples with the denatured enzyme. With the active enzyme the substrate concentration decreases and the product concentration increases steadily by the time. Until a reaction time of 30 minutes the largest changes in product concentration per time are detected (Figure 14). In that period of time the largest differences in product concentration between the different variations of a parameter can be expected. Therefore the reaction is stopped after 30 minutes in further experiments.

Buffer screening



Scheme 24: methylation of 4-hydroxythiophenol (10) catalyzed by *Bo*tmt in different buffers.

In order to find the ideal aqueous phase a set of buffers with different pH values is tested (Scheme 24).

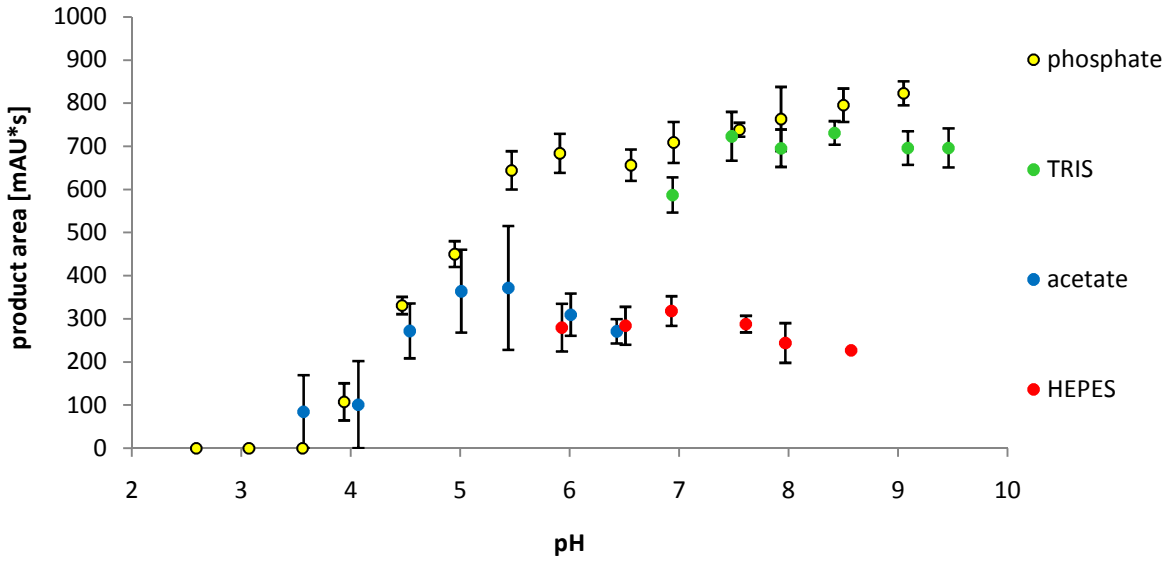
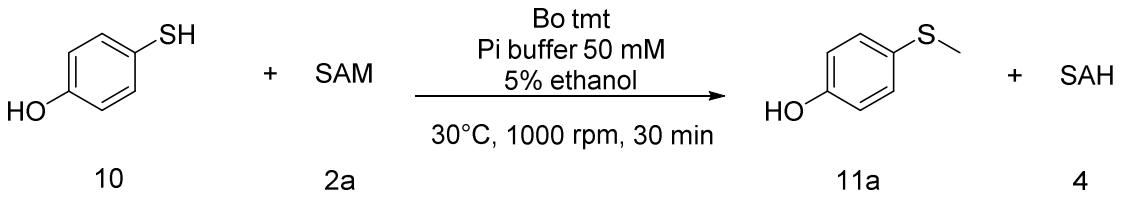


Figure 15: activity of *Bo*tmt in different buffer systems and at different pH values.

The reaction in 50 mM phosphate buffer and 50 mM TRIS buffer yields the highest concentration of 4-(methylthio)phenol (**11a**). In buffers with a high pH the product concentration is the highest (Figure 15). It is likely that this is not exclusively due to the enzyme activity but additionally to the fact that 4-hydroxythiophenol (**10**) has a pKa of 7 approximately. Therefore the nucleophilic substitution reaction might occur without enzyme catalysis.

At a pH below 7 the reaction in phosphate buffer yields the highest product concentration and therefore it is the optimal choice for the enzymatic assays.

pH screening



Scheme 25: methylation of 4-hydroxythiophenol (10) catalyzed by *Bo*tmt at different pH values.

In this experiment the pH optimum of the enzymatic reaction has been determined (Scheme 25). At the ideal pH the enzyme activity should reach a maximum and the product should be formed entirely in an enzyme catalyzed reaction. In order to prevent the non-enzymatic reaction the pH should be as low as possible. A very dramatic decrease of the pH might lead to the inactivation of the enzyme at a too acidic pH.

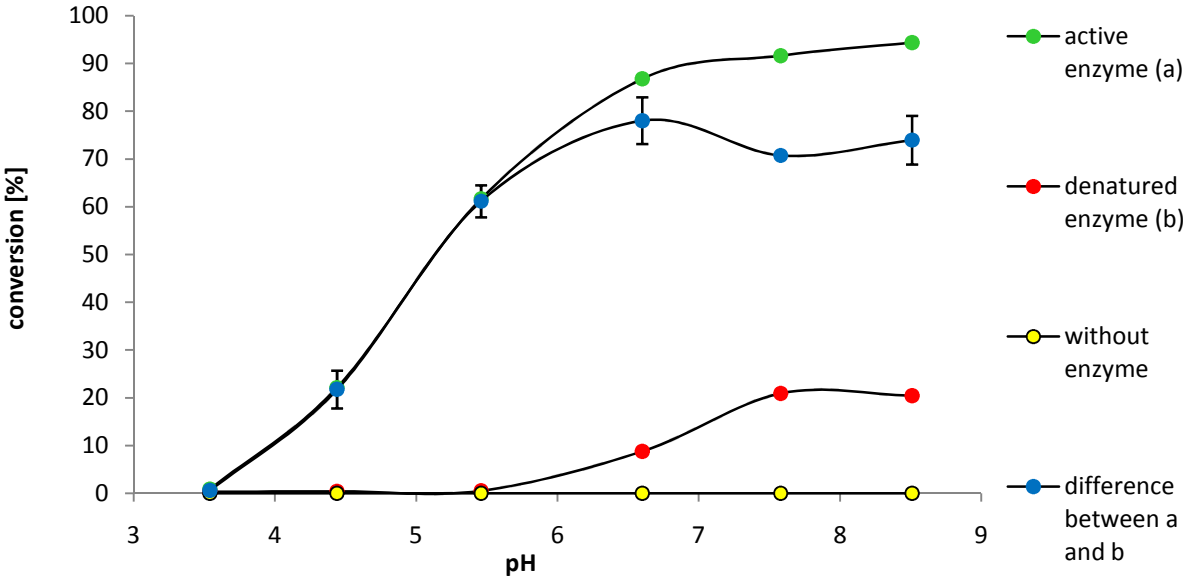
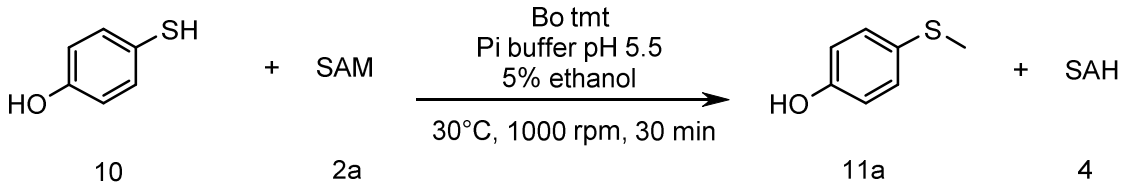


Figure 16: activity of *Bo*tmt at different pH values.

At a pH of 8.5 the highest product concentration is achieved but calculating the difference between the functional and the heat-denatured enzyme shows that from a pH of 6.5 unspecific catalysis occurs additionally to enzyme catalysis. There is no product formed when there are no cells in the sample which shows that the nucleophilic substitution reaction does not occur uncatalyzed at a pH of 6.5 and beyond. Certain cell components are required for the transfer of the methyl group from the cofactor to the substrate. Whether the reaction is catalyzed by amino acids of the enzyme *Bo*tmt or by another component of the *E.coli* cell cannot be

concluded from this experiment. What can be learned is that there is no product formed by the denatured enzyme at a pH of 5.5 and below (Figure 16). Therefore the reaction is most likely catalyzed by the enzyme active site and phosphate buffer pH 5.5 is used for further experiments.

Buffer concentration screening



Scheme 26: methylation of 4-hydroxythiophenol (10) catalyzed by *Bo*tmt at different phosphate buffer concentrations.

In order to find the ideal buffer concentration for the enzyme and the reaction phosphate buffers with a pH of 5.5 and concentrations ranging from 10 mM to 200 mM have been tested (Scheme 26).

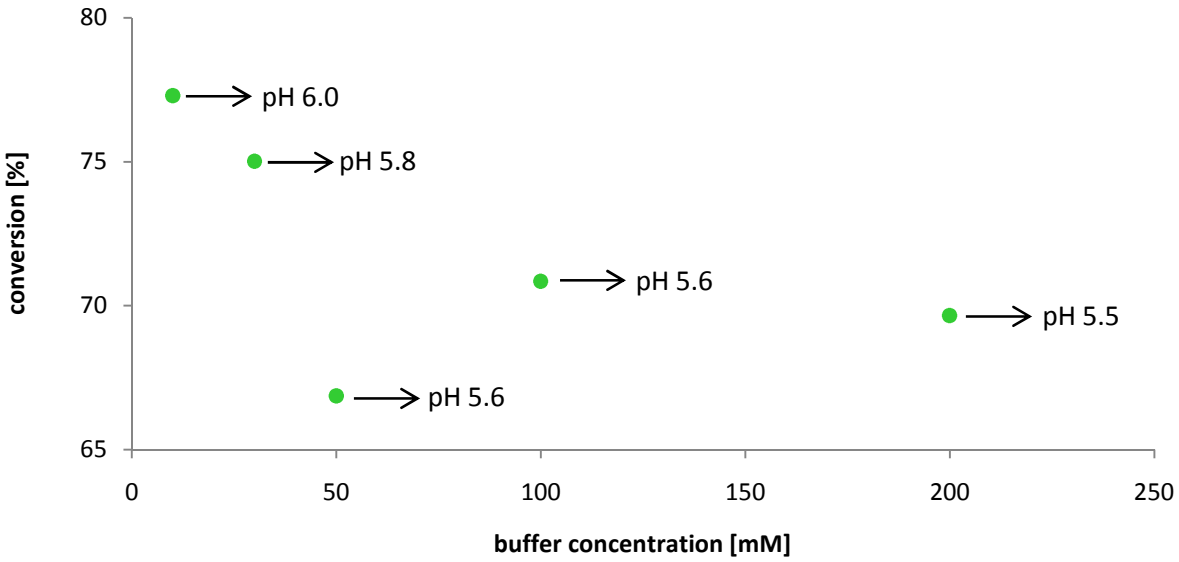
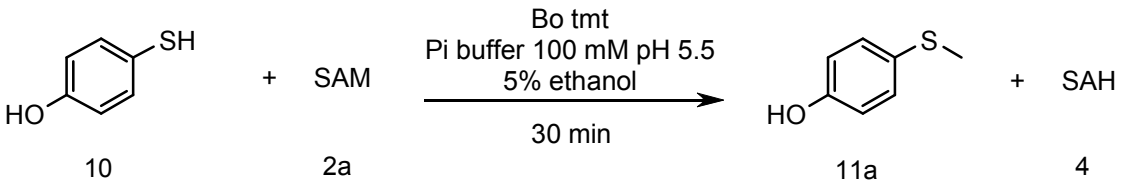


Figure 17: activity *Bo*tmt at different phosphate buffer concentrations and pH control before stopping the reaction.

With buffer concentrations of 10 mM and 30 mM a pH of 5.5 cannot be maintained. At a higher pH additional enzyme active site independent methylation is more likely to occur.

Therefore the conversion is higher than in the samples with higher buffer concentrations by which a pH of 5.5 can be maintained throughout the reaction. With a 100 mM pH 5.6 phosphate buffer most product was formed in an enzyme catalyzed methyl transfer reaction (Figure 17).

Temperature screening



Scheme 27: methylation of 4-hydroxythiophenol (10) catalyzed by *Bo*tmt at different temperatures.

In this screening the temperature optimum of the enzyme has been determined.

