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Characterisation of the *S*-methyltransferase *Bo*tmt

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1. Introduction

Methylation reactions are important transformations in the pharmaceutical industry for API (**A**ctive **P**harmaceutical **I**ngredient) modification and production. Methyl groups are often installed to optimise the properties of a new lead compound. The biological activity, bioavailability, solubility and binding affinity are main considerations in drug design. The so-called "methyl effect" can influence and in certain cases even improve all these characteristics. One example is the PLD2 inhibitor molecule where the insertion of a methyl group leads to a 590-fold boost in potency and lowers the IC_{50} value from 11800 nM to 20 nM. Chemo- or regio-selective introduction of a CH_3 group can be difficult and might require many synthetic steps and harsh conditions. Alkylating agents are often highly toxic compounds. A biocatalytic methylation might be a powerful alternative to the frequently used methods.[1]

In nature a methyl group transfer reaction is catalysed by methyltransferases (MTases). Most of these enzymes are *S*-adenosyl-L-methionine (SAM) dependent which is the second most ubiquitous cofactor after adenosine 5'-triphosphate (ATP) and the most important methyl group donor. SAM is a high energy compound and the transfer of a methyl group has a favourable enthalpy which enables high transfer efficiency.[1] Various MTases are known and they show next to structural variety very high sequential diversity (often less than 20 % amino acid identity). The cofactor *S*-adenosyl-L-methionine is very expensive and currently no efficient recycling system is available which is the main bottleneck for the application of SAM-dependent MTases.[2]

Methyltransferases catalyse the transfer of an activated methyl group to *N*-, *C*-, *O*-, *S*-nucleophiles in DNA, RNA, proteins and small molecules.[3–5] They are involved in many different processes like e.g. biosynthesis, protein repair, signal transduction and chromatin regulation.[4,5] A novel research field is focussed on SAM analogues which enable new analytical methods and assays besides many other possible applications, covalent labelling of DNA is only one of many examples.[2,3] Few research groups [2–5] investigate mainly DNA, RNA and protein methyltransferases. *S*-methyltransferases, especially those which catalyse the transfer of the methyl group to small molecules are mainly unexplored. The research in this field is still at the beginning but it might give insight into these highly potential enzymes.

2. Theoretical Part

2.1. The methyl effect

The methyl group is a small, uncharged, apolar group but the methylation of a molecule can have enormous impact on the properties of a compound. On the one hand it can be a "steric signal", where the addition of this small group can lead to specific recognition by cellular components. On the other hand it can act as a "chemical signal". Then it might lead to changes in chemical and/or physical properties such as e.g. H-bond rearrangements or formation of chiral centres.[5] The methyl group is the smallest but still one of the most ubiquitous functional groups in nature. The addition of CH_3 to lead compounds in drug discovery might improve the properties of these molecules. Biological activity, bioavailability, solubility and binding affinity can be influenced by this small group.[1,2]

The so-called "methyl effect" can, for example, improve the IC_{50} value of compounds more than 100-fold or increase or decrease the half-life. One reason for this effect is the reduction of the desolvatation energy. The transfer from aqueous environment to the binding cavity of a protein needs the removing of solvated water molecules around the substrate which in turn needs a lot of free energy. Increasing methylation pattern reduces the invested energy and favours binding to the protein.[1]

The effect is expected to lead to a three- to tenfold potency boost. Beyond this level it is referred to "magic methyl effect". The methyl group might increase the binding affinity of the enzyme to the substrate and mimic the conformation of the bound state which results in less reordering of the conformation upon binding. The following positions are reported to be preferred for higher activities upon methylation: *ortho* substitution, on substituted rings and between two freely rotatable bonds. These might serve as general rules to introduce a "magical methyl group".[1]

Therefore, methylations are important transformations especially for the pharmaceutical industry. The reaction might be difficult and might require many synthetic steps. Palladium catalysed methylation is one of the most common methods for carbon atoms.[1] Another common methylation method is the use of methyl iodide. Sometimes harsh conditions are required. Additionally most of them lack chemo-, regio- and stereoselectivity, hence need complex and/or multiple synthetic steps. The biocatalytic methylation might be a powerful

alternative to the established methods. Methyltransferases are able to methylate efficiently concerning yield, regio- and stereospecificity. Furthermore they are able to transfer the methyl group selectively to carbon, oxygen, nitrogen and sulphur atoms.[2]

Various methyltransferases are known. Biocatalytic methylation of synthetic or complex natural molecules can be applied for the production of pharmaceuticals, agrochemicals and fine chemicals. The cofactor *S*-adenosyl-L-methionine is very expensive and till now no efficient recycling system has been developed which limits the biotechnological application of these enzymes drastically.[2]

2.2. Methyltransferases (MTases EC 2.1.1.-)

A methyl group transfer reaction is catalysed by methyltransferases (MTases EC 2.1.1.-). Most MTases are *S*-adenosyl-L-methionine (SAM) dependent and catalyse the transfer of the methyl group to *N*-, *C*-, *O*-, *S*- nucleophiles or even halides. These enzymes can be categorised according to the nucleophile the methyl group is transferred to. They are able to methylate e.g. DNA, RNA, proteins and small molecules.[3–5] SAM-dependent methyltransferases are involved in many different natural processes, as for example in the biosynthesis, protein repair, signal transduction, chromatin regulation and more. [4,5]

There are five distinct structural folds (classes I-V) found for SAM-dependent methyltransferases.[4] The Class I MTases have a Rossmann-like protein fold and are the largest group among all MTase families.[2] Members of Class I act mainly on DNA and regulate gene expression, repair mutations or protect against bacterial restriction enzymes. Remarkably, these enzymes have a high structural consistency even when there is only 10 % primary amino acid sequence similarity.[2,4] This is reflected in the poor conservation of the SAM binding residues. The cofactor is bound in the same spherical position in most Class I MTases but the amino acid sequence of the pocket varies tremendously.[6]

2.2.1. DNA methyltransferases

DNA methyltransferases catalyse the transfer of an activated methyl group from *S*-adenosyl-L-methionine to specific DNA sequences. There are three DNA methyltransferase classes known, namely DNA adenine-*N*6 methyltransferase, DNA cytosine-*C*5 methyltransferase and DNA cytosine-*N*4 methyltransferase.[7] The methyl group acts mainly as a steric signal and plays an essential role in cellular differentiation, X-chromosome inactivation and more.[5]

DNA MTases mostly recognise palindromic DNA sequences. Furthermore doubly methylated, un-methylated and after DNA replication hemi-methylated DNA should be found in the cell. This can be seen as additional information content of DNA.[8]

Long time the exact interaction between the methyltransferases and the DNA helix was unclear. It seemed impossible to transfer the methyl group directly to the target atom. Eventually for *N*-DNA MTases nucleotide flipping – rotating out of the DNA helix towards the cofactor – was postulated. By rotating the target adenine together with its deoxyribose moiety and the neighbouring phosphodiester bonds out of the DNA helix facilitates the binding of the target nulceotides to methyltransferase in an extra helical formation.[8]

2.2.2. RNA methyltransferases

Methylation in RNA is more diverse and might occur at *N*1, *N*2 and *N*7 in guanine, *N*1 and *N*6 in adenine, *C*5 in cytosine or uracil and 2'*O* in ribose. These modifications have chemical functions in transforming RNA transcripts into biologically active species.[5] One example would be the maturation of the cap structure. All mRNAs in eukaryotes are capped at the 5' end and at least two methyltransferases are involved in the modification.[3]

2.2.3. Protein methyltransferases

In proteins there are following methylation targets possible: the carboxylate group of aspartate and glutamate, the sulphur of cysteine and methionine, the imidazole of histidine, the amide of glutamine and asparagine, the guanidinium of arginine, the ε-amino group of lysine and the terminal amino and carboxylate groups. Furthermore arginine and lysine can be methylated more than once.[5]

2.2.4. Small molecule methyltransferases

Many small molecules are involved in various processes in nature. The most prominent example for a methyltransferase acting on a small molecule is the catechol *O*-methyltransferase (COMT) which is responsible for the methylation of dopamine and other catecholamine neurotransmitters. [5] This enzyme is one of the major targets for drug development against Parkinson's disease. Furthermore it was used in the production of vanillin in the food industry.[2] In 1994 COMT was the first SAM-dependent methyltransferase where the structure could be solved.[6] Another example is the methylation of norepinephrine to epinephrine (adrenaline) catalysed by small molecule MTases and also the biosynthesis of vitamin B_{12} involves six different methyltransferases.[5]

2.3. Thiol methyltransferase from *Brassica oleracea*

Brassica oleracea belongs to common vegetables of the Cruciferae family and in specific comprises of cabbage, kale, Brussels sprouts, cauliflower, broccoli and kohlrabi. Cabbage-like vegetables are the major source of glucosinolates and their hydrolysis products for human nutrition. The glucosinolate content depends on growth conditions and environment and levels differ in various plant parts. *Brassica* vegetables have been regarded as anticarcinogenic because of their high amount of glucosinolates. Isothiocyanates and indoles, hydrolysis products of glucosinolates, might have an inhibitory effect on tumorgenesis. But this impact is dependent on many different factors and some isothiocyanates can even show a contrary, mutagenic effect on mammalian cells and bacteria.[9]

The exact role of thiol methyltransferases (TMT) in *Brassica oleracea* is still not known. A methyltransferase was purified and characterised by the group of Attieh *et al*. The molecular mass of this enzyme was 29.5 kDa and its pI value at pH 4.8.[10] In further surveys they isolated five functional isoforms of thiol methyltransferase from red cabbage. Three of them catalysed the SAM-dependent methylation of isothiocyanates, thiols and iodide. In addition there was a competitive inhibition between SAH and SAM and a non-competitive inhibition between SAH and iodide. With all these characterisations the main function of the enzyme is still unclear.[11]

The involvement of TMTs in the glucosinolate hydrolysis pathway might be an evidence for the main function of these enzymes. They are part of the detoxification of the hydrolysis products. Also the natural substrate of these thiol methyltransferases might be a compound of this pathway.[12] Previous studies showed that TMTs can methylate the halides iodide, bromide and chloride as wells as HS^- ions, but the K_m value for HS^- was much lower, indicating that this cabbage enzyme is involved in sulphur metabolism rather than halide detoxification.[10,12]

Attieh *et al.* revealed thiocyanates, 4,4'-thiobisbenzenethiol, thiophenol and thiosalicylic acid as possible substrates. The group further analysed the amount of thiol methyltransferase in different compartments of the cabbage plant. Based on activity measurements of iodide methylation, most activity was found in the cytosol but not in chloroplasts. Sulphur reduction mainly takes place in chloroplasts indicating that TMT is not directly involved in this process. Another survey revealed that TMT activity is only found in glucosinolate accumulating plants. All these results highly favour the theory that thiol methyltransferases from *Brassica oleracea* are involved in the detoxification of the hydrolysis products of glucosinolates.[12]

2.4. Glucosinolate hydrolysis pathway

In the first step of this pathway the enzyme myrosinase hydrolases glucosinolates. This happens only when the cells are damaged by chewing or cutting; then the enzyme is set free, comes in contact with its substrate and hydrolysis begins. The thereby produced products are toxic and protect the plant against herbivores. Figure 1 shows the whole pathway including the role of thiol methyltransferase (TMT).[12]

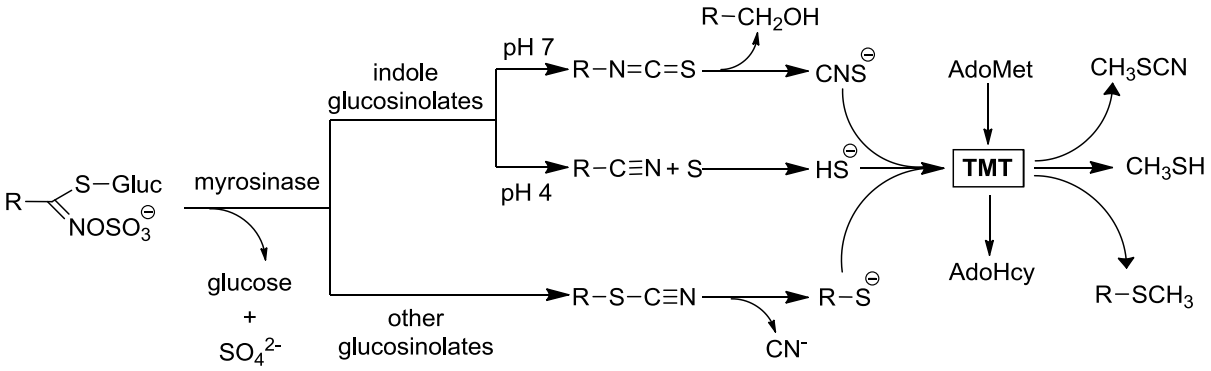


Figure 1: Glucosinolate hydrolysis pathway: glucosinolates are hydrolysed by the enzyme myrosinase and glucose and sulphate are released. Depending on the R-group glucosinolates can degrade to organic thiocyanates and further to thiols and cyanide. Indole glucosinolates at pH 7 form unstable isothiocyanates which degrade to the corresponding alcohol and isothiocyanate ions. At acidic pH indole glucosinolates form indole-3-acetonitrile and elemental sulphur. All these hydrolysis products, isothiocyanate ions, bisulfide ions and thiols can be methylated by thiol methyltransferase (TMT) with the cofactor *S*-adenosyl-L-methionine (SAM or AdoMet) to the corresponding products. This last step is the detoxification of the hydrolysis compounds.

All glucosinolates are composed of a thioglucose, a sulfonated oxime moiety and differing side chains. R can be alkyl, alkenyl, arylalkyl, alkylthioalkyl, β- hydroxyalkyl or indolmethyl.[9] After hydrolysis catalysed by myrosinase various degradation steps follow, depending on the pH value of the environment and depending on the nature of the side chain. The hydrolysis products can then be methylated by thiol methyltransferase to the corresponding products with the cofactor *S*-adenosyl-L-methionine.[13]

The products of this pathway can be methylthiocyanate, methylisothiocyanate, methanethiol, methylthioether and alkylmethyldisulfide. All these compounds are believed to have an effect on insect attacks, supress spreading of fungal pathogens or can attract predators of insects.[13] The glucosinolate hydrolysis pathway forms these toxic molecules for protection but they can also inhibit enzymes of aerobic respiration and oxidative metabolism

and are therefore also toxic to the plant itself. TMT plays a major role in detoxification of these compounds by methylation.[11]

The transcription level of the enzyme varies in different plant parts. The highest enzyme activity is found in 3-week old seedlings and leaves of young plants showed higher activity than mature plants. Also the enzyme myrosinase is highly abundant in young plants, which is another evidence for the involvement of TMTs in glucosinolate pathway. The thiol methyltransferases found in *Brassica oleracea* are distinct from known plant methyltransferases as they do not show *N*- or *O*-methyltransferase activity. In addition the enzyme also differs from the known *S*-methyltransferases as it does not methylate methionine or homocysteine. Thiol methyltransferases from *Brassica oleracea* are most probably involved in the detoxification of glucosinolate hydrolysis products, but the natural substrate has to be discovered yet and the main function of this enzyme has to be proved.[13]

2.5. *S*-adenosyl-L-methionine (SAM)

After adenosine 5'-triphosphate (ATP) is *S*-adenosyl-L-methionine (SAM) the second most ubiquitous cofactor in nature and the most important methyl group donor.[5,14] SAM is a high energy compound and the transfer of a methyl group has a favourable enthalpy which enables an efficient methylation. The sulfonium centre induces a partial positive charge on the adjacent three carbons, namely the methyl group, the 5'-carbon of ribose and the γ-carbon of methionine.[5]

In 1953 Cantoni was the first scientist who reported the discovery of SAM. He was able to unravel the biosynthesis and the structure of *S*-adenosyl-L-methionine. A methyl donating activity was assigned to the cofactor.[15] It took some more years to explore that this molecule is involved in many key metabolic reactions. The activated methyl group of SAM can be transferred to nitrogen, carbon, sulphur or oxygen in DNA, RNA, proteins and small molecules, as demonstrated in Figure 2.[14]

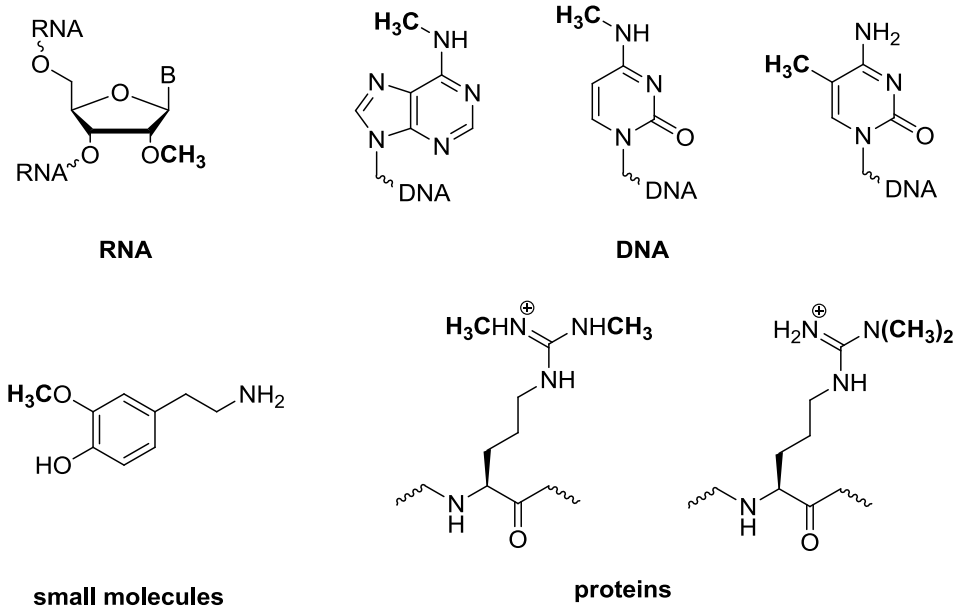


Figure 2: The activated methyl group of SAM can be transferred to the 2'-hydroxyl group of ribose in RNA, to *N*6 position of adenine, *N*4 and *C*5 position of cytosine in DNA, regioselectively to one of the hydroxyl groups in dopamine as an example for small molecules and symmetrically or unsymmetrically to nitrogen of arginine (example for proteins) by MTases.

After transfer reaction the cofactor *S*-adenosyl-L-methionine has to be recycled again. Two possible routes are known which are shown in Figure 3. After transferring a methyl group to a substrate, *S*-adenosyl-L-homocysteine (SAH) is the by-product which then enters the methyl cycle. When 3-amino-3-carboxypropyl group is cleaved, 5'-deoxy-5'-methylthioadenosine (MTA) is formed which enters the MTA cycle for recyclation of SAM. The MTA cycle is not present in mammalian cells. SAM is synthesised by the enzyme SAM synthetase (also known as methionine adenosyltransferase, MAT) from L-methionine and ATP. The formed triphosphate is hydrolysed to inorganic phosphate (P_i) and pyrophosphate (PP_i) which is the driving force for this reaction.[14]

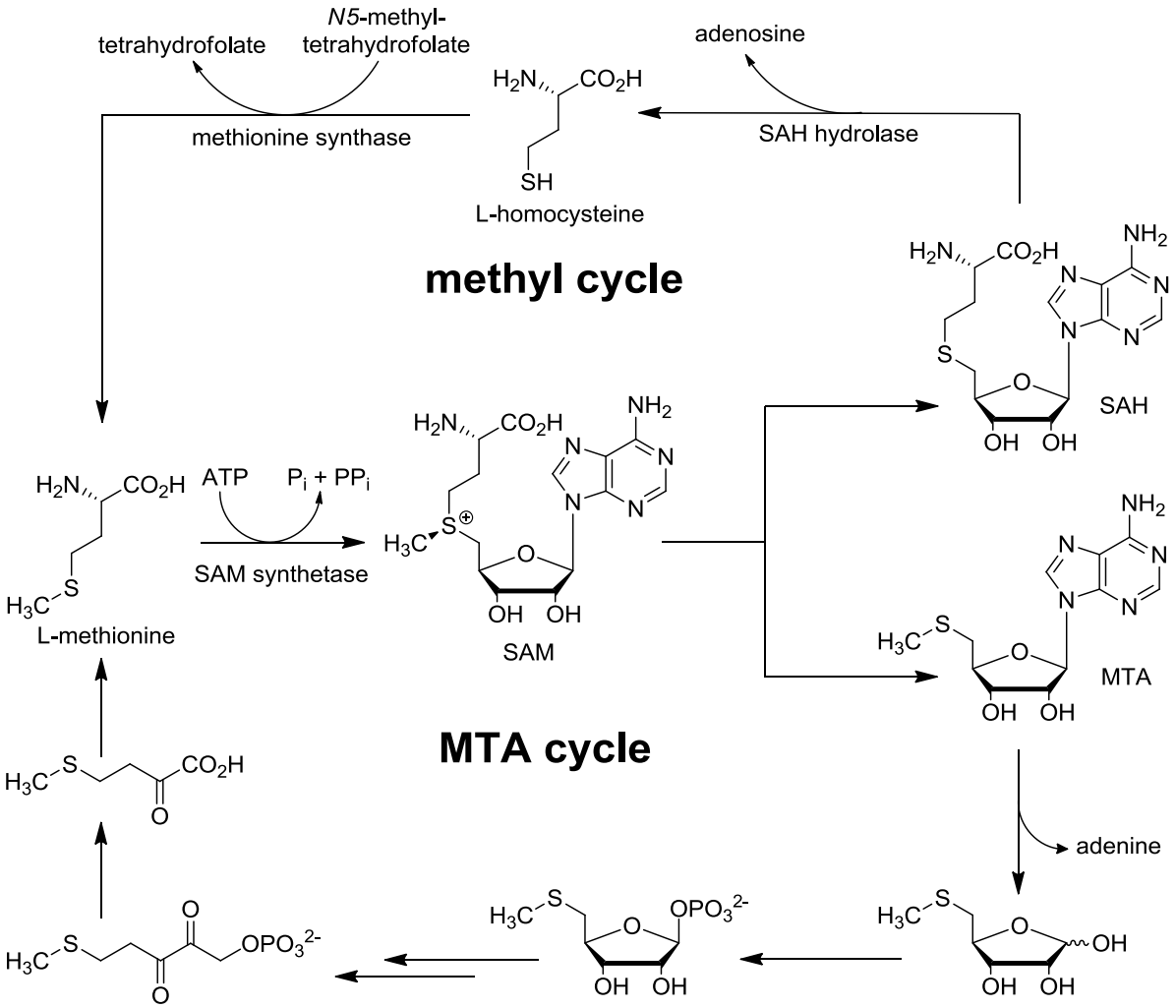


Figure 3: Synthesis of SAM and two possible recycling mechanisms: methyl cycle and MTA cycle.

