

Dissertation

Biotransformations from and to methylated flavo- noids

Subtitle

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noch nicht bekannt

It is what it is. Accept it and move on.

– *unknown* –

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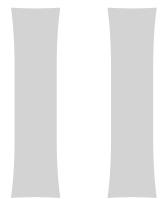


Preface

1 Abstracts

1.1 English Abstract

1.2 Deutsche Zusammenfassung



Thesis

2 Introduction

Some introductory text

2.1 Natural products and secondary metabolites

2.1.1 General

2.1.2 Classes of natural products

Terpenoids and Steroids

... here is some text

Polyketides and non-ribosomal peptides

... here is some text

Alkaloids

... here is some text

Phenylpropanoids

... here is some text Flavonoids and phenyl propanoids have important functions in nature and can function as protection against high UV-exposure, signaling molecules or transcriptional regulators [5, 31].

2.2 Alkylating reactions in nature

2.2.1 Methylation

2.2.2 Prenylation

2.2.3 Glycosylation

2.3 Usage and expansion of natures reaction tool-box

2.3.1 Terpene synthases and elongases

2.3.2 Methyl transferases

2.3.3 Glycosyl transferases

2.3.4 Other important enzymes in biotech research

BMVOs

Esterases/Lipases

Oxidases

Lyases

Transaminases

2.4 Conclusion

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3 Material And Methods

Within this section percentages refer to volume per volume (v/v) percentages unless otherwise specified.

3.1 Materials

3.1.1 Chemicals

Enzymes and buffers used for molecular cloning were obtained from Life Technologies (Darmstadt, Germany), unless otherwise noted. Flavonoid HPLC standards were purchased from Extrasynthese (Genay, France). Deuterated solvents were acquired from Deutero GmbH (Kastellaun, Germany). Solvents, purchased from VWR (Poole, England), were distilled in-house before use.

All other chemicals were obtained from either Sigma-Aldrich (Steinheim, Germany), Applichem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

3.1.2 Commonly used solutions and buffers

50× 5052	25 % glycerol, 2.5 % (w/v) glucose, 10 % (w/v) α -lactose
binding buffer	50 mM Tris/HCl, 500 mM NaCl, 10 % glycerol, 2.5 mM imidazole pH 7
elution buffer	50 mM Tris/HCl, 500 mM NaCl, 10 % glycerol, 250 mM imidazole pH 7

lysis buffer	50 mM Tris/HCl, 500 mM NaCl, 10 % glycerol, 2.5 mM imidazole, 0.2 % Tween-20 pH 7
1 M MMT pH 4 (10×)	26.8 g/l L-malic acid, 78.1 g/l MES, 26.8 g/l Tris, 2.1 % 10 M HCl
1 M MMT pH 9 (10×)	26.8 g/l L-malic acid, 78.1 g/l MES, 26.8 g/l Tris, 6.7 % 10 M NaOH
20× NPS	1 M Na ₂ HPO ₄ , 1 M KH ₂ PO ₄ , 0.5 M (NH ₄) ₂ SO ₄
1 M SSG pH 4 (10×)	14.8 g/l succinic acid, 60.4 g/l NaH ₂ PO ₄ · H ₂ O, 32.8 g/l glycine, 0.4 % 10 M NaOH
1 M SSG pH 10 (10×)	14.8 g/l succinic acid, 60.4 g/l NaH ₂ PO ₄ · H ₂ O, 32.8 g/l glycine, 10.3 % 10 M NaOH
5× SDS sample buffer	10 % (w/v) SDS, 10 mM β-mercaptoethanol, 20 % glycerol, 0.2 M Tris/HCl pH 6.8, 0.05 % (w/v) bromophenolblue
1000× trace elements	50 mM FeCl ₃ , 20 mM CaCl ₂ , 10 mM MnCl ₂ , 10 mM ZnSO ₄ , 2 mM CoCl ₂ , 2 mM CuCl ₂ , 2 mM NiCl ₂ , 2 mM Na ₂ MoO ₄ , 2 mM Na ₂ SeO ₃ , 2 mM H ₃ BO ₃

Preparation of natural deep eutectic solvent (NADES)

NADES were prepared by adding each component in a round-bottom flask with a stirrer and stirring the mixture at 50 °C with intermittent sonication treatments until a clear solution was obtained.

Table 3.1: NADES-mixtures used within this work.

name	composition	mole ratio	mass fraction (w/w)
PCH	propane-1,2-diol	1:1:1	0.326
	choline chloride		0.597
	water		0.077
GCH	L-glucose	2:5:5	0.314
	choline chloride		0.608
	water		0.078

3.1.3 Culture media used to grow bacteria

LB-medium	10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract, pH 7.5
LB-agar	LB + 1.5 % (w/v) agar-agar
TB-medium	12 g/l tryptone, 24 g/l yeast extract, 0.4 % glycerol, 72 mM K ₂ HPO ₄ , 17 mM KH ₂ PO ₄
ZY	10 g/l tryptone, 5 g/l yeast extract
ZYP-5052	volume fraction (v/v): 0.928 ZY, 0.05 20× NPS, 0.02 50× 5052, 0.002 1 M MgSO ₄ , 0.0002 1000× trace elements

3.1.4 Bacterial strains

E.coli

BL21(DE3)	F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm λ</i> (DE3) Invitrogen, Karlsruhe (Germany)
C41(DE3)	F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm λ</i> (DE3) Lucigen, Wisconsin (USA)
C43(DE3)	F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm λ</i> (DE3) Lucigen, Wisconsin (USA)
DH5α	F ⁻ Φ80lacZΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17(r_K⁻m_K⁺) phoA supE44 λ⁻ thi-1 gyrA96 relA1</i> Invitrogen, Karlsruhe (Germany)
JM110	<i>rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm glnV44 Δ(lac-proAB) e14-</i> [F' <i>traD36 proAB⁺ lacI^q lacZΔM15</i>] <i>hsdR17(r_K⁻m_K⁺)</i> Martin-Luther-University Halle-Wittenberg
JW1593 (BW25113 derivative)	<i>rrnB ΔlacZ4787 HsdR514 Δ(arabAD)568 rph-1 ΔydgG</i> (Kan ^R) Keio Collection, National Institute of Genetics (Japan)
MG1655	F ⁻ λ ⁻ <i>ilvG⁻ rfb-50 rph-1</i> DSMZ, Hamburg (Germany)

One Shot TOP10	F ⁻ Φ80lacZΔM15 Δ(<i>mrr-hsdRMS-mcrBC</i>) <i>recA1 endA1 mcrA</i> Δ <i>lacX74 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^R) λ ⁻ <i>nupG</i> Invitrogen, Karlsruhe (Germany)
Origami(DE3)	Δ(<i>ara-leu</i>)7697 Δ <i>lacX74 phoA Pvull phoR araD139 ahpC galE</i> <i>galK rpsL</i> F'[<i>lac + lacI q pro</i>] (DE3)gor522::Tn10 <i>trxB</i> (Kan ^R , Str ^R , Tet ^R) Novagen, Wisconsin (USA)
Rosetta(DE3)	F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm</i> λ(DE3) pRARE (Cam ^R) Novagen, Wisconsin (USA)
Rosetta(DE3) pLysS	F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm</i> λ(DE3) pLysSRARE (Cam ^R) Novagen, Wisconsin (USA)
T7 Express	<i>fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mer-73::miniTn10-Tet^S)2 [dcm] R(zgb-210::Tn10-Tet^S) endA1</i> Δ(<i>mcrC-mrr</i>)114::IS10 NEB, Massachusetts (USA)

Agrobacterium tumefaciens

GV3101	chromosomal background: C58, marker gene: <i>rif</i> , Ti-plasmid: cured, opine: nopaline Sylvestre Marillonet, IPB
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3.1.5 Plasmids

Table 3.3.: Plasmids used in this work.

name	supplier/source
pACYCDuet-1	Merck, Darmstadt (Germany)
pCDFDuet-1	Merck, Darmstadt (Germany)
pET-20b(+)	Merck, Darmstadt (Germany)
pET-28a(+)	Merck, Darmstadt (Germany)
pET-32a(+)	Merck, Darmstadt (Germany)
pET-41a(+)	Merck, Darmstadt (Germany)

name	supplier/source
pQE30	QIAGEN, Hilden (Germany)
pUC19	Invitrogen, Karlsruhe (Germany)

3.1.6 Oligonucleotides and synthetic genes

Oligonucleotides and primers were ordered from Eurofins Genomics (Ebersberg, Germany). The purity grade was *high purity salt free* (HPSF). Synthetic genes or gene fragments were obtained from GeneArt® (Life Technologies, Darmstadt, Germany) or Eurofins Genomics (Ebersberg, Germany).

Table 3.4.: Primers used in this work. Recognition sites for endonucleases are underlined. Positions used for site directed mutagenesis are in lower case font.

name	sequence (5'→3')	cloning site
somt1	TTG <u>AAG ACA</u> AAA TGG CTT CTT CAT TAA ACA ATG GCC G	BpiI
somt2	TTG <u>AAG ACA</u> AGG ACA CCC CAA ATA CTG TGA GAT CTT CC	BpiI
somt3	TTG <u>AAG ACA</u> AGT CCT TAG GAA CAC CTT TCT GGG AC	BpiI
somt4	TTG <u>AAG ACA</u> AAA GCT CAA GGA TAG ATC TCA ATA AGA GAC	BpiI
pfomt1.fw	CAG AGA GGC cTA TGA GAT TGG CTT GC	
pfomt1.rv	GCA AGC CAA TCT CAT AgG CCT CTC TG	
pfomt2.fw	<u>CAT ATG</u> GAT TTT GCT GTG ATG AAG CAG GTC	NdeI
pfomt2.rv	<u>GAA TTC</u> AAT AAA GAC GCC TGC AGA AAG TG	EcoRI
pRha1.fw	CTC TAG <u>CAG ATC</u> TCG GTG AGC ATC ACA TCA CCA CAA TTC	BglII
pRha1.rv	CAA TTG <u>AGG ATC</u> CCC ATT TTA ACC TCC TTA GTG	BamHI
pUC1.fw	GCG TAT TGG Gag aTC TTC CGC TTC CTC	
pUC1.rv	GAG GAA GCG GAA GAt ctC CCA ATA CGC	

3.1.7 Instruments

CD-spectrometer

Jasco J-815 (Eaton, USA)

electrophoresis (horizontal)	Biometra Compact XS/S (Göttingen, Germany)
electrophoresis (vertical)	Biometra Compact M (Göttingen, Germany) Biometra Minigel-Twin (Göttingen, Germany)
FPLC	ÄKTA purifier (GE Healthcare, Freiburg, Germany)
GC/MS	GC-MS-QP2010 Ultra (Shimadzu, Duisburg, Germany)
HPLC	VWR-Hitachi LaChrom Elite (VWR, Darmstadt, Germany)
ITC	MicroCal iTC200 (Malvern, Worcestershire, UK)
plate-reader	SpectraMax M5 (Molecular Devices, Biberach, Germany)
NMR-spectrometer	Varian Unity 400 (Agilent, Böblingen, Germany) Varian VNMRS 600 (Agilent, Böblingen, Germany)
photospectrometer	Eppendorf Biophotometer Plus (Hamburg, Germany) JASCO V-560 (Eaton, USA) Colibri Microvolume Spectrometer (Biozym, Hess. Oldendorf, Germany)
centrifuges	Eppendorf 5424 (Hamburg, Germany) Hettich Mikro 120 (Kirchlengern, Germany) Beckman Avanti J-E, Beckman Allegra X-30R (Krefeld, Germany)
centrifuge rotors	Beckman JA-10, JA-16.250, JS-4.3 (Krefeld, Germany)

3.1.8 Software

All mathematical and statistical computations and graphics were done with the R software (versions 3.1.X, <http://cran.r-project.org/>) [68]. Visualizations of macromolecules were arranged using the PyMol Molecular Graphics System, version 1.7.0.0 (Schrödinger, New York, USA).

Physicochemical calculations and calculations of different molecular descriptors were performed using Marvin Beans 15.4.13.0 (ChemAxon, Budapest, Hungary) and Molecular Operating Environment 2008.10 (Chemical Computing Group, Montreal, Canada). Special software used for X-ray crystal structure solution is discussed separately in the corresponding section (3.5).

3.2 Molecular Biology

Basic molecular biology methods like polymerase chain reaction (PCR), DNA restriction/ligation, DNA gel electrophoresis, preparation of competent cells and transformation were performed based on the protocols summarized by Sambrook and Russell [73].

Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

In vitro site-directed mutagenesis was set-up according to the protocol of the *QuikChange™ Site-Directed Mutagenesis* kit [2] offered by Agilent Technologies (Santa Clara, USA).

Nucleotide fragments obtained by PCR, restriction/ligation procedures or excision from electrophoresis gels were purified and concentrated using the *Nucleospin Gel and PCR Clean-up* kit provided by Machery-Nagel (Düren, Germany) according to the instructions provided by the manufacturer.

3.2.1 Golden Gate Cloning

The Golden Gate cloning procedure is a one-pot method, meaning the restriction digestion and ligation are carried out in the same reaction vessel at the same time [24, 44]. Consequently PCR-fragments, destination vector, restriction endonuclease and ligase are added together in this reaction. The methodology employs type II restriction enzymes, which together with proper design of the fragments allow for a ligation product lacking the original restriction sites.

For digestion/ligation reactions of fragments containing BpiI sites, 20 fmol of each fragment or vector, together with 5 U of BpiI and 5 U of T4 ligase were combined in a total volume of 15 µl 1× ligase buffer. For fragments to be cloned via BsaI sites, BpiI in the above reaction was substituted by 5 U BsaI.

The reaction mixture was placed in a thermocycler and incubated at 37 °C for 2 min and 16 °C for 5 min. These two first steps were repeated 50 times over. Finally, the temperature was raised to 50 °C (5 min) and 80 °C (10 min) to inactivate the enzymes.

3.2.2 Subcloning of genes

All subcloning procedures were performed according to section 3.2 and specifically subsection 3.2.1. Specific steps for the subcloning of any genes discussed can be found in the appendix (p.72). The *pfromt* gene was subcloned from the pQE-30 vector kindly provided by Thomas Vogt (Leibniz-Institute of Plant Biochemistry (IPB), Halle, Germany) into the pET-28a(+) vector. The *somt-2* gene was subcloned from the pQE-30 vector kindly provided by Martin Dippe (IPB, Halle, Germany) into the pET-28-MC vector.

3.2.3 Transformation of electrocompetent *Agrobacterium tumefaciens* cells

A 50 µl aliquot of electrocompetent *A. tumefaciens* cells was thawed on ice. (50 to 100) ng of plasmid were added, the solution was mixed gently and transferred to a pre-cooled electroporation cuvette. After pulsing (2.5 kV, 200 Ω) 1 ml of lysogeny broth (LB)-medium was added, the mixture transferred to a 1.5 ml tube and incubated for (3 to 4) hours at 28 °C. The culture was centrifuged (10 000 × *g*, 1 min) and 900 µl supernatant were discarded. The pellet was resuspended in the remaining liquid, plated onto LB-agar plates supplemented with 40 µg/ml rifampicin and 50 µg/ml carbencillin and incubated at 28 °C for (2 to 3) days.

3.3 Treatment of plant material

3.3.1 Infiltration of *Nicotiana benthamiana*

Before infiltration *N. benthamiana* plants were pruned, such that only leaves to be infiltrated remained with the plant (Figure 3.1). 5 ml cultures of transformed *A. tumefaciens* in LB-medium (with 40 µg/ml rifampicin and 50 µg/ml carbencillin) were grown over night at 28 °C and 220 rpm. OD₆₀₀ of the culture was measured and adjusted to 0.2 by dilution with infiltration buffer (10 mM MES/NaOH, 10 mM MgSO₄ pH 5.5). When multiple *A. tumefaciens* transformed with different constructs/plasmids were used for infiltration, the cultures were mixed and diluted using

infiltration buffer, such that OD_{600} of each culture in the mix was 0.2. The solution was infiltrated into the abaxial side of *N. benthamiana* leaves using a plastic syringe. The leaf material was harvested after 7 days.



Figure 3.1.: Pruned *N. benthamiana* plant, with two bottom and one top leaf, ready to be infiltrated.

3.3.2 Plant material harvest

Infiltrated/Infected areas of *N. benthamiana* leaf material were cut out and grouped by plant number, leaf position (top/bottom) and leaf side (right/left). The grouped clippings were weighed, frozen in liquid nitrogen, ground to a powder, freeze-dried and stored at -80°C .

3.3.3 Extraction of flavonoids from *N. benthamiana* leaves

Two tips of a small spatula of freeze-dried material ($\approx 6\text{ mg}$), were weighed exactly and extracted with $500\text{ }\mu\text{l}$ 75 % aqueous methanol containing 1 mM ascorbic acid, 0.2 % formic acid and 0.1 mM flavone (internal standard). Therefore the suspension was vortexed for 30 s, rotated on an orbital shaker for 10 min and vortexed again for 30 s. The suspension was centrifuged ($20\,000 \times g$, 4°C , 10 min) and the supernatant transferred to a new tube, to remove the insoluble plant material. The supernatant was centrifuged again ($20\,000 \times g$, 4°C , 10 min) and the resulting supernatant was transferred to a HPLC-vial and stored at -20°C until analysis.

3.4 Protein biochemistry

Stock solutions of antibiotics, IPTG or sugars were prepared according to the pET System Manual by Novagen [64], unless otherwise noted.