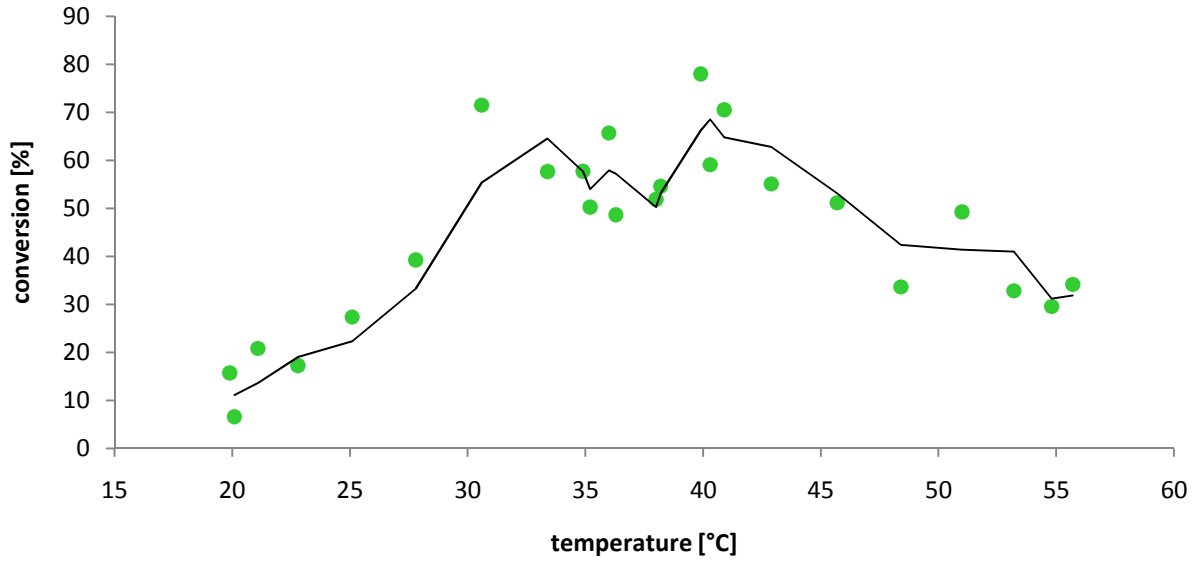
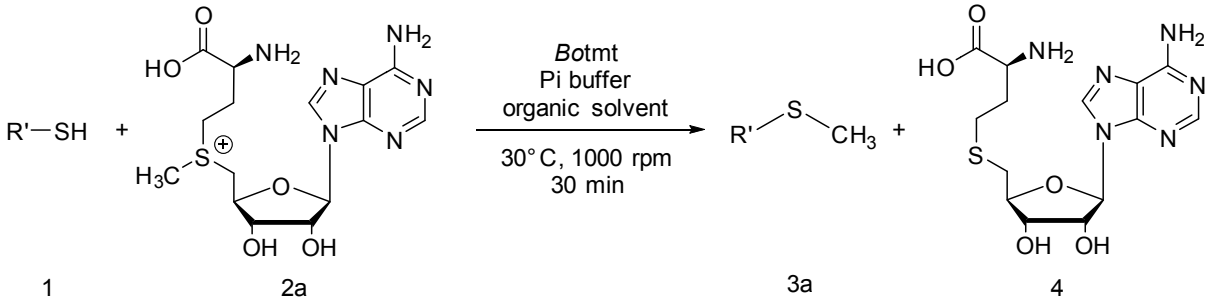


Figure 18: activity of *Bo*tmt at different temperatures.

The enzyme activity and hence the product concentration increase until a temperature of 30°C is reached. From 30°C to 40°C there is a maximum in activity and at a temperature of 40°C the product concentration starts decreasing. For enzyme stability's sake the lowest

temperature of the maximum which is 30°C is chosen for the substrate and cofactor scope screenings.

Substrate scope screening



Scheme 28: methylation of different thiols catalyzed by the *S*-methyltransferase *Bo*tmt.

The substrate scope screening should show whether the *S*-methyltransferase *Bo*tmt selectively transfers methyl groups to sulphur atoms and which types of thiol are accepted. Aromatic substrates with different substituents besides the thiol group are tested in order to determine the influence of electron withdrawing groups such as carboxy or nitro groups and electron donating groups such as hydroxyl, methoxy or methyl groups on the success of the reaction. The effect of a second substituent in different positions is studied. Furthermore the substrate promiscuity of *Bo*tmt regarding heteroaromatic and aliphatic substrates is be explored (Scheme 28).

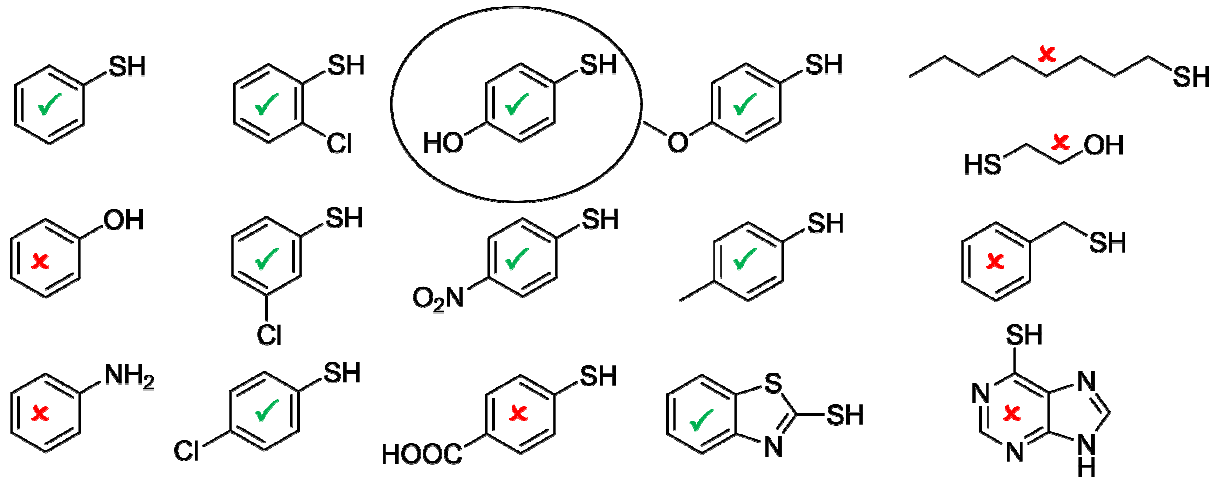


Figure 19: substrate scope of Botmt.

Thiophenol is methylated by the *S*-methyltransferase *Bo*tmt while phenol and aniline are not. In order to exclude *O*- and *N*-methylation a screening with further phenol and aniline derivatives additionally to 4-hydroxythiophenol (**10**) would be required.

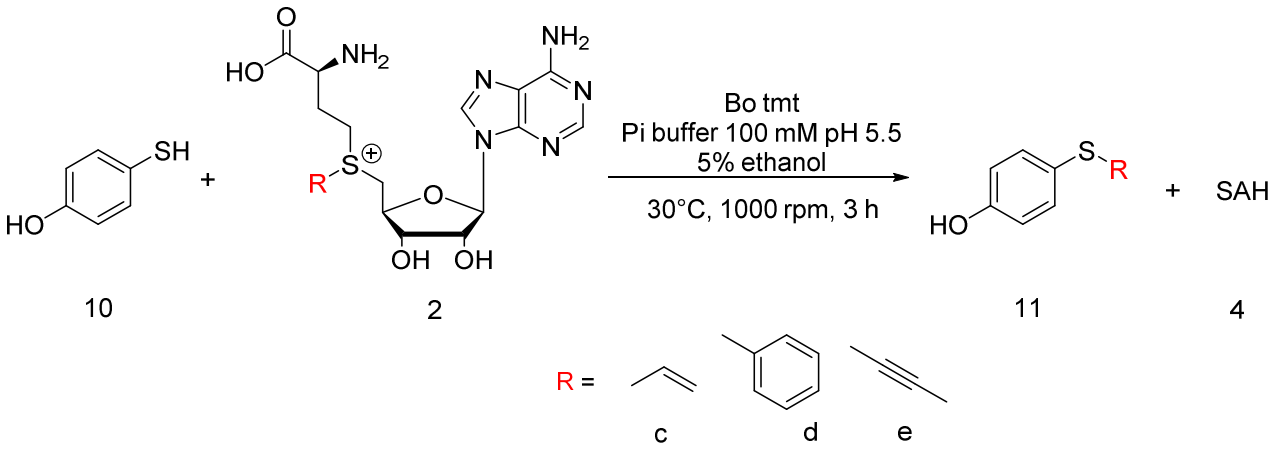
Electron withdrawing groups decrease the electron density of the aromatic ring and increase the acidity of the thiol. Therefore the activation of the substrate in the enzyme active site and subsequently the nucleophilic substitution reaction might be easier to accomplish. Interestingly, substrates with electron donation groups are preferred compared to substrates with electron withdrawing groups by the enzyme. 4-hydroxythiophenol (**10**) which has been chosen as a substrate at the beginning of the studies shows the highest conversion of all screened substrates. Additionally to the electronic properties of the substrates, electrostatic and hydrogen bonding effects can activate or deactivate the enzyme depending on the nature of the substituent. While the hydroxyl group of 4-hydroxythiophenol (**10**) exerts an activating effect on *Bo*tmt the carboxy group of 4-mercaptobenzoic acid deactivates the enzyme.

Since 2-chlorothiophenol, 3-chlorothiophenol and 4-chlorothiophenol show similar conversions to the corresponding products it can be concluded that the positions of a second substituent has no significant effect on the enzyme catalyzed reaction.

The aliphatic substrates are not converted. 2-mercaptoethanol is completely oxidized to the disulfide. Therefore it may be added to the assay in order to prevent the oxidation of the substrate 4-hydroxythiophenol (**10**) (Figure 19).

Cofactor scope screening

Cofactor screening with allyl, benzyl and 2-butynyl SAH (2 c-e)



Scheme 29: alkyation of 4-hydroxythiophenol (10) catalyzed by the *S*-methyltransferase *Bo*tmt.

In this screening the cofactor scope of *Bo*tmt has been studied. The substrate 4-hydroxythiophenol (**10**) was alkylated by utilizing the non-natural cofactors allyl, benzyl and 2-butynyl SAH (**2 c-e**) (Scheme 29).

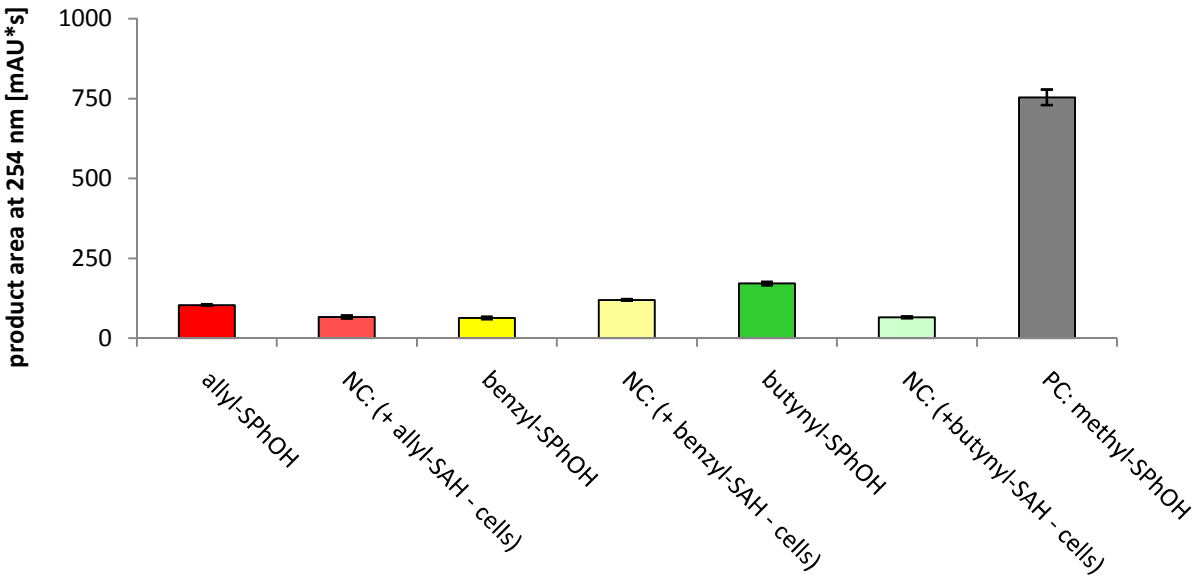


Figure 20: comparison of the catalyzed and uncatalyzed reactions. NC: no lyophilized cells expressing *Bo*tmt are added to the substrate and the respective cofactor.