The MTA cycle requires a lot of different steps and starts from the molecule 5'-deoxy-5'-methylthioadenosine (MTA). The methylthiol group is conserved and from the ribose the carbon backbone for L-methionine is formed. The second recycling pathway, called "methyl cycle", includes two steps. In the first *S*-adenosyl-L-homocysteine (SAH) is converted to L-homocysteine by SAH hydrolase. This product is then methylated by methionine synthase and the cofactor *N5*-methyltetrahydrofolate to L-methionine.[14]

2.6. Stability of *S*-adenosyl-L-methionine

S-adenosyl-L-methionine is a highly regulated molecule as it requires ATP for synthesis. The cofactor is very unstable and decomposes easily. The optimal condition to stabilise SAM is to keep the molecule at pH 3-5. Further a large and non-nucleophilic counter ion such as tosylate or sulphate have a positive effect on stability while halide ions decrease the stability.[16]

Three possible degradation pathways for (*S*,*S*)-SAM (biologically active epimer) are known today, which are represented in Figure 4. Two of them are pH dependent. The first possibility is the epimerisation of the molecule which is pH-independent - when the active *S*-configuration at the sulfonium centre changes to the biologically inactive *R*-configuration (pathway **A**).[14,17]

With increasing pH intramolecular nucleophilic attack of the carboxylate group to Cγ is the main process (pathway **B**). The products of the decomposition are 5'-deoxy-5'-methylthioadenosine (MTA) and homoserine lactone (HSL; also known as α-amino-γ-butyrolactone).[14,17]

At neutral pH or higher decomposition occurs via sulfonium ylide formation (pathway **C**). A proton is abstracted from the C5' carbon, the ribose ring is opened and adenine is eliminated. Shortly a double bond between C4' and C5' is formed and then loss of the double bond gives rise to S-ribosylmethionine.[14,17]

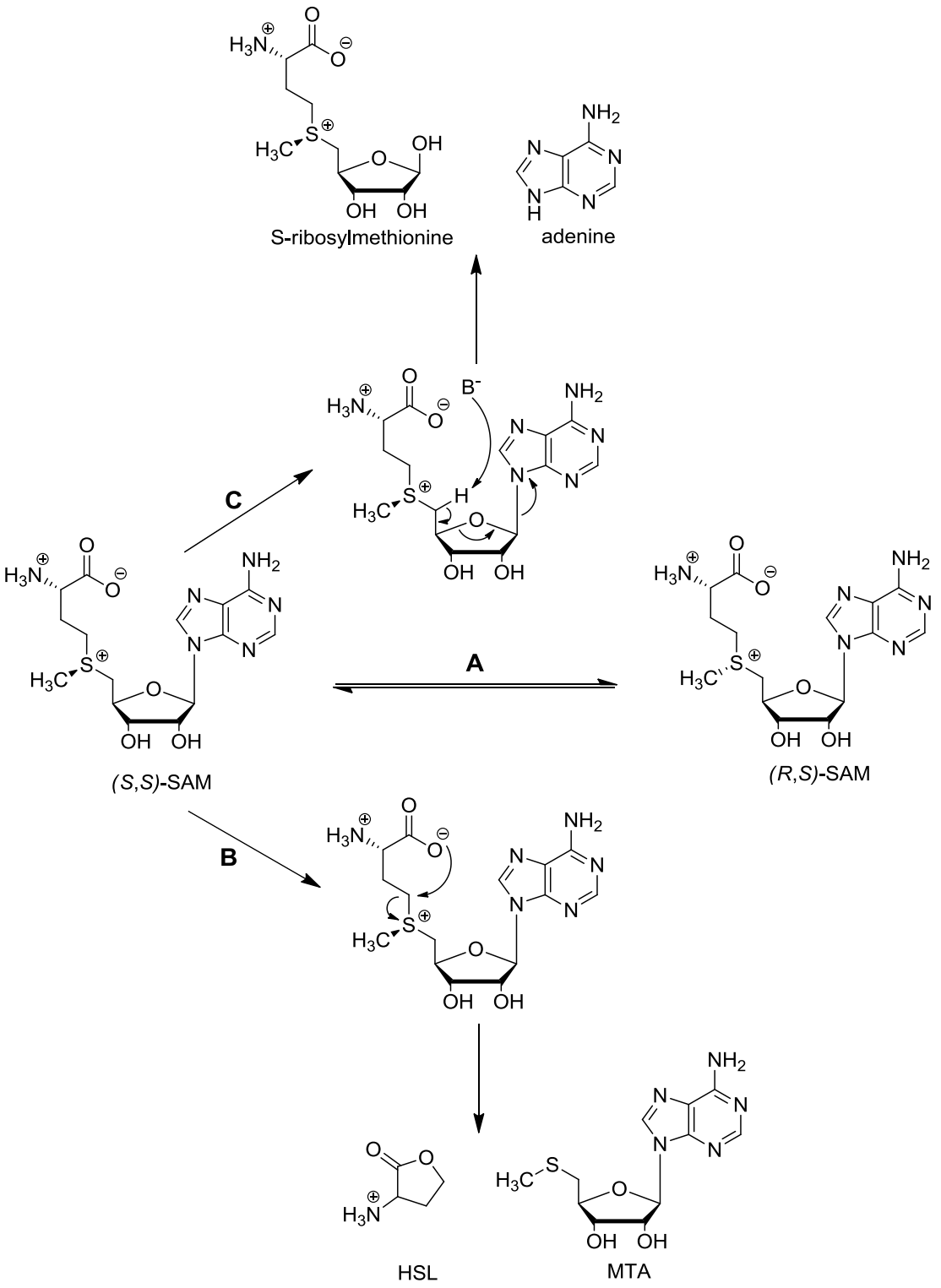


Figure 4: Three possible decomposition pathways of the cofactor SAM are known. Pathway A describes the epimerisation of the sulfonium centre from *S*- to *R*-configuration. Pathway B shows the intramolecular nucleophilic substitution resulting in MTA and HSL. Pathway C represents the abstraction of a proton by a base giving the products adenine and S-ribosylmethionine.

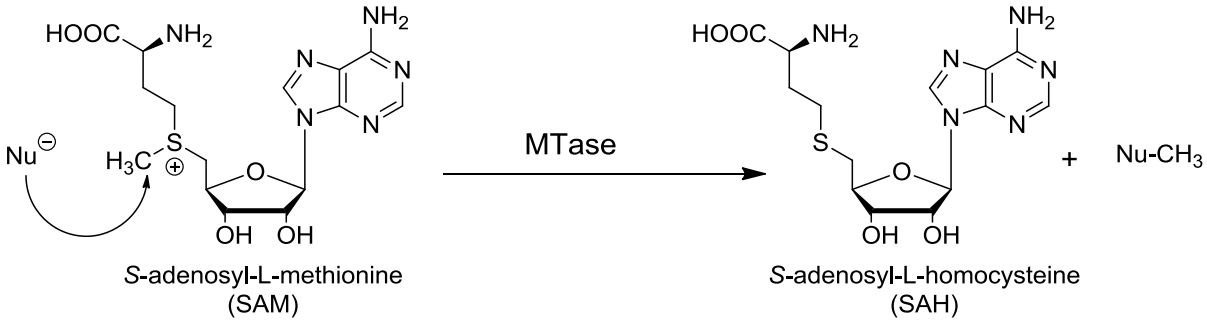
2.7. Reaction mechanisms of methyltransferases

The function of SAM-dependent methyltransferases is to catalyse the transfer of the activated methyl group from the cofactor *S*-adenosyl-L-methionine to the substrate. Attieh *et al.* postulated an ordered Bi Bi reaction mechanism for *Bo*tmt: SAM binds first to the enzyme TMT followed by the substrate. After catalysis the methylated product leaves first followed by SAH.[10,11] In most cases the enzyme activity of methyltransferases is inhibited by SAH and sinefugin. The latter is a natural antibiotic compound which strongly competes with binding of SAM.[18]

A completely different reaction mechanism is postulated for methyl transfer catalysis by SAM radical enzymes.[14] In the following chapters the two reaction mechanisms for the methyl transfer are explained in detail.

2.7.1. S_N2 type reaction

Most of the SAM-dependent methyl transfer reactions follow a S_N2 type reaction. The nucleophile attacks the carbon of the activated methyl group of the cofactor SAM catalysed by the enzyme methyltransferase and results in the methylated nucleophile and in SAH. (Scheme 1) **[3,5]**



Scheme 1: Reaction of SAM-dependent methyltransferases (MTases): a nucleophile attacks the activated methyl group of SAM catalysed by MTases resulting in SAH and the methylated nucleophile.

In Figure 5 the change in hybridization of the methyl carbon from sp^3 to sp^2 and back to sp^3 is shown. According to the S_N2 type reaction the nucleophile and the cofactor are involved in a single transition state.[3,5]

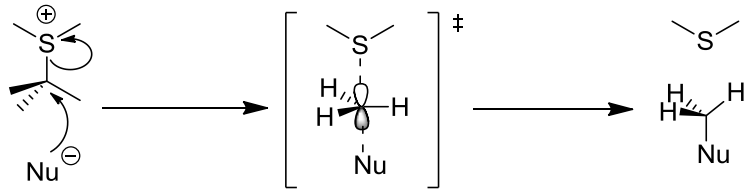


Figure 5: The methylation reaction follows a S_N2 type reaction where the nucleophile and the cofactor are involved in a single transition state. Thereby the hybridization of the carbon goes from sp^3 to sp^2 and back to sp^3 and results in inversion of the configuration.

In the transition state a *p* orbital at the carbon is formed which interacts with the nucleophile and the leaving group. The result is the inversion of the configuration of the carbon.[3]

2.7.2. Radical mechanism

The "radical SAM" superfamily enzymes combine an iron-sulphur cluster with the cofactor *S*-adenosyl-L-methionine to form a SAM radical for further catalysis. SAM binds to the iron in the cluster with its amino and carboxylate groups. After transfer of one electron to SAM, homolytic cleavage of the S-C5' bond occurs and leads to the formation of L-methionine and 5'-deoxyadenosyl radical (5'-dA•). This radical abstracts a proton from a substrate; the substrate becomes a radical itself for further reactions and 5'-deoxyadenosine (5'-dA) is formed.[14,17] The reaction mechanism is shown in Figure 6 with the example of *C*2-methylation of adenosine of ribosomal 23S rRNA.[19,20] The aromatic carbons of adenosine are sp^2 hybridized and therefore electrophilic and cannot undergo the S_N2 type reaction of SAM-dependent methyltransferases described before. In this case only the radical SAM mechanism can methylate this position.[19]

Two molecules of SAM have to be invested, one for the methylation of the cysteine residue and the second for the 5'-deoxyadenosyl radical formation.[19] Grove *et al.* observed five conserved cysteine residues in the protein RlmN, of which three are in the canonical radical SAM motif. Two conserved cysteines play a crucial role in this reaction mechanism, one carrying the methyl group and the other cleaving the cysteine residue from the alkylated substrate.[20]

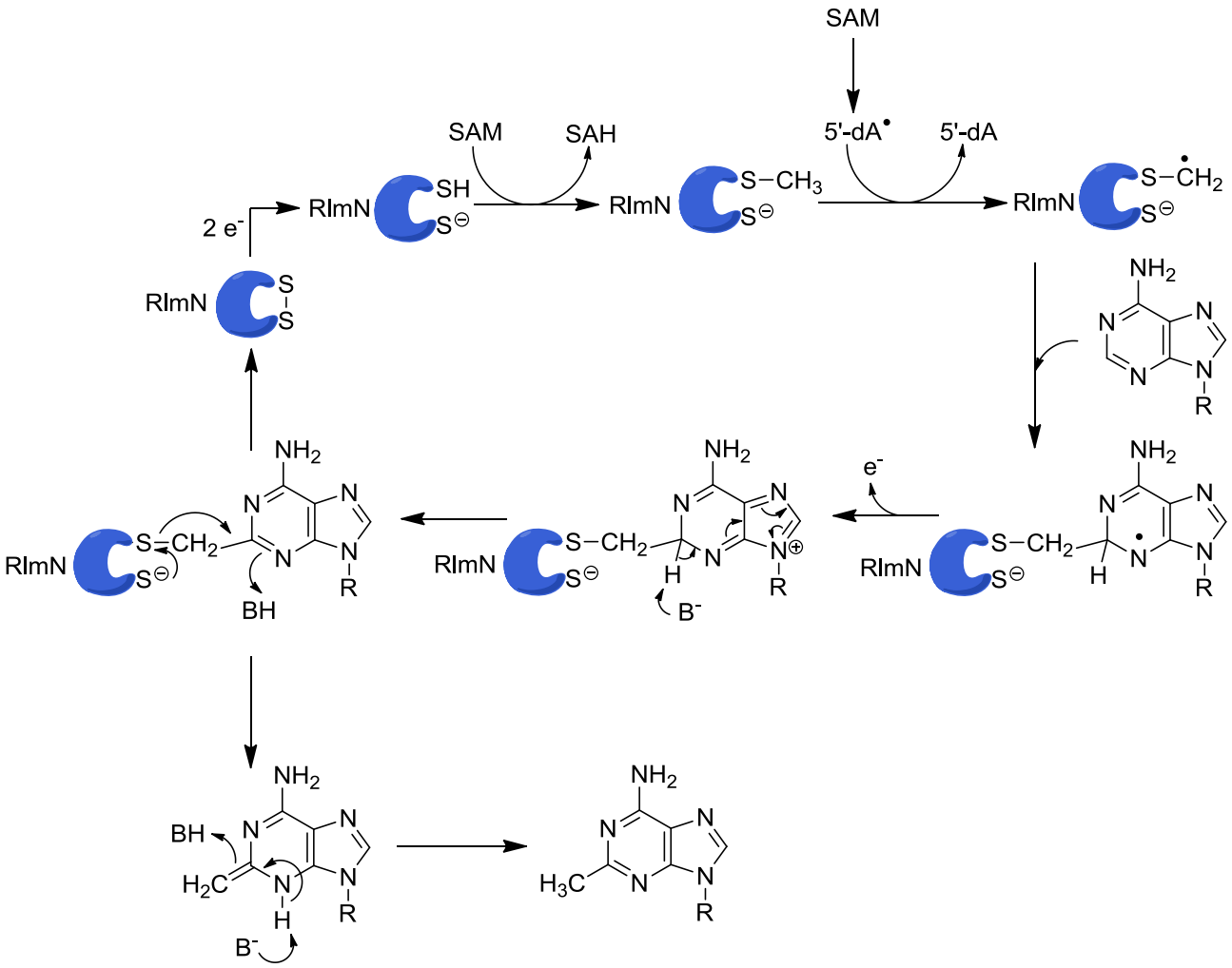


Figure 6: Postulated mechanism for the *C*2-methylation of adenosine catalysed by the radical SAM-dependent RNA-methyltransferase RlmN.

The initial step is the transfer of the methyl group from SAM to a cysteine residue of RlmN according to the S_N2 type reaction. Further a second SAM molecule is homolytically cleaved giving the 5'-deoxyadenosyl radical (5'-dA•). This radical abstracts a hydrogen from the cysteine-attached methyl group which becomes a radical itself. Then the protein radical binds covalently to *C*2-carbon of adenosine, forming a new carbon - carbon bond and delocalising the radical into the nucleotide base. After loss of one electron and abstraction of the proton from the *C*2-carbon the product is still linked to the cysteine residue of the protein. The second cysteine attacks the first one forming a disulphide bond and releasing the product. The last step is tautomerisation giving the methylated adenosine.[20]

Due to deuterium studies of RlmN, the proposed mechanism is only valid if BH does not exchange protons with the solvent and the hydrogen of the *C*2 carbon is recovered in the

attached methyl group. Still a lot of questions are unsolved concerning this radical SAM mechanism and are waiting to be elucidated.[19]

2.8. Cofactor modifications

Over the last years two feasible methods have been established to produce artificial cofactors. Either enzymatic or synthetic reactions can be applied.[3,14] One of the first scientists who was able to replace the methyl group by ethyl was L. W. Parks in 1958. He used ethionine-enriched growth medium and extracted the cofactor analogue *S*-adenosyl-ethionine from the *Torulopsis utilis* cells.[21]

The scientists F. Schlenk and J. L. Dainko were able to obtain the propyl analogue of SAM in 1975. They also applied the enzymatic reactions and used SAM synthetase for production.[22] One big advantage of this method is the high diastereoselectivity, which is missing in chemical synthesis.[17] Schlenk and Dainko also faced problems concerning the low amount of product. They further determined very small transfer rates for the propyl group. Moreover they figured out one big disadvantage of enzymatic reactions. SAM synthetase did not tolerate bigger or longer moieties.[22] Hence the second feasible method, chemical synthesis of artificial cofactors, became first choice.

Since then various analogues of the cofactor SAM have been synthesised, like selenium and tellurium, sulfoxide and sulfone, nitrogen and aziridine analogues. All those molecules change the characteristics of the cofactor. *N*-adenosylaziridine for example, binds covalently and sequence specifically to DNA and can be used in labelling studies.[14]

The transfer rates decrease drastically with the increasing size of the groups. Placing an unsaturated bond in β-position to the sulfonium centre is thought to stabilise the transition state of the reaction due to conjugation of the *p* orbitals. Hence the carbon atom between the sulfonium centre and the double or triple bond is activated for nucleophilic substitution by both functional groups. These so called double activated SAM analogues can transfer the side group of the artificial cofactor more efficiently.[3,5,23]

The research group of C. Dalhoff established a protocol for the chemical synthesis of propenyl and butynyl analogues of *S*-adenosyl-L-methionine. The substrate is unprotected *S*-adenosyl-L-homocysteine. No protecting groups are needed, because the one step reaction is performed under acidic conditions and therefore the substrate is fully protonated. The

products were obtained in an almost 1:1 diastereomeric ratio mixture at the sulfonium centre and were separated by preparative reversed phase HPLC.[24]

The same research group also synthesised ethyl and propyl cofactors which represent the "deactivated" SAM analogues. As expected these saturated groups showed poor reactivity and small to no transfer rates. Furthermore they were able to determine that all three classes of DNA methyltransferases accept SAM analogues as cofactor.[7] Lukinavičius *et al.* showed that even larger moieties including functional groups can be attached to SAH but need elaborate synthetic steps.[25]

H. Stecher *et al*. alkylated SAH with different alkyl bromides under acidic conditions. The cofactor analogues were also obtained as diastereomeric mixtures and used for the enzymes NovO and CouO as crude products which saved the intensive purification steps.[26]

Different SAM analogues can be applied in various reactions, surveys, assays and analysis methods and have a broad application area.

2.9. Applications of artificial cofactors

SAM mimics in combination with various MTases would provide a new biocatalytic method for targeted functionalisation of biomolecules.[5] These synthesised SAM analogues give rise to completely new experiments and analysis methods. The placement of bulky groups on DNA, for example, can be used for studying DNA-modifying enzymes, DNA-binding proteins or blockade of restriction endonuclease cleavage. The DNA MTase from *Thermus aquaticus* (M.TaqI) is able to transfer the extended groups of the cofactor analogues sequence specifically (5'-TCGA-3') to DNA. This can be proved by a DNA protection assay where the restriction endonuclease from *Thermus aquaticus* (REase R.TaqI) (same recognition site 5'-TCGA-3') is able to restrict unmodified DNA but not the parts which were modified by M.TaqI. The fragmentation pattern and the degree of DNA protection can then be analysed by agarose gel electrophoresis.[24]

As mentioned before the synthetic SAM analogues by Stecher *et al*. can be applied as crude products with the enzymes NovO and CouO which are able to perform biocatalytic Friedel-Crafts alkylation. This provides a new and specific method for carbon - carbon bond formation.[26]

Using modified cytosine residues with DNA methyltransferases are new approaches for sequence-specific derivatisation and labelling of DNA.[27] One common method for DNA labelling is the utilisation of aziridine cofactors. Nucleophilic attack on the aziridine ring leads to ring opening and coupling of the whole artificial cofactor to DNA. The method is very useful due to simple attaching of a reporter molecule to the cofactor.[3]

A more desirable approach would be the coupling of cofactor synthesis and utilisation in further reactions like methyl transfer in a single vessel. Current state-of-the-art for SAM analogues is multistep synthesis and requires HPLC purification of the diastereomeric mixtures and removal of *S*-adenosyl-L-homocysteine. Further the SAM analogues are very unstable which represents a limiting factor in practical developments. One possible route is the screening of different SAM synthetases in various organisms to find the ideal enzyme for the one pot reaction which will then produce SAM analogues and subsequently methylate complex natural products.[28]

3. Aim of the master thesis

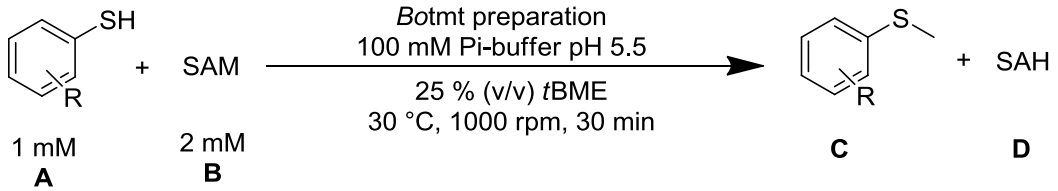
The methyl group is one of the most ubiquitous functional groups in biological molecules. When designing a drug, a methyl group is often installed to optimise the properties of the compound. Biological activity, sterically block or enhance metabolism, solubility and binding affinity can be influenced by this small group. Therefore, methylations became very important for the pharmaceutical industry. One of the frequently used methods is palladium catalysed methylation. The biocatalytic methylation might be a powerful alternative to the recently used reactions. [1]

S-Adenosyl-L-methionine (SAM) is the second most ubiquitous cofactor in nature and the most important methyl group donor. A methyl group transfer reaction is catalysed by methyltransferases (MTases EC 2.1.1.-). Most MTases are SAM-dependent and catalyse the transfer of the methyl group to nitrogen, oxygen, carbon or sulphur atoms in DNA, RNA, proteins and small molecules.[3–5] SAM-dependent methyltransferases are involved in various biological processes, as for example in the biosynthesis, protein repair, signal transduction and chromatin regulation.[4,5]

A new research field is concentrating on the production of SAM analogues by replacing the methyl group with bigger chemical moieties. One new application is to use modified SAM cofactors to covalently label biopolymers, for example DNA.[2,3] Many research groups [2–5] focus mainly on DNA and RNA methyltransferases next to protein MTases. Nevertheless the research in this field is still at the beginning.

The focus of this master thesis is on the *S*-methyltransferase from *Brassica oleracea* (*Bo*tmt). This enzyme belongs to the cabbage family and catalyses the transfer of a methyl group to thiol functionalities of small molecules. The expression, isolation and purification of *Bo*tmt are the basis for different enzyme preparations, like lyophilised cells, crude lysate and purified enzyme, which should be tested and compared with each other.

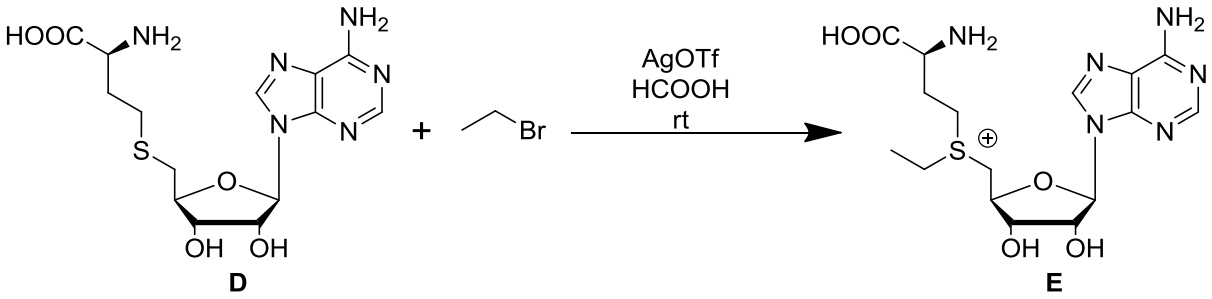
Further, a general assay setup has to be developed to characterise the enzyme. The first approach to a general reaction is shown in Scheme 2. The substrate scope of *Bo*tmt had to be analysed and the products quantified. For this quantification, the establishment of HPLC and GC methods is necessary.



Scheme 2: General reaction setup for the first assays with the *S*-methyltransferase *Bo*tmt: 1 mM substrate, 2 mM of cofactor SAM reacted with the enzyme preparation in 100 mM sodium phosphate buffer pH 5.5 and 25 % (v/v) *t*BME at 30 °C and 1000 rpm for 30 minutes.

Once the best substrate is found, further assays follow to characterise the enzyme. The establishment of an upscaling reaction setup is an important aim. With reaction optimisation a preparative scale transformation can be carried out in order to isolate the product for characterisation.

The synthesis of artificial cofactors is another subject in this master thesis. A general reaction procedure, according to Stecher *et al.*[26], is shown in Scheme 3. The compound **E** has to be synthesised and tested as cofactor for the *S*-methyltransferase from *Brassica oleracea*.



Scheme 3: Reaction scheme of the synthesis of artificial cofactor: SAH (D) will be alkylated with ethyl bromide in formic acid at room temperature to the product E.

The master thesis should provide methods and tools for the characterisation of *S*-methyltransferases in general. In order to evaluate the developed methods and protocols besides gaining knowledge, *Bo*tmt is investigated particularly.

4. Results and Discussion

4.1. Heterologous expression of *Brassica oleracea*

The thiol - methyltransferase from *Brassica oleracea* is referred to *Bo*tmt in this work. The wild type enzyme and the tagged variant were cloned in a pET 26b (+) vector and expressed in *E. coli* BL21 Gold (DE3) cells. The clones were kindly provided by Martin Tengg and Kerstin Steiner (ACIB, Graz). Various enzyme preparations were expressed and analysed by SDS-PAGE. Additionally, all of them were screened for activity.

E. coli B strain BL21 has very good growth characteristics and is lacking the proteases Lon and OmpT. Proteases are responsible for the degradation of misfolded proteins, prematurely terminated polypeptides and vulnerable folding intermediates. Lon is an ATP-dependent heat shock protease situated in the cytoplasm and is responsible for degradation of bulk and unstable proteins. OmpT is an outer membrane protein and adsorbs to inclusion bodies during cell lysis and remains active under denaturing conditions. Hence BL21 cells are frequently used expression systems.[29]

4.1.1. Lyophilised cells and crude lysate of *Bo*tmt

To control the quality of the expression of *Bo*tmt cells and to compare crude lysate and pellet, a SDS-PAGE was made which is shown in Figure 7. Crude lysate, pellet dissolved in urea and pellet dissolved in 100 mM sodium phosphate buffer pH 7.5 were applied in three different concentrations; 3 µg, 4.5 µg and 6 µg. Urea has a bursting effect and the enzymes enclosed in the pellet are set free. The method serves to resolubilise the insoluble proteins of the cells. In the crude lysate, the band for *Bo*tmt was clearly visible. The pellet dissolved in buffer showed nearly no bands which was the reassurance that the enzyme *Bo*tmt was soluble and found in the crude lysate.