3.4.1 Determination of protein concentration

Protein concentrations were estimated using the absorption of protein solutions at 280 nm, which is mainly dependent on the amino acid composition of the protein studied [29]. Extinction coefficients of proteins were calculated from the amino acid sequence using the ExpPASy servers's ProtParam tool [28].

Table 3.6.: Calculated extinction coefficients of proteins used in this work.

protein/enzyme	$\epsilon_{280\text{nm}}^{1\text{g/l}}$ in $\text{ml mg}^{-1} \text{cm}^{-1}$
PFOMT (reduced)	0.714
PFOMT Y51K N202W (reduced)	0.852
SOMT-2 (oxidized)	1.263
SOMT-2 (reduced)	1.247
COMT	

3.4.2 Protein production test (expression test)

The heterologous production of proteins in *E. coli* was assessed in a small scale protein production test, henceforth called expression test. Single colonies of *E. coli* transformed with the constructs to be studied were used to inoculate a 2 ml starter culture in LB-medium containing the appropriate antibiotics. The working concentrations of antibiotics used was as follows: 200 µg/ml ampicillin, 150 µg/ml kanamycin, 50 µg/ml chloramphenicol, 20 µg/ml tetracycline.

The starter culture was allowed to grow at 37 °C and 200 rpm over night. A 5 ml sampling culture of the medium to be studied containing the appropriate antibiotics was prepared. The media tested included LB, terrific broth (TB) and auto-induction media like ZYP-5052. The sampling culture was inoculated to an OD₆₀₀ of 0.075

using the starter culture and incubated at different temperatures and 200 rpm in a shaking incubator. 1 mM isopropyl-D-thiogalactopyranosid (IPTG) was added when the OD₆₀₀ reached 0.6–0.8, if appropriate for the studied construct. 1 ml samples were removed after different times of incubation (e.g. 4, 8, 12 hours), subfractionated (3.4.3) and analyzed via SDS-PAGE (3.4.6).

Exact specifications of growth conditions (e.g. temperature, time, constructs) are discussed in the individual sections.

3.4.3 Protein subfractionation

The protein subfractionation procedure described herein was adapted from the protocol described in the pET Manual [64]. Overall 5 protein subfractions can be obtained, including *total cell protein*, *culture supernatant (medium) protein*, *periplasmic protein*, *solute cytoplasmic protein* and *insoluble protein*.

The OD₆₀₀ of the culture sample was measured and the cells harvested by centrifugation at 10 000 × *g*, 4 °C for 5 minutes. The protein in the supernatant medium was concentrated by precipitation with trichloro acetic acid (TCA) (3.4.4) for SDS-PAGE analysis. The periplasmic protein was prepared (3.4.5) and also concentrated by TCA precipitation for SDS-PAGE. Cells were lysed by resuspending the cell pellet in (OD₆₀₀ × V × 50) µl of bacterial protein extraction reagent (B-PER) and vortexing vigorously for 30 s. The suspension was incubated at room temperature (RT) for 30 min to assure complete lysis. To separate insoluble protein and cell debris from the soluble cytosolic protein, the suspension was centrifuged at 10 000 × *g* and 4 °C for 10 min. Soluble cytoplasmic protein was contained in the supernatant, whereas insoluble protein remained in the pellet. For SDS-PAGE analysis of the insoluble protein, the pellet was resuspended in the same volume of B-PER. To obtain only the total cell protein fraction, the preparation of periplasmic and soluble cytosolic protein was omitted. Sample volumes of 10 µl of each fraction were used for SDS-PAGE analysis.

3.4.4 Protein sample concentration by TCA precipitation

Diluted protein samples were concentrated by TCA precipitation in microcentrifuge tubes. Therefore 0.1 volume (V) of 100 % (w/v) TCA in water was added to the clarified sample, which was then vortexed for 15 s and placed on ice for a minimum of 15 min. The sample was centrifuged at $14\,000 \times g$, 4 °C for 15 min. The supernatant was discarded and the pellet was washed twice with 0.2 V ice-cold acetone. The acetone was removed and the pellet set to air-dry in an open tube. After drying, the protein pellet was resuspended in 0.1 V phosphate buffered saline (PBS) containing 1 × SDS-sample buffer by heating to 85 °C and vigorous vortexing, to achieve a 10 × concentration. After resuspension the sample was analyzed by SDS-PAGE or stored at -20 °C until use.

3.4.5 Preparation of periplasmic protein

Target proteins may be directed to the periplasmic space by N-terminal signal sequences like *pelB* or *DsbA/C* [55]. The periplasma is, other than the cytosol, an oxidizing environment and often used for the production of proteins containing disulfide linkages. The preparation of periplasmic protein was accomplished by an osmotic shock protocol modified from Current Protocols in Molecular Biology [6]. The cell pellet was resuspended in the same volume as the culture sample of 30 mM tris-HCl, 20 % (w/v) sucrose, pH 8 and 1 mM ethylenediaminetetraacetic acid (EDTA) was added. The suspension was stirred for 10 min at RT and the cells were collected by centrifugation at $10\,000 \times g$, 4 °C for 10 min. The supernatant was discarded and the cell pellet was resuspended in the same volume of ice-cold 5 mM MgSO₄. The suspension was stirred for 10 min on ice, while the periplasmic proteins were released into the solution. The cells were collected by centrifugation as before. Periplasmic proteins were contained in the supernatant.

3.4.6 Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The analysis of samples via SDS-PAGE was realized via the discontinuous system first described by Laemmli, which allows separation of proteins based on their electrophoretic mobility, which in turn depends on their size [47].

The SDS-PAGE procedure was carried out according to standard protocols described by Sambrook and Russell [73]. Very dilute and/or samples with high ionic strength were concentrated and/or desalted by the TCA precipitation procedure described in subsection 3.4.4. Generally a 10 % (acrylamide/bisacrylamide) running gel combined with a 4 % stacking gel was used. Reducing SDS-PAGE sample buffer was added to the protein sample to be analyzed, whereafter the sample was heated to 95 °C for 5 min, to allow for total unfolding of the protein. After cooling to RT the samples were transferred into the gel pockets for analysis. The *PageRuler™ Prestained Protein Ladder* (Life Technologies GmbH, Darmstadt, Germany) was used as a molecular weight (MW) marker and run alongside every analysis as a reference. Gels were stained using a staining solution of 0.25 % Coomassie Brilliant Blue G-250 (w/v) in water:methanol:acetic acid (4:5:1) and destained by treatment with water:methanol:acetic acid (6:3:1).

3.4.7 Buffer change of protein samples

The buffer in protein samples was exchanged either by dialysis, or by centrifugal filter concentrators (Amicon® Ultra Centrifugal Filter; Merck, Darmstadt, Germany). Large volumes of highly concentrated protein solutions were preferably dialyzed. Respectively, very dilute samples were concentrated and rebuffed using centrifugal concentrators.

Dialysis was carried out at least twice against a minimum of 100 times the sample volume. Dialysis steps were carried out at RT for 2 hours, or over-night at 4 °C. Centrifugal concentrators were used according to the manufacturers instructions.

3.4.8 Production of recombinant protein

Heterologous production of PFOMT

PFOMT was produced as a N-terminally (His)₆-tagged fusion protein. A 2 ml starter culture of LB containing 100 µg/ml kanamycin was inoculated with a single colony of *E. coli* BL21(DE3) transformed with pET28-pfomt and incubated at 37 °C, 220 rpm for 6 hours. The main culture (N-Z-amine, yeast extract, phosphate (ZYP-5052) containing 200 µg/ml kanamycin) was inoculated with the starter culture such that OD₆₀₀ was 0.05. The culture was incubated in a shaking incubator at 37 °C, 220 rpm over night (\approx 16 h). Due to the autoinducing nature of the ZYP-5052 medium, addition of IPTG was not necessary. Cells were harvested by centrifugation at 10 000 $\times g$, 4 °C for 10 min and the supernatant discarded. The pellet was resuspended in 50 mM Tris/HCl, 500 mM NaCl, 2.5 mM imidazole, 10 % glycerol pH 7 using a volume of \approx 10 ml/g of cell pellet. The cells were lysed by sonication (70 % amplitude, 1 s on-off-cycle) for 30 seconds, which was repeated twice. The crude lysate was clarified by centrifugation at 15 000 $\times g$, 4 °C for 15 minutes followed by filtration through a 0.45 µm filter. Consequently, the His-tagged PFOMT was purified by immobilized metal affinity chromatography (IMAC) (3.4.10). The eluted PFOMT protein was dialyzed (3.4.7) against 25 mM HEPES, 100 mM NaCl, 5 % glycerol pH 7 and stored at -20 °C until use.

Heterologous production of SOMT-2

SOMT-2 was produced as a fusion protein with an N-terminal His-tag. A starter LB-culture (\approx 2 ml) containing 100 µg/ml kanamycin was inoculated with a single colony of *E. coli* BL21(DE3) transformed with pET28MC-somt and incubated at 37 °C, 220 rpm for 6 hours. The starter culture was used to inoculate the main culture (LB-medium containing 100 µg/ml kanamycin), such that OD₆₀₀ \approx 0.05. The culture was incubated at 37 °C, 220 rpm in a shaking incubator until OD₆₀₀ \approx 0.6. Expression was induced by addition of 1 mM IPTG. Incubation continued at 37 °C, 220 rpm for 6 hours. Cells were harvested by centrifugation (10 000 $\times g$, 4 °C, 10 min) and used, or stored at -20 °C until use. SOMT-2 was produced in inclusion bodies (IBs), which were prepared as laid out in subsection 3.4.9.

3.4.9 Preparation of inlusion bodies (IBs)

Often, when recombinant protein is produced in high levels in *E. coli* it is accumulated in so-called inlusion bodies (IBs) [71]. The accumulating IBs consist mainly of the overproduced target protein, which is inherently quite pure already. IBs can be selectively recovered from *E. coli* cell lysates and can consequently be refolded. IBs were prepared according to a modified protocol by Palmer [65].

The cells were resuspended in 5 ml/g_{cells} IB lysis buffer (100 mM Tris/HCl, 1 mM EDTA pH 7), 0.5 mM phenylmethylsulfonylfluoride (PMSF) was added as protease inhibitor. The solution was homogenized using a tissue grinder homogenizer (Ultra Turrax®; IKA®-Werke GmbH & Co. KG, Staufen, Germany). 200 µg/ml lysozyme was added to aid in the breakage of cells and the cells were lysed by sonicating thrice at 70 % amplitude (1 s on-off-cycle) for 30 seconds. DNase I (10 µg/ml) was added and the solution was incubated on ice for 10 min. The lysate was clarified by centrifuging for 1 h at 20 000 × *g*, 4 °C. The supernatant was discarded and the pellet was resuspended in 5 ml/g_{cells} IB wash buffer I (20 mM EDTA, 500 mM NaCl, 2 % (w/v) Triton X-100 pH), followed by thorough homogenization. The solution was centrifuged (30 min at 20 000 × *g*, 4 °C), the supernatant discarded and the pellet was washed twice more. To remove detergent, the pellet was washed twice again with IB washing buffer II (20 mM EDTA, 100 mM Tris/HCl pH 7). The IBs were resuspended in IB solubilization buffer (100 mM Tris/HCl, 5 mM DTT, 6 M GdmCl pH 7), such that the protein concentration was about 25 mg/ml and stored at -20 °C until use.

3.4.10 Purification of His-tagged proteins using immobilized metal affinity chromatography (IMAC)

N- or C-terminal oligo-histidine tags (His-tags) are a common tool to ease purification of recombinantly produced proteins. The free electron pairs of the imidazol nitrogens of histidines can complex divalent cations such as Mg²⁺ or Ni²⁺, which are usually immobilized on a matrix of nitrilo triacetic acid (NTA)-derivatives. The affinity of the His-tag is correlated with its length and tagged proteins can simply be eluted by increasing the concentration of competing molecules (e.g. imidazole).

His-tagged protein was purified by fast protein liquid chromatography (FPLC) via Ni²⁺- (HisTrap FF crude) or Co²⁺-NTA (HiTrap Talon FF crude) columns, obtained from GE Healthcare (Freiburg, Germany), following modified suppliers instructions. First the column was equilibrated with 5 column volumes (CV) of binding buffer (50 mM Tris/HCl, 500 mM NaCl, 10 % glycerol, 2.5 mM imidazole pH 7). The sample (generally clarified lysate) was applied to the column using a flow of 0.75 ml/min. Unbound protein was removed by washing with 3 CV binding buffer. Unspecifically bound proteins were washed away by increasing the amount of elution buffer (50 mM Tris/HCl, 500 mM NaCl, 10 % glycerol, 250 mM imidazole pH 7) to 10 % (constant for 3 to 5 CV). Highly enriched and purified target protein was eluted with 6 to 10 CV of 100 % elution buffer.

3.4.11 Refolding of SOMT-2 on a micro scale using design of experiments (DoE)

Design of experiments (DoE) and FFD have been successfully used to optimize the refolding conditions of several proteins [3, 7, 93]. Thus, an approach using FFD was used to find optimal refolding conditions for SOMT-2.

Factors studied were pH (buffer), arginine, glycerol, divalent cations, ionic strength, redox system, cyclodextrin and co-factor addition. The experimental matrix was constructed using the FrF2 package (<http://cran.r-project.org/web/packages/FrF2/index.html>) in the R software.

Table 3.7.: Factors used in the construction of the FFD.

factor	symbol	setting		unit
		-1	+1	
pH	A	5.5	9.5	-
arginine	B	0	0.5	M
glycerol	C	0	10	% (v/v)
divalent cations ¹	D	no	yes	-

¹no: 1 mM EDTA; yes: 2 mM CaCl₂, MgCl₂

factor	symbol	setting		unit
		-1	+1	
ionic strength ²	E	low	high	-
redox state ³	F	reducing	redox-shuffling	-
α -cyclodextrin	G	0	30	mM
SAH	H	0	0.5	mM

Table 3.8.: Experimental design matrix for the FFD.

Experiment	A	B	C	D	E	F	G	H
1	+	+	+	-	-	-	-	+
2	-	-	-	-	-	-	-	-
3	+	-	+	+	-	+	+	-
4	-	+	+	-	+	+	+	-
5	+	+	-	-	+	+	-	-
6	-	+	-	+	+	-	+	+
7	+	+	-	+	-	-	+	-
8	-	-	+	-	+	-	+	+
9	+	-	+	+	+	-	-	-
10	-	-	-	-	-	+	+	+
11	+	-	-	+	+	+	-	+
12	-	+	+	+	-	+	-	+

The buffers were mixed from stock solutions and prepared in 1.5 ml microcentrifuge tubes immediately prior to the experiment. 50 μ l of solubilized SOMT-2 (1 mg/ml) in IB solubilization buffer was added to each buffer followed by a short vortex boost for rapid mixing. The final protein concentration in the refolding reaction was 50 μ g/ml, whereas the remaining GdmCl concentration was \approx 286 mM. The refolding reactions were incubated at RT for 1 hour, followed by an over

²low: 10 mM NaCl, 0.5 mM KCl; high: 250 mM NaCl, 10 mM KCl³reducing: 5 mM DTT; redox-shuffling: 1 mM glutathione (GSH), 0.2 mM glutathione disulfide (GSSG)

night incubation at 4 °C. After incubation the refolding reactions were centrifuged ($10\,000 \times g$, 4 °C, 10 min) to separate insoluble and soluble protein fractions. The supernatant was transferred to a new tube, whereas the pellet was washed twice with 200 µl acetone and once with 400 µl methanol/acetone (1:1). The pellet was resuspended in 100 µl PBS with 20 µl SDS-PAGE sample buffer and 10 µl were used for SDS-PAGE analysis.

100 µl of the supernatant were concentrated using TCA precipitation (3.4.4) and analyzed by SDS-PAGE. The remaining supernatant was rebuffered into 50 mM 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (BisTris) pH 7.5 using Amicon® Ultra 0.5 ml centrifugal filters (Merck, Darmstadt, Germany) according to the manufacturers instructions. The pre-weighed collection tubes were re-weighed after recovery and the volume of recovered liquid calculated ($\rho \approx 1\text{ g/cm}^3$). The sample was filled up to 100 µl using 50 mM BisTris pH 7.5 and the protein concentration was assessed using the Roti®-Quant protein quantification solution (Carl Roth, Karlsruhe, Germany) according to the manufacturers description. 50 µl of each refolded sample was used for an activity test using naringenin as substrate (3.6.3). The reactions were incubated over night and stopped by the extraction method. However, before the actual extraction 1 µl of anthracene-9-carboxylic acid (AC-9) was added as internal standard. The samples were analyzed by high-performance liquid chromatography (HPLC).

Assessment of refolding performance

The performance of each buffer on the refolding of SOMT-2 was examined by comparing the SDS-PAGE results, as well as the amount of soluble protein and the conversion of substrate. Main effects were analyzed qualitatively using main effects plots [10].

Upscaling of refolding reactions

Refolding reactions were scaled up to 50 ml. Therefore 2.5 ml solubilized SOMT-2 (1 mg/ml) were added over 10 minutes to 50 ml of refolding buffer while stirring at

RT. The refolding reaction was allowed to complete over night at 4 °C.

3.4.12 Enzymatic production of SAM and SAE

SAM and SAE were prepared according to the method described by Dippe, et. al [19].