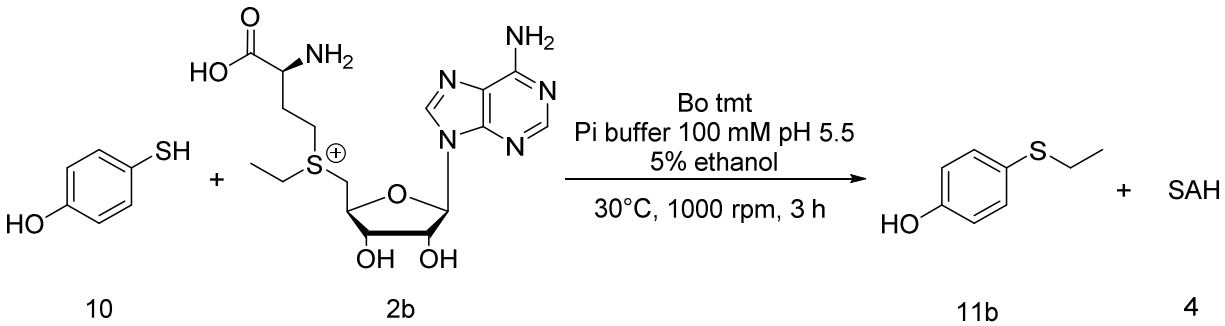
Allyl, benzyl and 2-butynyl SAH (**2 c-e**) are activated towards nucleophilic substitution reactions. The sulfur atom of the cofactor molecules is positively charged and therefore it has

an electron withdrawing effect on the reacting carbon of the substituents. The allyl, benzyl and 2-butynyl substituents are electron withdrawing groups themselves. This is due to the increased electronegativity of the carbon atom next to the reacting carbon. The neighboring carbon shows a higher tendency to withdraw electrons because as a part of a double or triple bond it is sp^2or sp hybridized. Together both electron withdrawing neighbors make the reacting carbon atom a good electrophile. Cofactors with a multiple bond next to the reacting carbon are named "double activated cofactors".[42]
Furthermore the unsaturated bond is considered to stabilize the transition state of the nucleophilic substitution reaction by conjugative stabilization.[43]

S-methyltransferases catalyze the alkylation of sulfur atoms. The sulfur atom of the thiol group of the substrate is a good nucleophile. Therefore the nucleophilic substitution reaction could occur without being catalyzed by the enzyme.

In the case of allyl and 2-butynyl SAH (**2 c+e**) there is more product formed in the catalyzed reactions. With benzyl SAH (**2d**) as a cofactor the uncatalyzed reaction yields more product. That may be due to the bulkier size of the benzyl substituent which probably does not fit well into the active site of the enzyme. Compared to the reaction with the natural cofactor SAM (**2a**) the enzyme shows a much lower activity towards the non-natural analogs (Figure 20).

Cofactor screening with ethyl SAH (2b)



Scheme 30: ethylation of 4-hydroxythiophenol (10) catalyzed by the *S*-methyltransferase *Bo*tmt.

The compatibility of *Bo*tmt towards the non-activated non-natural cofactor ethyl SAH (**2b**) has been studied (Scheme 30).

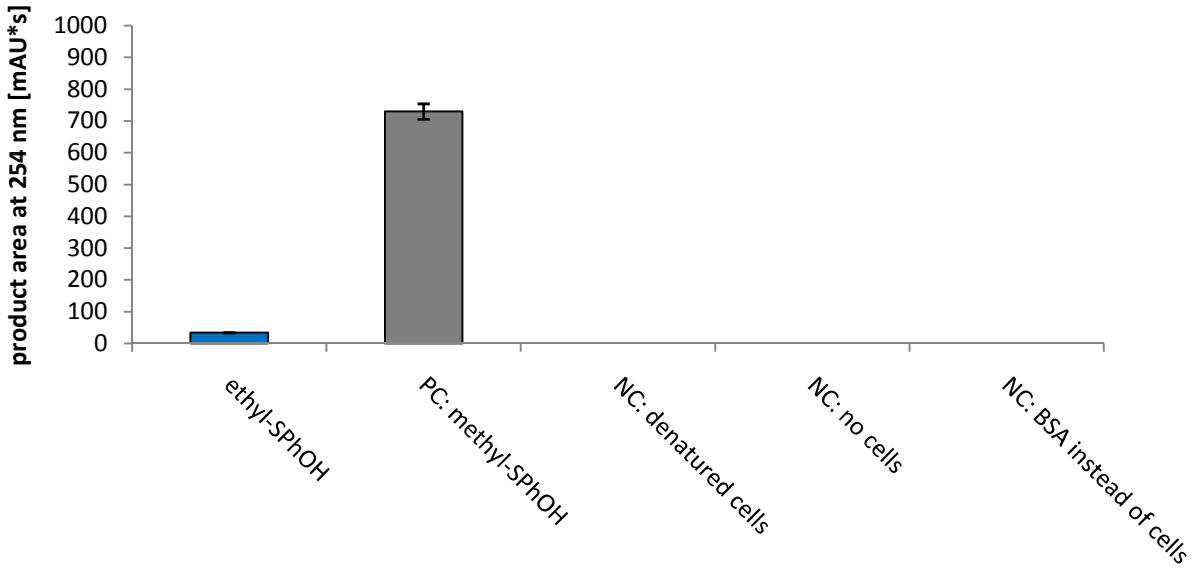


Figure 21: comparison of the catalyzed and uncatalyzed reactions. NC: either heat-denatured, no or bovine serum albumin instead of lyophilized cells expressing *Bo*tmt are added to the substrate and the cofactor ethyl SAH.

The ethyl SAH (**2b**) is not only deactivated from the electronic point of view but additionally due to its steric demand compared to the methyl group.

The ethyl group is transferred from the artificial cofactor to the substrate by the active enzyme. There is no product formed when the heat-denatured enzyme, no enzyme and serum albumin from bovine instead of the methyltransferase are added to the substrate and the cofactor ethyl SAH (**2b**) (Figure 21).

As expected the enzyme catalyzed ethylation does not yield as much product as the allylation and the 2-butynylation because the nucleophilic substitution should not occur as easily as with an activated cofactor. Therefore there is no product formed in the non-enzymatic reaction with ethyl SAH (**2b**) (Figure 22).

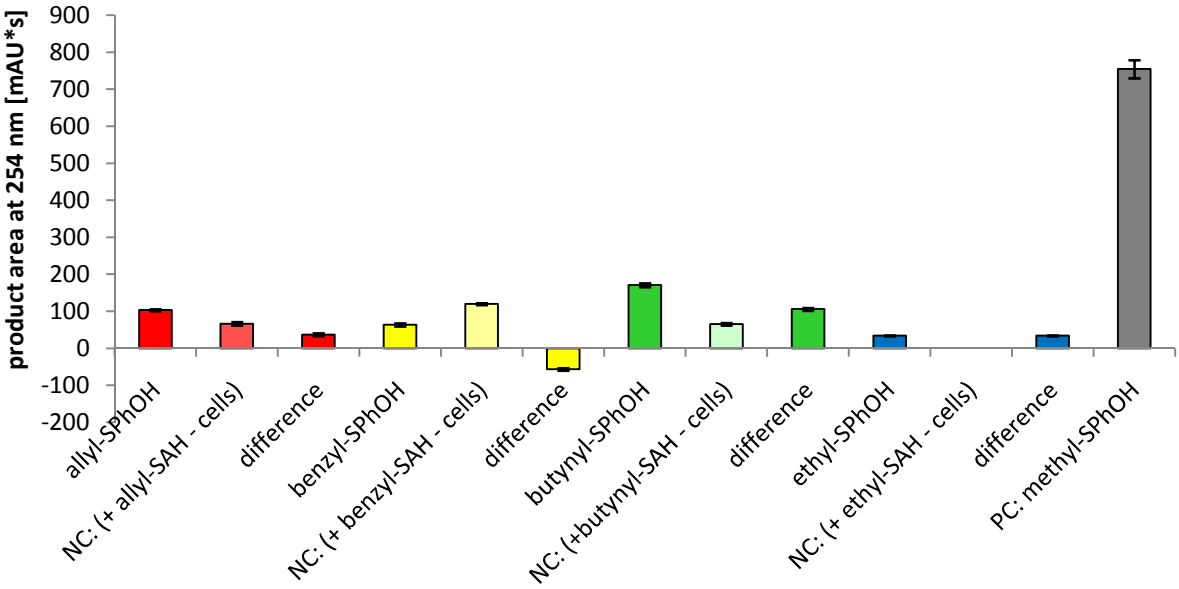
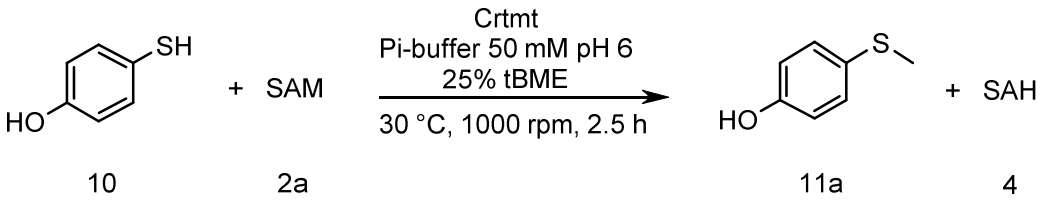


Figure 22: overview of the alkylation reactions with different cofactors. NC: no lyophilized cells expressing *Bo*tmt are added to the substrate and the respective cofactor. The differences between the catalyzed and the uncatalyzed reactions are depicted for each of the alkyl moieties.

In Figure 22 the cofactor scope screenings with allyl, benzyl, 2-butynyl and ethyl SAH (**2 b-e**) are summed up.

*Cr*tmt

Expression condition screening



Scheme 31: methylation of 4-hydroxythiophenol (10) catalyzed by the *S*-methyltransferase *Cr*tmt.

In order to identify the optimal conditions for the expression of functional *S*-methyltransferase *Cr*tmt the *E.coli* strains BL 21 Gold (DE 3) and C 43 and the media LB and 2xTY have been screened. Expression is induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) in one half of the cultures.

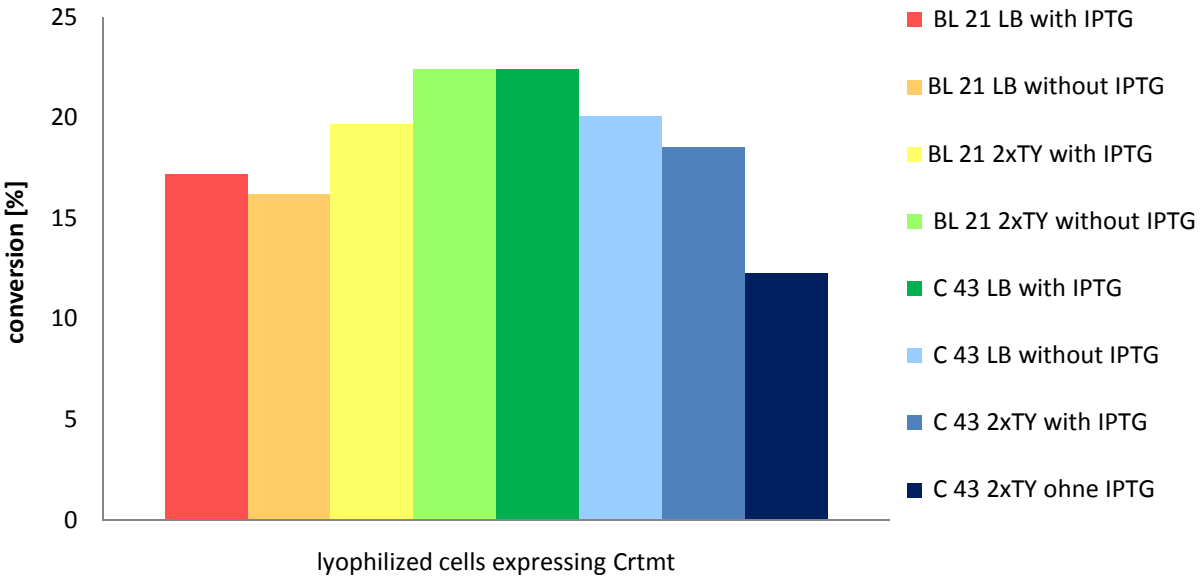
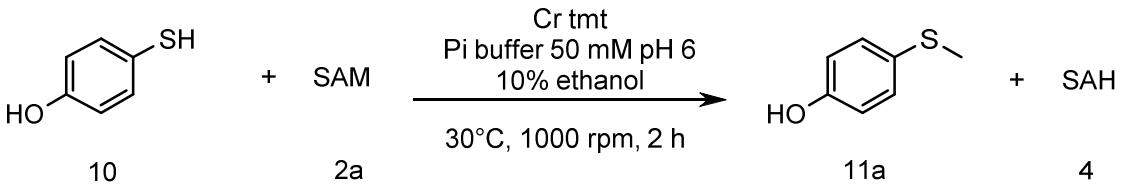


Figure 23: comparison of strains, media and induction.

For the formation of functional *S*-methyltransferase *Cr*tmt non-induced expression in *E.coli* BL 21 Gold (DE 3) and 2xTY medium and induced expression in *E.coli* C43 and LB medium are best suitable (Figure 23).

For further experiments *Cr*tmt cloned into the vector pET26b(+) is expressed in *E.coli* BL 21 Gold (DE 3) and 2xTY medium and the expression is not induced by IPTG.

Expression



Scheme 32: methylation of 4-hydroxythiophenol (10) catalyzed by *Cr*tmt.

The activity of *Cr*tmt expressed in *E.coli* BL 21 Gold (DE 3) and 2xTY medium has been tested in this assay (Scheme 32). The activities of cells, lysates and inclusion bodies as well as GC and HPLC analysis are compared. Lysates are tested to determine a positive or negative effect of cell disruption on the activity of the soluble enzyme. According to the SDS-PAGE (Figure 24) *Cr*tmt is mostly expressed as an insoluble enzyme. Therefore the activity of the inclusion bodies is tested.