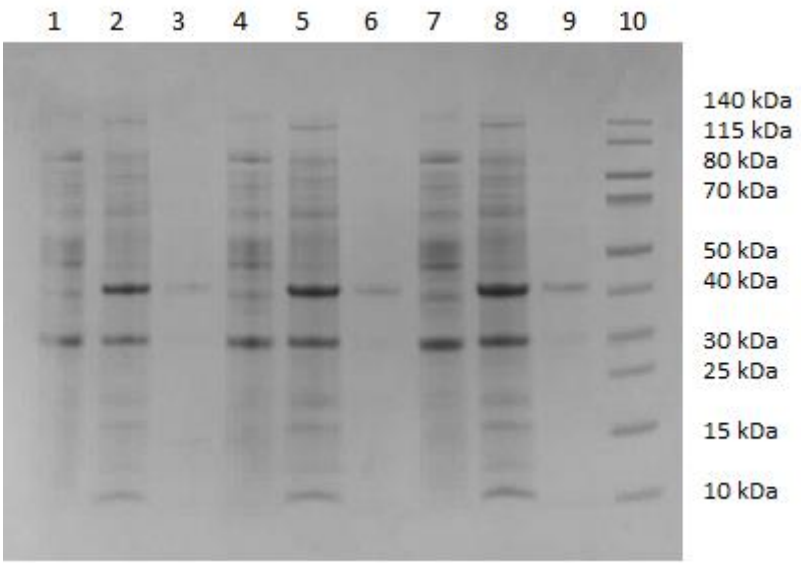


Figure 7: SDS-gel for expression analysis. Lane 1: lysate 3 µg, lane 2: pellet in urea 3 µg, lane 3: pellet in 100 mM sodium phosphate buffer 3 µg, lane 4: lysate 4.5 µg, lane 5: pellet in urea 4.5 µg, lane 6: pellet in buffer 4.5 µg, lane 7: lysate 6 µg, lane 8: pellet in urea 6 µg, lane 9: pellet in buffer 6 µg, lane 10: STD PageRuler® Prestained Protein Ladder. *Bo*tmt size: 26.5 kDa.

The in-gel quantification shown in Figure 8 helps to identify the approximate concentration of a protein. All identified gel bands are marked with yellow lines. The three red marked bands represent *Bo*tmt. The concentration of this enzyme compared to the total enzyme concentration in the crude lysate amounts to approximately 25 %.

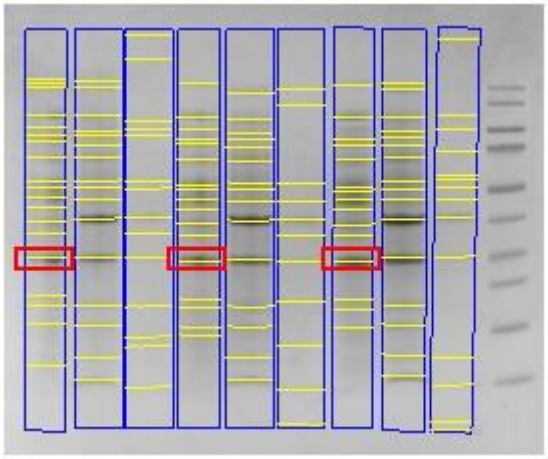


Figure 8: In-gel quantification of the SDS-PAGE. All recognised bands are marked with yellow lines. Red marked bands represent *Bo*tmt.

The total protein concentration of the crude lysate was 15 mg/mL. 25 % (w/w) of the total protein amount would mean 3.75 mg/mL *Bo*tmt content in the crude lysate.

4.1.2. *Bo*tmt_*Strep*

For purification of the enzyme *Bo*tmt, the *C*-terminal *Strep*-tagged *Bo*tmt was applied. With this tag, it is possible to purify the enzyme with a Strep-tactin column. The *Strep*-tagged enzyme has a molecular weight of 27.5 kDa. After expression of the cells, the crude lysate was obtained after cell disruption upon ultra sonication. *Bo*tmt_*Strep* was purified by a Strep-tactin column following the "(Twin-) *Strep*-tag® Purification Short Protocol" by iba. The crude lysate had a concentration of 8.5 mg/mL. A SDS gel, represented in Figure 9, was used to analyse the quality of the purification.

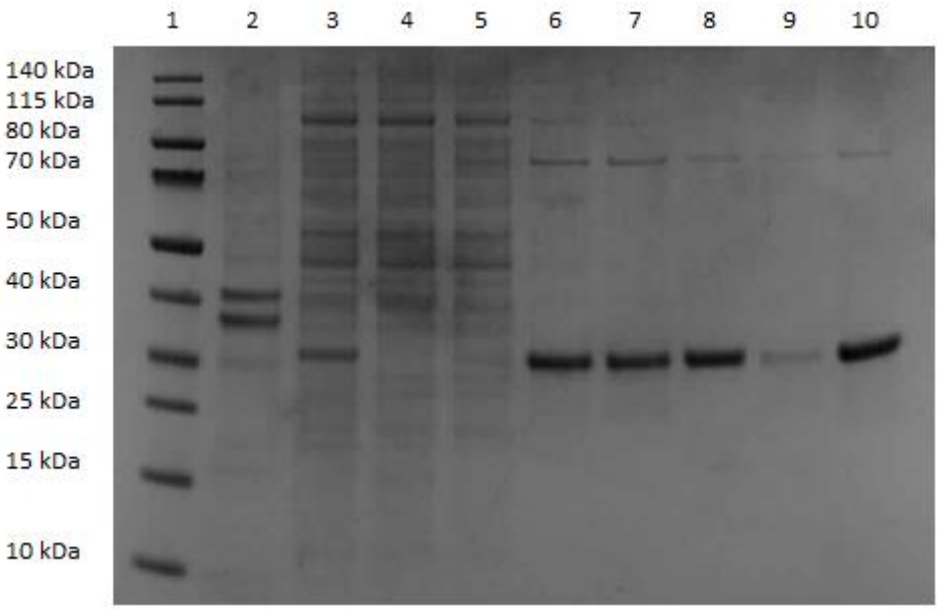


Figure 9: SDS gel of the purification of *Bo*tmt_*Strep*. 10 µL sample/slot, lane 1: STD PageRuler® Prestained Protein Ladder (3 µL), lane 2: *Bo*tmt_*Strep* pellet resuspended in buffer, lane 3: *Bo*tmt_*Strep* crude lysate, lane 4: flow through 1, lane 5: flow through 2, lane 6: wash 1, lane 7: wash 2, lane 8: eluate, lane 9: post wash, lane 10: eluate in the final buffer.

In lane 2, nearly no *Bo*tmt_*Strep* can be found which means that the enzyme is soluble and can be found in the lysate, as shown in lane 3. In lane 4 and 5 the desired enzyme cannot be found, which means that the column was not overloaded. Some protein was lost during the washing steps, shown in lane 6 and 7. The protein was eluted from the Strep-tactin column as represented in lane 8. In the post wash (lane 9) nearly no wanted *Bo*tmt_*Strep* was found, so the enzyme was eluted completely. Lane 10 displays *Bo*tmt_*Strep* in the final buffer (100 mM sodium phosphate buffer pH 7.5). The final concentration of the purified *Bo*tmt_*Strep* was 0.8 mg/mL.

4.1.3. *SBP_TEV_Bo*tmt

For purification of the enzyme *Bo*tmt, the *N*-terminal *SBP_TEV* tag (Strep binding protein_Tobacco etch virus) was cloned to the sequence. With the tag attached, the enzyme had a molecular weight of 32.4 kDa and 291 amino acid residues. After cutting the tag, the enzyme had a molecular weight of 26.5 kDa and 238 amino acid residues.

*SBP_TEV_Bo*tmt was purified by a Strep-tactin column. Further, the tag was cleaved by a Tev protease. The enzyme was refined by a His-column in order to remove the Tev protease and subsequently on a Strep-tactin column to eliminate the *Strep* tag and finally a buffer exchange was performed. Figure 10 shows the SDS gel of this purification procedure. The concentration of the crude lysate was 5.0 mg/mL.

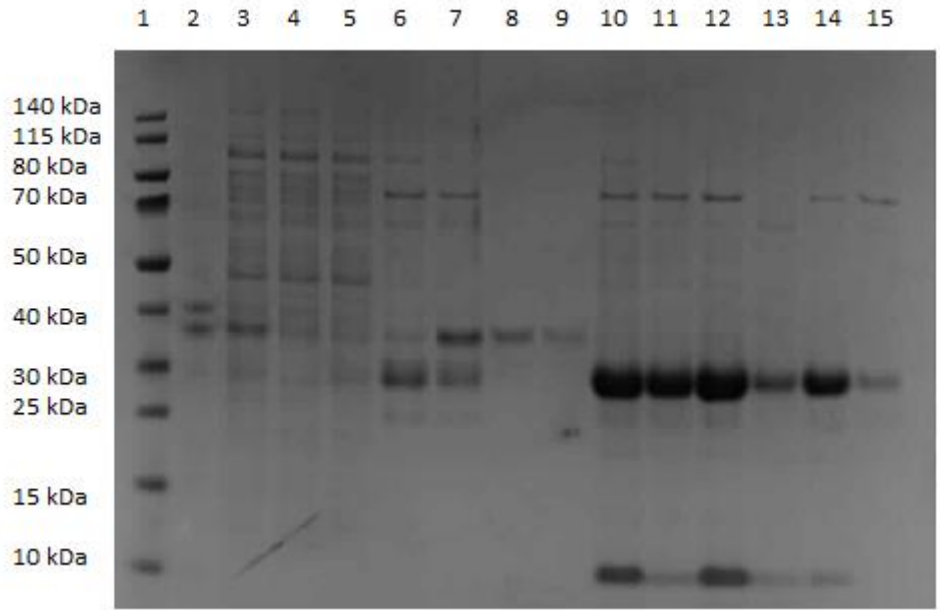


Figure 10: SDS gel of the purification of *SBP_TEV_Bo*tmt. 10 µL sample/slot, lane 1: STD PageRuler® Prestained Protein Ladder (3 µL), lane 2: *SBP_TEV_Bo*tmt pellet resuspended in buffer, lane 3: *SBP_TEV_Bo*tmt crude lysate, lane 4: flow through 1, lane 5: flow through 2, lane 6: wash 1, lane 7: wash 2, lane 8: eluate, lane 9: post wash, lane 10: TEV cleavage mixture, lane 11: wash His column, lane 12: eluate His column, lane 13: flow through Strep column, lane 14: eluate Strep column, lane 15: eluate in the final buffer.

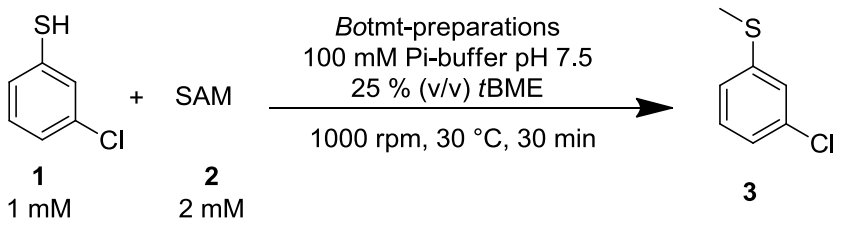
Lane 1 shows the STD PageRuler® Prestained Protein Ladder, lane 2 the pellet and lane 3 the crude lysate. In the pellet, a small amount of insoluble protein was found. However in the crude lysate, soluble enzyme can be found between 30 and 40 kDa. In the flow through (lane 4 and 5) of the Strep-tactin column nearly no *Bo*tmt band is visible. Hence the column was not overloaded with protein. Some of the protein has been lost at the washing step of the

Strep-tactin column as in lane 7 a *Bo*tmt band is clearly visible. The desired protein was eluted from the Strep-tactin column as determined in lane 8. Probably even more elution buffer should have been applied because there is still a protein band in lane 9 in the post wash sample. The Tev protease cut was successful. The mass of the protein was exactly reduced by the mass of the tag which is also clearly replicable on the SDS gel in lane 10. After cutting the tag, the enzyme preparation was purified by a His column. A huge amount of protein was lost during this step, because the enzyme was not washed from the column completely but eluted in the next step. This is apparent from lane 12. This was also the case with the following Strep-tactin column. In lane 13 the desired protein amount appears small, while in lane 14 there should not be a protein band. The desired protein should be found in the flow through of the last Strep-tactin column. A huge amount remained on the column and was not eluted. With the following elution buffer the rest of the protein was washed from the column. After buffer exchange to the final buffer, 100 mM sodium phosphate buffer pH 7.5, a small band corresponding to *Bo*tmt is visible in lane 15. The concentration of the purified *Bo*tmt in the final buffer was 0.27 mg/mL.

4.1.4. Comparison of various *Bo*tmt preparations and variants

Lyophilised cells and crude lysate showed high activity and all assays could be performed with these enzyme preparations. Using purified enzyme could even enhance the yield and increased the reaction velocity significantly. Some of the investigations were only possible with purified enzyme, like for example the influence of metal ions on the enzyme activity in order to exclude cross-contaminations and background activities. Three new enzyme preparations were expressed and analysed: *Bo*tmt_*Strep* and *SBP_TEV_Bo*tmt next to tag-cleaved purified *Bo*tmt.

After expression of a new batch of enzyme, an activity assay was made to compare old with new batches, crude lysates with lyophilised cells and purified enzyme. For this purpose, a general reaction scheme was applied as shown in Scheme 4.



Scheme 4: General reaction scheme for activity assays. 1 mM 3-chlorobenzenethiol (1) and 2 mM SAM (2) in 100 mM sodium phosphate buffer pH 7.5 and 25 % (v/v) *t*BME in the presence of various *Bo*tmt preparations at 1000 rpm and 30 °C for 30 minutes and transformation to the corresponding product 3-chlorothioanisole (3).

After expression, the cells were dried by lyophilisation to gain lyophilised cells. In the assay 10 mg dried cells per mmol substrate were used by resuspending 10 mg of dry cells in 400 µL of a 100 mM sodium phosphate buffer pH 7.5 for 30 minutes. To gain crude lysate, the cells were disrupted by ultra-sonication. For the assays, 400 µL were applied.

For purification, the enzyme was cloned with the *N*-terminal *SBP_TEV* tag or with the *C*-terminal *Strep* tag. Either 400 µL of the lysates of these enzyme preparations or 0.5 mg/mL of purified enzyme preparation were applied for the assays.

The concentrations of the samples were determined by in-gel quantification. The protein concentration in crude lysate was analysed by a Bradford assay. [30] Nano Drop measurements were applied for determination of the purified enzyme amount. All enzyme preparations are listed in Table 1.

Table 1: List of all different enzyme preparations, expression date in brackets, the isolated concentration [mg/mL] and the method used for concentration determination. Furthermore either 400 µL crude lysate or 10 mg/mL dry cells or 0.5 mg/mL purified enzyme were tested in the activity assay and the conversion is given in per cent. *The reaction with purified *Bo*tmt ran for one hour instead of 30 minutes.

enzyme preparations	isolated concentration [mg/mL]	concentration determination	conversion [%]
*Bo*tmt lyophilised cells (12.6.2013)			85
*Bo*tmt lyophilised cells (27.11.2013)			88
lysate *Bo*tmt (27.11.2013)	3.8	in gel quantification	79
lysate *Bo*tmt_*Strep* (13.1.2014)	8.5	Bradford Assay	56
lysate *SBP_TEV_Bo*tmt (13.1.2014)	5.0	Bradford Assay	65
purified *Bo*tmt_*Strep* (13.1.2014)	0.8	Nano Drop	77
purified *SBP_TEV_Bo*tmt (13.1.2014)	1.4	Bradford Assay	72
purified *Bo*tmt (13.1.2014)	0.3	Nano Drop	64*
purified *SBP_TEV_Bo*tmt (11.2.2014)	3.7	Nano Drop	86

All enzyme preparations were active but some showed higher conversions than others. The tag had almost no influence on the enzyme activity. In addition the necessary purification steps had no negative effect. Both tagged preparations, the *C*-terminal *Strep* tag and the *N*-terminal *SBP_TEV* tag, could be expressed and purified as active enzymes. The crude lysates were less active than the corresponding purified enzyme preparations, emphasising cross-interactions of the *S*-MTase with other enzymes present in the lysate which are able to metabolise the substrate. Lyophilised cells and the purified *SBP_TEV_Bo*tmt expressed on the 11th of February 2014 showed the highest, the lysate of *Bo*tmt_*Strep* showed the least conversion of all enzyme preparations. Figure 11 gives an overview on the activity of the enzyme preparations.

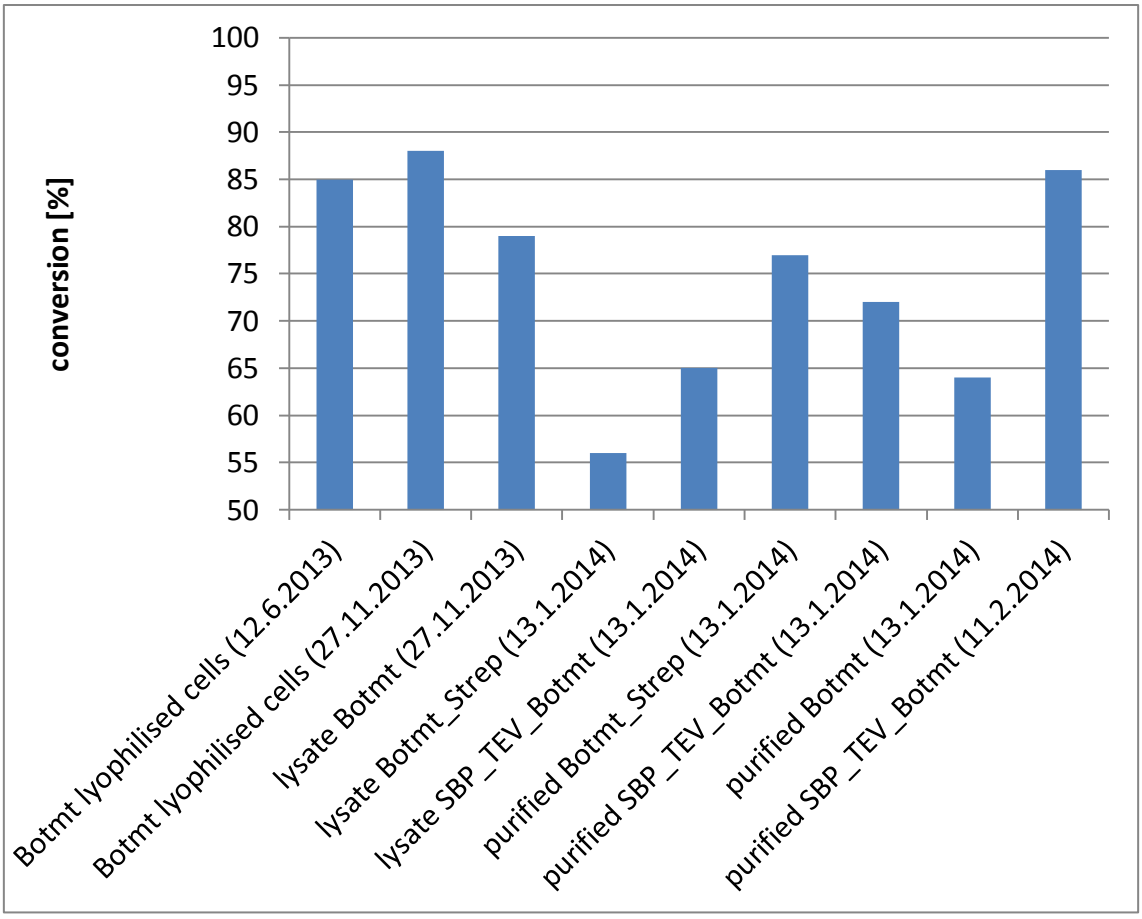


Figure 11: Comparison of the results of the activity assays with all *Bo*tmt enzyme preparations. The conversions of 3-chlorobenzenethiol (1) to the corresponding product 3-chlorothioanisole (3) are shown in per cent.

The purified *Bo*tmt without tag showed low conversion. This might be due to two reasons: First, the reaction ran for one hour instead of 30 minutes and this might be too long leading to product loss upon evaporation. Lyophilised cells and lysate preparations are stabilised by their environment and other cell compartments. Purified enzyme might be more sensitive and might lose activity faster. On the other hand the whole procedure of tag-cleavage and purification is a stress factor for the protein.

The second reason might be the concentration. Only 0.3 mg/mL of *Bo*tmt could be obtained after purification. The assay had to be adapted to the lower concentration. The applied concentrations of substrate, cofactor and enzyme were reduced by the factor of 3.7. These low amounts might influence the conversion due to K_M-values and lead to problems with detection limits.

For the metal ion assay the purified *SBP_TEV_Bo*tmt expressed on the 11th of February 2014 was applied, for all the other assays lyophilised cells were used, in few cases crude lysate. For each reaction the used enzyme preparation is noted.

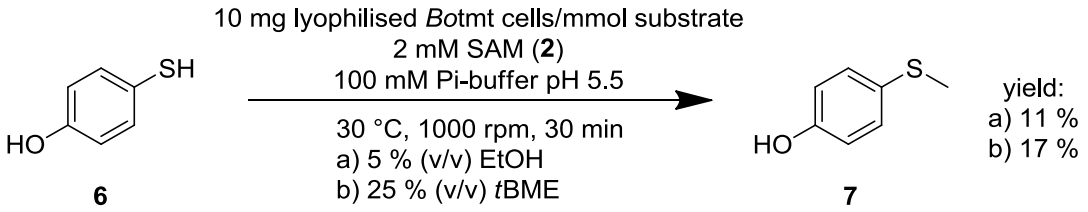
4.2. Substrate screening

Previously a thiol methyltransferase from *Brassica oleracea* was purified and characterised by Attieh *et al*. As part of various surveys, the substrate scope of the enzyme was analysed as well. Thiocyanates, 4,4'-thiobisbenzenethiol, thiophenol and thiosalicylic acid were determined as possible natural substrates. Further compounds such as 4-nitrothiophenol, aniline, phenol, quercetin and salicylic acid were tested but the enzyme showed no activity.[10]

In this master thesis eighteen different molecules were chosen and analysed with *Bo*tmt in order to determine the substrate scope of this *S*-methyltransferase. Before that, various reaction conditions were screened: In a first assay a high amount of enzyme (20 mg lyophilised *Bo*tmt cells per 10 mmol substrate) and 5 % (v/v) ethanol were applied. Three different substrates, 2-chlorothiophenol (**4**), 3-chlorobenzenethiol (**1**) and 4-chlorothiophenol (**5**), were tested. In addition, 2 % of internal standard 1,3,5-triisopropylbenzene was added to the substrate stocks. The reaction tubes were incubated for two hours at 30 °C and 1000 rpm. No conversion could be detected.

Soon it became clear that the enzyme concentration might be too high and the reaction time too long. The reaction conditions were changed to one hour and 10 mg lyophilised *Bo*tmt cells per mmol substrate. Also in this attempt, no conversion could be detected.

The substrate 4-mercaptophenol (**6**) was used to make a solvent test. Assay solutions containing 5 % (v/v) ethanol and 25 % (v/v) *t*BME respectively were compared with each other. Additionally the reaction time was lowered to 30 minutes. The reaction presented in Scheme 5, not only showed conversion but also led to the conclusion that *t*BME is a better solvent for the assay.

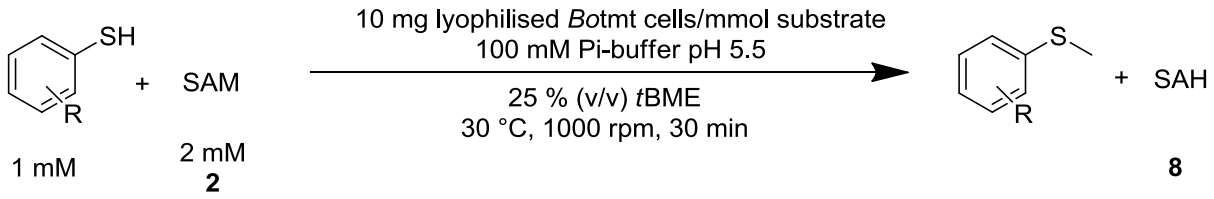


6 **7**

Scheme 5: Reaction conditions of 4-mercaptophenol (6) with lyophilised *Bo*tmt cells. Two different solvents were used in the assay: a) 5 % (v/v) EtOH or b) 25 % (v/v) *t*BME.

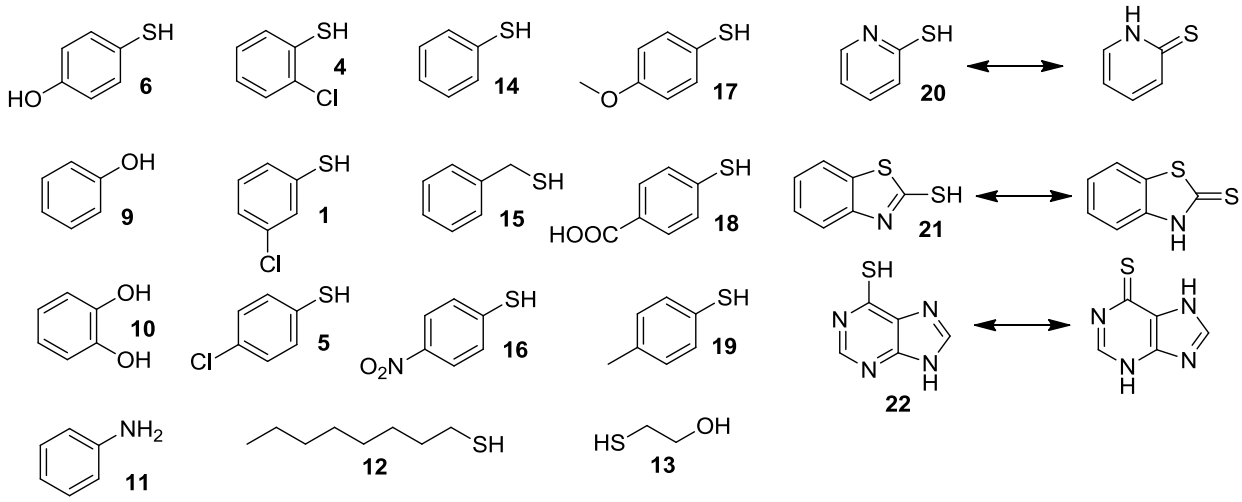
With 5 % (v/v) ethanol in the assay 11 % conversion was achieved while with 25 % (v/v) *t*BME in the assay 17 % conversion to the corresponding product (4-methylmercapto)phenol (**7**) was reached.