Preparative reactions (20 ml) were performed in 0.1 M Tris/HCl, 20 mM MgCl₂, 200 mM KCl pH 8.0 and contained 7.5 mM adenosine triphosphate (ATP), 10 mM D,L-methionine or D,L-ethionine, for the production of SAM or SAE respectively, and 0.2 U S-adenosylmethionine synthase (SAMS) variant I317V. The reaction was stopped by lowering the pH to 4 using 10 M acetic acid after 18 h of incubation at 30 °C, 60 rpm. After 10 min incubation on ice the solution was centrifuged (15 000 × g, 10 min) to remove insoluble matter. The supernatant was transferred to a round bottom flask, frozen in liquid nitrogen and lyophilized.

Crude products were extracted from the pellet using 73 % ethanol and purified using ion exchange chromatography (IEX). IEX was performed on a sulfopropyl sepharose matrix (25 ml) via isocratic elution (500 mM HCl). Before injection, the crude extract was acidified to 0.5 M HCl using concentrated hydrochloric acid. After elution, the product containing fractions were dried via lyophilization.

The amount of product was determined by UV/VIS-spectroscopy at 260 nm using the published extinction coefficient of SAM ($\epsilon_0 = 15\,400\,M^{-1}\,cm^{-1}$) after resuspension in water [78].

3.5 Crystallographic Procedures

3.5.1 Crystallization of proteins

Commercially available crystallization screens were used to find initial crystallization conditions. The tested screens included kits available from Hampton Research (Aliso Viejo, USA) and Jena Bioscience (Jena, Germany). Crystallization screens were processed in 96-well micro-titer plate (MTP)s, where each well possessed 4 subwells aligned in a 2×2 matrix. The subwells were divided into 3 shallow wells for sitting drop vapour diffusion experimental setups and a fourth subwell,

which was deep enough to act as buffer reservoir. This way the performance of each crystallization buffer could be assessed using three different protein solutions with varying concentrations, effectors etc. A pipetting robot (Cartesian Microsys, Zinsser-Analystik; Frankfurt, Germany) was used to mix 200 nl of each, protein and buffer solution, for a final volume of 400 nl. The crystallization preparations were incubated at 16 °C and the progress of the experiment was documented by an automated imaging-system (Desktop Minstrel UV, Rigaku Europe, Kent, UK). Furthermore, fine screens (e.g. for refinement of crystallization conditions) were set up by hand in 24-well MTPs using the hanging drop vapour diffusion method.

PFOMT

PFOMT protein was concentrated to (6 to 8) mg/ml and rebuffered to 10 mM Tris/HCl pH 7.5 using Amicon® Ultracel centrifugal concentrators (10 kDa MWCO). The concentrated protein solution was centrifuged at 14 000 × g, 4 °C for 10 min to remove any insoluble material or aggregates. Flavonoids and phenylpropanoid substrates were added to the protein solution from 10 mM stock solution in dimethyl sulfoxide (DMSO). Crystallization screens were set up as described above. *apo*-PFOMT was crystallized using the following conditions – 2 M (NH₄)₂SO₄, 20 %glycerol. The protein solution contained 0.25 mM SAE, 0.25 mM MgCl₂, 0.25 mM eriodictyol and 7.53 mg/ml (0.262 mM) PFOMT .

Crystallization of proteins using NADES

NADES have the potential to be excellent solvents for hydrophobic compounds such as flavonoids or cinnamic acids [14] and in addition they are able to stabilize and activate enzymes [33].

Four different model proteins (bovine trypsin, hen-egg white lysozyme, proteinase K and *Candida cylindrica* lipase B) were used to assess the capability of NADES for protein crystallization. PCH was tested in a full factorial grid layout using PCH concentrations of (20, 30, 40 and 50) % combined with buffers of different pH. The buffers included 0.1 M sodium acetate pH (4.5 and 5.5), 0.1 M sodium citrate pH 6.5, 0.1 M 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic

acid (HEPES)/NaOH pH (7 and 7.5) and 0.1 M Tris/HCl pH 8.5. Thus, the full factorial design had a size of $4 \times 6 = 24$ different conditions. Protein solutions were prepared from lyophilized protein and were as follows: 90 mg/ml trypsin in 10 mg/ml benzamidine, 3 mM CaCl₂; 75 mg/ml lysozyme in 0.1 M sodium acetate pH 4.6; 24 mg/ml proteinase K in 25 mM Tris/HCl pH 7.5 and 6 mg/ml lipase B in water. For crystallization 2 µl enzyme solution and 1 µl reservoir buffer were mixed and set up in a hanging drop experiment on a 24-well MTP. The experiments were set up at 4 °C.

3.5.2 Data collection and processing

Crystallographic data were collected at the beamline of the group of Professor Stubbs (MLU, Halle, Germany). The beamline was equipped with a rotating anode X-ray source MicroMax007 (Rigaku/MSC, Tokio, Japan), which had a maximum power of 0.8 kW (40 kV, 20 mA) and supplied monochromatic Cu-K_α-radiation with a wavelength of 1.5418 Å. Diffraction patterns were detected with a Saturn 944+ detector (CCD++, Rigaku/MSC, Tokio, Japan).

Indexing and integration of the reflexes via Fourier transformation (FT) was accomplished using *XDS* [38, 39, 40] or *MOSFLM* [67]. *Scala* [25], which is integrated in the Collaborative Computational Project No. 4 (CCP4)-Suite, was used for scaling of the intensities.

3.5.3 Structure solution

For the determination of the electron density $\rho(\mathbf{r})$, where \mathbf{r} is the positional vector, from the diffraction images by FT two terms are necessary as coefficients; the *structure factor amplitudes*, $F_{\text{obs}}(\mathbf{h})$ and the *phase angles or phases*, $\alpha(\mathbf{h})$, where \mathbf{h} is the reciprocal index vector. The structure factor amplitudes can be directly determined from the measured and corrected diffraction intensities of each spot. However, the phase information is lost during the detection of the diffracted photons and there is no direct way to determine the phases. This constitutes the so-called *phase problem*. Thus, additional phasing experiments are necessary in order to obtain the phases. A variety of phasing experiments are available, which include

marker atom substructure methods, density modification and molecular replacement (MR) techniques [72]. Phases of the structures herein were exclusively determined by MR [69, 70].

MR was performed using the software *Phaser* [57, 58], which is included in the CCP4-Suite [95]. A previously published PFOMT structure (PDB-code: 3C3Y [46]) was used as a template during MR procedure for the PFOMT structure solution. For the MR of the lysozyme structure the PDB-entry 4NHI was used.

3.5.4 Model building, refinement and validation

Macromolecular model building and manipulation, as well as real space refinement and Ramachandran idealization were performed using the Crystallographic Object-Oriented Toolkit (*Coot*) software [23]. Structure refinement was done using the software REFMAC5 [62, 89] as part of the CCP4-suite or the Phyton-based Hierarchial Environment for Integrated Xtallography (PHENIX) [1]. Validation of the structures was carried out using the web service MolProbity (<http://molprobity.biochem.duke.edu/>) [12]. Structure visualization and the preparation of figures was performed using PyMOL (Schrödinger, New York, USA).

3.5.5 *In silico* substrate docking

In silico molecular docking studies were performed using the AutoDock Vina 1.1.2 or AutoDock 4.2.6 software in combination with the AutoDockTools-Suite (<http://autodock.scripps.edu/>) [34, 60, 88]. Substrates were docked into the PFOMT structure with the PDB-code 3C3Y. The grid box, which determines the search space, was manually assigned to center at 1.581, 5.196 and 25.718 (x, y, z) and had size of (22, 20 and 25) Å (x, y, z). The exhaustiveness of the global search for AutoDock Vina was set to 25, whereas the rest of the input parameters were kept at their defaults.

3.6 Analytics

3.6.1 Recording of growth curves

Starter cultures (≈ 2 ml) of the transformed *E. coli* cells were prepared in the medium to be studied, containing the appropriate antibiotics. The cultures were incubated at 37°C , 200 rpm over night and harvested by centrifugation ($5000 \times g$, 4°C , 5 min). The pellet was resuspended in 15 ml PBS and the suspension centrifuged ($5000 \times g$, 4°C , 5 min). The supernatant was discarded and the washing step repeated once more. The washed pellet was resuspended in 2 ml of the medium to be studied with the appropriate antibiotics and the OD_{600} was measured. Three independent 50 ml cultures of the medium containing the appropriate antibiotics were inoculated such that $\text{OD}^{600} \approx 0.05$ using the washed cell suspension. The cultures were incubated at the conditions to be studied and sampled at appropriate intervals of time (≈ 1 h). One ml samples were kept on ice until all samples were acquired. 100 μl aliquots of the samples were transferred into a clear MTP and the OD_{600} was measured. Green fluorescent protein (GFP) fluorescence was measured accordingly, but the MTP used was opaque. Excitation (λ^{ex}) and emission (λ^{em}) wavelengths were (470 and 510) nm respectively.

3.6.2 *In vitro* determination of glucose

The glucose concentration in clarified, aqueous samples was determined by a modified version of the glucose assay kit procedure provided by Sigma-Aldrich [80]. Glucose oxidase (GOD) oxidizes D-glucose to gluconic acid, whereby hydrogen peroxide is produced. The hydrogen peroxide can be detected and quantified by horseradish peroxidase (HRP), which reduces the produced H_2O_2 and thereby oxidizes its chromogenic substrate *o*-dianisidine via consecutive one-electron transfers. The oxidized diimine form of *o*-dianisidine can then be measured photospectrometrically [13].

The methodology employs a coupled photospectrometric assay using GOD and HRP with *o*-dianisidine as reporter substrate. The assay was prepared in MTP-format. A reaction solution containing 12.5 U/ml GOD, 2.5 U/ml HRP and 0.125 mg/ml *o*-dianisidine dihydrochloride in 50 mM sodium acetate pH 5.1 was prepared.

Sample solutions from culture supernatants were typically diluted in 9 volumes of

water. The reaction was started, by adding 50 µl reaction solution to 25 µl of sample and was incubated at 37 °C and 200 rpm for 30 min in a shaking incubator. 50 µl 6 M sulfuric acid was added to stop the reaction and achieve maximum color development (full oxidation of any *o*-dianisidine charge transfer complexes) (Figure 3.2). The developed pink color was measured at 540 nm in a MTP-reader. A calibration curve of a standard D-glucose solutions (0 to 100 µg/ml), that was always part of the experiments, was used to quantify the sample measurements.

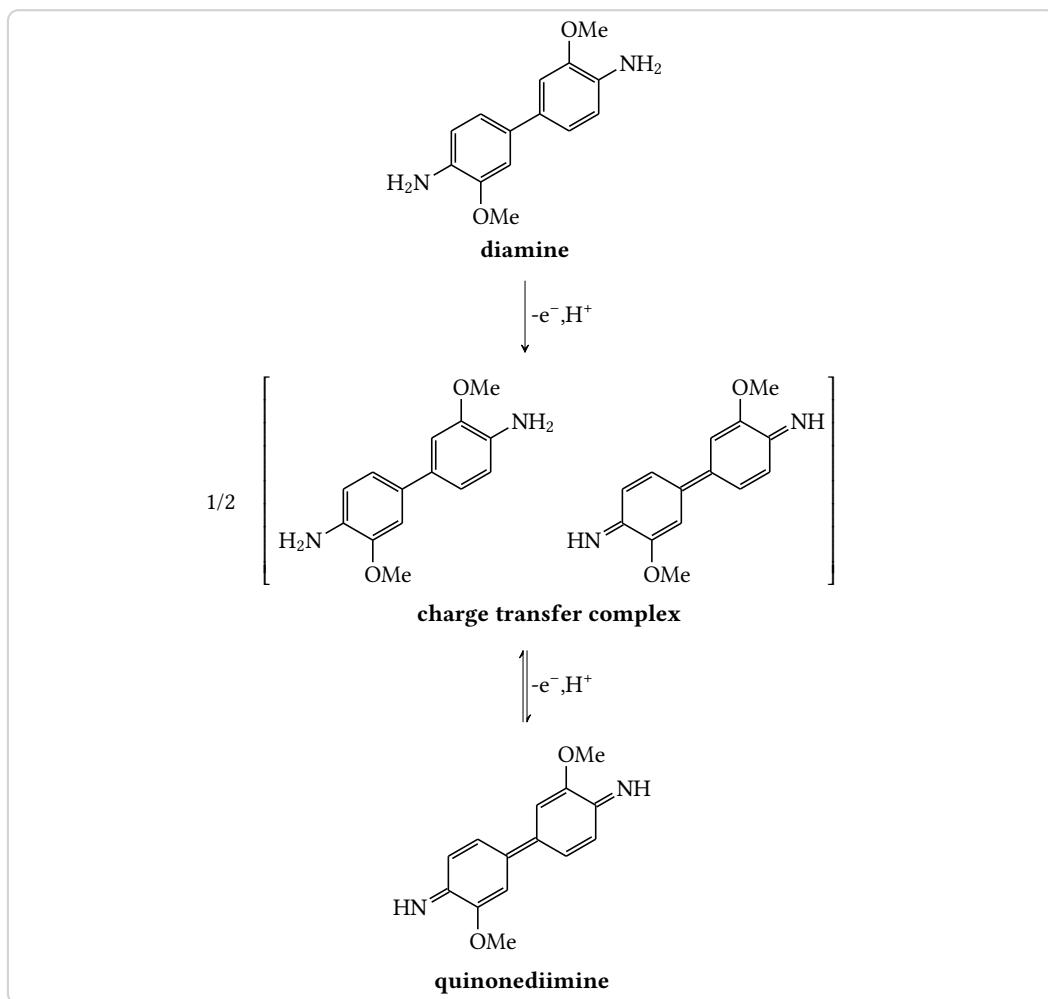


Figure 3.2.: Oxidation of the reporter substrate *o*-dianisidine. Consecutive one-electron transfers lead to the fully oxidized diimine form of *o*-dianisidine. The first electron transfer is believed to produce a charge transfer complex intermediate. [13, 37]

3.6.3 *In vitro O-methyl transferase (O-MT) assay*

O-methyl transferase (*O*-MT) assays were conducted in a total volume of (50 to 100) µl. The standard assay buffer was 100 mM Tris/HCl, 2.5 µM GSH pH 7.5. 1 mM MgCl₂, which was otherwise omitted, was added for reactions using cation dependent *O*-MTs (e.g. PFOMT). Reactions contained 0.5 mM alkyl donor (e.g. (S,S)-SAM) and 0.4 mM flavonoid or cinnamic acid substrate. Enzymatic reactions were started by addition of enzyme (usually 0.2 mg/ml) and incubated at 30 °C.

Reactions were stopped by addition of 500 µl ethyl acetate containing 2 % formic acid and vortexed for 15 s to extract the hydrophobic phenylpropanoids and flavonoids. After centrifugation (10 000 × *g*, 4 °C, 10 min) the organic phase was transferred into a new tube. The reaction was extracted once more with 500 µl ethyl acetate, 0.2 % formic acid and the pooled organic phases were evaporated using a vacuum concentrator (Concentrator 5301; eppendorf, Hamburg, Germany). The residue was dissolved in methanol and centrifuged at 10 000 × *g* for 10 min to remove unsoluble matter. The supernatant was transferred into a HPLC vial and analyzed by HPLC (3.6.8).

When detection of hydrophobic (e.g. flavonoids) and hydrophilic compounds (e.g. SAM, SAH) was performed simultaneously reactions were stopped by addition of 0.3 volumes 10 % (w/v) TCA in 50 % acetonitrile. The mixture was vortexed for complete mixing and incubated on ice for at least 30 min. After centrifugation (10 000 × *g*, 4 °C, 10 min) the supernatant was transferred into HPLC-sample vials and analyzed (see 3.6.8).

Measurement of activity/pH profiles

Assays to measure activity over larger pH ranges were set up in 50 mM L-malic acid/MES/Tris (MMT)- (pH 4 to 9) or succinate/sodium phosphate/glycine (SSG)-buffer (pH 4 to 10) to keep the concentrations of buffer salts constant for each pH [63].

The protein of interest was first extensively dialyzed against the reaction buffer (e.g. MMT, SSG) at pH 7 with added EDTA (5 mM) and then against the same buffer without EDTA. Standard reaction conditions were 50 mM buffer, 0.4 mM

alkyl acceptor (e.g. caffeic acid), 0.5 mM SAM, 2.5 µM GSH and 0.2 mg/ml enzyme. MgCl₂ was either omitted or added at 10 mM to assess influences of divalent cations. Assays were stopped as described in 3.6.3 and analyzed accordingly.

3.6.4 Photospectrometric assay for the methylation of catecholic moieties

Catecholic moieties can form stable complexes in the presence of heavy metals such as copper or iron [59, 76]. Hence, caffeic acid can complex ferric (Fe³⁺) ions and form a colored complex with $\lambda_{\text{max}} = 595 \text{ nm}$ [18]. Since the complex formation is specific for caffeic acid and methylated derivatives (i.e. ferulic and iso-ferulic acid) cannot complex Fe³⁺, this can be used as a measure for methylation reactions. O-MT assays were prepared as before (3.6.3). However, the reactions were stopped by addition of 0.1 volumes 1 M Tris/HCl pH 8, immediately followed by 0.5 volumes catechol reagent (2 mM FeCl₃ in 10 mM HCl). The complex formation reaction was allowed to equilibrate for 5 min at RT and the absorbance at 595 nm was measured.