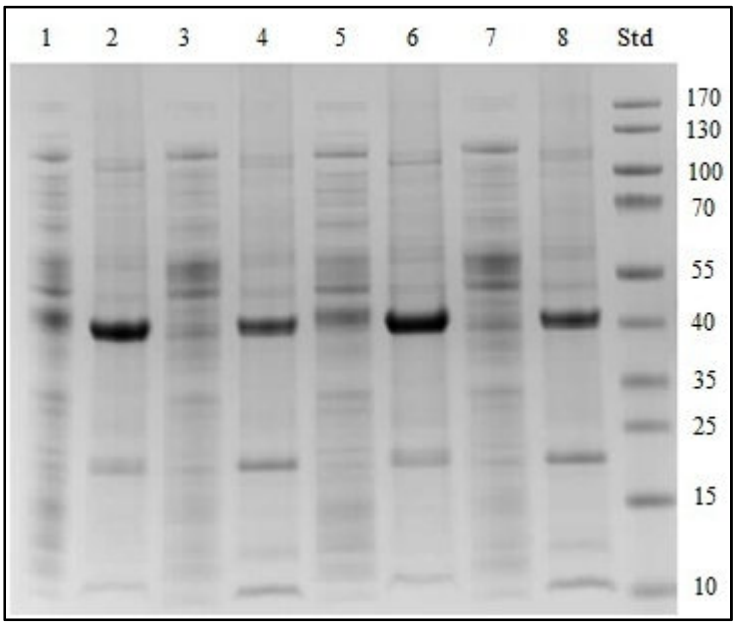


Figure 24: SDS-PAGE of *Cr*tmt (41 kDa). Lysates and pellets in 6 M urea (= inclusion body fraction) with a protein concentration of 0.75µg/µl (according to Bradford) were applied. 1+5 lysate – with IPTG, 2+6 pellet – with IPTG, 3+7 lysate – without IPTG, 4+8 pellet – without IPTG. 1-4: 4.5 µg (6 µl), 5-8: 6 µg (8 µl), 9 Standard PageRuler Prestained Protein Ladder.

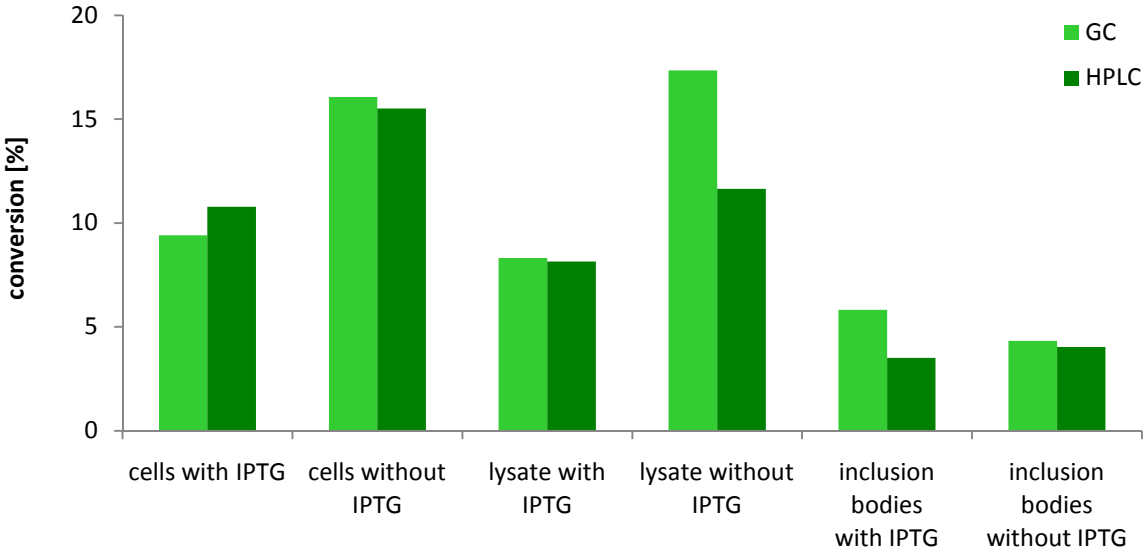


Figure 25: comparison of the activities of different enzyme preparations and analysis methods.

In bacterial expression systems protein misfolding followed by inclusion body formation constitutes a common problem of the high level expression of complex heterologous proteins from eukaryotes such as plants. These proteins fail to fold in their active form and aggregate with each other to form insoluble inclusion bodies. Stress-induced protein misfolding is due to

strong promoters and high inducer concentration. The rate of protein aggregation exceeds the rate of proper folding.[44]

In order to test the effect of the inducer on the biosynthesis of the enzyme expression is induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) in only one half of the cultures.

Non-induced expression is possible because *E.coli* BL 21 Gold (DE 3) cells host an additional gene for T7 RNA polymerase which is controlled by the Lac regulatory region. Since the Lac system is not tightly regulated there will be low levels of the T7 RNA polymerase present in the cell even when its expression is not induced by IPTG. The gene encoding for the target protein is cloned into a pET26b(+) vector system which is controlled by T7 promoter. The T7 RNA polymerase subsequently transcribes the target gene starting protein biosynthesis.

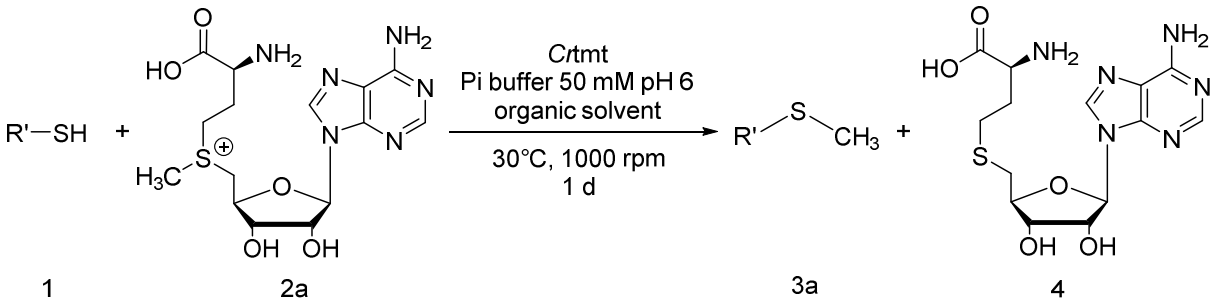
According to the SDS-PAGE more soluble as well as more insoluble enzyme is produced when expression is induced (Figure 24).

The product concentration in the cells and lysates is higher when expression is not induced. The inclusion bodies show similar activities with and without IPTG. The reaction with the lysates yields more product than with the inclusion bodies. The concentration of soluble *Cr*tmt in the lysate is lower and therefore its activity must be higher compared to that of *Cr*tmt in the inclusion bodies which consist of the insoluble, partially inactive enzyme (Figure 25).

Analysis by GC and HPLC yielded almost the same data and the deviations are not significant (Figure 25).

For further experiments *Cr*tmt cloned into the vector pET26b(+) is expressed in *E.coli* BL 21 Gold (DE 3) and 2xTY medium and the expression is not induced by IPTG. The samples are analyzed by HPLC when ethanol is used as organic solvent and by GC when tBME is used.

Substrate screening with aromatic and aliphatic thiols



Scheme 33: methylation of different thiols catalyzed by the *S*-methyltransferase *Cr*tmt.

In this screening the substrate promiscuity of the *S*-methyltransferase *Cr*tmt has been studied (Scheme 33).

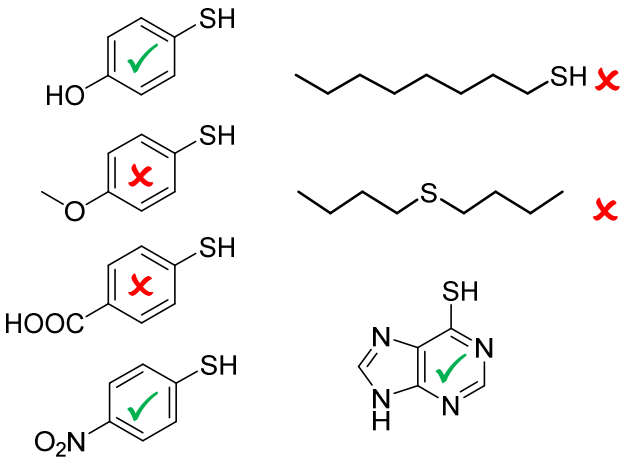
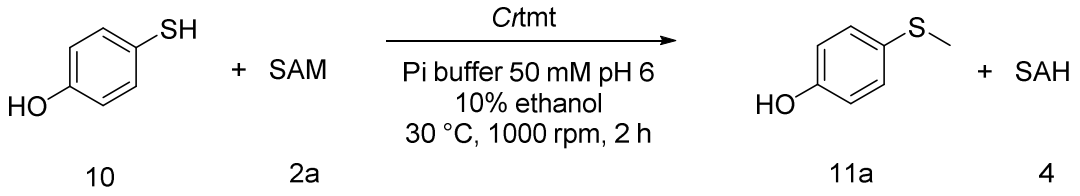


Figure 26: substrate scope of *Cr*tmt.

The *S*-methyltransferases *Bo*tmt and *Cr*tmt differ in their substrate scopes (Figure 19 and Figure 26). While *Bo*tmt is able to convert 4-methoxythiophenol *Cr*tmt cannot. *Cr*tmt converts 6-mercaptopurine while *Bo*tmt does not. Both enzymes do not catalyze the methylation of aliphatic substrates. Compared to *Bo*tmt, *Cr*tmt shows no preference for substrates with electron donating substituents. The substrates 4-hydroxythiophenol (**10**) and 4-nitrothiophenol are converted to the corresponding products with yields of 54 % and 25% respectively. Due to the higher yield and the better solubility in ethanol and tBME 4-hydroxythiophenol (**10**) is used as a substrate in the dry cell concentration screening and the kinetic studies.

Dry cell concentration screening



Scheme 34: methylation of 4-hydroxythiophenol (10) catalyzed by *E.coli* cells expressing *Cr*tmt.

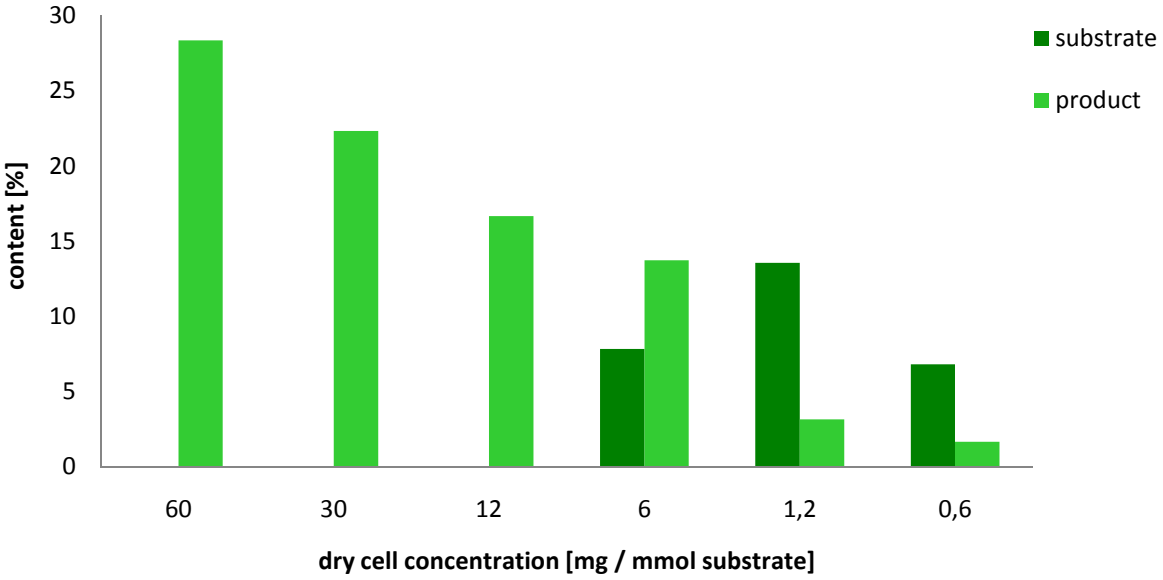
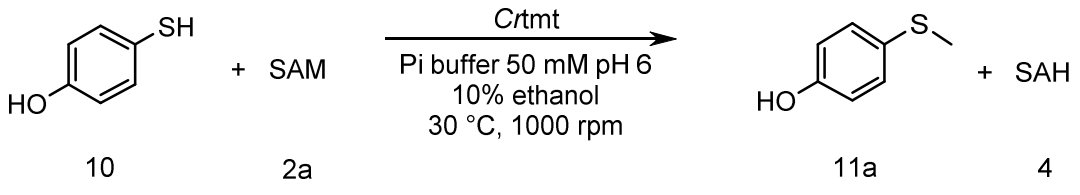


Figure 27: substrate and product contents at different dry cell concentrations.

The dry cell concentration screenings with *Bo*tmt and *Cr*tmt show similar results. Compared to *Bo*tmt only half of the product concentration is reached with *Cr*tmt in a twice as long reaction (Scheme 34 and Figure 27). Therefore the kinetic studies are performed with a dry cell concentration of 10 mg / mmol substrate.

Kinetics



Scheme 35: methylation of 4-hydroxythiophenol (10) catalyzed by *Cr*tmt.