With these previous analytical assays, it was possible to find a general reaction setup for all substrates which is shown in Scheme 6. Only 10 mg lyophilised *Bo*tmt cells per mmol substrate and 25 % (v/v) *t*BME as cosolvent in the assay were applied.



Scheme 6: General reaction scheme of the assay: 1 mM of substrate and 2 mM of SAM (2) incubated with 10 mg lyophilised *Bo*tmt cells per mmol substrate in 100 mM sodium phosphate buffer pH 5.5 and 25 % (v/v) *t*BME at 30 °C and 1000 rpm for 30 minutes.

To identify the substrate scope of the methyltransferase *Bo*tmt eighteen different molecules were screened as substrates, which are listed in Scheme 7.



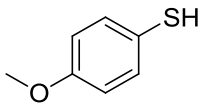
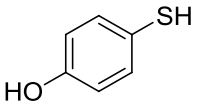
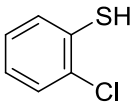
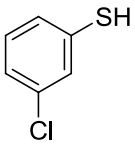
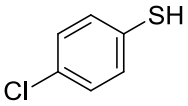
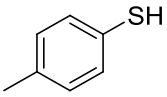
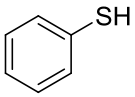
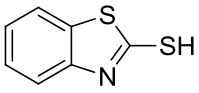
Scheme 7: Substrate scope: substrates screened for *Bo*tmt activity.

Most of them contain a thiol group, some only an alcohol (phenol (**9**) and 1,2-dihydroxybenzol (**10**)) or amine functional group (aniline (**11**)) which might be a possible methylation target. Some combine two functional groups, mostly in *para* position. With the example of chlorothiophenols (2-chlorothiophenol (**4**), 3-chlorobenzenethiol (**1**) and 4-chlorothiophenol (**5**)) the position of the additional functional group (*meta*, *ortho*, *para*) was

investigated. Two aliphatic molecules (octane-1-thiol (**12**) and 2-mercaptoethan-1-ol (**13**)) were among the chosen putative compounds. Additionally 4-mercaptophenol (**6**), mono substituted thiophenol (**14**) and the aliphatic thiol benzyl mercaptan (**15**) were selected for the screening. In order to examine the influence of the electron density the electron deficient aromatic thiol *p*-nitrothiophenol (**16**) and the electron rich aromatic thiol *p*-methoxythiophenol (**17**) were chosen. 4-mercaptobenzoic acid (**18**) and 4-methylbenzenethiol (**19**) might also possess a putative methylation site. Finally, heterocyclic aromatic ring systems with the examples of 2-mercaptopyridin (**20**), mercaptobenzothiazole (**21**) and 9*H*-purine-6-thiol (**22**) were investigated.

Only eight of these eighteen substrates were accepted by the enzyme under the defined assay conditions. In Table 2 eight substrates are listed with name, structure, the solvent used in the assay and the per cent conversion to the corresponding product measured by HPLC and GC-FID, respectively.

Table 2: List of accepted substrates for methyl transfer catalysed by *Bo*tmt.

substrate	structure	org. solvent	conversion product HPLC	conversion product GC-FID
p-methoxythiophenol (**17**)		25 % *t*BME	26%	28%
4-mercaptophenol (**6**)		25 % *t*BME	19%	22%
2-chlorothiophenol (**4**)		25 % *t*BME	50%	49%
3-chlorobenzenethiol (**1**)		25 % *t*BME	69%	68%
4-chlorothiophenol (**5**)		25 % *t*BME	27%	32%
4-methylbenzenethiol (**19**)		25 % *t*BME a	14%	5%
thiophenol (**14**)		25 % *t*BME	29%	b
mercaptobenzothiazole (**21**)		25 % *t*BME	7%	b

a: assay temperature reduced to 25 °C
b: compounds inapplicable for GC analysis

In Figure 12 the results are represented as bar diagram. 3-chlorobenzenthiol (**1**) showed the highest conversion with around 70 % and mercaptobenzothiazole (**21**) the lowest with only 7 %.

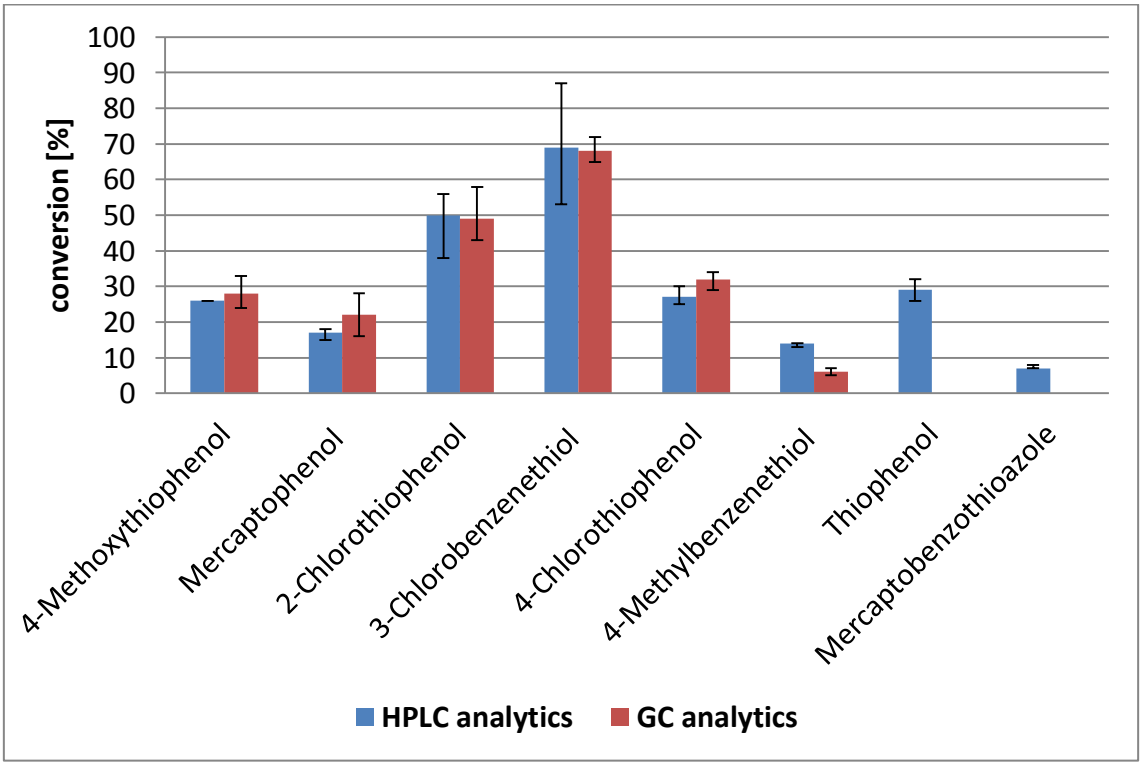


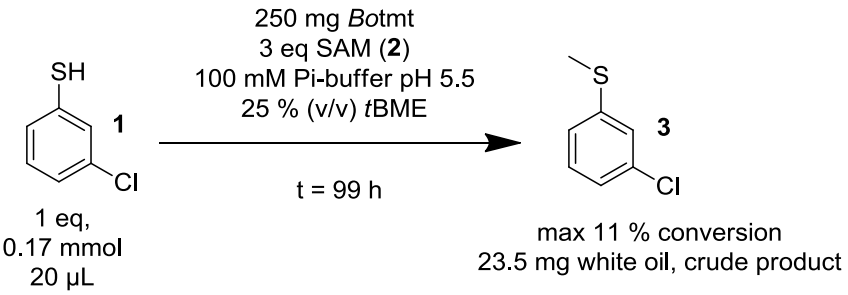
Figure 12: Results of the substrate assay: eight molecules were accepted by the enzyme *Bo*tmt and the conversion to the corresponding product is shown in per cent. The assays were analysed by two different methods, HPLC analytics in blue and GC analytics in red.

One aim of this substrate screening was not only to determine the substrate scope of the methyltransferase *Bo*tmt but also to find the best substrate for further analysis. Hence 3-chlorobenzenthiol (**1**) proved to be the substrate of choice for further investigations.

4.3. Upscaling with lyophilised *Bo*tmt cells

4.3.1. First attempts

In order to perform the reaction in preparative scale to isolate the product for characterisation, two different attempts were made. In the first attempt all used amounts and volumes were enhanced. 250 mg of lyophilised *Bo*tmt cells were added to 25 mL of 100 mM sodium phosphate buffer pH 5.5, emulsified and rehydrated in an Erlenmeyer flask. Subsequently 7.25 mL *t*BME was added. 0.27 g (3 eq, 0.52 mmol) *S*-(5'-adenosyl)-L-methionine chloride hydrochloride (SAM, **2**) and 20 µL (1 eq, 0.17 mmol) 3-chlorobenzenethiol (**1**) were added to the mixture. The Erlenmeyer flask was shaken in a rotary shaker at 25 °C and 120 rpm (Scheme 8). The reaction progress was controlled by GC-FID.



Scheme 8: Reaction conditions of the first attempt of an upscale reaction. 23.5 mg of crude product were isolated but not further purified.

After 21.5 hours 0.05 g SAM (**2**), 6.2 mL *t*BME, 50 mg of lyophilised *Bo*tmt cells and 12 mL of 100 mM sodium phosphate buffer pH 5.5 were added to the reaction mixture to push the reaction towards completion. The reaction was stopped after 99 hours with the addition of 5 mL *t*BME and 1.5 mL 0.33 M HCl. The reaction mixture was centrifuged for 10 min at 10000 rpm and 5 °C. The phases were separated and the organic solvent was removed by evaporation. The crude product was 23.5 mg (87 %) of colourless oil. It was a mixture of the substrate 3-chlorobenzenethiol (**1**), enzyme compartments and low amount (under 10 %) of product 3-chlorothioanisole (**3**) as indicated by GC-FID. Further purification of the product was not performed because of the low conversion.

Several problems were identified in upscaling. Erlenmeyer flask is not ideal for this reaction, as too much oxygen can enter the reaction mixture and lead to negative effects on the oxygen-sensitive substrate **1**. Additionally *t*BME, substrate **1** and product **3** are volatile

compounds and may evaporate during the long incubation time. The reaction is very slow and even after adding further enzyme, solvent and cofactor **2** the reaction was not pushed towards completion. The calculated conversion was maximum 11 %.

The next attempt was performed like an analytical setup approach in 24 Eppendorf tubes. Two of them were analysed by GC-FID as control. In the assays 2 mM SAM (**2**), 10 mg lyophilised *Bo*tmt cells per mmol substrate, 1 mM substrate 3-chlorobenzenethiol (**1**), 25 % (v/v) *t*BME and 100 mM sodium phosphate buffer pH 5.5 was used. The total volume of each tube was 1 mL. The 24 tubes were shaken in a thermo mixer at 1000 rpm and 30 °C for 30 minutes. The enzymatic reactions were quenched by addition of 0.33 M HCl and centrifuged for 5 minutes and 13000 rpm. The *t*BME phases were separated from the reaction mixture and subsequently combined. All aqueous phases were extracted twice with 500 µL *t*BME each and added to the organic phase which was dried over anhydrous $MgSO_4$, filtered, the drying reagent washed with additional 15 mL *t*BME and the solvent was evaporated. The crude product was 6.6 mg of a white-grey solid. The GC-FID samples showed a conversion rate of about 77 % which was expected from analytical studies. This attempt was successful but the amount of the product obtained was not enough for further analysis and the obtained product 3-chlorothioanisole (**3**) was not pure.

On the one hand it was impossible to just elevate all amounts of used reagents; on the other hand with a lot of small analytical setups, the amount of product was too low to perform NMR analysis. The setup of both reactions was not optimal for the purpose of an upscaling reaction. Further optimisation of the reaction conditions was necessary.

4.3.2. pH - screening of 3-chlorobenzenethiol (1)

In order to optimise the reaction conditions and to investigate the influence of different pH values on the conversion rates of 3-chlorobenzenethiol (**1**), a pH screening was performed. *Bo*tmt lyophilised cells were proportionated, emulsified and rehydrated in 100 mM sodium phosphate buffer with the corresponding pH value from 3.5 up to 8.5 for 30 minutes at 1000 rpm. To ensure that the pH of the solution did not influence side reactions or non-catalysed reactions, active enzyme, denatured enzyme and buffer as negative control have been used in three parallel experiments. The mean value of the conversion rate was determined as shown in Figure 13.

The enzyme is very stable over a huge pH range and very active in basic conditions. The enzyme shows no activity under acidic conditions. With buffer and denatured enzyme, no conversion could be detected. With the native enzyme, the conversion rises with increasing pH values. The highest conversion could be achieved with pH values between 6.5 and 8.5.

This effect might be due to deprotonation of the substrate **1** at higher pH and leads to faster reaction. To confirm this theory, more substrates have to be tested over a pH range and their pKa values have to be compared with the pH values at the highest conversion. The effect of the pH of the buffer on enzyme activity and reaction velocity would be an important aspect of this enzyme to be analysed in more detail.

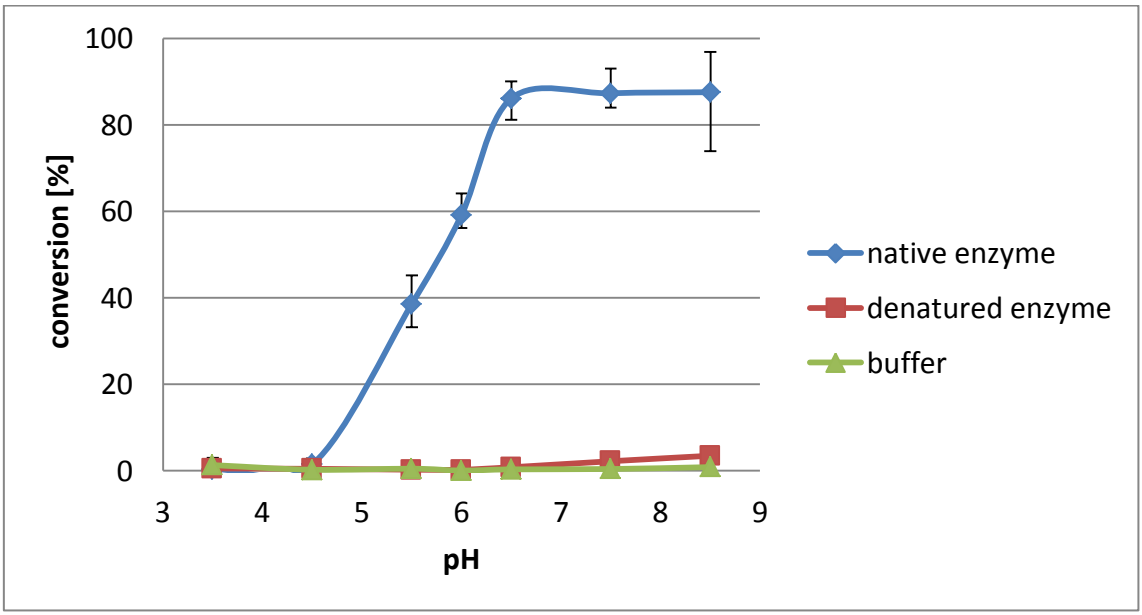


Figure 13: Results of the pH screening. Native enzyme is marked in blue, denatured enzyme in red and buffer in green as negative control.

Another highly interesting aspect would be the reaction in basic conditions. With sodium phosphate buffer, it was not possible to go to higher pH values, so the buffer system would have to be changed. This triggers more unknown effects. A buffer screening was done previously[31] and showed that different buffer systems had a positive or negative effect on the enzyme activity. Sodium phosphate buffer revealed the highest conversions followed by Tris buffer.

This pH screening showed that reactions can be performed at higher pH values to enhance the conversion rate. For an upscale reaction, it was possible to use a 100 mM sodium phosphate buffer pH 7.5.

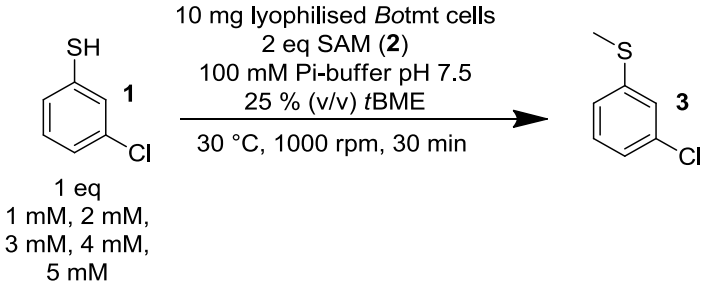
4.3.3. Substrate assay in basic conditions

The pH screening revealed that basic conditions can push the reaction to higher conversion rates. This effect is probably due to the deprotonation of the substrate and therefore faster reaction times, as mentioned before. The following experiment was an attempt to add the substrate 3-chlorobenzenethiol (**1**) as deprotonated thiolate to the reaction mixture. The substrate **1** was dissolved in a basic solution before adding to the reaction mixture. With this procedure, the substrate would be in the deprotonated form.

The substrate stock solution was prepared in a different manner. 2 µL of 3-chlorobenzenethiol (**1**) were diluted in 25 mM NaOH solution and added to the reaction mixture instead of a substrate stock in *t*BME. The buffer capacity of the used 100 mM sodium phosphate buffer pH 5.5 was very high and the pH remained around 5.6 after substrate addition. The positive effect of adding deprotonated substrate **1** was not achieved. After addition of the deprotonated form it was protonated again by the buffer environment. After 30 minutes approximately 66 % of the substrate **1** were converted to the corresponding product **3**, after one hour 69 %. Already the substrate scope analysis showed conversions around 69 % with substrate **1**, so no improvement could be achieved.

4.3.4. Molarity analysis

The molarity analysis was an essential step in optimising the reaction conditions in terms of upscaling.



Scheme 9: Reaction conditions of the molarity assay. The substrate 3-chlorobenzenethiol (1) was applied in five different concentrations. The cofactor SAM (2) was elevated according to the substrate.

The amount of lyophilised *Bo*tmt cells was constant (10 mg per mL of total assay volume). The concentration of the substrate 3-chlorobenzenethiol (**1**) was increased from 1 mM up to 5 mM and the cofactor SAM (**2**) was adjusted in a substrate to cofactor ratio of 1:2, represented in Scheme 9. The results of this screening are shown in the Figure 14.

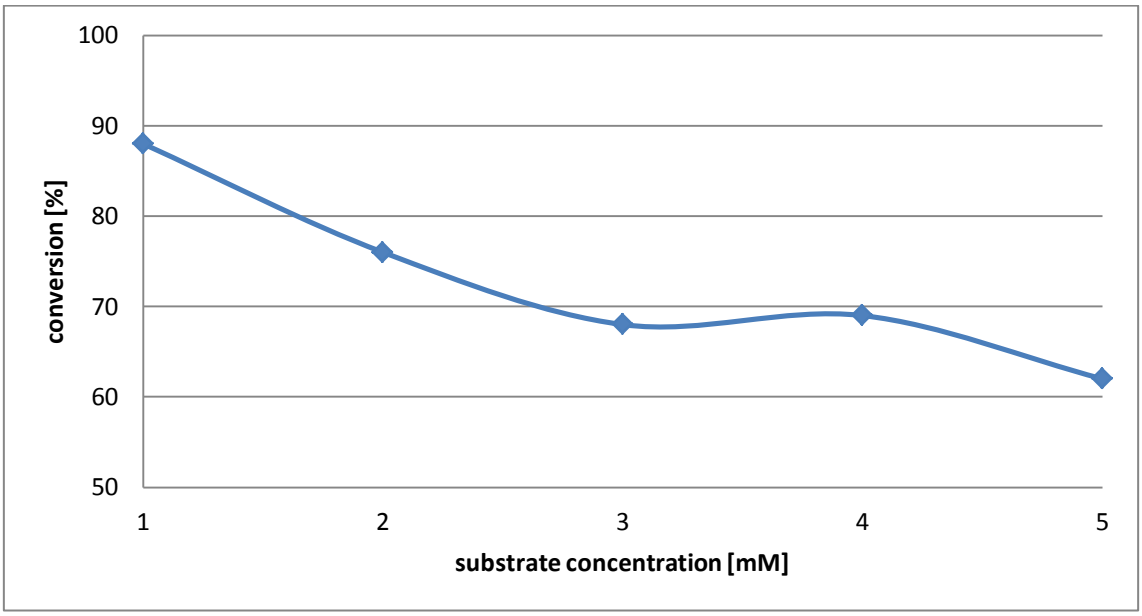


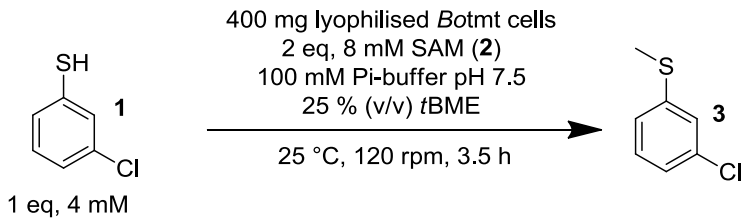
Figure 14: Results of the molarity assay. The conversion of substrate 1 to the product 3-chlorothioanisole (3) are given in per cent and decline with the concentration of substrate [mM] applied in the reaction.

1 mM substrate **1** showed the highest conversion to the corresponding product 3-chlorothioanisole (**3**), while 5 mM showed the lowest with 62 %. The trend of this screening was clearly visible; with higher substrate concentrations the conversion was decreased

probably due to substrate or enzyme inhibition. With around 4 mM substrate concentration, the conversion still amounts to 69 %. It was possible to use this concentration in upscaling without losing too much enzyme activity and enhancing yield.

4.3.5. Upscaling and purification of 3-chlorothioanisole (3)

After optimising the reaction conditions as described in the previous studies, upscaling was performed. Scheme 10 shows the conditions for this transformation.



Scheme 10: Conditions for upscaling with 4 mM 3-chlorobenzenethiol (1) and 400 mg lyophilised *Bo*tmt cells.

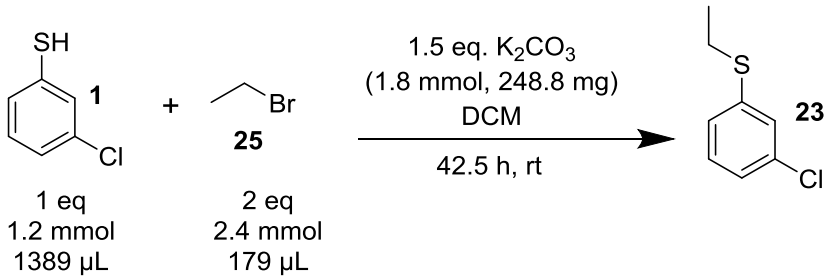
4 mM of the substrate 3-chlorobenzenethiol (**1**) was used in this mixture as determined in the molarity assay. The pH value of the buffer was elevated to 7.5 as investigated in the pH screening. The total volume of the reaction was 40 mL in a 50 mL closed tube. It was carried out in a rotary shaker at 25 °C and 120 rpm. Full conversion could be achieved after 3.5 hours. GC and HPLC analysis showed the product **3** and few undefined impurities.

Few problems appeared in work-up. The product 3-chlorothioanisole (**3**) has a vapour pressure of 0.253 mbar at 25 °C (according to the programme Scifinder determined vapour pressure). The drying of the compound was not possible in high-vacuum or by lyophilisation. For all extraction steps highly volatile solvents had been used. Some of the undefined impurities might be parts of cell materials which are not easy to separate. Due to many purification steps the product was partially lost. After a silica gel filtration and a flash chromatography 18.3 mg (72 %) of a white solid could be obtained. This was dissolved in 1 mL of methanol and purified by preparative reversed phase HPLC. The product was extracted with n-pentane from the HPLC eluent water/methanol mixture. After removing the solvent by evaporation the yield was 16.7 mg (66 %) of a white solid. The product was characterised by NMR. The upscaling was successful and enough product could be obtained for NMR analysis.

4.4. Synthesis of (3-chlorophenyl)(ethyl)sulfane (23)

(3-chlorophenyl)(ethyl)sulfane (**23**) was needed as reference compound for enzymatic reactions with the artificial cofactor ethyl-SAH (**24**). The prescription of Porto *et al.* was used for the synthesis.[32]

The synthesis of (3-chlorophenyl)(ethyl)sulfane (**23**) was performed as shown in the Scheme 11. After purification by flash chromatography, 87.4 mg colourless oil could be obtained and used as reference compound.