3.6.5 Concentration of SOMT-2 using hydrophobic interaction chromatography (HIC)

After refolding using rapid dilution protein samples are very dilute and a concentration step is required. Refolded SOMT-2 was concentrated directly from the refolding buffer using hydrophobic interaction chromatography (HIC).

The ammonium sulfate concentration of the protein sample was brought to 1 M using a 2 M (NH₄)₂SO₄ solution and the pH was adjusted to 7 using 5 M NaOH. The sample was centrifuged (20 000 × g, 4 °C, 30 min) to remove insoluble material and the clarified supernatant was applied to a 1 ml HiTrap Phenyl FF (Low Sub) (GE Healthcare, Freiburg, Germany), which had been equilibrated with high salt buffer (1 M (NH₄)₂SO₄, 50 mM HEPES pH 7). The target protein was eluted using a stepwise gradient ((1, 0.8, 0.6, 0.4, 0.2 and 0) M (NH₄)₂SO₄, 50 mM HEPES pH 7; 5 CV each) to remove the ammonium sulfate. The column was washed using 20 %

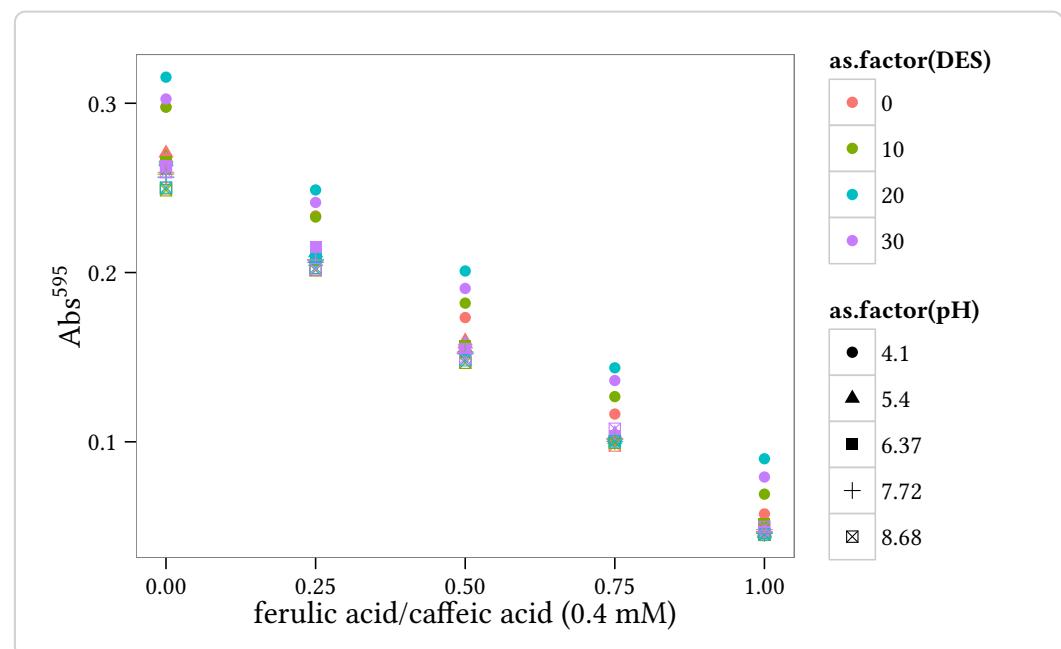


Figure 3.3.: Calibration curves of different relative compositions of ferulic acid to caffeic acid, that were taken as described in 3.6.4. The total concentration was always 0.4 mM. At lower pH values around 4, the method seems to overestimate the concentration of caffeic acid. However, the slope of the curves stays the same.

ethanol. Before SDS-PAGE analysis the eluted high salt fractions were desalting using TCA precipitation (3.4.4).

3.6.6 Analytical gel filtration

Analytical gel filtration was done using a Superdex 200 10/300 GL column (GE Healthcare, Freiburg, Germany) in combination with a FPLC system according to the manufacturers instructions. The column was equilibrated using an appropriate buffer (e.g. 0.1 M Tris/HCl pH 7.5) and 100 µl of sufficiently concentrated (≥ 1 mg/ml) protein sample were injected. The Gel Filtration Standard by Bio-Rad (München, Germany) was run separately to assess the size of the proteins in the analyzed sample.

3.6.7 Binding experiments using Isothermal Titration Calorimetry (ITC)

ITC can be used to directly characterize the thermodynamics of an observed process, be this a binding interaction or an enzymatic reaction [27].

ITC measurements to describe the interaction between PFOMT and its substrates/-effector were performed using a MicroCal iTC200 device (Malvern, Worcestershire, UK). PFOMT protein was extensively dialyzed against 50 mM MMT-buffer pH 7 prior to ITC experiments. The solution was subsequently centrifuged ($14\,000 \times g$, 4 °C, 10 min), to remove insoluble matter and aggregates. The dialysate was stored at 4 °C and used to prepare substrate and effector solutions. Generally 50 µM protein was provided in the ITC cell and the effectors/substrates to be titrated were loaded into the syringe. The substance concentration in the syringe was ten times higher than the protein solution. Experiments were carried out at 20 °C unless otherwise stated. The stirring speed was set to 500 rpm. The injection volume was set to (2 to 4) µl, amounting to a total of 10 to 19 injections.

3.6.8 High-performance liquid chromatography (HPLC) analytics

Due to their aromaticity, methanolic extracts of flavonoids exhibit two major absorption peaks in the UV/VIS region of the light spectrum in the range of (240 to 400) nm [54]. However, even the more simple phenyl propanoids (e.g. cinnamic acids) show absorption of light in the UV/VIS-region.

Methanolic extracts of flavonoids and phenyl propanoids were analyzed by HPLC using a photo diode array (PDA)-detector, which was set to record in the range of (200 to 400) nm. HPLC runs were performed on a reverse-phase C-18 end-capped column (YMC-Pack ODS-A; YMC Europe, Dinslaken, Germany) with a pore size of 120 Å. The mobile phase was aqueous acetonitrile supplemented with 0.2 % formic acid. The flow was kept constant at 0.8 ml/min. 10 µl *O*-MT enzyme assay extract (3.6.3) were injected and analyzed using an acetonitrile gradient starting with 5 % acetonitrile (4 min). The acetonitrile content was increased to 100 % in 21 min and was kept at 100 % for 5 min. Peaks were integrated from the 280 nm trace using the software provided by the manufacturer of the device.

3.6.9 liquid chromatography coupled mass-spectrometry (LC/MS) measurements

4 Evaluation of PFOMT towards the acceptance of long-chain SAM analogues

4.1 Introduction

Small changes to molecules can have profound influences on their chemical, physical and biological properties. Butyric acid esters differing only by a few methylene groups already exhibit quite divergent smells. However, not only the macroscopically qualitative properties can differ. The quantifiable psychotomimetic effect of methylated and ethylated lysergic acid amids differ by at least an order of magnitude [32, 79]. There are many more of these so-called structure activity relationships (SARs) and quantitative structure activity relationships (QSARs) studies on any number of compounds and situations [4, 56, 74].

Methylation reactions are one of the key tailoring steps during natural product biosynthesis and can in consequence greatly affect a molecules bio- and physico-chemical behavoir [51, 84]. However, between the highly complex core structures of natural products, which are produced by a plethora of enzymes (e.g. poly ketide synthases (PKSs), non-ribosomal peptide synthases (NRPSSs), terpene cyclases), and the rather simple alkyl-modification introduced by methylation nature is missing some medium-sized modifaction options, that proceed as elegantly as the methylation by MTs. Thus, natural products containing longer chain alkyl modifications

like ethyl or propyl moieties on O, N or S-centers have rarely, if ever been observed.¹

It has recently been shown however, that a wide array of SAM analogues are used as co-substrates by a variety of MTs [84]. The majority of the work so far has been done on P-MTs and DNA MTs (Figure 4.1), since epi-genetics and finding regions of gene-reulation is of great interest. There have been a great many of SAM analogues synthesized, both chemically and with the help of enzymes [15, 81].

However, the first description of novel synthetic SAM analogues with extended carbon chains, including SAE, allyl and propargyl derivatives, that were also shown to be useful in modifying DNA via the action of several DNA MTs was provided by Dalhoff, *et al.* [15, 16]. It was also noted, that allyl transalkylation reactions proceeded much faster than ethyl- or propyl transfers possibly due to conjugative stabilization of the transition state [15]. A whole variety of allyl derivatives was examined by different researchers and site-specific introductions of allyl, pent-2-en-4-ynyl and even 4-propargyloxy-but-2-enyl moieties into proteins (i.e. histones) was demonstrated using P-MTs [66, 92]. However, the larger substrate analogues were not necessarily accommodated by the native P-MTs making engineering efforts for the accommodation of larger substrates inevitable [92]. The specific introduction of alkyne functionalized groups made it then possible to use click chemistry for further functionalization and/or detection of the labelled proteins, DNA or RNA and has been studied extensively (Figure 4.2) [61, 66, 75, 92, 94].

In 2012 Bothwell and Luo even described the exchange of the sulfonium with a selenonium center, which afforded SeAM analogues that have since then been described as substrates for several P-MTs [8, 9]. SeAM analogues have the advantage of being more resistant to chemical decomposition than their sulfur counterparts, but also show enhanced transmethylation reactivity [8].

There have also been some reports on the use of SAM analogues by small molecule MTs. In 2009 Stecher *et al.* reported the use of the C-methyl transferases (C-MTs) NovO and CouO along with synthetic SAM analogues to accomplish biocatalytic

¹Reaxys searches for natural product isolates with a molecular mass between (150 and 1500) containing the substructures methyl, ethyl or propyl connected to a heteroatom return 66759, 2797 and 52 results respectively. However, it stands to note that 70 % of the propyl results were either esters or otherwise activated moieties. [22]

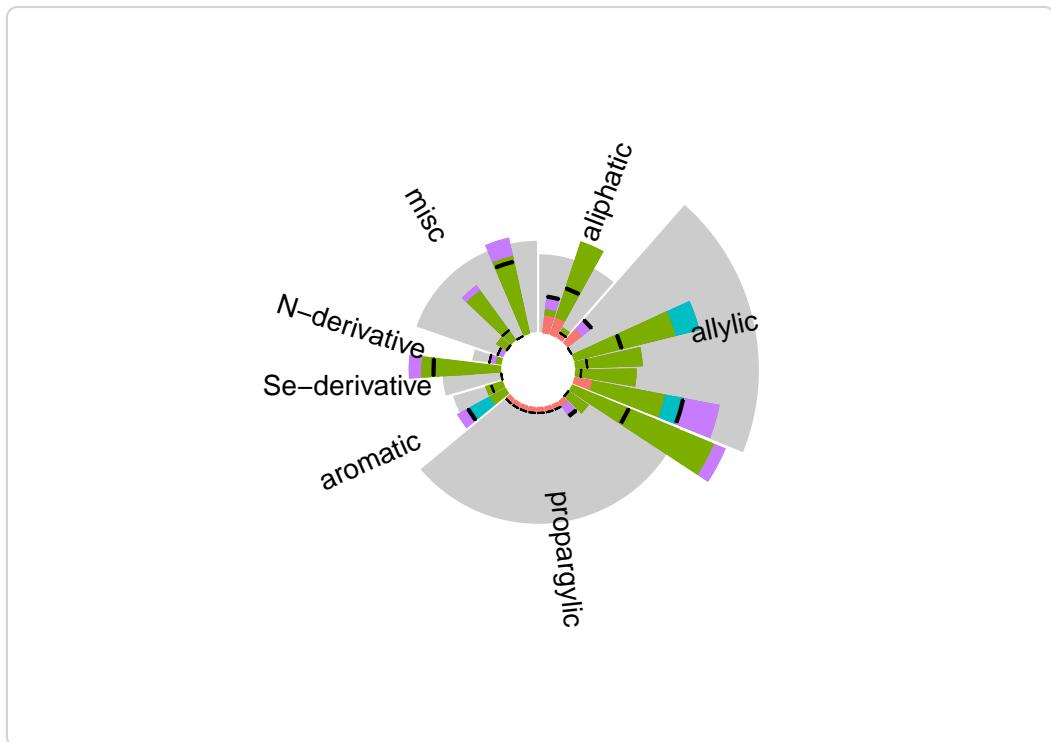


Figure 4.1: Graphical representation of the work that has been done on MTs in combination with SAM analogues. The grey areas represent individual groups of SAM analogues (aliphatic, allylic, propargylic, aromatic, SeAM analogues, nitrogen analogues and miscellaneous others). The height of the grey areas represents the number of times a member of the corresponding group has been described as tested in the MT literature. The height of the colored bars represents the times that individual substrate has been tested. The colors represent the different types of MT (red – DNA MT, green – P-MT, lilac – small molecule MT, blue – rna MT). The black dash across the bar shows the number of times this substrate was actually converted by either enzyme.

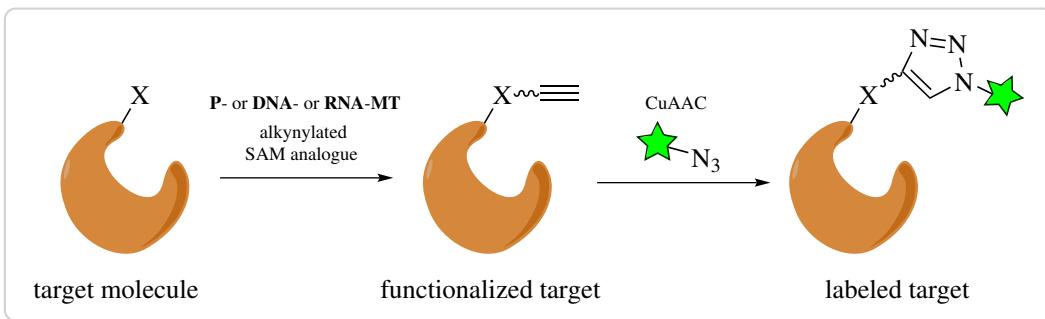


Figure 4.2.: Labelling of macromolecules by using a combination of novel alkyne-derivatized SAM analogues and Cu^I-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC). Depending on the type of label used, it can be employed for detection (e.g. through fluorophores, coupled assays) or affinity purification (e.g. biotin). This technique is also feasible for use in activity based protein profiling (ABPP) approaches.

Friedel-Crafts alkylations of some aminocoumarine antibiotics [83]. Lee *et al.* were the first ones to describe the transfer of a keto-group from an SAM derivative by means of the small molecule MTs catechol O-methyl transferase (EC 2.1.1.6) and thiopurine S-methyl transferase (EC 2.1.1.67) [49]. Furthermore there was work done on the O-MTs RebM and RapM, which modify the antitumor active natural products rebeccamycin and rapamycin respectively, that shows the general feasibility of using SAM analogues in combination with MTs to modify small molecules [48, 81, 98]. In all of these reports the specificity of the group transfer is retained despite the fact that SAM analogues are employed as substrates.

There is of yet no bioactivity data reported that shows the biological activity of the newly produced compounds.

PFOMT is highly promiscuous towards its flavonoid substrate [35, 46]. However, the promiscuity towards different SAM analogues has not yet been described. Combination of both, substrate and co-substrate promiscuity in the small molecule MT PFOMT could provide a powerful tool towards the biosynthetic production of novel small molecules with potentially new and promising biological activities. Functionalization/Detection of substrates could furthermore provide a means of finding new compounds/substrates in complex (e.g. biological) samples analogous to activity based protein profiling (ABPP) approaches. It was thus of interest, whether or not PFOMT would accept SAM analogues as alkyl donors. The already

extensively studied PFOMT was the prime candidate, since the preparation and crystallizability were established and lots of substrates had already been described [11, 35, 46, 90].

4.2 Substrate binding studies using ITC

The binding of different substrates by PFOMT was examined by ITC. SAH, SAM and SAE were selected to study the influence of the alkyl chain length on binding (??). Furthermore the binding of the substrate caffeic acid and the influence of Mg^{2+} addition on substrate binding was investigated.

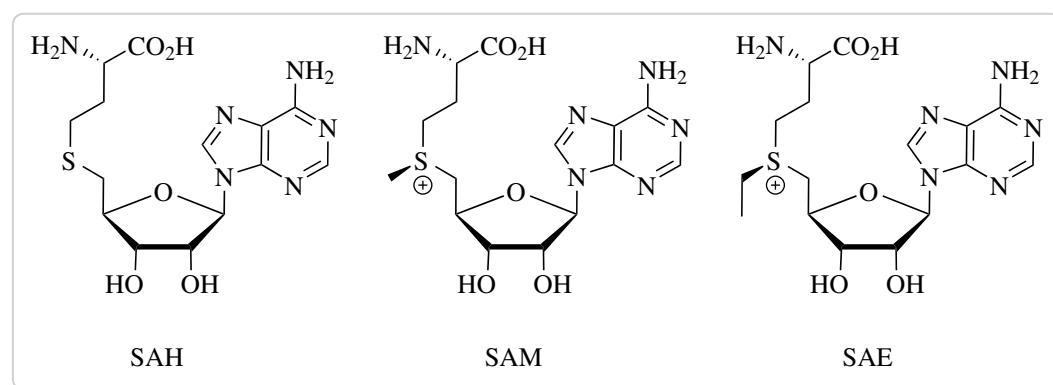


Figure 4.3.: The binding of different SAM analogues was measured via ITC, but also calculated using molecular modelling techniques.