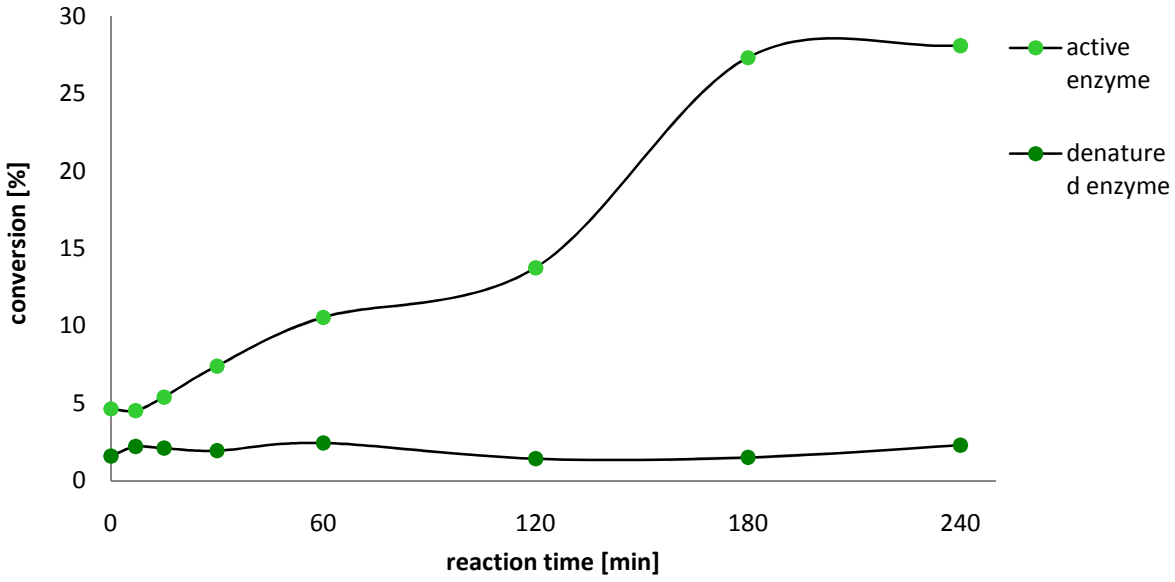


Figure 28: changes of product content by applying functional and heat-deactivated enzyme.

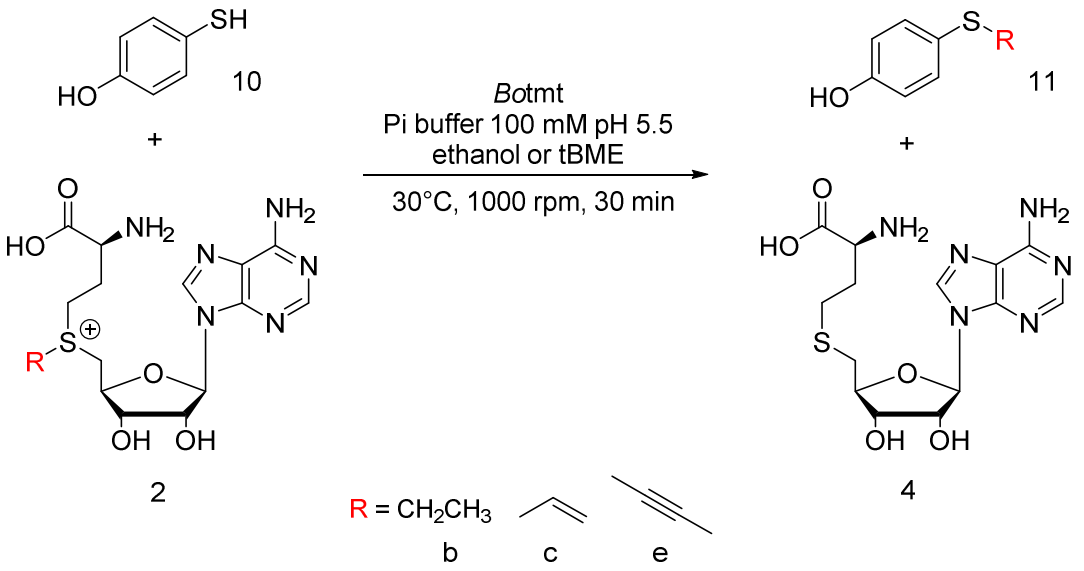
With the active enzyme the product concentration increases by the time while the product concentration is constant with the heat-denatured enzyme. Interestingly, there is 5 % of the product present in the samples in which the active enzyme has been denatured prior to the screening. For further experiments the reaction should be stopped after 180 minutes instead of 30 minutes as it has been in the case of *Bo*tmt (Figure 28).

CONCLUSION & OUTLOOK

The allyl, benzyl, 2-butynyl and ethyl analogs of S-adensoyl-L-methionine (**2 b-e**) were successfully synthesized. The artificial cofactors were used as alkyl donors for the experiments with the *C*- and *S*-methyltransferases.

L-tyrosine (**5**) could be alkylated with SAM (**2a**), allyl (**2c**) and 2-butynyl SAH (**2e**) by the *C*-methyltransferase SfmM2. The allylation, benzylation, butynylation and methylation of 4,5,7-trihydroxy-3-phenylcoumarin (**8**) could be achieved by enzyme catalysis with NovO but only the methylated product could be obtained as a pure compound. Purification of the other products by preparative silica gel TLC was not successful due to the instability of the compounds.

Non-induced expression of the *S*-methyltransferase *Bo*tmt in *E.coli* BL 21 Gold (DE 3) and 2xTY medium yielded the enzyme with the highest activity and for most experiments 10 mg dry cells / mmol substrate were used. Since the substrates were not soluble in water a variety of organic solvents was screening with the enzyme showing good activities in 5 – 10 % Vol. ethanol or 25 % Vol. tBME. As aqueous phase phosphate buffer 100 mM pH 5.5 proved to be best for the enzyme performance and the reaction since there was no unspecific catalysis additionally to enzyme catalysis. The temperature optimum of the enzyme was between 30°C and 40°C. *Bo*tmt is a promiscuous yet sulfur selective methyltransferase with a broad substrate spectrum converting aromatic and heteroaromatic compounds. Allyl, 2-butynyl and even non-activated ethyl groups could be transferred from the synthesized artificial cofactors (**2c, 2e, 2b**) to 4-hydroxythiophenol (**10**) (Scheme 36).



Scheme 36: alkylation of 4-hydroxythiophenol (10) catalyzed by the *S*-methyltransferase *Bo*tmt.

The second *S*-methyltransferase *Cr*tmt was partially characterized. In contrast to *Bo*tmt *Cr*tmt was mostly expressed as an insoluble enzyme. When expression in *E.coli* BL 21 Gold (DE 3) and 2xTY medium was not induced less but more active enzyme was formed. Both enzymes differ in their substrate scopes but for *Cr*tmt only few substrates have been tested to date. *Cr*tmt was less active than *Bo*tmt and therefore 10 mg dry cells / mmol substrate were applied for the kinetic studies.

Since the allylated, benzylated and butynylated 4,5,7-trihydroxy-3-phenylcoumarin (**9c, 9d, 9e**) could not be purified by preparative silica gel TLC other purification techniques such as preparative reversed phase HPLC should be applied.

The alkylation reactions catalyzed by the *S*-methyltransferase *Bo*tmt should be scaled up in order to be able to isolate the products. Besides the methylated 4-hydroxythiophenol (**11a**) we are especially interested in the ethylated product which is alkylated with a non-activated artificial cofactor (**2b**). Since *Bo*tmt has a broad substrate scope further substrates could be converted to the corresponding alkylated products.

The characterization of the *S*-methyltransferase *Cr*tmt should be completed. Therefore the parameters concentration of organic solvent, type of buffer, pH and concentration of the buffer, temperature and cofactor scope should be screened.

However, the ultimate aim is the establishment of methyltransferases as catalysts for alkylation reactions in industry. To date their application in biotechnological processes had not been economical because stoichiometric amount of the high priced cofactor are required. To decrease the costs of this biocatalytic transformation a recycling system for the alkyl donors *S*-adenosyl-*L*-methionine (**2a**) and its analogs has to be developed.

EXPERIMENTAL PART

Syntheses of artificial cofactors

For all reactions 50 ml two-necked round bottom flasks were dried in vacuo and flushed with argon. All steps of the syntheses were performed under an inert atmosphere (argon).

Synthesis of allyl SAH (2c)

100 mg (0.26 mmol, 1 eq) *S*-adenosyl-*L*-homocysteine (**4**) were dissolved in 10 ml formic acid and 134 mg (0.52 mmol, 2 eq) silver trifluoromethanesulfonate were added. 900 µl (10.41 mmol, 40 eq) allyl bromide were added slowly to the solution. Precipitation occurred immediately. The suspension was stirred with a magnetic stirring bar at room temperature (20°C) for 24 hours.

The reaction progress was controlled by HPLC-MS (method: allyl-SAH).

The reaction mixture was diluted with 15 ml water and 20 ml diethylether, shaken and filtered through a pleated paper filter to remove the precipitate. The filter cake was washed with 50 ml water and 25 ml diethylether. After phase separation the aqueous phase was washed three times with 20 ml diethylether each. The aqueous phase was lyophilized to remove the water.

The crude product (204.6 mg) was used for the synthesis of allylated 4,5,7-trihydroxy-3-phenylcoumarin (**9c**) and for the cofactor scope screening with SfmM2 and *Bo*tmt.

Synthesis of benzyl SAH (2d)

2d was synthesized according to the procedure described for **2c**. 1.245 ml (10.41 mmol, 40 eq) benzyl bromide were added slowly to the solution of SAH, formic acid and silver triflate.

The reaction progress was controlled by HPLC-MS (method: benzyl-SAH).

After 26 hours another equivalent (67 mg, 0.26 mmol) of silver triflate was added and the suspension was stirred at room temperature (20°C) for another 16 hours.

The reaction was worked up according to the procedure described before. The crude product (204.0 mg) was used for the synthesis of benzylated 4,5,7-trihydroxy-3-phenylcoumarin (**9d**) and for the cofactor scope screening with *Bo*tmt.

Synthesis of 2-butynyl SAH (2e)

2e was synthesized according to the procedure described for (**2c**). 911 µl (10.41 mmol, 40 eq) 1-bromo-2-butyne were added slowly to the solution of SAH, formic acid and silver triflate.
The reaction progress was controlled by HPLC-MS (method: butinyl-SAH).
After 25 hours the reaction was worked up according to the procedure described before. The crude product (223.3 mg) was used for the synthesis of 2-butynylated 4,5,7-trihydroxy-3-phenylcoumarin (**9e**) and for the cofactor scope screening with SfmM2 and *Bo*tmt.

Synthesis of ethyl SAH (2b)

30 mg (0.08 mmol, 1 eq) *S*-adenosyl-L-homocysteine (**4**) were dissolved in 3 ml formic acid and 40 mg (0.16 mmol, 2 eq) silver trifluoromethanesulfonate were added. 233 µl (3.12mmol, 40 eq) ethyl bromide were added slowly to the solution. Precipitation occurred immediately. The suspension was stirred with a magnetic stirring bar at room temperature (20°C). After 43 hours 40 mg (0.16 mmol, 2 eq) silver trifluoromethanesulfonate and 233 µl (3.12 mmol, 40 eq) ethyl bromide were added and the suspension was stirred at room temperature (20°C) for another 8 hours.
The reaction progress was controlled by HPLC-MS (method: ethyl-SAH).
2b was worked up according to the procedure described for **2c**. The crude product (84.8 mg) was used for the cofactor scope screening with *Bo*tmt.

C-Methyltransferases

SacF and SfmM2

Expression

The cells were taken from a glycerol stock solution of SacF or SfmM2 genes cloned into the vector pET26b(+) and transformed into *E.coli* BL21 Gold (DE3) and grown over night at 37°C on LB medium Lenox containing 40 mg/l of the antibiotic kanamycin. A pre-culture containing 40 mg/l kanamycin was inoculated to an OD_{600} of 0.1 with the over-night-culture and grown to an OD_{600} of 1.0 at 30°C. The main-culture containing 40 mg/l kanamycin was inoculated to an OD_{600} of 0.1 with the pre-culture and grown to an OD_{600} of 1.0 at 30°C.

Expression was induced at an OD_{600} of 1.0 by 100 µM isopropyl β-D-1-thiogalactopyranoside and the temperature was decreased to 25°C. After 20 hours the cells were harvested, washed with sodium phosphate buffer 50 mM pH 7.0, suspended in the buffer and lyophilized.

Aqueous phase screening and pH tests with SacF

5.5 mg lyophilized cells expressing SacF were suspended in 900 µl water and the pH was measured. 0.8 mg (1 eq) *L*-tyrosine (**5**) were dissolved in 100 µl of a 30 mM aqueous NaOH solution and added. 2.4 mg (1 eq) *S*-adenosyl-*L*-methionine (**2a**) were added. The pH was measured after the addition of *L*-tyrosine (**5**) and *S*-adenosyl-*L*-methionine (**2a**).