Scheme 11: Synthesis of (3chlorophenyl)(ethyl)sulfane (23). To 1 eq of 3-chlorobenzenethiol (1) and 1.5 eq of potassium carbonate in DCM, 2 eq of ethyl bromide (25) were added and stirred at room temperature for 42.5 hours.

Porto *et al.* mentioned no time indication. The reaction took 42.5 hours. The only difference to the prescription was the addition of triethyl amine to the reaction mixture by this research group.[32]

This was the first attempt to synthesise this reference compound and enough product could be obtained as reference material. Hence no further reaction optimisation was made.

4.5. Synthesis of artificial cofactors

In general, there are two possible ways of gaining artificial cofactors. Either the molecule is formed via enzymatic reaction with SAM synthetase or it is synthesised chemically.[14] The advantage of the enzymatic formation of the cofactor is the higher diastereoselectivity, while the chemical reactions show no or poor diastereoselectivity.[17]

Already in 1958, Parks has replaced the methyl group in SAM by ethyl via enzymatic synthesis using ethionine-enriched growth medium for *Torulopsis utilis*.[21] In 1975, Schlenk and Dainko were able to produce the propyl analogue of SAM (**2**) in a similar approach, although only low amounts of product and very small transfer rates could be observed. Bigger or longer moieties were not tolerated by SAM synthetase.[22]

Dalhoff *et al.* published a protocol for the chemical synthesis of ethyl, propyl, propenyl and butynyl analogues of *S*-adenosyl-L-methionine (**2**). These reactions were performed under acidic conditions (1:1 mixture of formic acid and acetic acid), where no protection groups for *S*-adenosyl-L-homocysteine (SAH, **8**) are needed because of protonation of nucleophilic functionalities in SAH. The substrate **8** reacts either with alkyl triflate or allyl bromide to the corresponding analogue. The products were obtained in a nearly 1:1 diastereomeric mixture at the sulfonium centre and separated by preparative reversed phase HPLC.[7,24]

Stecher *et al.* alkylated SAH (**8**) with different alkyl bromides under acidic conditions resulting in diastereomeric mixture of the product as well. The research group was able to use them as crude products for the enzymes NovO and CouO to perform biocatalytic Friedel-Crafts alkylations.[26]

All research groups faced the problem of rapid degradation of the artificial cofactors. In order to improve stability a large and non-nucleophilic counter ion such as tosylate or sulphate can be applied.[16]

In this work, the focus lies on the synthesis of ethyl-SAH (**24**). Not many groups have synthesised this molecule and were more interested in the activated rather than the deactivated SAM analogues.

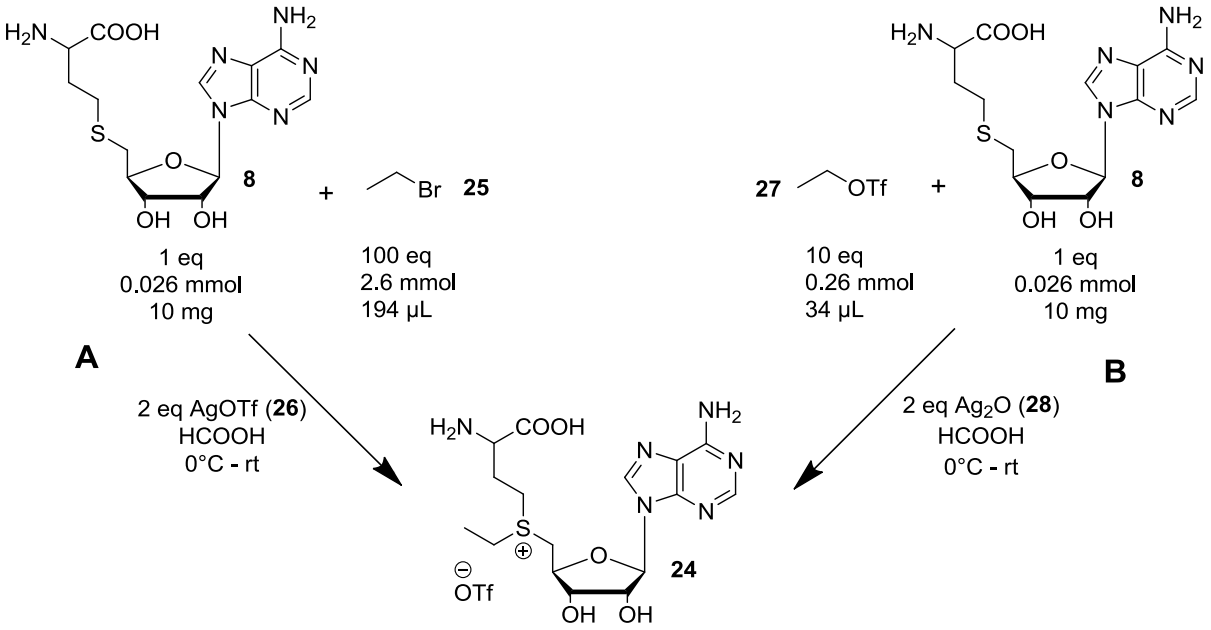
4.5.1. Screening of reaction conditions for ethyl-SAH (24) synthesis

Two different synthesis routes were applied and compared with each other. In one reaction ethyl bromide (**25**) and silver trifluoromethane sulfonate (**26**) were used, in the other reaction ethyl trifluoromethane sulfonate (**27**) and silver oxide (**28**) were applied as shown in Scheme 12. In both cases the reaction was performed under inert atmosphere. The flasks were dried in vacuum and flushed with nitrogen. All chemicals were added under nitrogen flow.

Condition A: 10 mg (0.026 mmol, 1 eq) *S*-adenosyl-L-homocysteine (**8**) were diluted in 5 mL formic acid. 13.36 mg (0.052 mmol, 2 eq) silver trifluoromethane sulfonate (**26**) were added. The flask was cooled in an ice bath and then 200 µL (2.6 mmol, 100 eq) ethyl bromide (**25**) were added and the reaction was stirred at room temperature for 68 hours.

Condition B: 10 mg (0.026 mmol, 1 eq) *S*-adenosyl-L-homocysteine (**8**) was diluted in 5 mL formic acid. 12.05 mg (0.052 mmol, 2 eq) silver oxide (**28**) was added. The flask was cooled in an ice bath and 34 µL (0.26 mmol, 10 eq) ethyl trifluoromethane sulfonate (**27**) were added and the reaction was stirred at room temperature for 68 hours.

The reaction progress was controlled by HPLC-MS after 2, 4, 18, 24, 48 and 68 hours before it was stopped. The peak areas for adenine (**29**) (common degradation product), the substrate SAH (**8**) and the product ethyl-SAH (**24**) were compared with each other.



Scheme 12: Screening of reaction conditions for ethyl-SAH (24) synthesis. Two routes, A and B, were compared with each other. Either 100 eq ethyl bromide (25) and 2 eq silver trifluoromethane sulfonate (26) [A] or 10 eq ethyl trifluoromethane sulfonate (27) and 2 eq silver oxide (28) [B] were added to 1 eq of SAH (8) in the reactions.

With route A the maximum conversion to the product ethyl-SAH (**24**) was 31 % after 48 hours. After 24 hours, already 30 % conversion were achieved and only the decomposition compound adenine (**29**) was elevated as listed below in Table 3.

Table 3: Results of Route A. The reaction was analysed at defined time points [h] and the measured peaks were compared with each other and are given in per cent.

time [h]	adenine (29) [%]	SAH (8) [%]	ethyl-SAH (24) [%]	recovery [%]
2		82	3	85
4	2	84	6	92
18	15	51	24	90
24	22	37	30	89
48	39	13	31	83
68	50	9	25	84

With route B, the maximum yield of ethyl-SAH (**24**) was 23 % after 24 hours. The conversion to the product was faster at the beginning but stopped after 24 hours and decreased again. A much higher percentage of adenine (**29**) was formed as shown in Table 4.

Table 4: Results of Route B. The reaction was analysed at defined time points [h] and the measured peaks were compared with each other and are given in per cent.

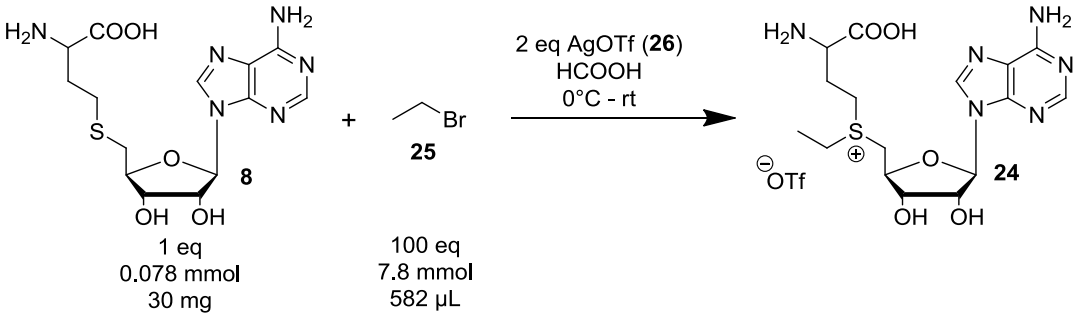
time [h]	adenine (29) [%]	SAH (8) [%]	ethyl-SAH (24) [%]	recovery [%]
2	5	78	8	91
4	15	57	18	90
18	*	*	*	
24	54	7	23	84
48	69	3	12	84
68	76	2	8	86

This screening showed that none of the routes gave full conversion and degradation of the newly synthesised cofactor already starts after short time. Therefore, the reaction has to be stopped after 24 hours. For further investigations, route A was applied.

4.5.2. Synthesis of ethyl-SAH (24)

Stecher *et al.* synthesised artificial cofactors according to route A as mentioned before. They were able to attach methyl (natural cofactor SAM), allyl, butenyl, propinyl, butinyl and phenyl groups to SAH (**8**) with this reaction system. Silver trifluoromethane sulfonate (**26**) served as stabilising counter ion (OTf^-) and as catalyst activator pushing the reaction towards product side due to precipitation of silver bromide.[16,26]

The artificial cofactor ethyl-SAH (**24**) had to be synthesised freshly prior to use. The cofactor is not stable in the freezer and decomposes fast. Scheme 13 shows the reaction conditions for the synthesis.



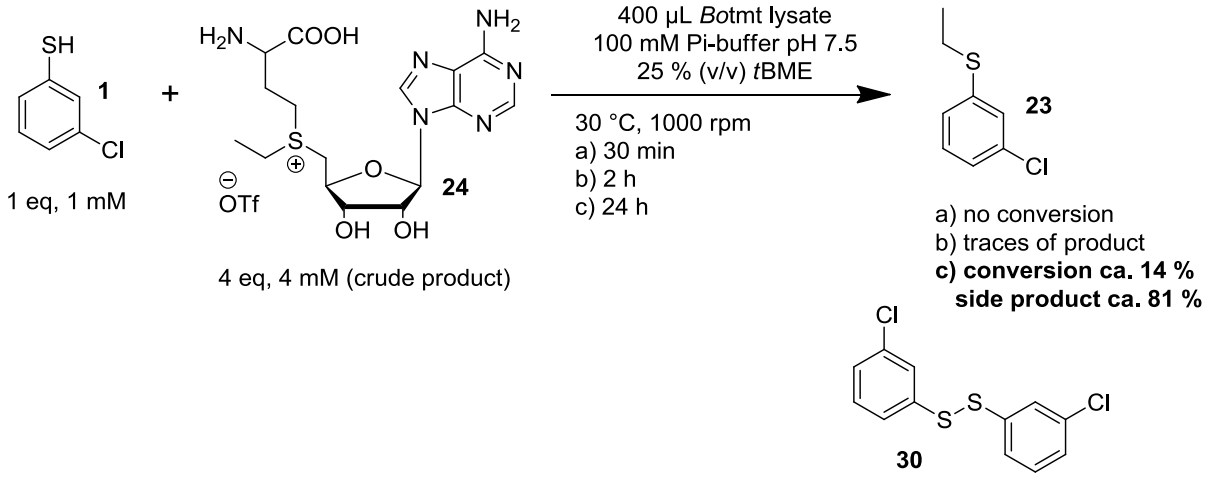
Scheme 13: Reaction scheme of the synthesis of ethyl-SAH (24) according to Stecher *et al*.

The reaction was quenched after 24 hours. Approximately 28 % product **24** was built, 31 % educt **8** was left and 31 % adenine (**29**) was formed. The product **24** was subjected to purification by preparative HPLC. 3.7 mg (11 %) ethyl-SAH (**24**) was isolated and 3.3 mg (11 %) SAH (**8**) could be regained.

4.5.3. Assays with ethyl-SAH (24)

In 1958 Parks was able to obtain the cofactor analogue *S*-adenosyl-ethionine via enzymatic synthesis, as mentioned before. He tested this artificial cofactor for transfer reactions.[21] Singh *et al.* screened different SAM synthetases in various organisms to produce SAM analogues and subsequently methylate complex natural products. This research group was successful to settle a one pot system where the SAM analogue is formed by SAM synthetase and then this newly formed cofactor subsequently transfers its alkyl groups to the substrate molecule. SAH hydrolase was added to the reaction mixture to prevent the inhibition by SAH. One major advantage of this method is the possibility to reduce the rapid degradation of SAM analogues in the cascade setup.[28] With this knowledge in mind, different assays were developed to test the newly synthesised cofactor ethyl-SAH (**24**) with the enzyme *Bo*tmt.

In the first attempt, 1 mM 3-chlorobenzenethiol (**1**), 4 mM crude ethyl-SAH (**24**), 400 µL *Bo*tmt crude lysate, 100 mM sodium phosphate buffer pH 7.5 and 25 % (v/v) *t*BME were mixed as shown in Scheme 14. The reaction mixture was incubated for 30 minutes, 2 hours and 24 hours.



Scheme 14: Reaction conditions of an assay with ethyl-SAH (24). After 30 minutes no conversion, after 2 hours traces of product 23 and after 24 hours a conversion of approximately 14 % could be detected. In addition 81 % of side product 30 was measured.

In the second attempt, the same reaction conditions were performed again but with purified *SBP_TEV_Bo*tmt in hopes of getting higher yield. 1 mM 3-chlorobenzenethiol (**1**), 400 µL purified *SBP_TEV_Bo*tmt and 25 % (v/v) *t*BME were applied. As cofactor, 56 mg freshly

synthesised crude ethyl-SAH (**24**) dissolved in 1.5 mL 100 mM sodium phosphate buffer pH 7.5 were used. The reaction mixture was shaken at 30 °C and 1000 rpm for 24 hours. The reaction progress was controlled by GC-MS and GC-FID. Again, the prominent side product 1,2-bis(3-chlorophenyl)disulfane (**30**) was formed in high amount. This time, no product **23** could be detected. The results from the first attempt could not be reproduced.

In the third attempt, the reaction conditions were modified as it was done before for the upscaling with lyophilised *Bo*tmt cells and SAM (**2**). The amount of lyophilised cells and substrate **1** could be enough to produce a higher amount of desired product **23** to isolate and analyse it. The reaction was abandoned after around 140 hours. The reaction progress was constantly controlled by GC-MS but no product **23** was detected, only substrate **1** and side product **30**.

In the last attempt, 0.7 mM 3-chlorobenzenethiol (**1**), 1 mg/mL purified *SBP_TEV_Bo*tmt and 25 % (v/v) *t*BME were applied. As cofactor, 3.7 mg freshly synthesised and purified ethyl-SAH (**24**) dissolved in 0.5 mL 100 mM sodium phosphate buffer pH 7.5 were used. In addition, 20 % β- mercaptoethanol (**31**) was used as reducing agent to avoid the dimerisation of the substrate **1** to the side product **30**. The reaction mixture was shaken at 30 °C and 1000 rpm for 24 hours. The prior synthesised reference (3-chlorophenyl)(ethyl)sulfane **23** was dissolved in *t*BME and used for spike experiments with HPLC. One unknown peak was detected but the spiking with the reference compound revealed that it was not the product peak. The side product **30** could not be detected. Hence adding a reducing agent (**31**) helps avoiding dimerisation. Still no product could be detected.

Currently, no successful reaction conditions with ethyl-SAH (**24**) as cofactor could be found. The desired product (3-chlorophenyl)(ethyl)sulfane (**23**) could only be detected once in traces. This reaction could not be reproduced. Further condition optimisation is necessary for further investigations regarding transformations with deactivated cofactors.

4.5.4. 24 hours stability test of ethyl-SAH (24) and SAM (2)

An important aspect concerning cofactors is their stability under assay conditions. *S*-adenosyl-L-methionine (SAM, **2**) is very unstable and decomposes easily. Three possible degradation pathways which are pH dependent are known till today. Acidic pH triggers epimerisation of the molecule, where the active *S*-configuration changes to the biologically inactive *R*-configuration at the sulphur centre. With increasing pH, intramolecular nucleophilic attack of the carboxylate group at Cγ results in 5'-deoxy-5'-methylthioadenosine (MTA) and homoserine lactone (HSL; also known as α-amino-γ-butyrolactone) increases. At neutral pH or higher, decomposition occurs via sulfonium ylide formation. After abstraction of a proton, adenine (**29**) is eliminated.[14]

Ethyl-SAH (**24**) and SAM (**2**) were compared with each other within 24 hours regarding stability. Both were dissolved either in 100 mM sodium phosphate buffer pH 7.5 or 0.005 M H_2SO_4 containing 10 vol % ethanol and stored at room temperature. All four samples were measured by HPLC-MS at defined time points: 0, 0.5, 1, 3, 6 and 24 hours. The peaks are determined at 260 nm.

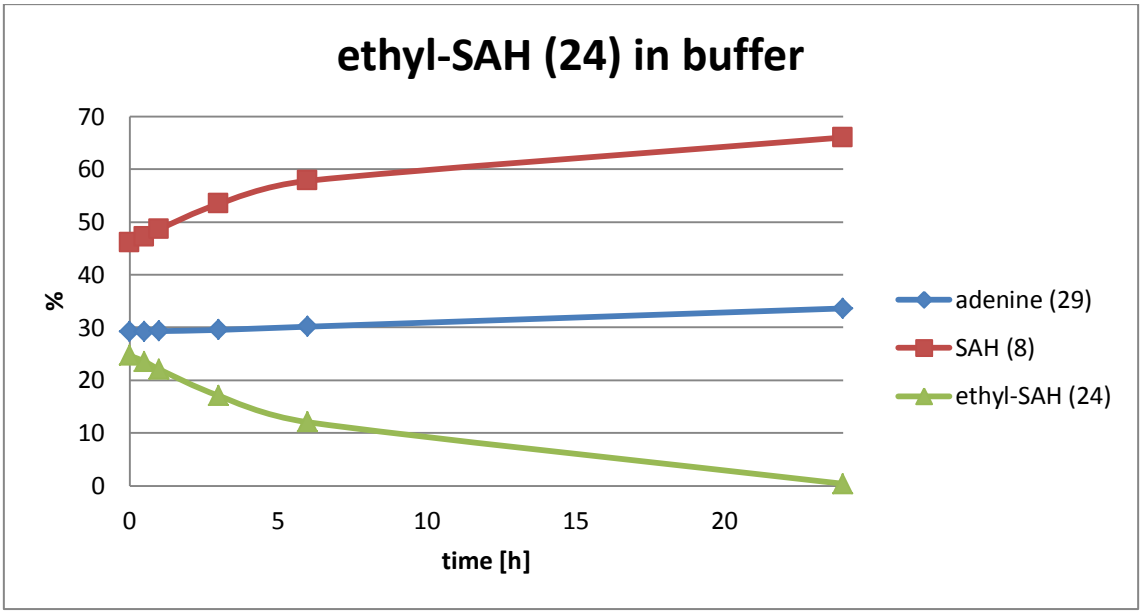


Figure 15: Crude ethyl-SAH (24) dissolved in 100 mM sodium phosphate buffer pH 7.5 and measured by HPLC-MS at defined time points. The areas of the three main peaks, adenine (29), SAH (8) and ethyl-SAH (24), were compared with each other by calculating the relative percentage.

At time point zero the mixture comprises of 25 % ethyl-SAH (**24**), 29 % adenine (**29**) and 46 % SAH (**8**), after 24 hours of 0 % ethyl-SAH (**24**), 34 % adenine (**29**) and 66 % SAH (**8**) which means that ethyl-SAH (**24**) has decomposed almost exclusively to SAH (**8**).

The crude ethyl-SAH (**24**) dissolved in 100 mM sodium phosphate buffer pH 7.5 showed always the same three peaks in the chromatogram: adenine (**29**) at 5.25 minutes, SAH (**8**) at 5.74 minutes and ethyl-SAH (**24**) at 6.19 minutes as shown in Figure 15. Sometimes a peak around 6.7 minutes and a peak around 12.6 minutes appeared but they were small and disappeared again. After 24 hours, no cofactor **24** could be detected any more.

The crude ethyl-SAH (**24**) dissolved in 0.005 M H_2SO_4 containing 10 vol % ethanol revealed similar results, similar retention times and same peak distribution as in the buffer, shown in Figure 16. The latter solvent mixture stabilised the cofactor **24** because after 24 hours ethyl-SAH (**24**) could still be detected and even after 72 hours traces of the cofactor were found in the chromatogram.

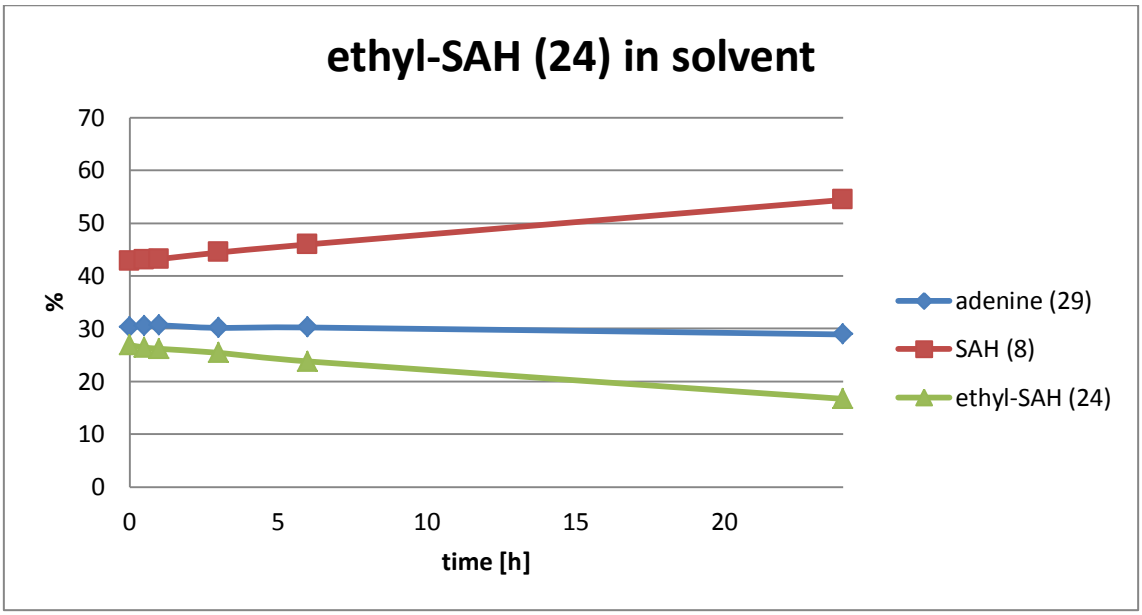


Figure 16: Crude ethyl-SAH (24) dissolved in 0.005 M H_2SO_4 containing 10 vol % ethanol and measured by HPLC-MS at defined time points. The areas of the three main peaks, adenine (29), SAH (8) and ethyl-SAH (24), were compared with each other by calculating the relative percentage.

The commercially available *S*-adenosyl-L-methionine chloride dihydrochloride (**2**) was dissolved either in 100 mM sodium phosphate buffer pH 7.5 or in 0.005 M H_2SO_4 containing 10 vol % ethanol. The retention time of SAM (**2**) was around 4 minutes (3.95 – 4.14 min). Three additional peaks could be found in all chromatograms. These unknown peaks with retention times of 5.89 minutes, 7.52 minutes and 12.86 minutes were probably decomposition compounds, as the areas of the peaks were getting larger as shown in Figure 17.