The K_D values of SAH, SAM and SAE were all in the low micromolar range, around $2\text{ }\mu\text{M}$. However, the binding enthalpy clearly decreased with the length of the aliphatic chain connected to the sulfur atom (Figure 4.4a). The binding of SAH, gave off more heat than the binding of SAM, which in turn gave off more heat than the binding of SAE. Thus, the entropic influence must get larger with increasing chain length in order for (4.1) and (4.2) to still hold true. Indeed, the value for ΔS was negative for binding of SAH and got positive for the binding of SAM and SAE (Table 4.1). This relationship between the change of entropy and the change of enthalpy has been found for many biological systems and is also called enthalpy-entropy compensation (EEC) [21, 30, 77]. The stoichiometry for the binding process is given by the parameter N (Table 4.1). For all the ligands

SAH, SAM and SAE this value was found to be about 0.5, which corresponds to one bound molecule ligand per dimer of PFOMT.

Upon titration of caffeic acid to PFOMT small amounts of heat were given off by the system (Figure 4.4c). When the enzyme was incubated with SAH prior to addition of caffeic acid this heat increased slightly. The slope of the ITC profile also got steeper. However, the data obtained could not be fitted to afford a sensible solution. When caffeic acid and Mg²⁺ were incubated with PFOMT prior to addition of SAH, the process of heat production as observed by ITC also had a steeper slope. Nonetheless, the thermodynamic parameters did not differ significantly. On its own Mg²⁺, in the form of an MgCl₂ solution, titrated to the enzyme solution did not cause signals during the ITC experiments.

$$\Delta G = \Delta H - T\Delta S \quad (4.1)$$

$$\Delta G = \Delta G^0 - RT \ln K \quad (4.2)$$

Table 4.1.: Results of fitting a simple one-site binding model to the data obtained from ITC experiments.

	K_D [μM]	ΔH [cal mol ⁻¹]	ΔS [cal mol ⁻¹ K ⁻¹]	N
SAH	2.06 ± 4.27	$-10\ 380 \pm 1025$	-9.41	0.505 ± 0.038
SAM	1.08 ± 3.50	-4606 ± 242	11.6	0.492 ± 0.018
SAE	2.22 ± 3.79	-1338 ± 190	21.3	0.513 ± 0.050

4.3 Study of variants for long-chain alkylations

The work described in this section was done in cooperation with Dr. Martin Dippe. Dr. Dippe did most of the work on the PFOMT variants described herein.

Since the ability to bind the elongated analogue SAE was present in wildtype PFOMT, the activity of the PFOMT protein was tested. Activity tests were performed with caffeic acid as substrate under standard reaction conditions. The wildtype

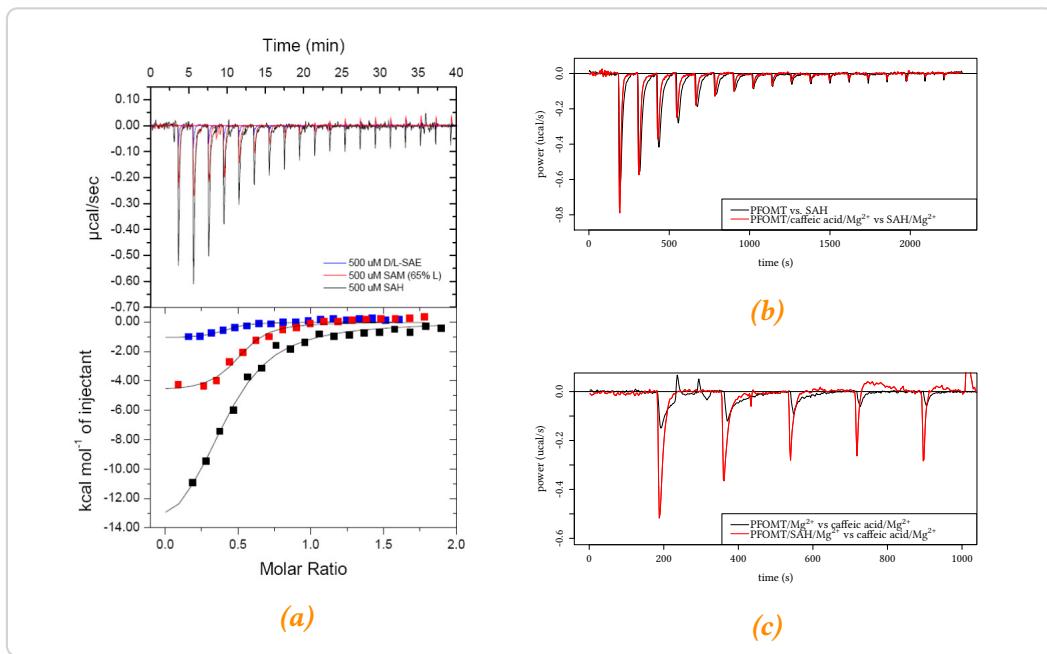


Figure 4.4: Exemplary ITC measurements. **a** – Binding of SAH, SAM and SAE to PFOMT. **b** – SAH is injected into a PFOMT solution, with (red) or without (black) addition of Mg²⁺ and caffeinic acid. When Mg²⁺ and caffeinic acid were already present, the binding process seems to happen quicker, but is less enthalpic. **c** – Upon addition of caffeinic acid to the protein heat is produced, however no sensible binding curve could be obtained.

of PFOMT was able to use SAE as a co-substrate for the ethylation of caffeic acid, albeit the amount of detected product was very minute. The site of ethylation was determined by liquid chromatography coupled mass-spectrometry (LC/MS) measurements. It was found that ethylation occurs on the catecholic group, however it could not be determined whether at the 3- or 4-position. Nonetheless it is highly likely that ethylation occurs at the same position as methylation and thus the product was annotated as 3-ethoxy-4-hydroxy cinnamic acid.

Enzyme variants were prepared to further test the ethylation reactivity of PFOMT, since a number of groups were able to accomplish transalkylation with larger substrates by expanding the available space in the active site [92]. Residues that were exchanged were selected based upon their position in the active site and in relation to the substrate(s) (Figure 4.5). Fortunately a crystal structure of PFOMT was available to help with the selection.

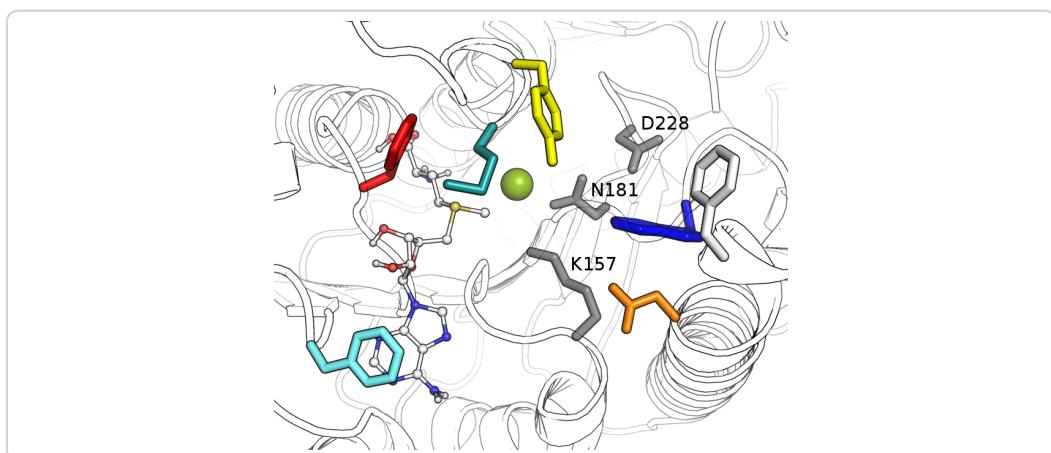


Figure 4.5.: The active site of PFOMT. The outline of the protein backbone is displayed, with active site residues portrayed as colored sticks (cyan – F103, red – F80, turquoise – M52, yellow – Y51, white – F198, blue – W184, orange – N202, grey – as labelled). The co-substrate SAM (ball-and-stick model) was docked into the structure.

Over 20 enzyme variants were prepared to assess, whether PFOMT ethylation activity would improve over the wildtype. Be that as it may, an improved ethylation activity was not observed. Some of the new variants however displayed an increased methylation activity with the substrates caffeic acid and SAM. The methylation activity of some of the variants increased by over 4-fold. Interestingly most amino

acid substitutions proved as beneficial, rather than detrimental.

Methylation activity benefited greatly from the replacement of bulky hydrophobic residues by smaller and/or charged residues in the vicinity of the acceptor substrates (Tyr51, Trp184 and Phe198). However, this was not a general trend since the substitutions N202W and Y51W also improved methylation activity. Looking more closely at residue Tyr51, the activity enhancing effect was greatest, when the tyrosine was substituted by the basic amino acids lysine or arginine. In addition to an enhanced activity the selectivity for the hydroxyl position to be methylated was also altered in these variants. This was not apparent, when caffeic acid was used as a substrate. However when a flavonoid, especially eriodictyol, was used not only the 3' hydroxyl, but to some extent the 4' hydroxyl was methylated. This effect was improved in some double variants, where also position 202 was altered. For example the variant Y51R N202W almost exclusively methylated flavonoid substrates at the 4' position. A detailed discussion of the results was published in a peer reviewed journal.

4.4 Crystallization of PFOMT

The binding of the non-natural substrate SAE to PFOMT could be shown. However, a transethylation reactivity was not observed. The question was to the chemical reasons behind these observations. Previous work on the crystal structure of PFOMT had been done, but only SAH could be co-crystallized [46]. But since the crystallizability of PFOMT had already been shown, this method was chosen to answer the aforementioned question.

At first the already crystallization procedures were evaluated to start with [46]. Albeit, reproduction of the results could not be accomplished and new crystallization conditions had to be found. This was done using commercially crystallization screening kits and a semi-automated pipetting robot along with an automated imaging system for the observation of the crystallization plates.

Each buffer solution was screened in combination with three different protein solutions (*A* – 0.25 mM SAH, 0.25 mM MgCl₂, 0.25 mM ferulic acid, 0.262 mM PFOMT; *B* – 0.25 mM SAE, 0.25 mM MgCl₂, 0.25 mM eriodictyol, 0.262 mM PFOMT and *C*

– 0.25 mM SAH, 0.25 mM MgCl₂, 0.25 mM ferulic acid, 0.219 mM PFOMT Y51R N202W) to obtain protein crystals co-crystallized with the substrates.

During the preparation of the protein solutions it was noted, that upon addition of the flavonoids or phenyl propanoids from DMSO stocks these tended to precipitate. Thechniques meant to circumvent this problem are discussed in chapter 7. Crystals began to appear in various wells after a few days and were observed for each tested protein solution at least once. The crystal shape varied from very smooth and almost cubic (high ammonium sulfate) over sphreulites and intergrown crystals (CaCl₂, PEG-4000) to brittle and ragged needles (LiCl, PEG-6000) (Figure 4.6).

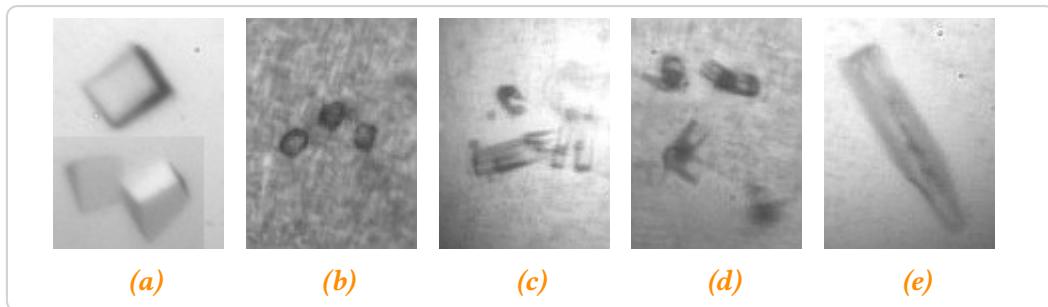


Figure 4.6.: Some crystal and pseudo-crystal shapes that were observed during the crystallization screen. a – high (NH₄)₂SO₄, b-c – CaCl₂, PEG-4000, e – LiCl, PEG-6000

Crystals that were large enough ($\geq 50 \mu\text{m}$), where screened for diffraction right away. A rough estimate of the resolution, cell parameters and the space group was aquired, if the diffraction images could be indexed. The screened crystals all had similar cell parameters and belonged to the same space group, *P2₁2₁2₁*. However, the unit cell of crystals that grew out of high ammonium sulfate concentrations ($\geq 1.8 \text{ M}$) was approximately four times as large as that of the published structure 3C3Y and crystals that developed under different crystallization conditions. Several datasets were collected of crystals from high (NH₄)₂SO₄, since these possessed different cell parameters than the previously reported structure and therefore seemed to be promising candidates for bound substrates.

The crystal structure of *apo*-PFOMT

Most of the collected datasets were partly solved. As it turned out however the substrates were not co-crystallized. Rather, the *apo*-form of PFOMT had been crystallized. Thus, one dataset was solved to completion to obtain a novel PFOMT structure with no substrate bound and a resolution of 1.95 Å (Table ??). The asymmetric unit of *apo*-PFOMT contained two homodimers (4 monomers) (Figure 4.7a), rather than just one homodimer (3C3Y). The active site of each monomer was found to be empty except for a sole sulfate ion, which was positioned where the amino- and carboxylate groups of the SAH reside in the 3C3Y structure (Figure 4.7b). Shifts in the structure of some loops were observed and contrary to the previously published structure the entire N-terminus was resolved up to the His-tag.

The resolved N-terminus contained another N-terminal α -helix, which was positioned in a cleft on the surface, where substrates may be bound [46]. This interaction extends up to the His-tag. Considerable movement was observed in different parts of the protein, when no substrate was bound, some of which can be attributed to SAM and metal ion binding residues (Figure 4.8 and Figure A.1) as is obvious for the loop region between β -sheet 1 and α -helix 4. Nonetheless, most of the movement seemed to be restricted to areas, which are not directly involved in the binding of either SAM or metal ions. However, all of the regions that moved are located at or near the active site.

4.5 Conclusion/Discussion

Whereas the binding of SAH was solely dependent on the large negative enthalpy, the binding of SAE was almost entirely driven by entropy, since ΔH was close to 0 (4.1). Entropy gain can be a major driving force for ligand-protein interactions and in some cases ligand binding can be entirely attributed this gain in entropy [52]. Displacement of protein-bound water molecules contributes strongly to the entropic gain. There were some waters present in the active site of PFOMT in the crystal structure developed herein. However, no metal ion was present in the active site in the *apo*-PFOMT structure. Furthermore Mg^{2+} titration via ITC did not afford significant signals, suggesting the notion, that the metal is only bound

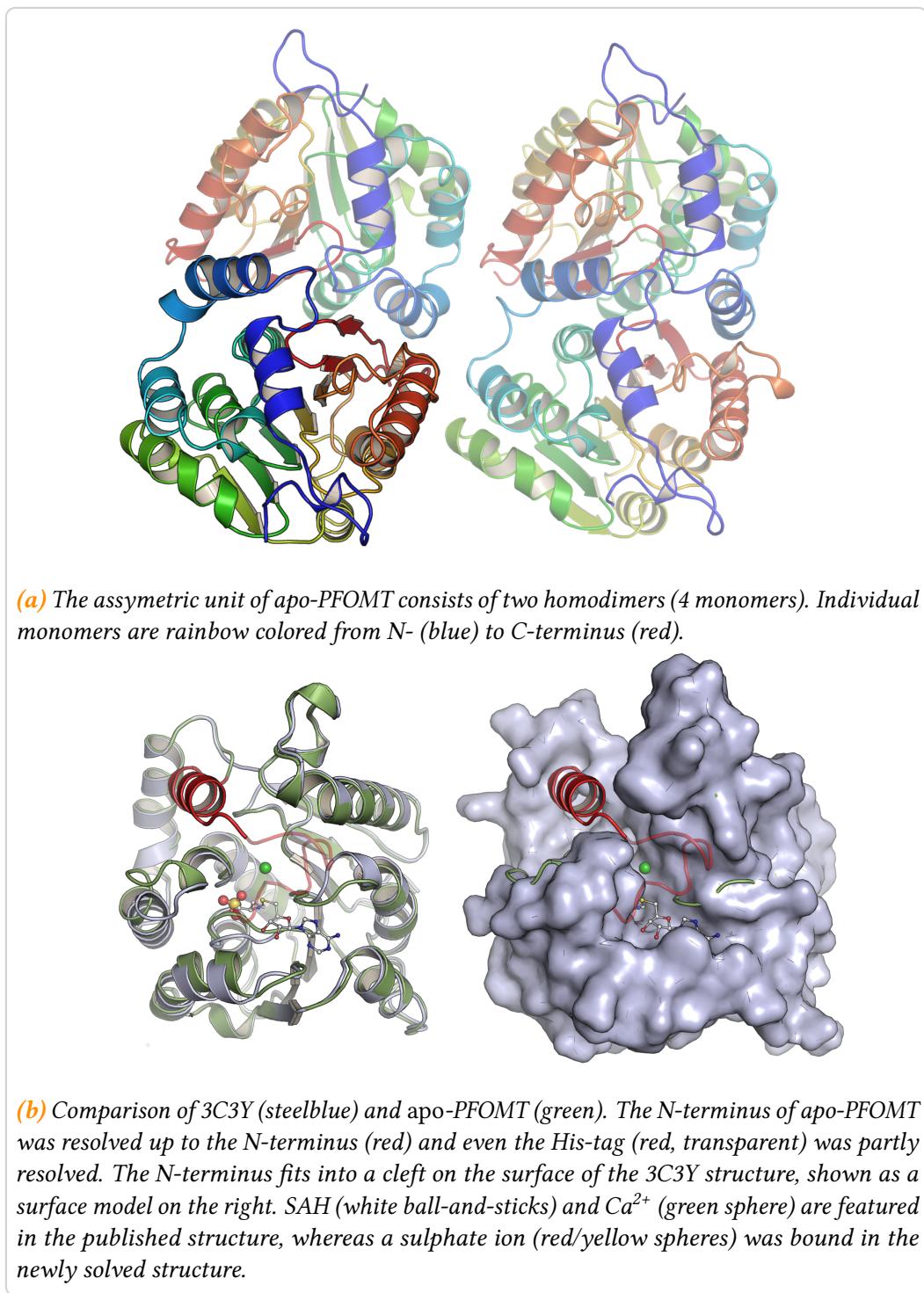


Figure 4.7.: An overview of the features in the apo-PFOMT structure.