To simulate the conditions after a second portion of cells and cofactor was added the experiment was repeated: 10 mg lyophilized cells expressing SacF were suspended in 900 µl water and the pH was measured. 0.8 mg (1 eq) *L*-tyrosine (**5**) were dissolved in 100 µl of a 30 mM aqueous NaOH solutionand added. 4.8 mg (2 eq) *S*-adenosyl-*L*-methionine (**2a**) were added. The pH was measured after the addition of *L*-tyrosine (**5**) and *S*-adenosyl-*L*-methionine (**2a**).

The experiment was repeated but the lyophilized cells were suspended in sodium phosphate buffer (50 mM pH 7.0) instead of water and the pH was measured after each step.

Crude lysate preparation with SacF

100 mg lyophilized cells expressing SacF were suspended in 25 ml sodium phosphate buffer (50 mM pH 7.0) and lysed by sonication.

Activity assay of SacF and SfmM2

50 mM buffer (sodium phosphate pH 7.0), 0.7 mg enzyme / mmol substrate (SacF crude lysate or purified SfmM2-Strep), 1 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 0.5mM substrate (*L*-tyrosine (**5**) in 10 mM aqueous NaOH solution) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After 3 and 5 hours respectively the reaction was stopped by adding 0.33 M aqueous HCl solution. The denatured enzyme was separated by centrifugation at 10000 rpm for 5 minutes. The supernatant was filtered prior to HPLC-MS analysis applying the method MeTyr AA MeOH iso pos.

Cofactor scope screening

50 mM buffer (sodium phosphate pH 7.0), 4.4 mg enzyme / mmol substrate (purified SfmM2-Strep in buffer), 2 mM cofactor (allyl SAH (**2c**) or 2-butynyl SAH (**2e**) in 10 % Vol. ethanol and 5 mM H_2SO_4 or *S*-adenosyl-*L*-methionine in buffer) and 1 mM substrate (*L*-tyrosine (**5**) in 25 mM aqueous NaOH solution) were combined in that order in a 1.5 ml Eppendorf tube. Five samples for each of the cofactors were incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After 24 hours buffer, enzyme, cofactor or enzyme and cofactor were added to one of the five samples respectively. The fifth sample of each cofactor was stopped by adding 0.33 M aqueous HCl solution. The other four samples were stopped after 48 hours. The denatured enzyme was separated by centrifugation at 13000 rpm for 10 minutes. The supernatant was filtered prior to HPLC-MS analysis applying the methods allylTYR H2O-MeOH 99-1 Nucleodur, butinylTYR H2O-MeOH 99-1 Nucleodur and MeTYR H2O-MeOH 99-1 Nucleodur.

Cofactor concentration screening

50 mM buffer (sodium phosphate pH 7.0), 0.7 mg enzyme / mmol substrate (purified SfmM2-Strep in buffer), 0.5 mM (1 eq) or 1 mM (2 eq) cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 0.5 mM substrate (*L*-tyrosine (**5**) in 10 mM aqueous NaOH solution) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After one day the reaction was stopped by adding 0.33 M aqueous HCl solution. The denatured enzyme was separated by centrifugation at 10000 rpm for 5 minutes. The supernatant was filtered prior to HPLC-MS analysis applying the method MeTyr AA MeOH iso pos.

NovO

Activity assay

NovO (crude lysate in 100 mM sodium phosphate buffer pH 6.5), 1 mg bovine serum albumin / mmol substrate (in water), 2 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in 50 mM sodium phosphate buffer pH 6.0) and 1 mM substrate (4,5,7-trihydroxy-3-phenylcoumarin (**8**) in DMSO) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 35°C and 1000 rpm in an Eppendorf thermomixer. After 2 and 24 hours

respectively the reaction was stopped by heating at 80°C for 10 minutes. The denatured enzyme was separated by centrifugation at 13000 rpm for 10 minutes. The supernatant was filtered prior to HPLC-MS analysis applying the method methyl-PhCoum.

Syntheses of alkylated 4,5,7-trihydroxy-3-phenylcoumarins

Synthesis of allylated 4,5,7-trihydroxy-3-phenylcoumarin (9c)

25 mg (1.0 eq) 4,5,7-trihydroxy-3-phenylcoumarin (**8**) were dissolved in 2.5 ml DMSO. 204.6mg (max. 2.8 eq) crude allyl SAH (**2c**) were dissolved in 2.5 ml sodium phosphate buffer 50 mM pH 6.0 and added to the solution. Subsequently 25 ml NovO lysate in sodium phosphate buffer 100 mM pH 6.5 and 30 mg BSA were added. pH control indicated a pH of 5.0 which was set to 6.5 with aqueous NaOH solution. The suspension was shaken at 30°C and 100 rpm for 24 hours.

After the reaction progress was controlled by HPLC-MS (method: allyl-PhCoum) the suspension was shaken at 30°C and 100 rpm for another 29 hours.

The reaction was stopped by adding 2.5 ml 5 M aqueous HCl solution. The suspension was diluted with 20 ml ethyl acetate and after shaking the mixture was centrifuged at 4000 g for 5 minutes. The phases were separated, 20 ml ethyl acetate were added to the aqueous phase and the suspension was shaken. After centrifugation and phase separation the aqueous phase was washed once again with 20 ml ethyl acetate. The combined organic phases were dried over Na_2SO_4 which was removed by filtration. The solvents were evaporated to obtain the crude product.

Synthesis of benzylated 4,5,7-trihydroxy-3-phenylcoumarin (9d)

(**9d**) was synthesized according to the procedure described for (**9c**). 204.0 mg (max. 2.8 eq) crude benzyl SAH (**2d**) were dissolved in 2.5 ml sodium phosphate buffer 50 mM pH 6.0 and added to the solution.

The reaction progress was controlled by HPLC-MS (method: benzyl-PhCoum gradient). After 53 hours the reaction was worked up according to the procedure described before.

Synthesis of 2-butynylated 4,5,7-trihydroxy-3-phenylcoumarin (9e)

(**9e**) was synthesized according to the procedure described for (**9c**). 223.3 mg (max. 2.8 eq) crude 2-butynyl SAH (**2e**) were dissolved in 2.5 ml sodium phosphate buffer 50 mM pH 6.0 and added to the solution.

The reaction progress was controlled by HPLC-MS (method: butinyl-PhCoum). After 53 hours the reaction was worked up according to the procedure described before.

Synthesis of 4,5,7-trihydroxy-8-methyl-3-phenylcoumarin (9a)

25 mg (1.0 eq) 4,5,7-trihydroxy-3-phenylcoumarin (**8**) were dissolved in 2.5 ml DMSO. 80.5mg (0.19 mmol, 2 eq) *S*-adenosyl-*L*-methionine (**2a**) were dissolved in 2.5 ml sodium phosphate buffer 50 mM pH 6.0 and added to the solution. Subsequently 25 ml NovO lysate in sodium phosphate buffer 100 mM pH 6.5 and 30 mg BSA were added. pH control indicated a pH of 6.4. The suspension was shaken at 30°C and 100 rpm for 21 hours.

The reaction progress was controlled by HPLC-MS (method: methyl-PhCoum).

The reaction was stopped by adding 2.5 ml 5 M aqueous HCl solution. The suspension was diluted with 20 ml ethyl acetate and after shaking the mixture was centrifuged at 4000 g for 15 minutes. The phases were separated, 20 ml ethyl acetate were added to the aqueous phase and the suspension was shaken. After centrifugation and phase separation the aqueous phase was washed once again with 20 ml ethyl acetate. The combined organic phases were dried over Na_2SO_4 which was removed by filtration. The solvents were removed by evaporation.

Due to its high purity according to TLC the crude product (62.6 mg, 238 % brown solid) was purified by silica filtration (50 g silica, ethyl acetate / methanol 10:1, Rf 0.46). The solvents were removed in membrane pump vacuum and subsequently in oil pump vacuum. Yield: 17.5mg, 0.06 mmol, 67 % beige solid.

^{1}H-NMR (499.89 MHz, DMSO-d_6): δ = 1.97 (s, 3H, H-9), 5.94 (s, 1H, H-6), 6.99 (t, ^{3}J = 7.3 Hz, 1H, H-aromatic *para*), 7.17 (t, ^{3}J = 7.8, 2H, H-aromatic *meta*), 7.61 (d, ^{3}J = 7.0, 2H, H-aromatic *ortho*), 9.55 (s, 1H, OH).

^{13}C-NMR (125.70 MHz, DMSO-d_6): δ = 8.1 (C-9), 94.8 (C-3), 96.3 (C-6), 98.9 (C-8), 101.2 (C-4a), 123.8 (C-aromatic *para*), 126.8 (C-aromatic *meta*), 131.1 (C-aromatic *ortho*), 137.7 (C-aromatic *ipso*), 153.0 (C-8a), 158.3 (C-5), 160.3 (C-7), 164.2 (C-4), 177.3 (C-2).

S-Methyltransferases

*Bo*tmt

General procedure

A general procedure was applied for the characterization of the S-methyltransferase *Bo*tmt. Details differing from the procedure are described for each experiment.

50 mM buffer (sodium phosphate pH 6.0.), organic solvent (5 or 10 % Vol. ethanol or 25 % Vol. tBME), 10 mg enzyme / mmol substrate (lyophilized cell suspension in buffer), 2 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 1 mM substrate (4-hydroxythiophenol (**10**) in ethanol or tBME) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After 30 minutes the reaction was stopped by adding 0.33 M aqueous HCl solution. The denatured enzyme was separated by centrifugation at 13000 rpm for 10 minutes. The supernatant was analyzed by HPLC-MS applying the method HOPhSH FA MeOH pos.

Expression condition screening

The plasmid pET26b(+) with the gene encoding *Bo*tmt was transformed into *E.coli* BL21 (DE3) and *E.coli* C43. The cells were grown on agar medium on two separate plates. Two colonies were taken from each agar plate and grown over night at 37°C on either LB or 2xTY medium containing 40 mg/l of the antibiotic kanamycin. The pre-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the over-night-cultures and grown to an OD_{600} of 1.0 at 30°C. The main-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the pre-cultures and grown to an OD_{600} of 1.0 at 30°C. Each main-culture was divided in half. In one half of the main-cultures expression was induced by 100 µM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 1.0 and the temperature was decreased to 20°C. In the other half of the main-cultures expression was not induced and the temperature was decreased to 20°C at an OD_{600} of 1.0. After one day the cells were harvested. They were separated from the medium by centrifugation at 4000 g and 4°C for 10 minutes, washed with sodium phosphate buffer (50 mM pH 6.0), suspended in the buffer and lyophilized.

For the expression condition screening the general procedure was applied.

The substrate was dissolved in ethanol (10 % Vol.), dry cells were suspended in water and hydrated for 30 minutes while shaking prior to the screening. The reaction time was an hour.

Expression

The plasmid pET26b(+) with the gene encoding *Bo*tmt was transformed into *E.coli* BL21 Gold (DE 3). The cells were grown on agar medium. One colony was taken from the agar plate and grown over night at 37°C on 2xTY medium containing 40 mg/l of the antibiotic kanamycin. The pre-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the over-night-cultures and grown to an OD_{600} of 1.0 at 30°C. The main-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the pre-cultures and grown to an OD_{600} of 1.0 at 30°C. At an OD_{600} of 1.0 the temperature was decreased to 20°C. After one day the cells were harvested. They were separated from the medium by centrifugation at 4000 g and 4°C for 10 minutes, washed with sodium phosphate buffer (50mM pH 6.0), suspended in the buffer and lyophilized.

Solubility screening

10 mM substrate solutions were prepared by dissolving 4-hydroxythiophenol (**10**) in water, methanol, ethanol, acetonitrile, dimethylsulfoxide, tetrahydrofuran, acetone, ethyl acetate, *tert*-butyl methyl ether, dichloromethane, diethylether, toluene and cyclohexane.
10 mM product solutions were prepared by dissolving 4-(methylthio)phenol (**11a**) in water, methanol, ethanol, acetonitrile, dimethylsulfoxide, tetrahydrofuran, acetone, ethyl acetate, *tert*-butyl methyl ether, dichloromethane, diethylether, toluene and cyclohexane.

The different substrate and product solutions were mixed with a minishaker and centrifuged at 13000 rpm for 10 minutes. The supernatants were analyzed by HPLC-DAD applying the method HOPhSH FA MeOH.

Solvent screening

For the solvent screening the general procedure was applied.
The substrate was dissolved in methanol (5 % Vol.), the assay solutions contained 50 %, 25 %, 12.5 % or 5 % ethanol, acetonitrile, ethyl acetate or tBME respectively, 1.5 mg/ml enzyme (crude lysate in buffer) was employed and the reaction time was an hour. The supernatant of the samples with the water miscible solvents and the aqueous and organic phases of the

samples with the water immiscible solvents were analyzed by HPLC-DAD applying the method HOPhSH FA MeOH.

Dry cell concentration screening

For the dry cell concentration screening the general procedure was applied.

The substrate was dissolved in tBME, 0.3, 0.6, 3, 6, 15 and 30 mg/ml enzyme were employed and the reaction time was an hour. The organic phase was diluted 1:10 with tBME and analyzed by GC-MS applying the method MGK 50 M 100 kurz.