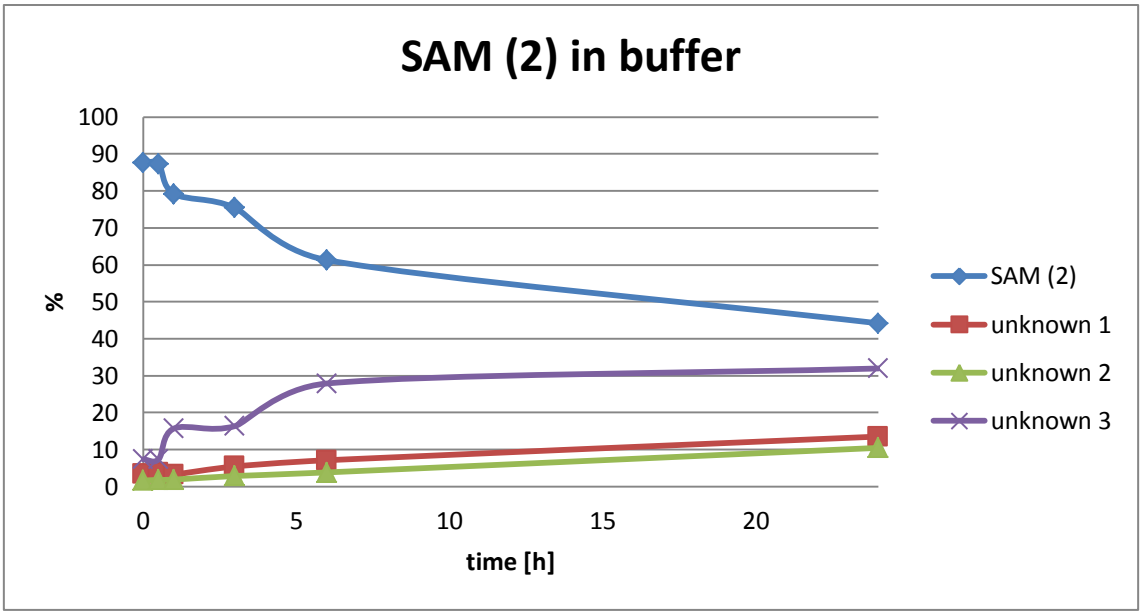


Figure 17: SAM (2) dissolved in 100 mM sodium phosphate buffer pH 7.5 and measured by HPLC-MS at defined time points. The area of the main peak SAM (2) and three unknown peaks were compared with each other by calculating the relative percentage.

S-adenosyl-L-methionine (**2**) was not fully decomposed in 24 hours and even longer. The degradation of SAM (**2**) was more slowly than from ethyl-SAH (**24**). The result of the measurement in acidic solvent after 24 hours was an outlier and is therefore not shown in Figure 18. After 6 hours approximately 20 % of the starting material SAM (**2**) was decomposed.

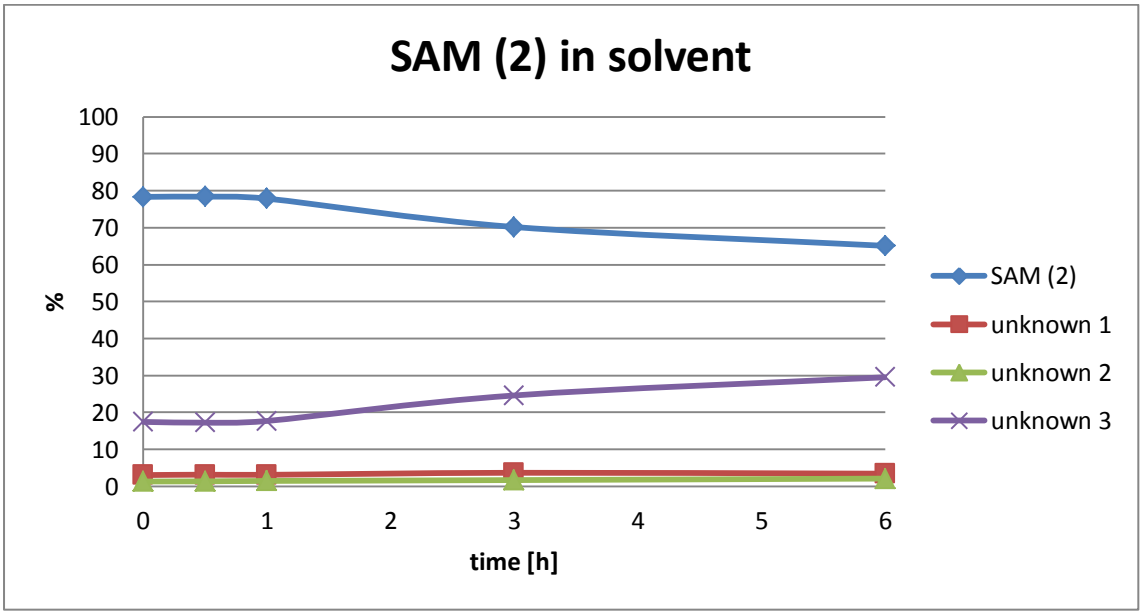


Figure 18: SAM (2) dissolved in 0.005 M H_2SO_4 containing 10 vol % ethanol and measured by HPLC-MS at defined time points. The area of the main peak SAM (2) and three unknown peaks were compared with each other by calculating the relative percentage.

The investigation shows that SAM (**2**) is much more stable than ethyl-SAH (**24**) and the solvent mixture (0.005 M H_2SO_4 containing 10 vol % ethanol) has a stabilising effect, while the decomposition in 100 mM sodium phosphate buffer pH 7.5 occurs much faster.

4.5.5. Inhibition study

For some methyltransferases it is known that SAH inhibits the enzyme activity. The research group of Attieh *et al.* found a methyltransferase in *Brassica oleracea* and postulated that SAH was a competitive inhibitor with respect to SAM.[10] Later surveys showed that there is a competitive inhibition between SAH and SAM and a non-competitive inhibition between SAH and iodide.[11] The natural product sinefungin has a very similar structure to SAM and is a highly competitive inhibitor for many methyltransferases.[14,18] The inhibition constants are even lower than SAH. This molecule has antiviral, antifungal and antiparasitic activities, but also a high *in-vivo* toxicity, which excludes its use in pharmaceutical products.[14,18]

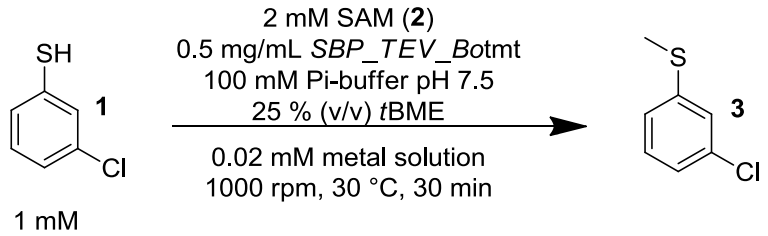
When using the artificial cofactor ethyl-SAH (**24**) without purification, a high amount of SAH (**8**) was introduced to the reaction. This might cause a negative effect and could lead to inhibition of the enzyme activity.

With the general reaction conditions which were developed during this work, an inhibition study was not possible. In our studies the solubility of *S*-adenosyl-L-homocysteine (**8**) posed a problem. SAH (**8**) was only soluble in acid or DMSO. The enzyme *Bo*tmt was not stable in acidic solution. Also dissolving SAH (**8**) in DMSO was not feasible. An inhibition study could not be performed and it remains unclear whether SAH has an inhibitory effect but it is most likely the case.

4.6. Influence of metal ions on enzyme activity

Metal ions can have a great influence on the enzyme activity and can accelerate or inhibit transformations. These effects might be due to stabilisation of the transition state, binding to the enzyme, cofactor or substrate. Many enzymes require metal ions, which can be covalently bound, as Fe, Ni, Cu, Co, V, Zn, Ca, Mg or Mn. On the contrary, highly charged inorganic ions can negatively affect the stability and the activity of enzymes.[33] Hence analysing the influence of metal ions on the enzyme activity was of high interest. The structure of *Bo*tmt and therefore the relevance of metal ions are not known. The assay might give some interesting insight to the reaction mechanism.

In the first metal screening, eight different metal ions were screened: Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Ni^{2+}. All metals were utilised as chloride salt and dissolved in distilled water. In the assay, 0.02 mM metal solution was applied. This corresponds to the enzyme concentration which was also 0.02 mM. The reaction conditions are shown in Scheme 15. The results of this screening were analysed by HPLC and GC and are shown in Figure 19.



Scheme 15: Reaction conditions of the metal assay: To 1 mM 3-chlorobenzenethiol (1) 2 mM SAM (2), 0.5 mg/mL *SBP_TEV_Bo*tmt and 0.02 mM metal solution was added.

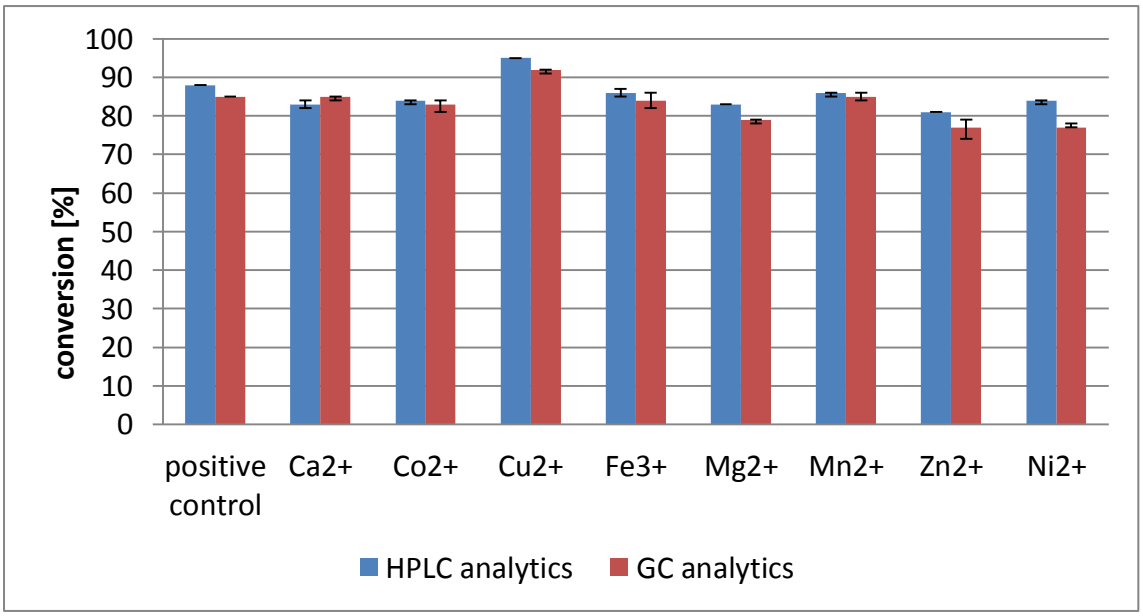


Figure 19: Results of the metal screening. HPLC analysis is marked in blue, GC analysis in red. The conversion is shown in per cent.

None of the eight metal ions had a great influence on the enzyme activity as all show a conversion in the same range as the positive control where no metal was added. Only copper additive seemed to have a slight positive effect and lead to a higher conversion than the others. Copper showed over 90 % conversion while the rest remained under 90 % as listed in Table 5.

Table 5: Results of the metal screening analysed by HPLC and GC analysis and listed in per cent.

	conversion product [%]	
	HPLC	**GC**
positive control	88	85
Ca^{2+}	83	85
Co^{2+}	84	83
Cu^{2+}	95	92
Fe^{3+}	86	84
Mg^{2+}	83	79
Mn^{2+}	86	85
Zn^{2+}	81	77
Ni^{2+}	84	77

Because of the result of the first screening, a concentration dependent copper screening was performed. Copper ions were added with 0.01 mM, 0.02 mM or 0.04 mM concentration to the assay. The enzyme preparation had a concentration of 0.02 mM in the assay. The rest of the reaction conditions were the same as in the previous assay.

Figure 20 shows that with higher concentration of copper additive, the conversion also rises. The negative control (without enzyme in the assay) showed no conversion (not shown). Copper seems to have a positive effect on the enzyme activity and more substrate was converted to the corresponding product in the same time.

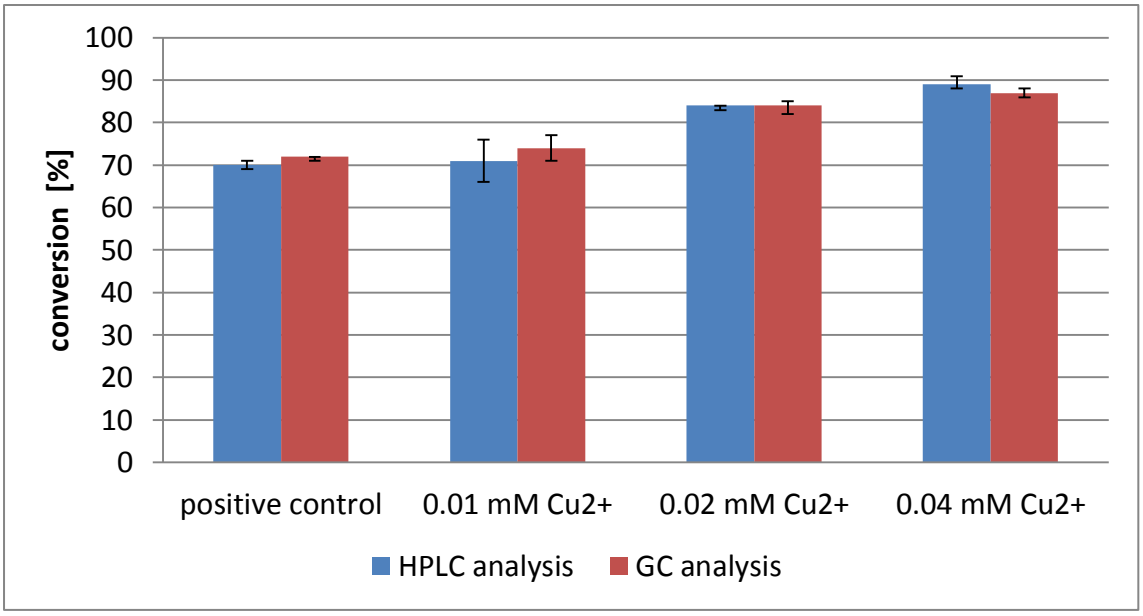


Figure 20: Results of the concentration dependent copper assay. Copper was used in three different concentrations and a positive control with no copper additive was performed. The results are shown in per cent.

The tendency in both screenings is the same. With the addition of an equimolar amount of copper (0.02 mM) approximately 15 % increase of conversion could be detected. But with double the amount of copper (0.04 mM) no essential enhancement of enzyme activity could be achieved.

5. Conclusion and Outlook

The *S*-methyltransferase from *Brassica oleracea* (*Bo*tmt) was cloned in a pET 26b (+) vector and expressed in *E.coli* BL21 Gold (DE3) cells without induction. Four main enzyme preparations were investigated: *Bo*tmt as lyophilised *E.coli* cells, *Bo*tmt as crude lysate, *Strep*-tagged and *SBP_TEV*-tagged *Bo*tmt.

Lyophilised cells were gained through drying by lyophilisation. Disrupting the cells by ultra sonication and separation of insoluble fractions provided the crude lysate. For the two other enzyme preparations, either the *C*-terminal *Strep* tag or the *N*-terminal *SBP_TEV* tag was applied. These modified enzymes were used as crude lysates or purified enzymes. The latter preparation required many purification steps. The *SBP_TEV* tag was cut off and the pure *Bo*tmt enzyme could be obtained as well. Some protein was lost during the purification procedure but all enzyme preparations could be screened for activity. To control each step of the purification, SDS-PAGE analyses were performed. The concentration of the enzyme was determined by in - gel quantification and Bradford Assay for crude lysate preparations or Nano Drop measurements in case of purified enzymes.

All enzyme preparations and batches were compared with each other in an activity assay. Lyophilised cells and the purified *SBP_TEV_Bo*tmt expressed on the 11th of February 2014 showed the highest conversions, the lysate of *Bo*tmt_*Strep* showed the lowest conversion. The tag had no negative influence on the activity, and purification of the enzyme in active form was possible. For the investigation of the influence of metal ions on enzyme activity the purified *SBP_TEV_Bo*tmt (11.2.2014) was applied, for all the other assays lyophilised cells were used, in some cases crude lysate.

The first analysis was the identification of the substrate scope of *Bo*tmt. After testing several reaction conditions, 1 mM of substrate, 2 mM of cofactor SAM (**2**), 100 mM sodium phosphate buffer pH 5.5, 25 % (v/v) *t*BME and 10 mg lyophilised cells per mmol substrate were used in the assay. Eighteen different substrates were screened by HPLC and GC analytics but only eight showed a conversion to methylthioether catalysed by *S*-MTase. With a conversion of 69 %, 3-chlorobenzenethiol (**1**) was considered as best substrate for *Bo*tmt.

Another aim was an upscale of the reaction. The reaction conditions had to be optimised.

The pH screening with the substrate 3-chlorobenzenethiol (**1**) showed that *Bo*tmt was very stable over a broad pH range. In acidic conditions the enzyme was inactivated, but in basic conditions, the conversion rises. 100 mM sodium phosphate buffer pH 7.5 was applied for upscaling.

The molarity assay contributed to the reaction optimisation in terms of upscaling. The substrate 3-chlorobenzenethiol (**1**) was applied in five different concentrations. The higher the substrate concentration in the assay, the lower was the conversion. But with 4 mM substrate **1** still a conversion of 69 % could be detected. This concentration was applied in the upscale reaction.

Upscaling was possible with the optimised reaction conditions. 4 mM of substrate 3-chlorobenzenethiol (**1**) and 8 mM SAM (**2**), in 100 mM sodium phosphate buffer pH 7.5 and 25 % (v/v) *t*BME catalysed by 400 mg lyophilised *Bo*tmt cells at 25 °C and 120 rpm yields the product. After 3.5 hours full conversion was detected. Some difficulties occurred during the work-up and after various purification steps; 16.7 mg (66 %) of a white solid could be obtained. NMR analysis of the product 3-chlorothioanisol (**3**) was possible.

The artificial cofactor ethyl-SAH (**24**) was synthesised according to Stecher *et al*. The screening of two different synthesis routes confirmed this prescription. The product **24** was very unstable, it had to be synthesised freshly for all assays and was difficult to purify. Preparative reversed phase HPLC led to product decomposition.

The reference compound (3-chlorophenyl)(ethyl)sulfane (**23**) was synthesised. Different assay setups for the ethyl transfer from the artificial cofactor ethyl-SAH (**24**) to the substrate 3-chlorobenzenthiol (**1**) catalysed by *Bo*tmt were tested but a conversion to the desired product **23** in traces was detected only once. This reaction could not be reproduced and also attempts to optimise the reaction conditions failed. Only the prominent side product 1,2-bis(3-chlorophenyl)disulfane (**30**) was formed. By adding 20 % β- mercaptoethanol (**31**) as reducing agent the formation of **30** could be suppressed but still no desired product **23** could be detected.

The instability of the artificial cofactor ethyl-SAH (**24**) was a major problem during the purification procedure and analytic assays. A 24 hours stability test of freshly synthesised crude ethyl-SAH (**24**) and the commercially available *S*-adenosyl-*L*-methionine chloride dihydrochloride (**2**) in two different solvent compositions was performed. Either the cofactors were dissolved in 100 mM sodium phosphate buffer pH 7.5 or in 0.005 M H_2SO_4

containing 10 vol % ethanol and analysed by HPLC-MS at defined time points. The latter solution stabilised both cofactors in contrast to buffer solution. The commercial SAM (**2**) preparation (Sigma A7007) probably contained stabilisers as it was stable over 24 hours and longer. The crude ethyl-SAH (**24**) degraded very fast. In buffer, no cofactor was left after 24 hours while in solvent, traces of ethyl-SAH (**24**) could still be detected in the same timeframe.

The purified enzyme *SBP_TEV_Bo*tmt was screened with eight different metal ions to analyse their influence on the enzyme activity. Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Ni^{2+} were applied in the reaction mixture in the same concentration as the enzyme; 0.02 mM. Only copper seemed to have a positive influence on the enzyme activity. A concentration dependent assay with copper metal (0.01 mM, 0.02 mM and 0.04 mM) confirmed the positive effect and conversions increased up to 15 %.

The *S*-methyltransferase *Bo*tmt was investigated in detail. It would be of high interest to compare these results with other *S*-methyltransferases and propose general characteristics of these enzymes. The substrate scope should be analysed further to make a broader application possible. With enzyme engineering, this enzyme could be used for chemo- and regiospecific methylation of various small molecules. One possible application field of *S*-methyltransferases would be in industrial processes and pharmaceutical industry as a biological alternative to harsh reaction conditions of recently used alkylation reactions.

6. Experimental Part

6.1. Chemicals

All chemicals and culture media were purchased from commercial suppliers, such as Sigma Aldrich, Fluka, abcr, Merck, Roth, Alfa Aesar and Acros. They were used without further purification. *S*-adenosyl-L-methionine chloride dihydrochloride was employed as cofactor for all enzyme assays. Deviations are noted otherwise.

6.2. Analytical methods

6.2.1. Thin - layer chromatography (TLC)

Thin - layer chromatography was performed using TLC-plates from Merck (TLC aluminium foil, silica gel 60 F_{254}). For analysing the TLC plates, a UV lamp with λ = 254 nm (fluorescence quenching) and λ = 366 nm (immanent fluorescence of the analytes) next to derivatisation reagents and heating was used.

Immersion reagent: 150 mL H_20, 20 mL H_2SO_4 conc., 125 mL ethanol and 1.5 g vanillin.

6.2.2. High Performance Liquid Chromatography (HPLC)

For HPLC analysis, three different machines were used. The methods are listed below according to the column which was applied. For all substrates and products, the retention time is given in minutes at the corresponding wavelength.

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

The **Agilent Technologies HPLC-System 1100 (Hewlett Packard)** composed of the degasser "G1322A", the quaternary pump "G1311A", the auto sampler "G1313A", the column compartment "G1316A" and the DAD detector "G1315A", was used.

HPLC-MS analysis was performed also on 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.

6.2.3. HPLC methods

Agilent Poroshell C18 ec 100 mm x 3 mm x 2.7 µm

"METHYLBENZENETHIOL": 0.1 % HCOOH/MeOH 35:65, flow: 0.5 mL/min, 30 °C, 10 min run, 2 µL injection, detection at: 200 nm, 242 nm, 254 nm

"MERCAPTOPYRIDIN POS": 0.1 % HCOOH/MeOH 70:30, flow: 0.3 mL/min, 30 °C, 10 min run, 2 µL injection, detection at: 242 nm, 254 nm, 325 nm, ESI-MS, positive mode

"CLPHSH FAMEOH POS" ("CLPHSH FAMEOH"): 0.1 % HCOOH/MeOH 35:65; flow: 0.5 mL/min, 30 °C, 9 min run, 2 µL injection, detection at: 242 nm, 254 nm, ESI-MS, positive mode

"CLPHSET FAMEOH POS": 0.1 % HCOOH/MeOH 35:65; flow: 0.5 mL/min, 30 °C, 15 min run, 2 µL injection, detection at: 242 nm, 254 nm, ESI-MS, positive mode

"CLPHSET FAMEOH POS INJ": 0.1 % HCOOH/MeOH 35:65; flow: 0.5 mL/min, 30 °C, 15 min run, 2 µL injection, detection at: 242 nm, 254 nm, ESI-MS, positive mode, injection program: draw 10 µL sample, draw 0 µL MeOH, draw 5 µL reference, draw 0 µL MeOH, mix in seat, eject 13 µl into waste, inject rest

"OHPHSH FAMEOH POS": 0.1 % HCOOH/MeOH 65:35, flow: 0.5 mL/min, 30 °C, 12 min run, 2 µL injection, detection at: 242 nm, 254 nm, ESI-MS, positive mode

"METHOXYTHIOPHENOL": 0.1 % HCOOH/MeOH 50:50, flow: 0.5 mL/min, 30 °C, 16 min run, 2 µL injection, detection at: 200 nm, 242 nm, 320 nm

"NITROTHIOPHENOL" ("NITRO FAMEOH POS"): 0.1 % HCOOH/MeOH 70:30, flow: 0.5 mL/min, 30 °C, 45 min run, 2 µL injection, detection at: 254 nm, 325 nm

"2MERCAPTOPYRIDIN": 0.1 % HCOOH/MeOH 70:30, flow: 0.3 mL/min, 30 °C, 10 min run, 2 µL injection, detection at: 242 nm, 254 nm

Macherey-Nagel Nucleodur C18 ec 150 mm x 4 mm x 5 µm

"Nucleodur_C18_isokratisch 65 % MeOH_30 °C": 0.01 % HCOOH/MeOH 35:65, flow: 1 mL/min, 30 °C, 15 min run, 2 µL injection, detection at: 242 nm, 254 nm, ESI-MS, positive mode

"MeTYR H2O-MEOH 99-1 nucleodur neu": 0.01 % HCOOH/MeOH, gradient: 0 min at 1 % MeOH, 0 - 5 min to 100 % MeOH, 5 - 11 min at 100 % MeOH, 11 – 12 min to 1 % MeOH, flow: 0.7 mL/min, 40 °C, 12 min run, 2 µL injection, detection at: 254 nm, 260 nm, ESI-MS, positive and negative mode

"MeTYR H2O-MEOH 99-1 nucleodur": 0.01 % HCOOH/MeOH, gradient: 0 min at 1 % MeOH, 0 - 10 min to 100 % MeOH, 10 - 11 min at 100 % MeOH, 11 – 12 min to 1 % MeOH, flow: 0.7 mL/min, 40 °C, 12 min run, 5 µL injection, detection at: 254 nm, 260 nm, ESI-MS, positive and negative mode

Merck LiChroCART® Purospher® STAR RP-18e 250 mm x 4 mm x 5 µm

"ETHYL-SAH" ("SAH TFAACCN"): 0.1 % aqueous TFA/AcCN, gradient: 0 min at 0 % AcCN, 0 - 10 min to 50 % AcCN, 10 - 11 min to 80 % AcCN, 11 – 15 min to 0 % AcCN, flow: 1 mL/min, 25 °C, 25 min run, 5 µL injection, detection at: 210 nm, 254 nm, 260 nm, ESI-MS, positive mode

"ETHYL-SAH-SHORT": 0.1 % aqueous TFA/AcCN, gradient: 0 min at 0 % AcCN, 0 - 10 min to 50 % AcCN, 10 - 11 min to 80 % AcCN, 11 – 15 min to 0 % AcCN, flow: 1 mL/min, 25 °C, 15 min run, 5 µL injection, detection at: 254 nm ,260 nm, ESI-MS, positive mode

"SAM": 0.1 % aqueous TFA/AcCN, gradient: 0 min at 0 % AcCN, 0 - 10 min to 50 % AcCN, 10 - 11 min to 80 % AcCN, 11 – 15 min to 0 % AcCN, flow: 1 mL/min, 25 °C, 15 min run, 5 µL injection, detection at: 254 nm, 260 nm, ESI-MS, positive mode

6.2.4. Preparative High Performance Liquid Chromatography

For preparative HPLC purification, the Thermo Scientific Dionex UltiMate 3000 LC system was used. The device was equipped with the pump HPG-3200BX, the sampler WPS-3000, the column compartment TCC-3000, the detector MWD-3000 and the fraction collector AFC-3000.