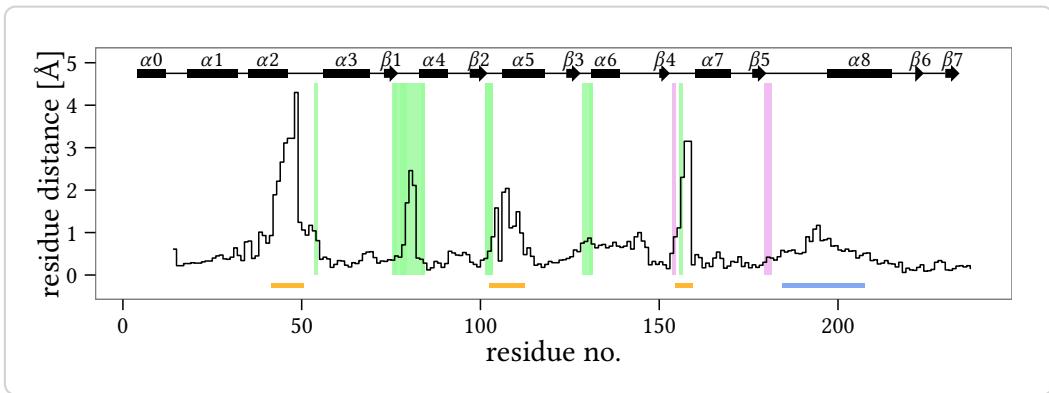


Figure 4.8.: Positional differences between the individual residues of the solved apo-PFOMT and the structure with bound SAH (pdb: 3C3Y). The diffraction precision indicator [17] (DPI) of the structures was (0.137 and 0.064) Å respectively. The overall rmsd amounted to 0.9034 Å. The secondary structure of apo-PFOMT is displayed at the top. Helices are displayed as rectangles and sheets are shown as arrows. Graphical background annotations are used to display the binding sites of SAH (green) and the metal ion (plum). The orange bars indicate regions, where much movement seems to happen upon binding or release of the co-substrate. The blue bar shows the region that was annotated as "insertion loop" in previous studies [46].

along with the co-substrate (Figure 4.9). It has been suggested, that the entropy cost to transfer one water molecule from bulk to the protein-bound state can be up to 7 cal mol⁻¹ K⁻¹ [20]. The replacement of ordered waters from the active site or from a hydrated metal ion by a growing aliphatic chain could therefore explain the gain in entropy, and SAH is positioned in a way to warrant exactly that (Figure 4.9). Also, the hydrogen and metal complexing bonds consequently lost could explain the less negative enthalpy. However, this is purely hypothetical since more evident data is missing. Additional insight might be gained by expanding the ITC experiments to even longer SAM analogues. The limited space in the active site, which forces the growing side chain to expel water might also be the reason for the inactivity of PFOMT towards SAE. If the metal ion is blocked from complexing moieties, activation of the substrate hydroxyl would be hindered.

Comparison of the novel *apo*-PFOMT and the published structure suggests a lot of movement along multiple parts of the backbone all proximal to the active site pocket, upon ligand binding (Figure 4.8). The N-terminus of PFOMT seems to acts

as a lid, which is closed in the *apo*-form, but highly flexible and therefore unresolved in the ligand bound form. Furthermore, the native enzyme has been shown to be truncated, starting only at residue 12 and being less catalytically efficient than the full length protein [46, 90]. The work presented here consequently supports the notion that the N-terminus plays an important role on the regulation of the enzymatic activity.

The laid out findings provide novel hints for regions in the PFOMT structure that can be studied using site-directed mutagenesis approaches in order to work towards a variant that can in fact employ SAE for transalkylation reactions. Furthermore variation of these regions might provide enzymes with altered substrate specificities which are of high interest.

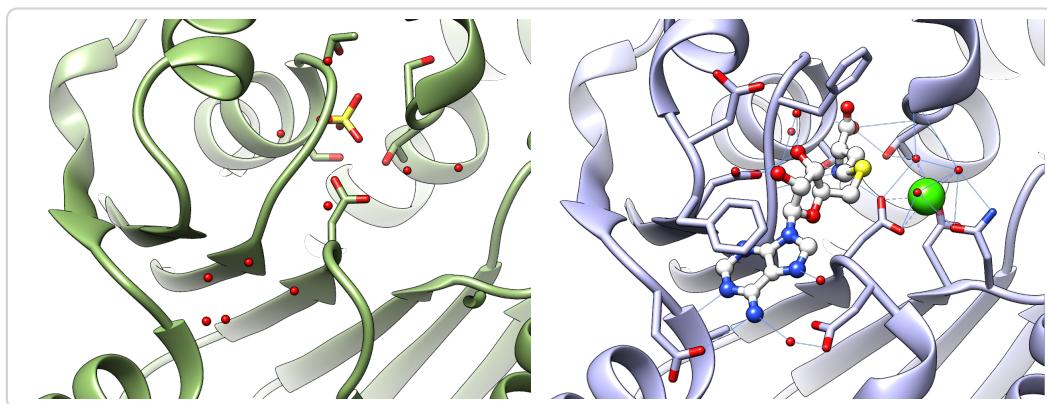


Figure 4.9.: Comparison of the active sites of the apo-structure (left, green) and the ligand-bound structure (right, steelblue). Waters are represented as small red spheres, calcium as a green sphere (complexing bonds are dashed) and SAH is displayed as a white ball-and-stick model. A possible hydrogen bond network (blue lines) for the ligand-bound state is displayed.

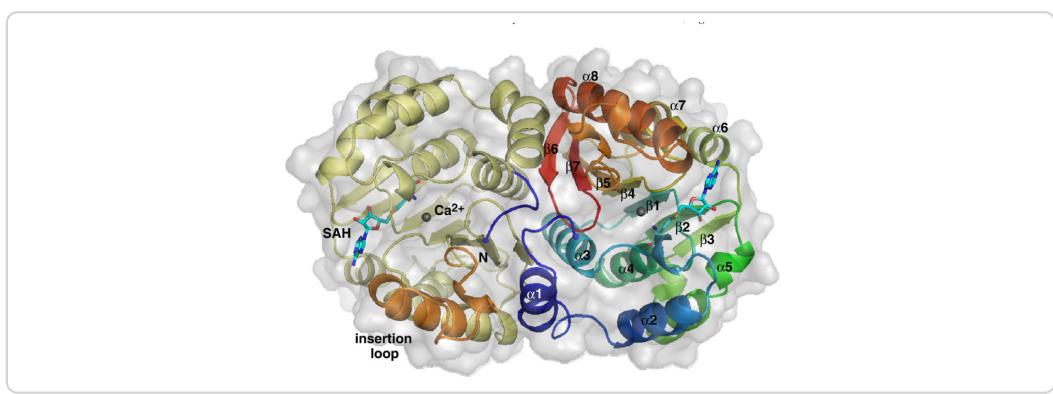


Figure 4.10: from: Kopycki, J. G., Rauh, D., Chumanovich, A. a., Neumann, P., Vogt, T., & Stubbs, M. T. (2008). Biochemical and Structural Analysis of Substrate Promiscuity in Plant Mg²⁺-Dependent O-Methyltransferases. *Journal of Molecular Biology*, 378(1), 154–164. <http://doi.org/10.1016/j.jmb.2008.02.019>

5 Enzymatic methylation of Non-catechols

5.1 Introduction

Non-catechols in nature (biosynthesis, mode of action?), chemical methylation???
phenol vs methoxyphenols vs catechols

The 4'-hydroxyl of naringenin is non-catecholic in nature. However it is much more acidic than the 4'-hydroxyl of eriodictyol (pK_a 9.8 vs 12.7) and thus about equally as acidic as the 3'-hydroxyl of eriodictyol (pK_a 9.7)¹ [Schweigert2001a].

5.2 SOMT-2

SOMT-2 has been described in the literature to methylate multiple flavonoids at the 4'-position of the B-ring [42, 43]. There it also showed the highest activity towards naringenin, to produce ponciretin (also known as isosakuranetin). Furthermore it has been the only characterized enzyme of this nature described in literature. Thus, SOMT-2 was selected as a model candidate for enzymes that can methylate 4'-hydroxyls of non-catecholic flavonoids.

¹ pK_a values were calculated using ChemAxon's MarvinBeans 15.2.16.0

5.2.1 *In vivo* biotransformation in *N. benthamiana*

The group of Sylvestre Marillonet (IPB) established an efficient system to clone and assemble multi enzyme pathways in *N. benthamiana*, using a modular cloning toolbox, which has already been used to produce flavonoids [45]. The group had already cloned all the enzymes required to establish the pathway up to naringenin in *N. benthamiana* (Figure 5.1). However, the *SOMT2* gene needed to be cloned into suitable vectors to be transiently expressed by *N. benthamiana*. The gene was first cloned into a level 0 module using *BpiI* and consequently subcloned into a level 1 module flanked by a promoter and a terminator using *BsaI* (Figure 5.2). The 35S-promoter was used alongside the nopaline synthase (*nos*)-terminator to achieve the highest possible transcription rates.

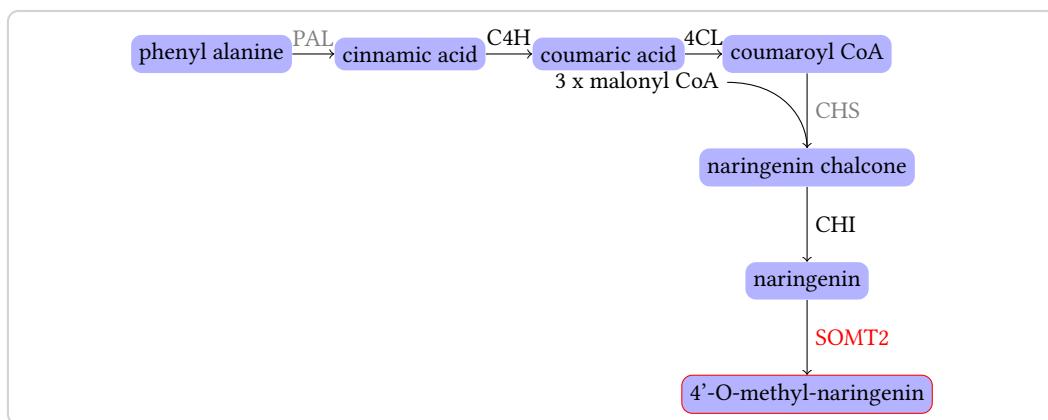
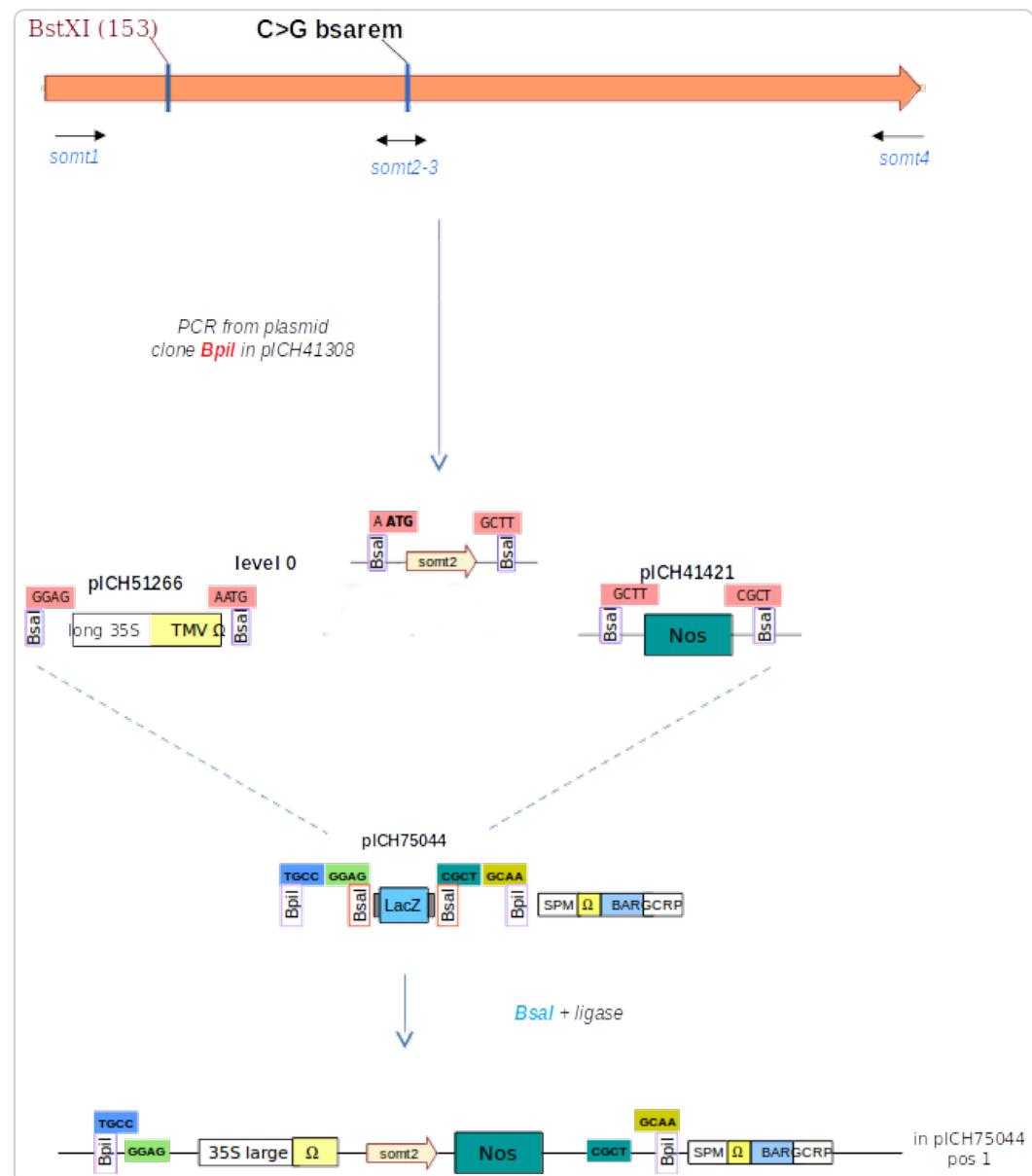


Figure 5.1.: Semi-synthetic pathway to naringenin and 4'-O-methyl naringenin in *N. benthamiana*. Enzymes not endogenous to *N. benthamiana* are in gray. PAL - phenylalanine ammonia lyase, C4H - cinnamic acid 4-hydroxylase, 4CL - 4-coumaric acid:CoA ligase, CHS - chalcone synthase, CHI - chalcone isomerase, SOMT2 - soy O-methyl transferase 2

Both sides of *N. benthamiana* leaves were infiltrated with different samples. The left side was infiltrated with *A. tumefaciens* cultures transformed with pAGM10733 (phenylalanine ammonia-lyase (PAL)), pAGM10406 (chalcone synthase (CHS)) and pBEW107 (SOMT-2). For the right side the *A. tumefaciens* culture containing pBEW107 was replaced by a control: *A. tumefaciens* transformed with the empty vector pICH75044. After 7 days, the plant material was harvested. The average weight loss after freeze drying was 87.5 %.

**Figure 5.2.: Cloning of SOMT-2**

The dried material was extracted and analyzed via HPLC to determine whether ponciretin or related compounds were produced (Table 5.1). However, through comparison with authentic standards it was apparent, that none of the expected compounds were detected. This finding suggest, that neither naringenin, nor any resulting flavonoids (ponciretin, poncirin, didymine) were present in detectable amounts in the plant tissue at the time of harvest. Although unlikely, it cannot be excluded that higher amounts of the compounds of interest were present at some point in the tissue. Numerous more experiments were required to address the issue of non-detection, which however was outside the scope of this work.

Table 5.1: Naringenin and 4'-methylated derivatives.

	R ¹	R ²	name
	H	H	naringenin
	CH ₃	H	ponciretin
	CH ₃	rutinose ¹	poncirin
	CH ₃	neohesperidose ²	didymine

The HPLC chromatograms were analyzed by principal component analysis (PCA) after the data were aligned, centered and scaled, to assess whether the collected plant material samples were different from one another (Figure 5.3 and A.2). The PCA-plot shows that the samples of the different leaf sides do not separate, indicating no difference between infiltration with the *SOMT* gene and vector control between the first two principal components, which account for 80 % of the variance. However, there is a slight separation between top and bottom leaves in the second principal component and between plant 3 and plants 1/2 in the first principal component. This suggest, that the chemical composition as detected by HPLC is slightly different in the top and bottom leaves, as well as between plants.