Kinetics

For the kinetic studies the general procedure was applied.

The substrate was dissolved in tBME and 6 mg/ml functional and denatured enzyme were employed. The enzyme was denatured at 96°C and 1000 rpm in an Eppendorf thermomixer for 30 minutes prior to the screening. For the "0 minutes samples" the functional and the heat-denatured enzyme were added to the assay mixture containing 0.33 M aqueous HCl solution. The other samples were incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After the respective reaction time 0.33 M aqueous HCl solution was added to the samples. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes. The organic phase was diluted 1:10 with tBME and analyzed by GC-MS applying the method MGK 50 M 100 kurz.

Buffer screening with phosphate and TRIS buffer

For the buffer screening the general procedure was applied.

10 mM substrate was dissolved in tBME. 6 mg/ml enzyme, 50 mM sodium phosphate buffer with a pH of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 or 9.0 and 50 mM TRIS buffer with a pH of 7.0, 7.5, 8.0, 8.5, 9.0 or 9.5 were employed. The organic phase was analyzed by GC-FID applying the method HOPhSH Me 100.

Buffer screening with acetate and HEPES buffer

For the buffer screening the general procedure was applied.

5 mM substrate was dissolved in tBME. 6 mg/ml enzyme, 50 mM ammonium acetate buffer with a pH of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 and 50 mM HEPES buffer with a pH of 6.0, 6.5, 7.0, 7.5, 8.0 or 8.5 were employed.The organic phase was analyzed by GC-FID applying the method HOPhSH Me 100.

pH screening

For the pH screening the general procedure was applied.

The substrate was dissolved in ethanol (5 % Vol.). 50 mM phosphate buffer with a pH of 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5 and active and denatured enzyme were employed. The enzyme was denatured at 96°C and 1000 rpm in an Eppendorf thermomixer for 30 minutes prior to the screening.

Buffer concentration screening

For the buffer concentration screening the general procedure was applied.

The substrate was dissolved in ethanol (5 % Vol.) and 10 mM, 30mM, 50 mM, 100 mM or 200mM phosphate buffer pH 5.5 were employed. Before the reaction was stopped by adding 0.33 M aqueous HCl solution the pH was controlled.

Temperature screening

For the temperature screening the general procedure was applied.

10 mM substrate was dissolved in ethanol (5 % Vol.) and 100 mM buffer pH 5.5 was employed. The components were combined in a 200 µl Eppendorf tube and the mixture was incubated in an Eppendorf PCR device.

Substrate scope screenings

Screening with aromatic substrates I

For the substrate screening the general procedure was applied.

1 mM or 10 mM substrates 4-methoxythiophenol, 4-nitrothiophenol and 9H-purine-6-thiol were dissolved in DMSO. 2 mM or 20 mM cofactor and 5 mg/ml enzyme (crude lysate) were employed and the reaction time was 22 hours. The supernatant was analyzed by HPLC-MS applying the methods MeOSPhenol FA MeOH pos, NO_2SPhenol FA MeOH pos and Spurin FA MeOH 95_5 pos.

Screening with aromatic substrates II

For the substrate screening the general procedure was applied.

The substrates 4-hydroxythiophenol (**10**) and 4-mercaptobenzoic acid were dissolved in methanol. 3 mg/ml enzyme (crude lysate) was employed and the reaction time was 2 hours. The supernatant was analyzed by HPLC-MS applying the methods HOPhSH FA MeOH pos and COOHPhSH FA MeOH pos.

Screening with aromatic substrates III

For the substrate screening the general procedure was applied.

10 mM substrates thiophenol, phenol, aniline, 2-cholorothiophenol, 3-chlorothiophenol, 4-chlorothiophenol, 4-methylthiophenol, benzylmercaptan and 2-mercaptobenzothiazole were dissolved in tBME. 6 mg/ml active and denatured enzyme were employed. The enzyme was denatured at 96°C and 1000 rpm in an Eppendorf thermomixer for 30 minutes prior to the screening. The organic phase was analyzed undiluted by GC-FID applying the method HOPhSH Me 100 and 1:10 diluted with tBME by GC-MS applying the method MGK 100 M 100 kurz.

Screening with aliphatic substrates

For the substrate screening the general procedure was applied.

10 mM substrates 1-octanethiol and 2-mercaptoethanol were dissolved in ethanol (5 % Vol.). 100 mM buffer pH 5.5 and active and denatured enzyme were employed. The enzyme was denatured at 96°C and 1000 rpm in an Eppendorf thermomixer for 30 minutes prior to the screening. The reaction time was an hour and the organic phase was analyzed by GC-MS applying the method MGK 100 M 10 kurz.

Cofactor scope screenings

Cofactor screening with allyl, benzyl and 2-butynyl SAH (2 c-e)

For the cofactor screening the general procedure was applied.

The substrate was dissolved in ethanol (5 % Vol.). 2 mM SAM (**2a**), allyl SAH (**2c**), benzyl SAH (**2d**) and 2-butynyl SAH (**2e**) were suspended in buffer. The reaction time was 3 hours and the supernatant was analyzed by HPLC-MS and GC-MS applying the methods HOPhSH FA MeOH pos, allyl-SPhOH, benzyl-SPhOH or butinyl-SPhOH and MGK 100 M 100 kurz.

Cofactor screening with ethyl SAH (2b)

For the cofactor screening the general procedure was applied.

The substrate was dissolved in ethanol (5 % Vol.). 2 mM SAM (**2a**) and ethyl SAH (**2b**) were suspended in buffer. 100 mM buffer pH 5.5 and active and denatured enzyme were employed. The enzyme was denatured at 96°C and 1000 rpm in an Eppendorf thermomixer for 30 minutes prior to the screening. The reaction time was 3 hours and the supernatant was analyzed by HPLC-MS applying the methods HOPhSH FA MeOH pos and ethyl-SPhOH.

*Cr*tmt

Expression condition screening

The plasmid pET26b(+) with the gene encoding *Cr*tmt was transformed into *E.coli* BL21 Gold (DE 3) and *E.coli* C43. The cells were grown on agar medium on two separate plates. Two colonies were taken from each agar plate and grown over night at 37°C on either LB or 2xTY medium containing 40 mg/l of the antibiotic kanamycin. The pre-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the over-night-cultures and grown to an OD_{600} of 1.0 at 30°C. The main-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the pre-cultures and grown to an OD_{600} of 1.0 at 30°C. Each main-culture was divided in half. In one half of the main-cultures expression was induced by 100µM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 1.0 and the temperature was decreased to 20°C. In the other half of the main-cultures expression was not induced and the temperature was decreased to 20°C at an OD_{600} of 1.0. After one day the cells were harvested. They were separated from the medium by centrifugation at 4000 g and 4°C for 10 minutes and washed with sodium phosphate buffer (50mM pH 6.0), suspended in the buffer and lyophilized.

50 mM buffer (sodium phosphate pH 6.0), 25 % Vol. tBME, 20 mg enzyme / mmol substrate (lyophilized cell suspension in buffer), 2 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 1 mM substrate (4-hydroxythiophenol (**10**) in tBME) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After 2.5 hours the reaction was stopped by adding 0.33 M aqueous HCl solution. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes. The organic phase was analyzed by GC-MS applying the method MGK 100 M 100 kurz.

Expression

The plasmid pET26b(+) with the gene encoding *Cr*tmt was transformed into *E.coli* BL21 Gold (DE 3). The cells were grown on agar medium. One colony was taken from the agar plate and grown over night at 37°C on 2 x TY medium containing 40 mg/l of the antibiotic kanamycin. The pre-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the over-night-cultures and grown to an OD_{600} of 1.0 at 30°C. The main-cultures containing 40 mg/l kanamycin were inoculated to an OD_{600} of 0.1 with the pre-cultures and grown to an OD_{600} of 1.0 at 30°C. In one half of the main-cultures expression was induced by 100 µM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 1.0 and the temperature was decreased to 20°C. In the other half of the main-cultures expression was not induced and the temperature was decreased to 20°C at an OD_{600} of 1.0. After one day the cells were harvested. They were separated from the medium by centrifugation at 4000 g and 4°C for 10 minutes and washed with sodium phosphate buffer (50 mM pH 6.0).

One half of the cells was lysed by sonication. The lysates and the inclusion bodies were obtained by differential centrifugation. The other half of the cells, the lysates and the inclusion bodies were lyophilized.

50 mM buffer (sodium phosphate pH 6.0), 10 mg enzyme / mmol substrate (lyophilized cell, lysate or inclusion body suspensions in buffer), 2 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 10 mM substrate (4-hydroxythiophenol (**10**) in 10 % Vol. ethanol) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After 2 hours the reaction was stopped by adding 0.33 M aqueous HCl solution. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes. The supernatant was analyzed by HPLC-MS and GC-MS applying the methods HOPhSH FA MeOH pos and MGK 100 M 100 kurz.

Screening with aromatic substrates

50 mM buffer (sodium phosphate pH 6.0), 10 mg enzyme / mmol substrate (lyophilized cell suspension in buffer), 2 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 1 mM substrates (4-hydroxythiophenol (**10**), 4-mercaptobenzoic acid, 4-nitrothiophenol, 4-methoxythiophenol and 9H-purine-6-thiol in 5 % Vol. ethanol) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an

Eppendorf thermomixer. After 20 hours the reaction was stopped by adding 0.33 M aqueous HCl solution. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes. The supernatant was analyzed by HPLC-MS applying the methods HOPhSH FA MeOH pos, COOHPhSH FA MeOH pos, MeOSPhenol FA MeOH pos, NO_2SPhenol FA MeOH pos and Spurin FA MeOH 95_5 pos.

Screening with aliphatic substrates

50 mM buffer (sodium phosphate pH 6.0), 25 % Vol. tBME, 10 mg enzyme / mmol substrate (lyophilized cell suspension in buffer), 10 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 10 mM substrates (1-octanthiol and dibutylthioether in tBME) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After 24 hours the reaction was stopped by adding 0.33 M aqueous HCl solution. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes. The organic phase was analyzed by GC-MS applying the method MGK 50 M.

Dry cell concentration screening

50 mM buffer (sodium phosphate pH 6.0), 0.6, 1.2, 6, 12, 30 or 60 mg enzyme / mmol substrate (lyophilized cell suspension in buffer), 2 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 1 mM substrate (4-hydroxythiophenol (**10**) in 10 % Vol. ethanol) were combined in that order in a 1.5 ml Eppendorf tube and the mixture was incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After 2 hours the reaction was stopped by adding 0.33 M aqueous HCl solution. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes. The supernatant was analyzed by HPLC-MS applying the method HOPhSH FA MeOH pos.

Kinetics

50 mM buffer (sodium phosphate pH 6.0), 10 mg active and denatured enzyme / mmol substrate (lyophilized cell suspension in buffer), 2 mM cofactor (*S*-adenosyl-*L*-methionine (**2a**) in buffer) and 1 mM substrate (4-hydroxythiophenol (**10**) in 10 % Vol. ethanol) were combined in that order in a 1.5 ml Eppendorf tube. For the “0 minutes samples” the functional and the heat-denatured enzyme were added to the assay mixture containing 0.33 M aqueous HCl solution. The other samples were incubated at 30°C and 1000 rpm in an Eppendorf thermomixer. After the respective reaction time 0.33 M aqueous HCl solution was added to

the samples. The precipitate was separated by centrifugation at 13000 rpm for 10 minutes. The supernatant was analyzed by HPLC-MS applying the method HOPhSH FA MeOH pos.

Chemicals

All chemicals and culture media were purchased from commercial suppliers. They were utilized without further purification. *S*-adenosyl-*L*-methionine chloride dihydrochloride was employed for all experiments.

High Performance Liquid Chromatography– Mass Spectrometry (HPLC-MS)

For HPLC-MS analysis the Agilent Technologies HPLC-System 1200 consisting of the vacuum degasser "G1379B", the binary pump "G1312B", the autosampler "G1367C" and the column compartment "G1316B" was used. For detection the multi wavelength detector (MWD) "G1365C"and the quadrupole mass detector"6120" with an electrospray ionization (ESI) ion source was used.

Methods

HOPhSH FA MeOH pos: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1 % HCOOH / MeOH 65:35, 0.5 ml/min, 30°C, MS-ESI positive mode, 242 nm, 254 nm.

allyl-SPhOH, butinyl-SPhOH and ethyl-SPhOH: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1 % HCOOH / MeOH, 0.5 ml/min, gradient: 0-5 min 35 % MeOH, 5-10 min to 80% MeOH, 10-11 min to 35 % MeOH, 11-15 min 35 % MeOH, 30°C, MS-ESI positive mode, 242 nm, 254 nm.

benzyl-SPhOH: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1 % HCOOH / MeOH, 0.5 ml/min, gradient: 0-5 min 35 % MeOH, 5-9 min to 80 % MeOH, 9-11 min 80 % MeOH, 11-12 min to 35% MeOH, 12-15 min 35 % MeOH, 30°C, MS-ESI positive mode, 242nm, 254 nm.

allyl-, benzyl-, butinyl- and ethyl-SAH: Merck LiChrocart Purosphere 250 mm x 4.6 mm x 5µm; 0.1 % TFA / MeCN,1 ml/min, gradient: 0-10 min from 0 % MeCN to 50 % MeCN, 10-11 min to 80 % MeCN, 11-15 min to 0 % MeCN, 25°C, MS-ESI positive mode, 260 nm.

methyl-PhCoum, allyl-PhCoum and butinyl-PhCoum: Merck Chromolith 100 mm x 4.6 mm, NH_4OAc 10mM pH 5.5 / MeCN 9:1, 1 ml/min, 40°C, MS-ESI positive mode, 316 nm.

benzyl-PhCoum gradient: Merck Chromolith 100 mm x 4.6 mm, NH_4OAc 10 mM pH 5.5 / MeCN, 1 ml/min, gradient: 0-10 min 10 % MeCN, 10-15 min to 50 % MeCN, 15-25 min to 100 % MeCN, 25-30 min to 10 % MeCN, 40°C, MS-ESI positive mode, 316 nm.