Macherey-Nagel Nucleodur C18 ec 125 mm x 21 mm x 5 µm

"EBR_NucleodurC18_001HCOOH_1to95MeOH": 0.01 % HCOOH/MeOH, gradient: 0 min at 1 % MeOH, 0 – 19 min to 95 % MeOH, 19 – 21 min at 95 %, 21 – 22 min to 1 % MeOH, 22 – 24 min at 1 % MeOH, flow: 12 mL/min, 30 °C, 24 min run, detection at: 210 nm, 260 nm, collection according 260 nm

"EBR_NucleodurC18_001HCOOH_iso65MeOH": 0.01 % HCOOH/MeOH 35:65, flow: 20 mL/min, 30 °C, 25 min run, detection at: 242 nm, 254 nm, collection according to 254 nm

6.2.5. Gas chromatography (GC)

For GC analysis, two different machines were used, one with MS- the other with FID-detection. The retention times are given in minutes.

The GC-MS analysis was performed with a **Agilent Technologies GC-System "7890A"** with the injector "7683B". 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 0.25 mm and a coating thickness of 0.25 µm were used. The gas chromatograph was combined with a mass spectrometer. An EI ion source was used for ionisation. The ions were separated by a quadrupole mass analyser and detected by the mass selective detector "5975C". For MS measurements, the fragments are given as m/z values; the basis peaks and the mass peak (M^+) are noted.

"MGK_100_M_100_KURZ": split ratio 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

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 ionisation detector (FID) with hydrogen 5.0 and air as detector gases was used.

"RPHSH_ME 100_SPLITLESS": Agilent HP5 30 m x 0.32 mm x 0.25 µm, splitless injection mode, inlet/injector temperature 250 °C, temperature program 100 °C for 1 min, 40 °C/min to 260 °C, hold for 2 min, detector temperature 250 °C, constant flow 1 mL/min, 6 min run

6.2.6. Nuclear Magnetic Resonance (NMR)

The NMR-spectra were recorded with a Varian Inova-500 (500 MHz) spectrometer or a Bruker AVANCE III spectrometer equipped with an auto sampler (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 certain compounds two - dimensional spectra such as COSY, ^{1}H^{13}C-HSQC and ^{1}H^{13}C-HMBC were obtained in addition.

The chemical shifts are given in δ as ppm (parts per million) and the coupling constants *J* in Hz (Hertz). Following abbreviations are used: s for singlet, d for doublet, t for triplet, q for quadruplet and m for multiplet.[34]

6.2.7. Calculation and analysis

Concentrations of the formed product and substrate are determined by correlation of integrated product- and substrate peaks at the respective wavelength with calibrated data of the reference compounds. Concentrations are determined by plotting peak areas [mAU/s] (y) against concentration [mM] (x) and calculation of the slope within the linear area. The measured peak areas are corrected with the slope of the corresponding calibration curve at the corresponding wavelength and divided by four to calculate the concentration of the formed product. (25 % of the total assay volume consisted of the water immiscible organic solvent *t*BME which was analysed. It was assumed that the total amount of substrate and product are dissolved in the organic solvent layer.) For the ethanol assay, this last step was not necessary. The conversion is given in per cent.

Either double or triple determination of the assays was performed. In addition, blank reactions without enzyme were always performed to exclude non-enzymatic catalysis (negative control). Positive controls were performed in the investigations regarding the metal ion influence on the enzyme activity.

The deviations of the results are given as bars representing the maximum and minimum per cent conversion of the double or threefold determination.

6.3. Experimental procedures

6.3.1. Heterologous expression of *Brassica oleracea*

6.3.1.1. Preparation of lyophilised cells

E. coli BL21 Gold (DE3) cells expressing the thiol-methyltransferase from *Brassica oleracea* (cabbage) cloned in pET 26b (+) vector is referred to *Bo*tmt cells through the work. The plasmids, *E.coli* strains, medium and antibiotics were kindly provided by Kerstin Steiner (ACIB, Graz).

The cells were grown on agar medium. For the overnight culture (ONC), three times 30 mL of 2xTY medium, for the pre culture five times 100 mL 2xTY medium and for the main culture eight times 500 mL 2xTY medium were prepared. Therefore, 155 g of 2xTY (tryptone 16 g/L, yeast extract 10 g/L and NaCl 5 g/L) concentrate were dissolved in 5 L of distilled water and mixed. The medium was sterilised by autoclaving.

30 µL of kanamycin antibiotic and a single colony from the agar plate but no isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to autoclaved 2xTY medium (ONC). The medium was shaken overnight at 130 rpm and 37 °C. One flask with autoclaved 2xTY medium was used for a sterility check.

For the measurement of the optical density (OD_{600}) a 1:10 dilution of the ONC was used. For the second pre culture, an OD_{600} of 0.1 was needed. The pre-cultures containing 40 mg/L kanamycin were inoculated to an OD_{600} of 0.1 with the overnight cultures. The flasks were shaken at 150 rpm and 30 °C for three hours. The growing of the culture was controlled by measuring the OD_{600}. The main cultures containing 40 mg/L kanamycin were inoculated to an OD_{600} of 0.1 with the pre cultures and grown at 150 rpm and 30 °C. When the OD_{600} reached 0.8 – 1.0, the temperature was decreased to 25 °C.

After 20 hours, the medium was centrifuged at 4500 g and 4 °C for 10 minutes. The pellets were washed with 30 mL of 100 mM sodium phosphate buffer pH 7.5 each and subsequently centrifuged. 3.61 g of the wet pellet were disrupted by ultra sonication for SDS – PAGE analysis. The rest was resuspended in approximately 50 mL of 100 mM sodium phosphate buffer pH 7.5, transferred into a round bottom flask, frozen in liquid nitrogen and lyophilised overnight. The dry weight of the lyophilised cells was 6.35 g. The lyophilised cells were stored in the freezer.

6.3.1.2. Preparation of crude lysate

For the preparation of the crude lysate, the cell pellet was resuspended in 100 mM sodium phosphate buffer pH 7.5 in five times the amount of the weight and disrupted by ultra sonication for 6 minutes. The samples were then centrifuged for one hour at 20000 rpm and 4 °C. The supernatant was filtered through a 0.45 µm and a 0.2 µm filter, aliquoted and stored in the freezer.

6.3.1.3. Preparation of purified *SBP_TEV_Bo*tmt

The thiol-methyltransferase *Bo*tmt was cloned with the *N*-terminal *SBP_TEV* tag which was kindly provided by Kerstin Steiner (ACIB, Graz). The enzyme was cultivated as described before, but with addition of IPTG.

The obtained pellet (18.5 g) was resuspended in 120 mL 100 mM sodium phosphate buffer pH 7.5 and then disrupted by ultra sonication for 6 minutes. The suspensions were centrifuged for one hour at 50000 rpm at 4 °C. The obtained lysate was filtered through a 0.45 µm and a 0.2 µm filter.

For purification a Strep-Tactin Superflow high capacity column from iba (Göttingen, Cat No. 2-1209-101; Lot No. 1209-0012) with 10 mL bed volume was used. The "(Twin-) *Strep*-tag® Purification Short Protocol" from iba was applied. The described volumes, buffers and steps were followed in the same order. D-desthiobiotin solution (10x buffer E) consisted of 1 M Tris-Cl pH 8.0, 1.5 M NaCl, 10 mM EDTA and 25 mM desthiobiotin. Strep-Tactin regeneration buffer (10x buffer R) was composed of 1 M Tris-Cl pH 8.0, 1.5 M NaCl, 10 mM EDTA and 10 mM hydroxyl-azophenol-benzoic acid (HABA). Strep-Tactin washing buffer (10x buffer W) was made of 1 M Tris-Cl pH 8.0, 1.5 M NaCl and 10 mM EDTA. From each step one or two samples were taken for the SDS-PAGE analysis for purification control.

The enzyme solution was concentrated with ultrafiltration spin columns from Vivaspin, and then a buffer exchange was done with PD-10 column (GE healthcare, Chalfont St Giles, Buckinghamshire). The concentration of the purified enzyme was measured by Nano Drop. 3.74 mg/mL *SBP_TEV_Bo*tmt in 15 mL 100 mM sodium phosphate buffer pH 7.5 were obtained and stored in the freezer.

6.3.1.4. Preparation of purified *Bo*tmt enzyme

All steps described in the previous section remained the same, but in a smaller scale. The obtained pellet had a weight of 1.89 g. The pellet was disrupted by ultra sonication, filtered and purified by a Strep-Tactin Superflow high capacity column from iba (Göttingen). A Bradford assay[30] determined the concentration of the eluate to be 1.4 mg/mL *SBP_TEV_Bo*tmt.

Following additional steps had to be performed: TEV protease incubation, then removal of TEV-protease by His Tag purification and finally removal of *SBP_TEV* tag by Strep Tag purification. To cut off the *SBP_TEV* tag, 1 mg Tev protease, 0.5 mM EDTA and 1 mM DTT were added to the enzyme and incubated at 4 °C and 20 rpm overnight. The Tev protease was removed by purification over a His column. After this step the enzyme was dissolved in buffer E. Buffer exchange via PD-10 column (GE healthcare, Chalfont St Giles, Buckinghamshire) to buffer W was made. Then the *SBP_TEV* tag was removed by Strep-Tactin column. Finally, a buffer exchange via PD-10 column (GE healthcare, Chalfont St Giles, Buckinghamshire) to 100 mM sodium phosphate buffer pH 7.5 was performed. The purified *Bo*tmt had a concentration of 0.27 mg/mL (Nano Drop measurement).

6.3.1.5. Bradford Assay

A Bradford Assay was performed to quantify the total enzyme concentration according to Bradford.[30] For the pellet samples, small amounts of the pellet were transferred into Eppendorf tubes, weighed and then dissolved in the corresponding amount of 100 mM sodium phosphate buffer pH 7.5 and urea, respectively. All samples were diluted with double distilled water to gain a measurable concentration. For the Bradford assay, the BioRad stock solution was diluted with distilled water in a ratio 1:5. Cuvettes were filled with 950 µL of the BioRad solution and 50 µL of the prepared samples, incubated for 10 minutes and then analysed in a photometer twice.

6.3.1.6. SDS-PAGE

The SDS-PAGE was used to qualify the preparations and the purity of the enzyme. Proteins were separated on NuPAGE 4 - 12 % (w/v) Bis-Tris polyacrylamide gels which are commercially available. A MES buffer was used for electrophoresis. The electrophoresis took 35 minutes at 200 V and 120 mA. The gel was stained with Coomassie solution (0.25 % (w/v) Coomassie Brilliant Blue, 10 % (v/v) acetic acid, 45 % (v/v) ethanol) and destained with destain solution (10 % (v/v) acetic acid, 40 % (v/v) ethanol) and then quantified. STD PageRuler® Prestained Protein Ladder from Thermo Scientific (Waltham, Massachusetts) served as molecular weight standard. For cultivation control and in - gel quantification 10 µL of 4x loading dye and 30 µL of 1 mg/mL samples were mixed and then denatured at 93 °C for 10 minutes. The samples were applied in three different amounts: 3 µg, 4.5 µg and 6 µg.

For purification control, the gel protocol of NuPAGE® was applied. A small amount of the pellet was transferred into Eppendorf tube, weighed and then dissolved in the corresponding amount of urea and then diluted 1:2 with double distilled water. The crude lysate and the flow - through were diluted 1:10; all other samples were applied directly without further dilution. 6.5 µL of the prepared sample, 2.5 µL of loading dye and 1 µL of reducing agent were mixed and then denatured at 95 °C for 10 minutes. 10 µL of the samples and 4 µL of the standard PageRuler® were applied on the gel.

6.3.1.7. Activity assays

To test the activity of lyophilised cells, crude lysate or purified enzyme, an assay with the standard reaction conditions and standard stock solutions was performed. 1 mM 3-chlorobenzenethiol (**1**) was used as standard substrate. Either 10 mg of lyophilised cells per mmol substrate were emulsified in 400 µL 100 mM sodium phosphate buffer pH 7.5 or 400 µL of crude lysate or 0.5 mg/mL purified enzyme were used in the assays. 2 mM SAM (**2**), 100 mM sodium phosphate buffer pH 7.5, 25 % (v/v) *t*BME were applied and the reaction performed at 30 °C and 1000 rpm for 30 minutes. The reaction was stopped by adding 30 µL of 0.33 M HCl, mixed, centrifuged for 5 minutes at 13000 rpm and the upper *t*BME phase was measured by GC-FID (method: “RPHSH_ME 100_SPLITLESS”). The conversion of the substrate 3-chlorobenzenethiol (**1**) to the corresponding product 3-chlorothioanisole (**3**) by different batches and enzyme preparations were compared to each other.

6.3.2. Substrate assay with *Bo*tmt enzyme preparations

Eighteen different substrate molecules were tested with the enzyme *Bo*tmt: 3-chlorobenzenethiol (**1**), 2-chlorothiophenol (**4**), 4-chlorothiophenol (**5**), 4-mercaptophenol (**6**), phenol (**9**), 1,2-dihydroxybenzol (**10**), aniline (**11**), octane-1-thiol (**12**), 2-mercapotethanol (**13**), thiophenol (**14**), benzyl mercaptan (**15**), *p*-nitrothiophenol (**16**), *p*-methoxythiophenol (**17**), 4-mercaptobenzoic acid (**18**), 4-methylbenzenethiol (**19**), 2-mercaptopyridin (**20**), mercaptobenzothiazole (**21**) and 9*H*-purine-6-thiol (**22**). Therefore, a general reaction procedure was developed.

General stock solutions:

- 20 mM cofactor SAM (**2**) stock solution in 100 mM sodium phosphate buffer pH 5.5
- 10 mg dry cells – per mmol substrate – were rehydrated in 400 µL of 100 mM sodium phosphate buffer pH 5.5 for 30 minutes
- for *t*BME: 100 mM substrate stock solution, 10-fold dilution
- for ethanol: 200 mM substrate stock solution, 10-fold dilution
- 100 mM product reference stock solution, 10-fold dilution

General reaction conditions:

- 1 mM substrate
- 2 mM cofactor SAM (**2**)
- 10 mg lyophilised *Bo*tmt cells (400 µL resuspended cells)
- 100 mM sodium phosphate buffer pH 5.5
- 25 % (v/v) *t*BME or 5 % (v/v) ethanol
- 30 °C
- 1000 rpm
- 30 minutes
- threefold reactions and blank

All components – buffer, *t*BME or ethanol, lyophilised *Bo*tmt cell preparation, substrate and cofactor - were mixed in Eppendorf tubes in this order. The reaction tubes were incubated for 30 minutes at 30 °C and 1000 rpm.

There were some small deviations to the general procedure: 4-mercaptophenol (**6**) was tested in ethanol and *t*BME solvent. The assay with *p*-nitrothiophenol (**16**) was performed in 25 % (v/v) *t*BME, also the substrate was dissolved in *t*BME, but the product reference *p*-nitrophenylmethylthioether (**32**) was dissolved in DMSO due to solubility issues. The assay with 4-methylbenzenethiol (**19**) had to be shaken at 25 °C as the product reference methyl *p*-tolyl sulphide (**33**) has a very low boiling point and might vaporise during reaction time. The assay with 2-mercaptopyridin (**20**) had to be performed in 5 % (v/v) ethanol due to solubility reasons. All other substrates were screened with 25 % (v/v) *t*BME in the assay.

The work - up procedure differs depending on the solvent:

For ethanol assays, the reactions were stopped with addition of 25 µL of a 0.33 M HCl solution and shaken thoroughly. The tubes were centrifuged at 13000 rpm for 5 minutes. The solution was filled into fresh tubes with a filter insert and centrifuged through the filter at 13000 rpm for 10 minutes. The solutions were transferred into a glass vial and analysed by HPLC and GC, respectively.

The assay with *t*BME is a two phase solution mixture. The reactions were stopped with addition of 30 µL of 0.33 M HCl and then centrifuged for 5 minutes at 13000 rpm. The upper *t*BME phase was transferred into glass vials and analysed.

Calibration of substrate and product were performed by diluting the stock solutions to 2 mM, 1 mM, 0.5 mM and 0.1 mM and analysed by HPLC and GC, respectively. Few reactions were also analysed by using TLC with an immersion reagent and/or UV detection.

Following HPLC methods were applied: “METHYLBENZENETHIOL”, “MERCAPTOPYRIDIN POS”, “CLPHSH FAMEOH POS”, “OHPHSH FAMEOH POS”, “METHOXYTHIOPHENOL”, “NITROTHIOPHENOL” and “2MERCAPTOPYRIDIN”.

From eighteen substrates, eight showed conversion to methylthioether. The column, method and retention times of these eight substrates and corresponding products are listed in Table 6.

Table 6: List of eight substrates which showed conversion to methylthioether catalysed by the enzyme *Bo*tmt. All were analysed by HPLC with the corresponding method. The retention time of substrates and products are given in minutes.

compound	column	method name	Rt [min]	
			substrate	product
***p*-methoxythiophenol (17)**	Agilent Poroshell	METHOXYTHIOPHENOL	8.32	12.29
4-mercaptophenol (6)	Agilent Poroshell	OHPHSH FAMEOH POS	5.41	8.11
2-chlorothiophenol (4)	Agilent Poroshell	CLPHSH FAMEOH POS	4.10	4.97
3-chlorobenzenethiol (1)	Agilent Poroshell	CLPHSH FAMEOH POS	4.58	6.92
4-chlorothiophenol (5)	Agilent Poroshell	CLPHSH FAMEOH POS	4.38	6.47
4-methylbenzenthiol (19)	Agilent Poroshell	METHYLBENZENETHIOL	4.20	5.64
thiophenol (14)	Agilent Poroshell	CLPHSH FAMEOH POS	2.74	3.60
mercaptobenzothioazole (21)	Agilent Poroshell	METHOXYTHIOPHENOL	3.96	14.97

The substrates 2-chlorothiophenol (**4**), 3-chlorobenzenethiol (**1**), 4-chlorothiophenol (**5**), *p*-methoxythiophenol (**17**), 4-mercaptophenol (**6**), and 4-methylbenzenethiol (**19**) were additionally analysed by GC-FID (method: "RPHSH_ME 100_SPLITLESS") and the retention times are listed in Table 7.

Table 7: List of substrates analysed by GC-FID with the corresponding method and the retention times of substrate and product given in minutes.

compound	method name	Rt [min]	
		substrate	product
***p*-methoxythiophenol (17)**	RPHSH_ME 100_SPLITLESS	3.34	3.65
4-mercaptophenol (6)	RPHSH_ME 100_SPLITLESS	3.57	3.89
2-chlorothiophenol (4)	RPHSH_ME 100_SPLITLESS	3.16	3.56
3-chlorobenzenethiol (1)	RPHSH_ME 100_SPLITLESS	3.16	3.49
4-chlorothiophenol (5)	RPHSH_ME 100_SPLITLESS	3.17	3.49
4-methylbenzenthiol (19)	RPHSH_ME 100_SPLITLESS	2.95	3.25

6.3.3. pH - screening of 3-chlorobenzenethiol (1)

In the assay, 1 mM substrate **1**, 2 mM cofactor SAM (**2**), 10 mg lyophilised *Bo*tmt cell preparations per mmol substrate, 25 % (v/v) *t*BME and 100 mM sodium phosphate buffer with certain pH value were used. 100 mM sodium phosphate buffer solutions with pH of 3.5, 4.5, 5.5, 6.0, 6.5, 7.5 and 8.5 were prepared and used.

The lyophilised *Bo*tmt cells were proportionated, emulsified and rehydrated in the buffer with the corresponding pH value for 30 minutes. Native enzyme, denatured enzyme and pure buffer as negative control have been used in three parallel test tubes. For denaturation 500 µL of the rehydrated *Bo*tmt enzyme preparation with different pH values were denatured at 90 °C and 1000 rpm for 30 minutes. A 40 mM stock solution of the cofactor SAM (**2**) was prepared by dissolving 22 mg SAM (**2**) in 1.1 mL 100 mM sodium phosphate buffer pH 5.5. For the substrate **1** a 100 mM stock solution was prepared by dissolving 12 µL of 3-chlorobenzenethiol (**1**) in 1 mL *t*BME and then diluting 1:10.

The reactions were performed for 30 minutes at 30 °C and 1000 rpm in a thermo mixer. By adding 30 µL of 0.33 M HCl solution to the reaction mixture, the enzymatic transformation was quenched, mixed thoroughly and centrifuged for 5 minutes at 13000 rpm. The organic layer was transferred into glass vials and analysed. The samples were analysed by HPLC (method: “CLPHSH FAMEOH POS”) and by GC-FID (method: “RPHSH_ME 100_SPLITLESS”), respectively. The reaction was performed three times.

6.3.4. Molarity assay

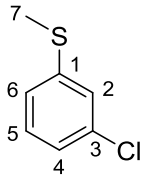
The substrate 3-chlorobenzenethiol (**1**) was used in five different concentrations: 1 mM, 2 mM, 3 mM, 4 mM and 5 mM in the assay. According to the substrate concentration, the cofactor concentration was elevated in a ratio of 1:2. For each reaction mixture the same amount of enzyme, 10 mg of lyophilised *Bo*tmt cells were used.

For the 40 mM cofactor stock solution 22.3 mg of SAM (**2**) was dissolved in 1.1 mL of 100 mM sodium phosphate buffer pH 7.5. For the 100 mM substrate stock solution 6 µL of 3-chlorobenzenethiol (**1**) were dissolved in 500 µL *t*BME. Two different dilutions were made to gain 50 mM and 10 mM stock solutions. Each concentration was tested twice and a negative control was made.

All reactions were performed in 100 mM sodium phosphate buffer pH 7.5 and 25 % (v/v) *t*BME at 30 °C and 1000 rpm for 30 minutes. The reactions were stopped by adding 30 µL of

0.33 M HCl solution, shaking and centrifuging for 5 minutes at 13000 rpm. The organic phase was transferred into glass vials and analysed by GC-FID (method: “RPHSH_ME 100_SPLITLESS”).

6.3.5. Upscaling with lyophilised *Bo*tmt cells and 3-chlorobenzenethiol (1)



400 mg lyophilised *Bo*tmt cells were emulsified and rehydrated in 30 mL of 100 mM sodium phosphate buffer pH 7.5 in a 50 mL Greiner tube at 120 rpm and 25 °C for 30 minutes. 19 µL (0.16 mmol, 1 eq) of 3-chlorobenzenethiol (**1**) were added. Additionally, 10 mL *t*BME (25 % of the total assay volume) were added. Finally, 162.5 mg (0.32 mmol, 2 eq) *S*-(5’-adenosyl)-L-methionine chloride dihydrochloride (SAM) (**2**) was added. The reaction was shaken in a rotary shaker at 120 rpm and 25 °C. The reaction progress was controlled by GC-FID (method: “RPHSH_ME 100_SPLITLESS”) and GC-MS (method: “MGK_100_M_100_KURZ”). After 3.5 hours full conversion was detected. The reaction mixture was centrifuged at 10000 rpm and 5 °C for 10 minutes. The upper organic phase was transferred into a round bottom flask. The aqueous phase was extracted three times with 10 mL *t*BME each and all organic phases were combined in the round bottom flask. The organic phase was analysed by GC-FID (method: “RPHSH_ME 100_SPLITLESS”), GC-MS (method: “MGK_100_M_100_KURZ”) and HPLC (method: “CLPHSH FAMEOH POS”); the aqueous phase was analysed by HPLC (method: “CLPHSH FAMEOH POS”). The organic phase was dried over $MgSO_4$, filtered and the filter cake was washed twice with 10 mL *t*BME each. The combined organic solvent was then removed under reduced pressure while cooling in an ice bath. The crude product was 87.8 mg of a light yellow oil.