¹α-L-rhamnopyranosyl-(1→ 6)-β-D-glucopyranose

²α-L-rhamnopyranosyl-(1→ 2)-β-D-glucopyranose

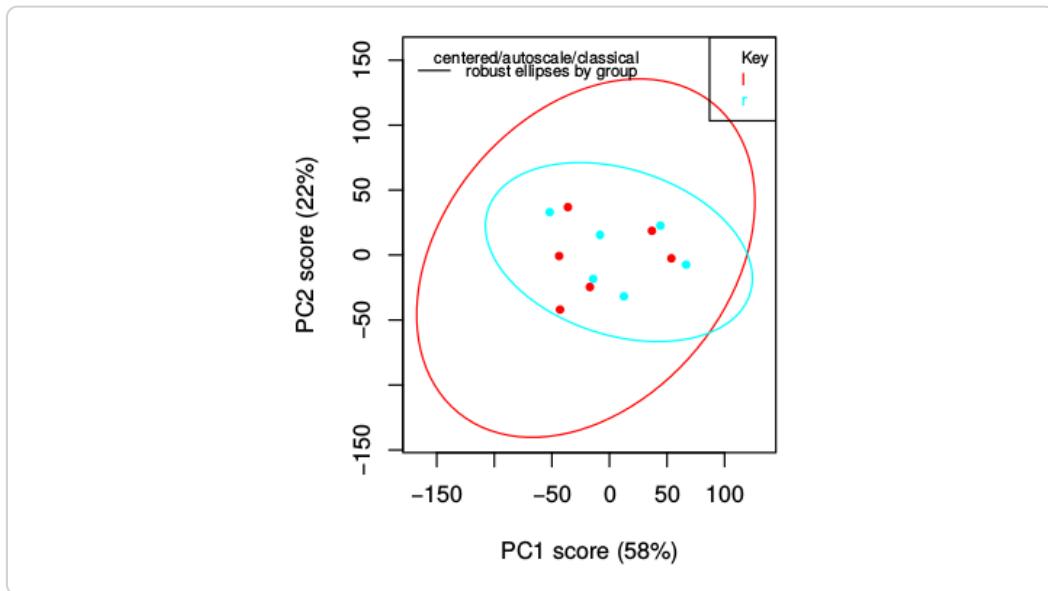


Figure 5.3.: PCA of leaf material. The samples are colored by leaf side.

5.2.2 *In vivo* biotransformation in *E. coli*

Kim *et al.* already showed, that SOMT-2 could be used for the biotransformation of different flavonoids in *E. coli* live cultures [42, 43]. Their technique was to be studied and expanded upon to gain additional insight into the enzyme. The SOMT-2 gene was cloned into the pET28a(+) and pET41a(+) vectors, to obtain constructs for the production of SOMT-2 without and with a N-terminal Glutathion S-transferase (GST)-tag, respectively since both have been used successfully by Kim *et al.*. However, methylated flavonoids were not detected when biotransformations were prepared according to the methods of the aforementioned authors (Figure 5.4).

Thus, the biotransformation medium was changed to auto-induction medium (ZYP-5052) [85]. 0.05 % glucose were present in the medium. Growth curves were measured, alongside glucose depletion, to establish the time at which substrate addition would be most beneficial. Furthermore, SDS-PAGE samples were prepared to observe protein accumulation. The glucose present in the medium was depleted after about 5 hours into growth. At that time expression of the SOMT-2 gene is expected to begin, because the catabolite repression on the *lac* promoter is relieved.

Thus, 0.1 mM flavonoid were added at 4 hours to minimize its influence on

growth and possible degradation. Although SDS-PAGE samples were prepared throughout the course of the experiment, accumulating SOMT-2 could not be clearly distinguished from endogenous *E. coli* protein in the SDS-PAGE gels (Figure A.3). Nonetheless, methylation of some of the tested substrates was observed over a course of 30 hours (Table 5.2).

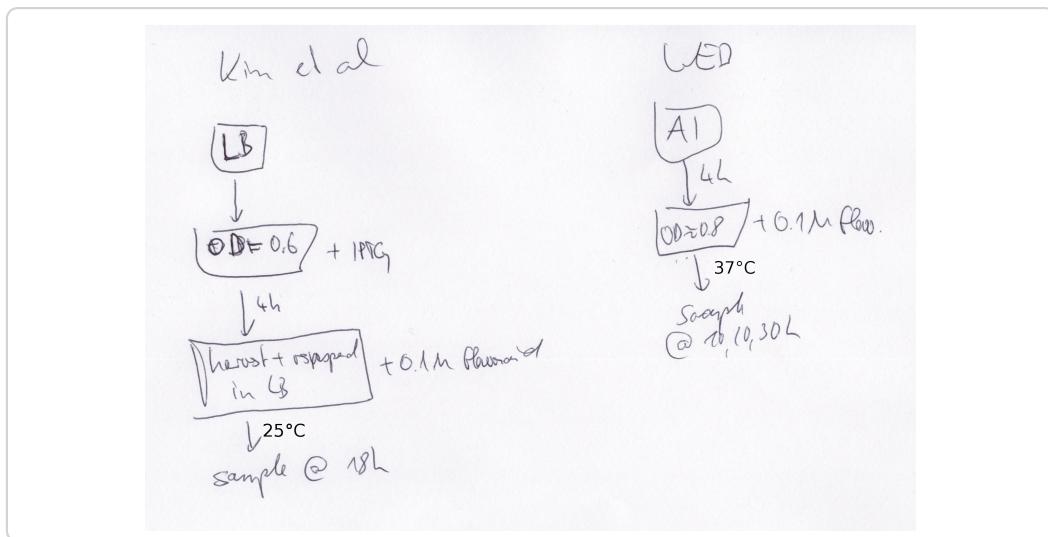


Figure 5.4: Biotransformation methods as described by Kim et al. (left) and developed in this work (right).

LC/MS was employed to determine the site of methylation, since this method is highly sensitive and numerous structural studies on flavonoids using tandem-mass spectrometry experiments have highlighted the feasibility of this approach [26, 50]. Collision induced dissociation (CID) was used to obtain structural information about the target molecules, since soft ionization techniques (e.g. electrospray ionization (ESI)) used in LC/MS instruments primarily produce protonated and deprotonated molecular ions, but rarely yield fragments [82]. The CID method collides the precursor ions with a neutral target gas while increasing the energy to induce fragmentation. The produced fragments vary depending on the energy chosen for fragmentation. Flavonoids follow certain different fragmentation pathways [26, 50]. The fragmentation of interest in this work, was the one along the C-ring, which produces two fragments (A- and B-ring) (Figure 5.6b). The mass of the A- and B-ring fragments gives strong evidence for the position (ring) at which

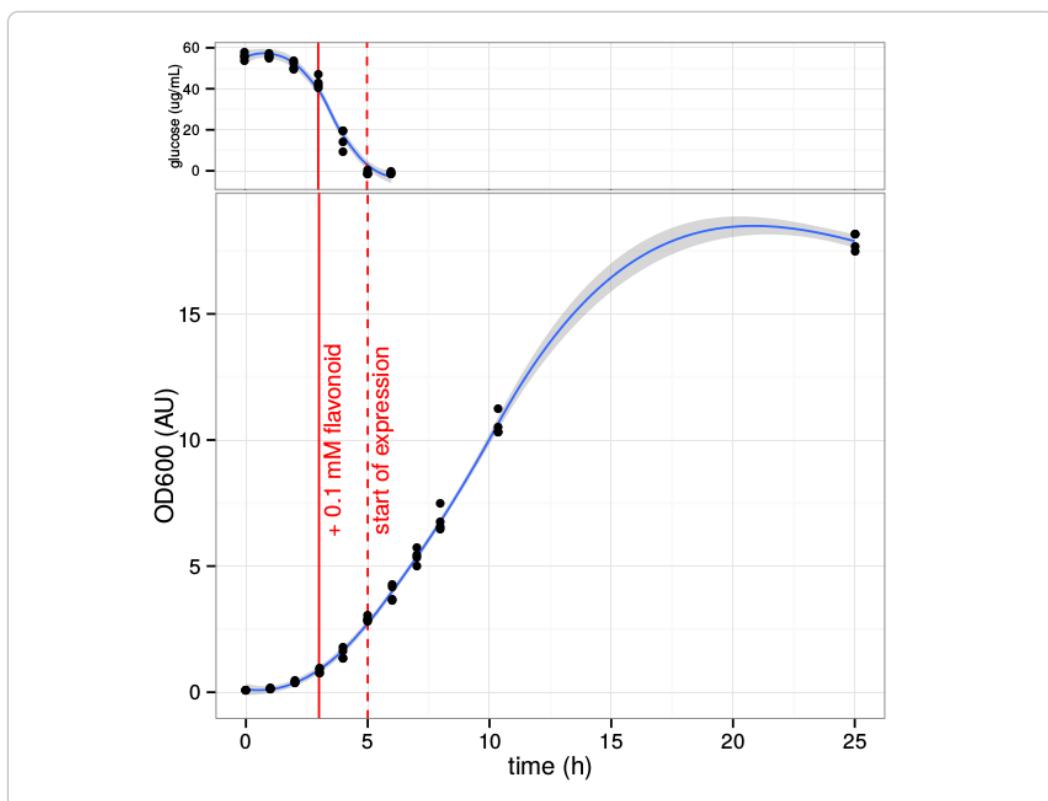


Figure 5.5: Growth curve of *E. coli* BL21(DE3) expressing SOMT-2 at 37 °C. Glucose is depleted about 5 hours into growth, at which point the start of SOMT-2 expression is expected. The OD₆₀₀ after inoculation was about 0.1.

methylation occurred. Using the CID technique, an energy of 30 eV proved sufficient to fragment most flavonoids along the C-ring as is shown here for the methylated naringenin (Figure 5.6). The molecular ion $[M+H]^+$ of the methylated naringenin has a mass-to-charge ratio (m/z) of 287.092. The fragments helping to derive structural information are m/z 133 and m/z 153, which can only be explained if the B-ring was methylated (Figure 5.6b). If the A-ring was methylated, the expected fragment ions of A and B-ring would have m/z -values of 167 and 119 respectively.

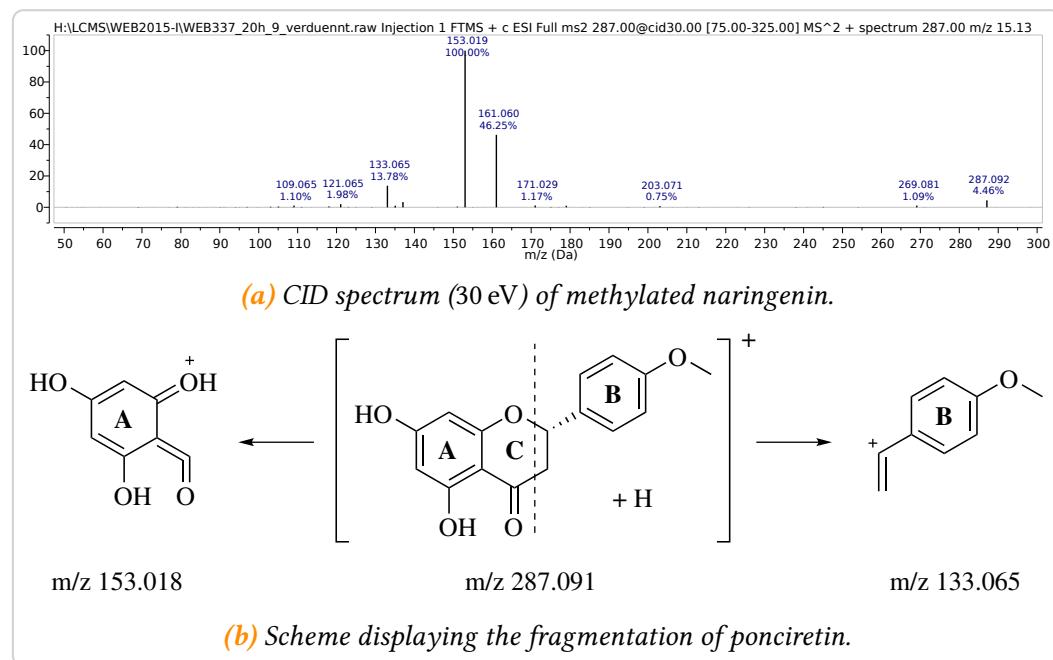


Figure 5.6.: The masses resulting from the fragmentation into A- and B-ring along the C-ring (dashed line, b) are evidence, that the 4'-hydroxyl on the B-ring is methylated by SOMT-2

The LC/MS results suggest, that methylation occurred exclusively at the 4'-hydroxyl. There was no conversion detected, when a 4'-hydroxyl was absent (Table 5.2). A free 4'-hydroxyl seems therefore necessary for a substance to be a substrate for SOMT-2, which confirms the previous results by Kim *et al.* [43]. Conversion was observed for flavonoids and the stilbene resveratrol, although conversion rates of the isoflavone genistein were very low. No conversion of anthraquinones, cinnamic acid derivates or chalcones was detected, which is also

in accordance with previously published data. SOMT-2 acts on phenolic, catecholic as well as (4-hydroxy-3-methoxy-phenyl)-moieties, as is suggested by the assay results that showed methylation of naringenin, eriodictyol and homoeriodictyol respectively. The methylation of (4-hydroxy-3-methoxy-phenyl)-moieties and of stilbenes are properties of SOMT-2 that have not been described before.

The conversion ratios were assessed, but are beset with large errors due to the nature of *E. coli* rich medium extracts. The highest conversions were observed for flavanones and flavones (up to $\geq 55\%$). The tested isoflavones and flavonols showed much lesser conversion ratios (less than 10%). The conversion ratios of apigenin ($\geq 54\%$) and naringenin ($\geq 55\%$) are comparable to the ones published by Kim *et al.*. However, genistein only showed minute conversions, which is converse to the data published by their group [43]. Conversion of eriodictyol, homoeriodictyol and kaempferol were not reported before.

The biotransformation of resveratrol to 3,5-dihydroxy-4'-methoxy-stilbene showed a conversion ratio of $\geq 86\%$ in 30 hours. This is roughly double the conversion which was recently reported for *in vivo* biotransformations using the specific resveratrol O-MT sbCOM1, which only achieved 42% conversion in 36 hours [41].

Table 5.2.: In vivo biotransformation of different flavonoids, phenylpropanoids and anthraquinones by SOMT-2 in *E. coli*

substrate	class	4'-OH	conversion ³	product ⁴
alizarin	anthraquinone	no	no	–
purpurin	anthraquinone	no	no	–
apigenin	flavone	yes	yes ($\geq 54\%$)	4'-O-methyl apigenin
chrysin	flavone	no	no	–
genistein	isoflavone	yes	yes (<1%)	Biochanin A ⁵
galangin	flavonol	no	no	–
kaempferol	flavonol	yes	yes ($\geq 6\%$)	kaempferide ⁶
naringenin	flavanone	yes	yes ($\geq 55\%$)	ponciretin

⁴as determined by LC/MS

⁵4'-O-methyl genistein

⁶4'-O-methyl kaempferol

eriodictyol	flavanone	yes	yes ($\geq 40\%$)	hesperetin
homoeeriodictyol	flavanone	yes	yes ($> 6\%$)	3',4'-(<i>O,O</i>)-dimethyl eriodictyol
hesperetin	flavanone	no	no	–
phloretin	chalcone	yes	no	–
resveratrol	stilbene	yes	yes ($\geq 86\%$)	4'- <i>O</i> -methyl resvera- trol
<i>p</i> -coumaric acid	cinnamic acid	yes	no	–
caffeic acid	cinnamic acid	yes	no	–
reosmin	cinnamic acid ⁷	yes	no	–

5.2.3 *In vitro* studies using recombinantly produced SOMT-2

In vivo biotransformations are an important tool for the primary characterization of enzymes. However, because live organisms are used and lots of variables are unknown, these systems can cause large errors and are not fit to thoroughly characterize an enzyme. Initially SOMT-2 was to be purified to homogeneity to be later thoroughly characterized *in vitro*, since Kim *et al.* had showed the recombinant production of SOMT-2 in *E. coli* as a fusion protein with an N-terminal T7-tag, but had missed to characterize the recombinant enzyme.

Initial protein production tests were carried out using *SOMT-2* cloned into pET28a(+) with an N-terminal His₆-tag. However, SOMT-2 was not produced in soluble form (Figure 5.7). Numerous systems were tested for the expression of *SOMT-2*. *E. coli* strains used for the trials included BL21(DE3), Rosetta(DE3), Origami(DE3), C41(DE3), C43(DE3), C41(DE3) pLys, C43(DE3) pLys and DH5 α . The *SOMT-2* gene was cloned into multiple other vectors, including pET20b for periplasmic protein production, pET32 for expression with an Trx-tag and vectors that carry promoters for induction by rhamnose. Multiple media, including TB, LB and autoinduction media were used along with different inducers (e.g. lactose,

³Calculation of conversion percentages are only rough estimates, because of the nature of crude medium extracts.

⁷homo-dihydro cinnamic ketone

rhamnose, IPTG) at different temperatures. Nonetheless SOMT-2 could not be produced in a soluble form and expression only resulted in IBs.