MeTyr AA MeOH iso pos: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, NH_4OAc 10mM pH 5.5 / MeOH 95:5, 0.7 ml/min, 25°C, MS-ESI positive mode, 230 nm, 270 nm.

COOHPhSH FA MeOH pos: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1 % HCOOH / MeOH1:1, 0.5 ml/min, 30°C, MS-ESI positive mode, 280 nm.

MeOSPhenol FA MeOH pos: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1% HCOOH / MeOH1:1, 0.5 ml/min, 30°C, MS-ESI positive mode, 242 nm.

NO_2SPhenol FA MeOH pos: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1% HCOOH / MeOH 7:3, 0.5 ml/min, 30°C,MS-ESI positive mode, 325 nm.

Spurin FA MeOH 95_5 pos: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1% HCOOH / MeOH 95:5, 0.5 ml/min, 30°C, MS-ESI positive mode, 330 nm.

HPLC-MS analysis was also performed with a Shimadzu Nexera HPLC-MS system. The device was equipped with the Nexera LC-30AD pump, the Nexera SIL-30AC auto sampler, the CTO-20AC prominence column oven, the SPD-M20A prominence diode array detector and the LCMS-2020 quadrupole mass detector.

Methods

allylTYR H2O-MeOH 99-1 Nucleodur, butinylTYR H2O-MeOH 99-1 Nucleodur and MeTYR H2O-MeOH 99-1 Nucleodur: Macherey-Nagel Nucleodur C18 ec 150/4 100 mm x 5mm, 0.01 % HCOOH / MeOH, 0.7 ml/min, gradient: 0-1 min 1 % MeOH, 1-10 min to 100 % MeOH, 10-11 min 100 % MeOH, 11-11.1 min to 1 % MeOH, 11.1-12 min 1% MeOH, 40°C, MS-ESI positive and negative mode, 200 – 400 nm.

High Performance Liquid Chromatography– Diode Array Detector (HPLC-DAD)

The Agilent Technologies HPLC-System 1100 consisting of the degasser "G1322A", the quaternary pump "G1311A", the autosampler "G1313A", the column compartment "G1316A" and the DAD detector "G1315A" was used.

Methods

HOPhSH FA MeOH: Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm, 0.1 % HCOOH / MeOH 65:35, 0.5 ml/min, 30°C, 242 nm, 254 nm.

Gas Chromatography – Mass Spectrometry (GC-MS)

For GC-MS analysis the Agilent Technologies GC-System "7890A" with the Injector "7683B" was used. The inert carrier gas Helium 5.0 served as mobile phase. As stationary phase a nonpolar Agilent HP-5MS capillary column with a length of 30 m, a diameter of 0.25mm and a coating thickness of 0.25 µm was used. The gas chromatograph was combined with a mass spectrometer. For ionization an EI ion source was used. The ions were separated by a quadrupole mass analyzer and detected by the mass selective detector "5975C".

Methods

MGK 50 M 100 kurz: split 1:100, inlet / injector temperature 250°C, temperature program 50°C for 1 min, 40°C / min to 300°C, 300°C for 0.5 min.

MGK 100 M 100 kurz: split 1:100, inlet / injector temperature 250°C, temperature program 100°C for 1 min, 40°C / min to 300°C, 300°C for 0.5 min.

MGK 100 M 10 kurz: split 1:10, inlet / injector temperature 250°C, temperature program 100°C for 1 min, 40°C / min to 300°C, 300°C for 0.5 min.

Gas Chromatography – Flame Ionization Detector(GC-FID)

The Agilent Technologies GC-System "6890N" with the Injector "7683" was used for GC-FID analysis. The inert carrier gas Nitrogen 5.0 served as mobile phase. For detection a flame ionization detector (FID) with Hydrogen 5.0 and air as detector gases was used.

Method

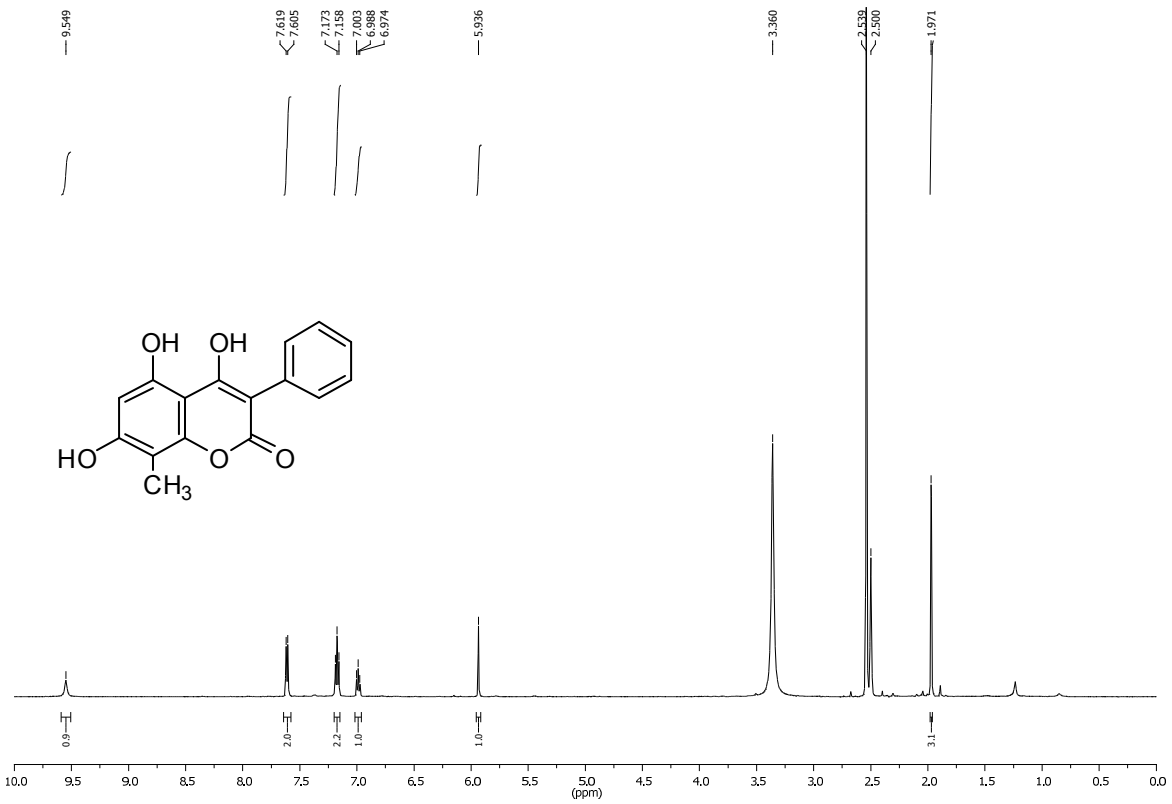
HOPhSH Me 100: Agilent HP5 30 m x 0.32 mm x 0.25 µm, splitless, injector temperature 250°C, temperature program 100°C for 1 min, 40°C / min to 260°C, 260°C for 2 min.

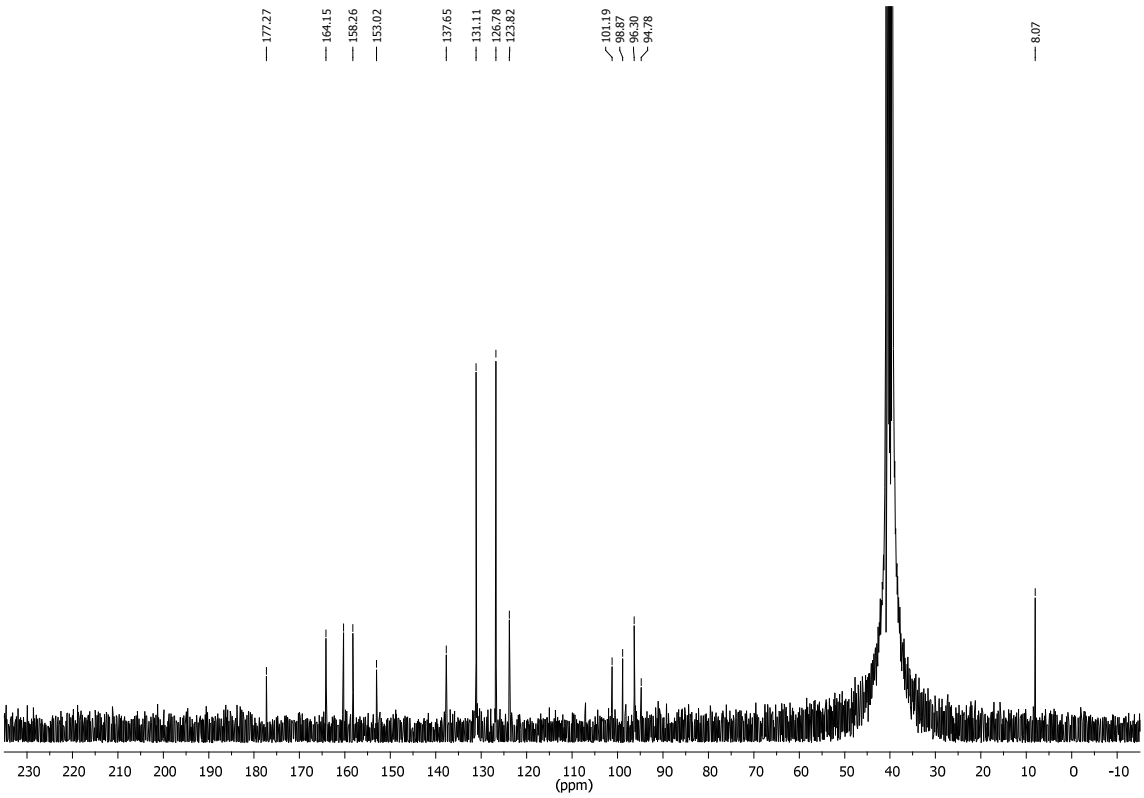
Nuclear Magnetic Resonance Spectroscopy (NMR)

The NMR-spectra were recorded with a Varian Inova-500 (500MHz) spectrometer or with a Bruker AVANCE III spectrometer equipped with an autosampler (300.36 MHz-^{1}H-NMR, 75.53 MHz-^{13}C-NMR).

The ^{1}H, ^{13}C and ATP chemical shifts were measured relative to the rest proton signal of the deuterated solvents as internal reference. For the explicit identification of certain compounds two-dimensional spectra such as ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC were recorded.

^{1}H and ^{13}C NMR of 4,5,7-trihydroxy-8-methyl-3-phenylcoumarin in DMSO-d_6





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ABBREVIATIONS

% Vol.	volume percent
(Het)Ar	heteroarene or arene
°C	degree Celsius
µm	micrometer
AA	ammonium acetate
acac	acetylacetonate
aq	aqueous
ATP	adenosine triphosphate
BSA	bovine serum albumin
d	dublet (NMR)
d	day
Da	dalton
DAD	diode array detector
DIPEA	*N,N*-diisopropylethylamine
DMSO	dimethyl sulfoxide
DMSO-d_6	fully deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
e. g.	exempli gratia (for example)
eq	equivalent/s
ESI	electrospray ionization
et al.	et alii (and others)
EtCN	propionitrile
FA	formic acid
FID	flame ionization detector
GC	gas chromatography
h	hour
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
IPTG	isopropyl β-D-1-thiogalactopyranoside
J	coupling constant (NMR)

LB	lysogeny broth
max	maximum
MeCN	acetonitrile
MeOH	methanol
MeTHF	methyltetrahydrofolate
mg	milligram
MHz	megahertz
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mmol	millimol
MS	mass spectrometry
NMR	nuclear magnetic resonance
OMT	O-methyltransferase
Pi	phosphate
pKa	logarithmic acid dissociation constant
pos	positive mode (MS)
PPh_3	triphenyl phosphine
PPPi	triphosphate
RNA	ribonucleic acid
rpm	revolutions per minute
s	singulet (NMR)
SAH	*S*-adenosyl-*L*-homocysteine
SAM	*S*-adenosyl-*L*-methionine
SDS	sodium dodecyl sulfate
Strep	streptavidin
t	triplet (NMR)
*t*BME	*tert*-butyl methyl ether
TFA	trifluoroacetic acid
THF	tetrahydrofolate
TLC	thin layer chromatography