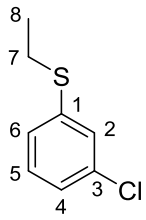
Various purification procedures were applied. Each purification step was analysed by HPLC (method: “CLPHSH FAMEOH POS”, “Nucleodur_C18_isokratisch 65 % MeOH_30 °C”) and NMR analysis in $CDCl_3$ (^1H, APT). The crude product was first dissolved in n-pentane: diethyl ether 100:1 and filtered through a silica column. Then it was purified by a flash chromatography (n-pentane: diethyl ether 100:1). Finally, 18.3 mg (72 %) of a white solid

were dissolved in 1 mL of methanol and purified by preparative HPLC (method: "EBR_NucleodurC18_001HCOOH_iso65MeOH"). The collected fractions were analysed by HPLC-MS (method: "Nucleodur_C18_isokratisch 65 % MeOH_30 °C"). The product **3** was extracted with n-pentane. The water/methanol mixture was washed three times with n-pentane and the phases were separated. The two phases were again analysed by HPLC (method: "Nucleodur_C18_isokratisch 65 % MeOH_30 °C"). The pentane phase was dried over $MgSO_4$ and the solvent was removed by evaporation. The yield was 16.7 mg (66 %) of a white solid. The product 3-chlorothioanisole (**3**) was determined by NMR.

C_7H_7ClS [158.65]

yield:	16.7 mg (66 %) white solid
GC-FID (RPHSH_ME 100_SPLITLESS):	t_R = 3.49 min
GC-MS (MGK_100_M_100_KURZ):	t_R = 3.60 min; m/z = 157.9 (100 % M^+= BP)
HPLC (CLPHSH FAMEOH POS, 254 nm):	t_R = 7.02 min
HPLC (Nucleodur_C18_isokratisch65%MeOH_30°C)	t_R = 11.70 min
^{1}H-NMR (300 MHz, $CDCl_3$):	δ (ppm) = 7.25 - 7.08 (m, 4H, C-2, C-4, C-5, C-6), 2.47 (s, 3H, C-7)
APT-NMR (75.7 MHz, $CDCl_3$):	δ (ppm) = 140.8 (Cq-S, C-1), 134.9 (Cq-Cl, C-3), 129.9 (CH, C-5), 126.0 (CH, C-6), 125.2 (CH, C-2), 124.6 (CH, C-4), 15.7 (CH_3, C-7)

6.3.6. Synthesis of (3-chlorophenyl)(ethyl)sulfane (23)



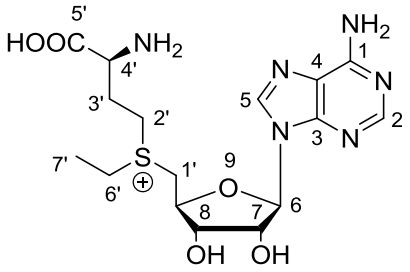
The reaction was performed under inert atmosphere. Therefore, the two-neck round bottom flask was dried in vacuum and flushed with nitrogen. All chemicals were added under nitrogen flow.

248.8 mg (1.8 mmol, 1.5 eq) potassium carbonate was suspended in 3 mL DCM. 139 µL (1.2 mmol, 1 eq) 3-chlorobenzenethiol (**1**) and 179 µL (2.4 mmol, 2 eq) ethyl bromide (**25**) were added and stirred at room temperature. The progress was controlled by GC-MS (method: "MGK_100_M_100_KURZ"). After 42.5 hours, the reaction was stopped due to full conversion. The reaction solution was diluted with 7 mL distilled water. The water phase was extracted three times with 10 mL ethyl acetate each. The combined organic solutions were dried over sodium sulphate and the solvent removed by evaporation. The crude product was purified by flash chromatography (cyclohexane). The product **23** was analysed by GC-MS (method: "MGK_100_M_100_KURZ"), HPLC (method: "CLPHSET FAMEOH POS") and NMR analysis in $CDCl_3$ (^1H). 87.4 mg (43 %) of a colourless oil was obtained.

C_8H_9ClS [172.68]

yield:	87.4 mg (43 %) colourless oil
GC-MS (MGK_100_M_100_KURZ):	t_R = 3.83 min; m/z = 172.0 (100 % M^+ = BP), 157.0 (55 %, 3-chlorothioanisole), 144.0 (76 %, 3-chlorobenzenthiol)
HPLC (CLPHSET FAMEOH POS, 254 nm):	t_R = 11.49 min
^1H-NMR (300 MHz, $CDCl_3$):	δ (ppm) = 7.27 – 7.10 (m, 4H, C-2, C-4, C-5, C-6), 2.95 (q, $^3J_{HH}$ = 7.4 Hz, 2H, C-7), 1.32 (t, $^3J_{HH}$ = 7.4 Hz, 3H, C-8)

6.3.7. Synthesis of ethyl-SAH (24)



The reaction was performed under inert atmosphere. Therefore the flask was dried in vacuum and flushed with nitrogen. All chemicals were added under nitrogen flow.

30 mg (0.078 mmol, 1 eq) *S*-(5'-adenosyl)-L-homocysteine (**8**), short SAH, was solved in 2 mL formic acid. 40 mg (0.156 mmol, 2 eq) silver trifluoromethane sulfonate (**26**) was added. The flask was cooled with an ice bath and 600 µL (7.8 mmol, 100 eq) ethyl bromide (**25**) was added. The reaction mixture was stirred at room temperature and precipitation occurred after half an hour. The reaction progress was controlled by HPLC (method: "Ethyl-SAH", "MeTYR H2O-MEOH 99-1 nucleodur"). The reaction ran for 24 hours in total.

The reaction mixture was diluted with 10 mL distilled H_2O and 10 mL diethyl ether, shaken and filtered through a frit with a 2 cm celite pad to remove the precipitate. The filter cake was washed with 20 mL distilled H_2O and 10 mL diethyl ether. The phases were separated and the organic phase was washed twice with 10 mL H_2O each and the aqueous phase was washed twice with 10 mL diethyl ether each. The aqueous and the organic phase were also analysed by HPLC (method: "Ethyl-SAH", "MeTYR H2O-MEOH 99-1 nucleodur"). The rest of diethyl ether in the aqueous phase was removed by evaporation. The water was removed by lyophilisation. The crude product (65.1 mg) was a yellow-greenish solid which was dissolved in 600 µL distilled water, filtered and transferred in a glass vial. The purification was made by preparative HPLC (method: "EBR_NucleodurC18 _001HCOOH_1to95MeOH"). The collected fractions were analysed by HPLC (method: "ETHYL-SAH", "MeTYR H2O-MEOH 99-1 nucleodur"). 3.3 mg of SAH (**8**) were regained and stored in the freezer. 3.7 mg ethyl-SAH (**24**) were gained.

$C_{16}H_{25}N_6O_5S^+$ [413.47]

yield:	3.7 mg (11 %) white solid
HPLC (Ethyl-SAH, 260 nm):	t_R = 6.35 min
HPLC (MeTYR H2O-MEOH 99-1 nucleodur, 254 nm):	t_R = 4.12 min

6.3.8. 24 hours stability test of ethyl-SAH (24) and SAM (2)

Ethyl-SAH (**24**) was freshly synthesised (10 mg of SAH (**8**) as starting material) and the crude product (34 mg of white-yellowish solid) separated into two parts. 19.3 mg of crude ethyl-SAH (**24**) was dissolved in 1 mL of 100 mM sodium phosphate buffer pH 7.5 and 14.7 mg of crude ethyl-SAH (**24**) was dissolved in 1 mL of 0.005 M H_2SO_4 containing 10 vol % ethanol. Both samples were stored at room temperature and analysed by HPLC (method: "ETHYL-SAH-SHORT") after 0, 0.5, 1, 3, 6 and 24 hours.

For *S*-adenosyl-L-methionine chloride dihydrochloride (SAM) (**2**) a 20 mM stock solution was prepared by dissolving 5 mg **2** in 500 µL of either 100 mM sodium phosphate buffer pH 7.5 or 0.005 M H_2SO_4 containing 10 vol % ethanol. A tenfold dilution was done and analysed by HPLC (method: "SAM") after 0, 0.5, 1, 3, 6 and 24 hours.

6.3.9. Metal ion assay

The influence of metal ions on the enzyme activity was screened by adding different metal solutions to the assay. To 1 mM substrate 3-chlorobenzenethiol (**1**) and 2 mM SAM (**2**), 0.02 mM of metal solution was added. The assay was performed in 100 mM sodium phosphate buffer pH 7.5, 25 % (v/v) *t*BME and with 0.5 mg/mL (0.02 mM) purified *SBP_TEV_Bo*tmt. All metals were tested twice. For each metal solution a blank reaction (without enzyme and metal) and one positive control (enzyme without metal in addition) were performed.

For the 20 mM cofactor stock solution 28 mg of SAM (**2**) were dissolved in 2.8 mL 100 mM sodium phosphate buffer pH 7.5. For the 100 mM substrate stock solution 6 µL of 3-chlorobenzenethiol (**1**) were dissolved in 500 µL *t*BME. For the 100 mM product stock solution 7 µL of 3-chlorothioanisole (**3**) were dissolved in 500 µL *t*BME. Both, substrate and product stock solutions, were diluted tenfold.

For the metal solutions, 2 mM stock solutions were prepared: 0.54 mg of copper (II) chloride (**34**), 0.95 mg of nickel (II) chloride hexahydrate (**35**), 0.52 mg of cobalt (II) chloride (**36**), 0.55 mg of zinc (II) chloride (**37**), 0.50 mg of manganese (II) chloride (**38**), 0.59 mg calcium (II) chloride dihydrate (**39**), 1.08 mg of iron (III) chloride hexahydrate (**40**) and 0.38 mg of magnesium (II) chloride (**41**) were dissolved in 2 mL of distilled water, respectively.

The reactions were shaken at 30 °C and 1000 rpm for 30 minutes. They were stopped by adding 30 µL of a 0.33 M HCl solution, shaking and centrifuging for 5 minutes at 13000 rpm. The organic phase was transferred into glass vials and analysed by GC-FID (method: “RPHSH_ME 100_SPLITLESS”) and HPLC-MS (method: “CLPHSH FAMEOH POS”).

Because copper showed a slight influence on the enzyme, copper solution was investigated in a concentration dependent manner. The reaction conditions remained the same. Copper was added in 0.01 mM, 0.02 mM and 0.04 mM concentration. All three concentrations were analysed twice and additionally two blank reactions and two positive controls were performed. The samples were analysed by GC-FID (method: “RPHSH_ME 100_SPLITLESS”) and HPLC-MS (method: “CLPHSH FAMEOH POS”).

7. References

[1] H. Schönherr, T. Cernak, *Angew. Chem. Int. Ed.* **2013**, *52*, 12256–12267.

[2] A.-W. Struck, M. L. Thompson, L. S. Wong, J. Micklefield, *ChemBioChem* **2012**, *13*, 2642–2655.

[3] S. Klimašauskas, E. Weinhold, *Trends Biotechnol.* **2007**, *25*, 99–104.

[4] H. L. Schubert, R. M. Blumenthal, X. Cheng, *Trends Biochem. Sci.* **2003**, *28*, 329–335.

[5] S. Klimašauskas, G. Lukinavičius, in *Encycl. Chem. Biol.* (Ed.: T.P. Begley), Wiley-VCH, Weinheim, **2009**, pp. 8–17.

[6] J. L. Martin, F. M. McMillan, *Curr. Opin. Struct. Biol.* **2002**, *12*, 783–793.

[7] C. Dalhoff, G. Lukinavičius, S. Klimašauskas, E. Weinhold, *Nat. Chem. Biol.* **2006**, *2*, 31–32.

[8] K. Goedecke, M. Pignot, R. S. Goody, A. J. Scheidig, E. Weinhold, *Nat. Struct. Biol.* **2001**, *8*, 121–125.

[9] D. T. H. Verhoeven, H. Verhagen, R. A. Goldbohm, P. A. van den Brandt, G. van Poppel, *Chem. Biol. Interact.* **1997**, *103*, 79–129.

[10] J. M. Attieh, A. D. Hanson, H. S. Saini, *J. Biol. Chem.* **1995**, *270*, 9250–9257.

[11] J. Attieh, S. A. Sparace, H. S. Saini, *Arch. Biochem. Biophys.* **2000**, *380*, 257–266.

[12] J. Attieh, K. F. Kleppinger-Sparace, C. Nunes, S. A. Sparace, H. S. Saini, *Plant Cell Environ.* **2000**, *23*, 165–174.

[13] J. Attieh, R. Djiana, P. Koonjul, C. Étienne, S. A. Sparace, H. S. Saini, *Plant Mol. Biol.* **2002**, *50*, 511–521.

[14] C. Dalhoff, E. Weinhold, in *Modif. Nucleosides Biochem. Biotechnol. Med.* (Ed.: P. Herdewijn), Wiley-VCH, Weinheim, **2008**, pp. 557–581.

[15] G. L. Cantoni, *J. Biol. Chem.* **1953**, *204*, 403–416.

[16] J. R. Matos, C.-H. Wong, *Bioorganic Chem.* **1987**, *15*, 71–80.

[17] D. F. Iwig, S. J. Booker, *Biochemistry (Mosc.)* **2004**, *43*, 13496–13509.

[18] G. Schluckebier, M. Kozak, N. Bleimling, E. Weinhold, W. Saenger, *J. Mol. Biol.* **1997**, *265*, 56–67.

[19] M. Fontecave, *Chem. Biol.* **2011**, *18*, 559–561.

[20] T. L. Grove, J. S. Benner, M. I. Radle, J. H. Ahlum, B. J. Landgraf, C. Krebs, S. J. Booker, *Science* **2011**, *332*, 604–607.

[21] L. W. Parks, *J. Biol. Chem.* **1958**, *232*, 169–176.

[22] F. Schlenk, J. L. Dainko, *Biochim. Biophys. Acta* **1975**, *385*, 312–323.

[23] M. Tengg, H. Stecher, P. Remler, I. Eiteljörg, H. Schwab, M. Gruber-Khadjawi, *J. Mol. Catal. B Enzym.* **2012**, *84*, 2–8.

[24] C. Dalhoff, G. Lukinavičius, S. Klimašauskas, E. Weinhold, *Nat. Protoc.* **2006**, *1*, 1879–1886.

[25] G. Lukinavičius, V. Lapienė, Z. Staševskij, C. Dalhoff, E. Weinhold, S. Klimašauskas, *J. Am. Chem. Soc.* **2007**, *129*, 2758–2759.

[26] H. Stecher, M. Tengg, B. J. Ueberbacher, P. Remler, H. Schwab, H. Griengl, M. Gruber-Khadjawi, *Angew. Chem. Int. Ed.* **2009**, *48*, 9546–9548.

[27] Z. Liutkevičiūtė, E. Kriukienė, I. Grigaitytė, V. Masevičius, S. Klimašauskas, *Angew. Chem. Int. Ed.* **2011**, *50*, 2090–2093.

[28] S. Singh, J. Zhang, T. D. Huber, M. Sunkara, K. Hurley, R. D. Goff, G. Wang, W. Zhang, C. Liu, J. Rohr, et al., *Angew. Chem. Int. Ed.* **2014**, *53*, 3965–3969.

[29] F. Baneyx, M. Mujacic, *Nat. Biotechnol.* **2004**, *22*, 1399–1408.

[30] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

[31] L. R. Offner, Bioalkylation Catalyzed by Methyltransferases, master thesis, Graz University of Technology, **2013**.

[32] A. L. M. Porto, F. Cassiola, S. L. P. Dias, I. Joekes, Y. Gushikem, J. A. R. Rodrigues, P. J. S. Moran, G. P. Manfio, A. J. Marsaioli, *J. Mol. Catal. B Enzym.* **2002**, *19-20*, 327–334.

[33] K. Faber, *Biotransformations in Organic Chemistry*, Springer, New York, **2011**.

[34] G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, K. I. Goldberg, *Organometallics* **2010**, *29*, 2176–2179.

8. Abbreviations

AcCN	acetonitrile
API	**A**ctive **P**harmaceutical **I**ngredient
aq	aqueous
ATP	adenosine triphosphate
*Bo*tmt	thiol-methyltransferase from *Brassica oleracea*
CH_3	methyl group
$CDCl_3$	deuterated chloroform
COMT	catechol *O*-methyltransferase
conc.	concentrated
d	dublet (NMR)
d	day
Da	Dalton
DAD	diode array detector
DCM	dichloromethane
DMSO	dimethyl sulfoxide
DMSO-d_6	fully deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e. g.	exempli gratia (for example)
EI	electron ionisation
eq	equivalent
ESI	electrospray ionisation
et al.	et alii (and others)
FA	formic acid
FID	flame ionization detector
g	gramm
g	earth's gravitational acceleration
GC	gas chromatography

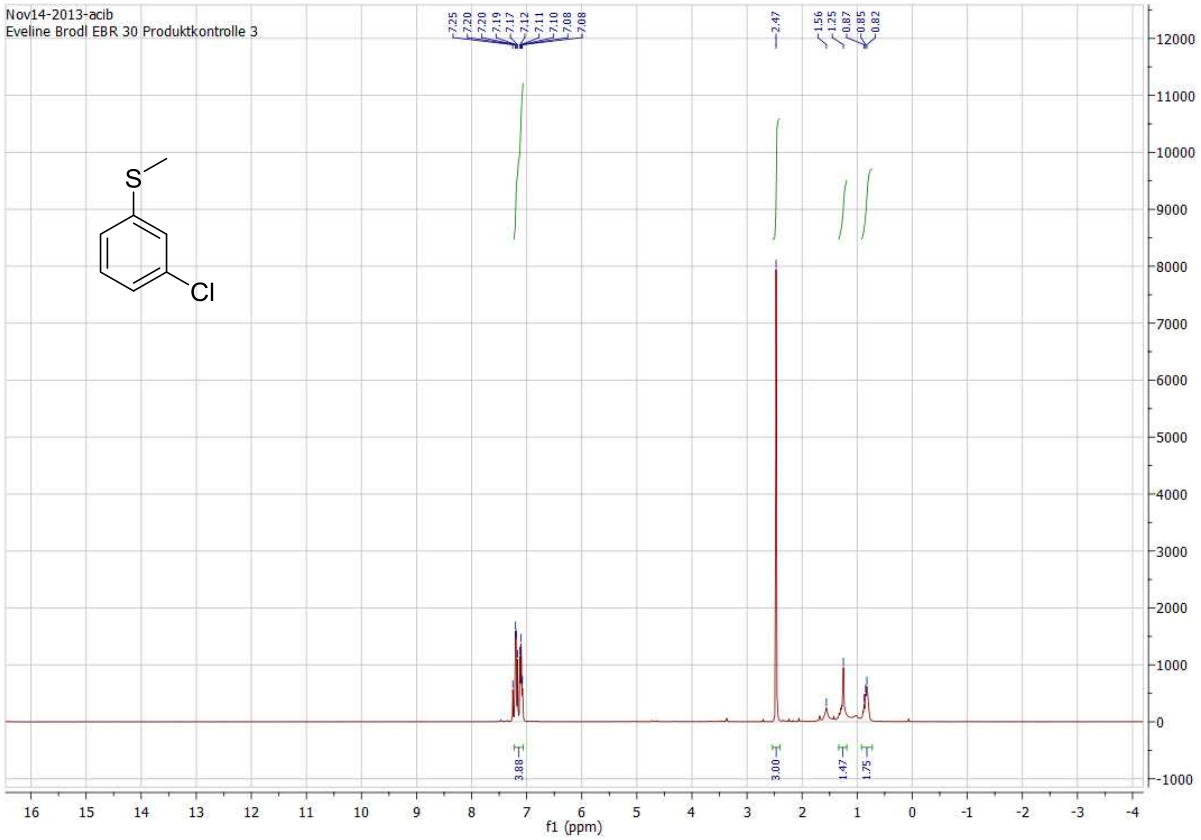
h	hour
H-bond	hydrogen bond
H_2O	water
H_2SO_4	sulfuric acid
HABA	hydroxyl-azophenol-benzoic acid
HCOOH	formic acid
HCl	hydrogen chloride
HIS	histidine
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HS^-	bisulfide ion
HSL	homoserine lactone
HSQC	heteronuclear single quantum coherence
IC_{50}	half maximal inhibitory concentration
IPTG	isopropyl β-D-1-thiogalactopyranoside
J	coupling constant (NMR)
kDa	kilo Dalton
K_m	Michaelis Menten constant
L	litre
max	maximum
MeOH	methanol
MES	2-(*N*-morpholino)ethanesulfonic acid
mg	milligram
$MgSO_4$	magnesium sulphate
MHz	megahertz
min	minute
mL	millilitre
mM	millimolar
mm	millimetre
mmol	millimol
mRNA	messenger ribonucleic acid
MS	mass spectrometry

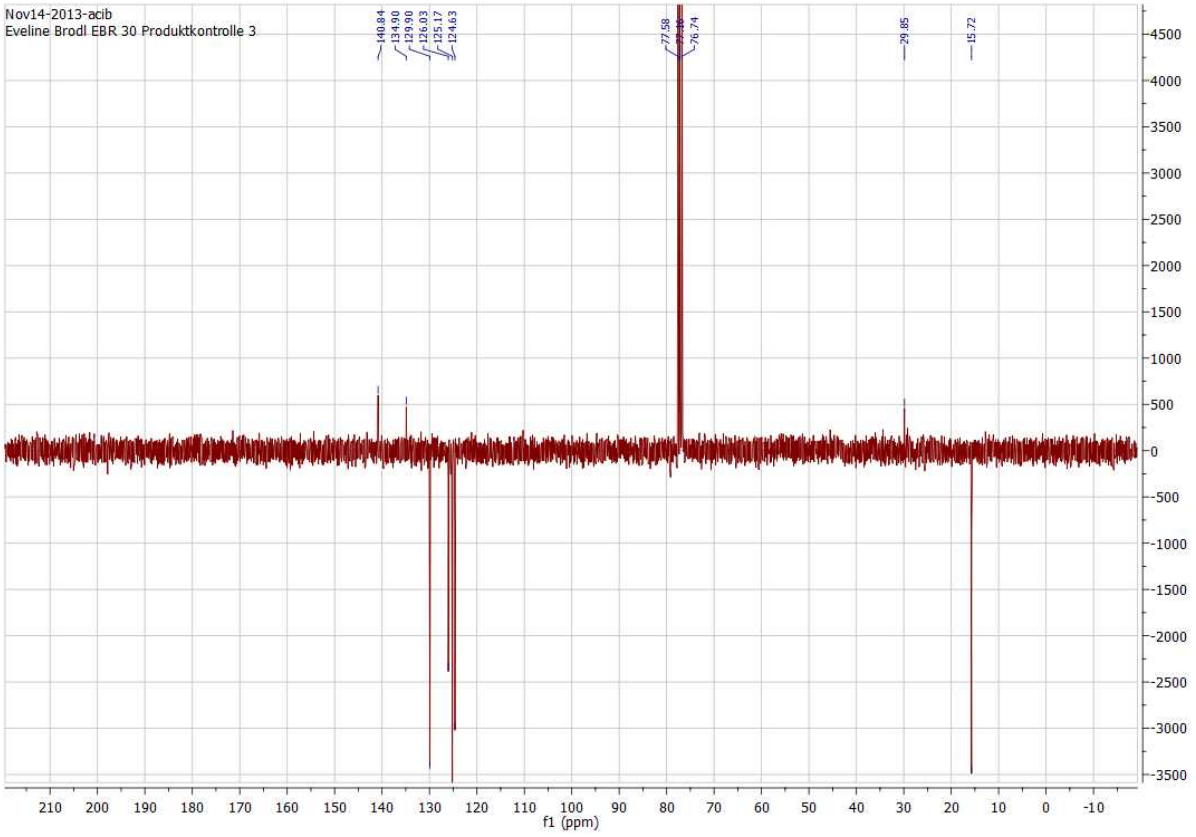
MTA	5’-deoxy-5’-methylthioadenosine
MTases	methyltransferases
NaCl	sodium chloride
nm	nanometre
NMR	nuclear magnetic resonance
OD	optical density
ONC	overnight culture
PAGE	polyacrylamide gel electrophoresis
pH	“power of hydrogen”
pI	isoelectric point
Pi	phosphate
pKa	logarithmic acid dissociation constant
pos	positive mode (MS)
ppm	parts per million
PPi	pyrophosphate
PPPi	triphosphate
q	quadruplet (NMR)
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
s	singulet (NMR)
s	second
SAH	*S*-adenosyl-L-homocysteine
SAM	*S*-adenosyl-L-methionine
SDS	sodium dodecyl sulfate
S_N2	nucleophilic substitution type 2
Strep	streptavidin
t	triplet (NMR)
*t*BME	*tertiary* butyl methyl ether
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TMT	thiol methyltransferase

V	volt
λ	lambda; wavelength
%	per cent
µm	micrometre
µL	microliter
(v/v)	volume/volume
(w/w)	weight/weight
%	per cent
% vol.	volume per cent
°C	degree Celsius

9. Appendix

^{1}H and APT NMR of 3-chlorothioanisole (**3**) in $CDCl_3$





^{1}H NMR of (3-chlorophenyl)(ethyl)sulfane (**23**) in $CDCl_3$