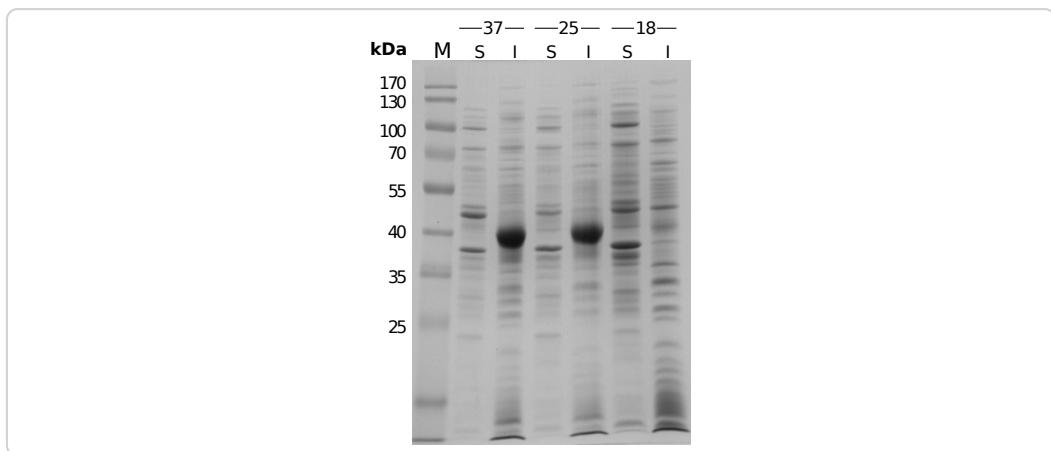


Figure 5.7.: SDS-PAGE of *pET28a(+)* SOMT-2 expressed in *E. coli* BL21(DE3) in autoinduction medium at different temperatures (shown above). The insoluble fractions show a protein band the same height as the 40 kDa marker band, which corresponds to the SOMT-2 protein (40 425 Da). M – protein size marker, S – soluble fraction, I – insoluble fraction

Since the SOMT-2 protein could not be obtained in soluble formen, when recombinantly expressed in *E. coli*, the IBs were prepared and used for *in vitro* refolding studies. Protein refolding is no trivial task. The refolding process competes with misfolding and aggregation processes and refolding buffers have to be optimized in order to obtain an efficient refolding system with the best possible results [71, 93, 97]. Refolding efficiency is best measured via biological activity, but even with adequate assays refolding studies are a time-consuming process of trial-and-error. The number of experiments required to even test only four variables, for example pH, salt, temperature and protein concentration with 3 states each (e.g. low, medium, high) in all possible combinations results in $3^4 = 81$. An experimental setup, which accounts for all possible variable (factor) combinations is also called a *full factorial design*.

Refolding studies were performed using a fractional factorial design (FFD).

5.3 PFOMT

5.3.1 Acidity and Nucleophilicity of phenolic hydroxyl-groups

5.3.2 pH-Profiles of PFOMT-catalysis

5.3.3 Influence of Mg^{2+} on PFOMT activity

5.4 Structural studies of flavonoids using tandem mass-spectrometry (MS/MS)

5.5 Conclusion/Discussion

6 Development of an whole cell methyl transferase screening system

6.1 Introduction

6.2 Theoretical considerations / design of system

6.3 Detectability of *S*-adenosyl-L-homocysteine (SAH)

SAM

6.4 Usage of the lsr-promoter for true autoinduction

6.5 Conclusion/Discussion

7 DES in protein crystallography

7.1 Introduction

7.2 Solubility enhancement of hydrophobic substances by addition of DES

7.3 Enzymatic *O*-methylation in DES

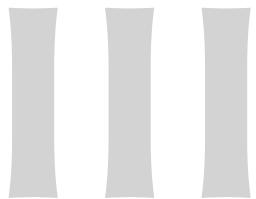
7.4 DES as precipitants in protein crystallization

7.5 Conclusion/Discussion

8 Acknowledgements

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Appendix

A Figures

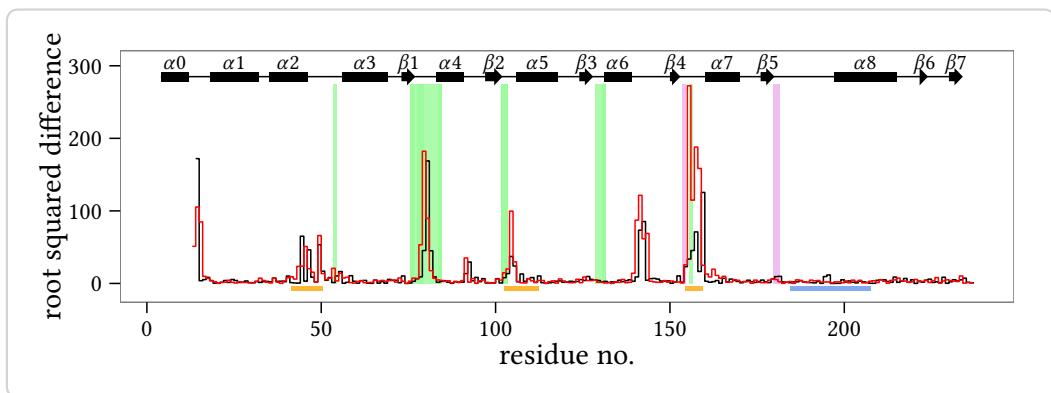


Figure A.1.: Differences in the dihedrals ψ (red) and ϕ (black) of the solved apo-PFOMT and the structure with bound SAH (pdb: 3C3Y). The secondary structure is displayed at the top. Helices are displayed as rectangles and sheets are shown as arrows. Graphical background annotations are used to display the binding sites of SAH (green) and the metal ion (plum). The orange bars indicate regions, where much movement seems to happen upon binding or release of the co-substrate. The blue bar shows the region that was annotated as "insertion loop" in previous studies.

Appendix A. Figures

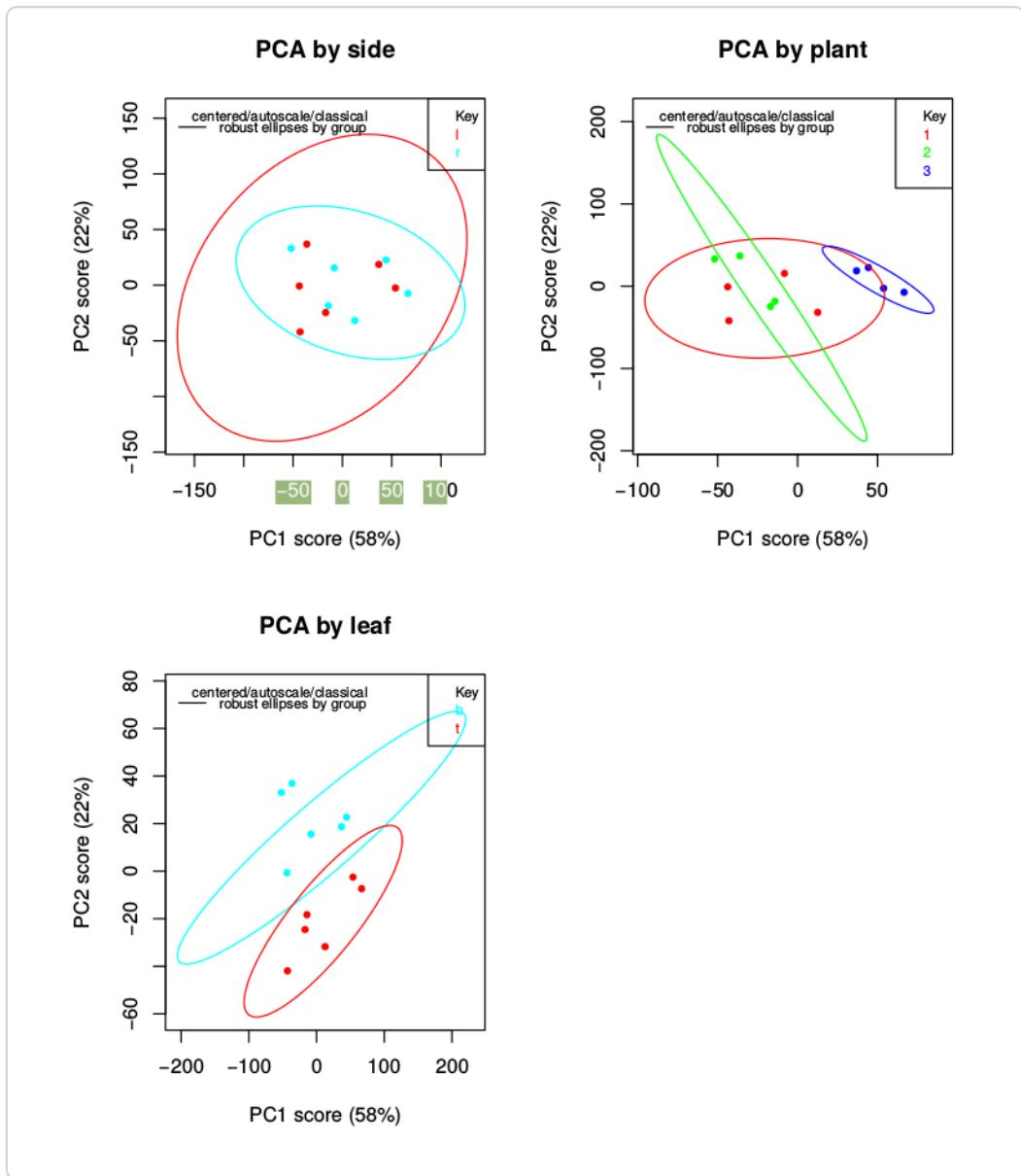


Figure A.2.: PCA of *N. benthamiana* leaves infiltrated by *A. tumefaciens* carrying different genes.

Appendix A. Figures

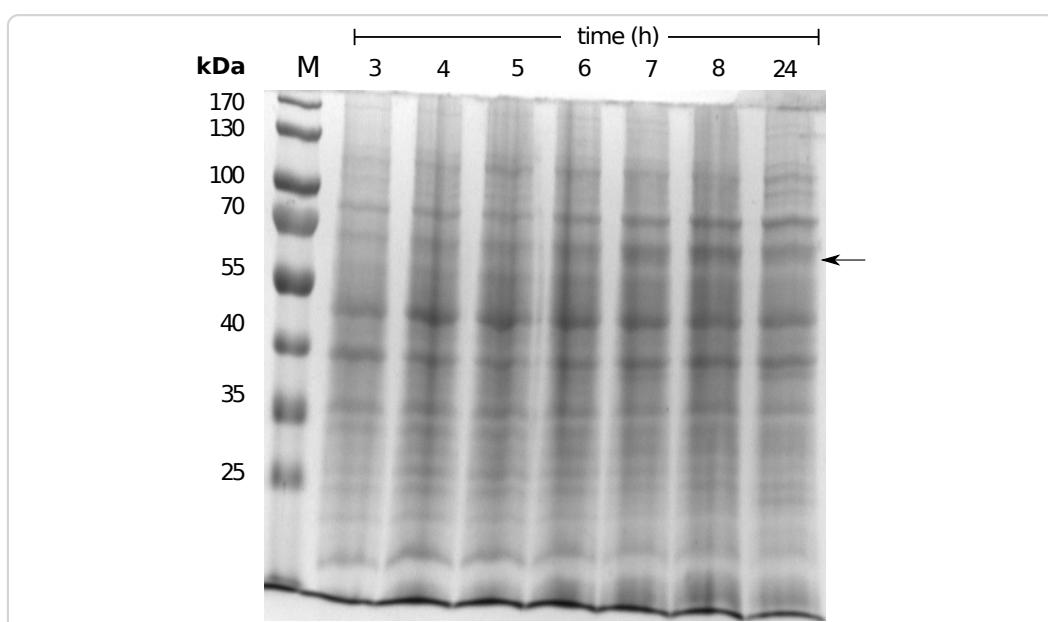


Figure A.3: SDS-PAGE gel of samples acquired during growth curve measurements.

B Tables

Table B.3.: SAM analogues that have been used with MTs. Targets: P – peptide/protein, D – DNA, R – RNA, S – small molecule.

analogue	enzyme	target	references
<i>SAM</i>			
-CH ₂ -CH ₃	PRMT1, M.TaqI, M.HhaI, M.BcnIB, RebM, RapM	S,P,D	[15, 48, 81, 87] ¹
-CH ₂ -CH ₂ -CH ₃	PRMT1, M.TaqI, M.HhaI, M.BcnIB	P,D	[15, 87]
-CH ₂ -CH ₂ -CH ₂ -CH ₃	PRMT1	P	[87]
-CH ₂ -C ₆ H ₅	NovO, CouO,	S,P	[83, 87]
-CH ₂ -C(=O)-CH ₃	PRMT1 COMT, TPMT, CazF	S	[49, 96]

¹Singh *et al.* (2014) published a series of 44 biocatalytically synthesized SAM and SeAM derivatives, most of which were not tested towards their alkyl donation potential in MT reactions.

Appendix B. Tables

analogue	enzyme	target	references
$-\text{CH}_2-\text{CH}=\text{CH}_2$	NovO, CouO, RapM, PRMT1, M.TaqI, M.HhaI, M.BcnIB, RebM, Tgs	P,S,D	[15, 48, 75, 81, 83, 86, 87, 91, 92]
$-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_3$	NovO, CouO	S	[83]
$-\text{CH}_2-\text{C}\equiv\text{CH}$	Dim-5, HsMLL, TRM1, NovO, CouO, PRMT1	P,R,S	[36, 83, 91, 92, 94]
$-\text{CH}_2-\text{C}\equiv\text{N}$	RebM	S	[81]
$-\text{CH}_2-\text{CH}_2-\text{C}\equiv\text{CH}$	PKMT	P	[36]
$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{C}\equiv\text{CH}$	PKMT	P	[36]
$-\text{CH}_2-\text{C}\equiv\text{C}-\text{CH}_3$	NovO, CouO, M.HhaI, M.TaqI, M.BcnIB	S,D	[15, 53, 83]
$-\text{CH}_2-\text{C}\equiv\text{C}-\text{CH}_2-\text{CH}_3$	M.HhaI	D	[53]
$-\text{CH}_2-\text{C}\equiv\text{C}-\text{CH}_2-\text{NH}_2$	M.HhaI	D	[53]
$-\text{CH}_2-\text{C}\equiv\text{C}-\text{CH}_2-\text{NH}-\text{C}(=\text{O})(-\text{CH}_2-)_3-\text{NH}_2$	M.HhaI	D	[53]
$-\text{CH}_2-\text{C}\equiv\text{C}(-\text{CH}_2-)_3-\text{NH}_2$	M.HhaI	D	[53]
$-\text{CH}_2-\text{C}\equiv\text{C}(-\text{CH}_2-)_3-\text{NH}-\text{C}(=\text{O})(-\text{CH}_2-)_3-\text{NH}_2$	M.HhaI	D	[53]
$-\text{CH}_2-\text{C}\equiv\text{C}(-\text{CH}_2-)_3-\text{C}\equiv\text{CH}$	M.HhaI	D	[53]
$-\text{CH}_2-\text{C}\equiv\text{C}(-\text{CH}_2-)_3-\text{N}_3$	M.HhaI	D	[53]
$-\text{CH}_2-\text{CH}=\text{CH}-\text{C}\equiv\text{CH}$	Dim-5, HsMLL, TRM1, PRMT1, Tgs	P,R	[36, 61, 66, 75, 91, 92, 94]
$-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{C}\equiv\text{CH}$			[36, 91]

Appendix B. Tables

analogue	enzyme	target	references
$-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{C}\equiv\text{CH}$	PRMT1	P	[91, 92]
<i>SeAM</i>			
$-\text{CH}_3$			
$-\text{CH}_2-\text{C}\equiv\text{CH}$	Dim-5, <i>HsMLL</i> , TRM1, RebM, CazF	P,R,S	[9, 81, 94, 96]
<i>N</i> -mustard derivatives			
$-\text{CH}_2-\text{CH}_2-\text{I}$	RebM	S	[98]

Appendix B. Tables

Table B.1: Overview over the constructs produced for the present thesis. Each step during the production of the construct is given in the workflow steps column. Primers (*italic font*) or restriction sites used during each step are displayed in parenthesis.

construct name	description	entry constructs	destination	workflow steps (primers/cloning sites)
pBEW101				
pBEW102	lsrA promoter			
pBEW103	pBEW102 with BamHI cloning site	pBEW102 pBEW4b	pBEW103	amplification (<i>pRhal1fw/rv</i>), cloning (BglII, BamHI)
pBEW104	rhaP_BAD promoter			
pBEW106	pICH413038-somt	pET28MC-somt	pICH413038	amplification (<i>somt1/2/3/4</i>), cloning (BpiI) golden gate cloning (BsAl)
pBEW107		pICH51266, pBEW106, pICH41421	pICH75044	golden gate cloning (BsAl)
pBEW1a				
pBEW1b				
pBEW2a				
pBEW2b				
pBEW3a				
pBEW3b				
pBEW4a				
pBEW4b				
pET28-pfomt	<i>pfomt</i> gene in pET-28a(+), endogenous NdeI site removed	pQE30-pfomt	pET-28a(+)	mutagenesis (<i>pfomt1fw/rv</i>), amplification (<i>pfom2fw/rv</i>), cloning (NdeI, EcoRI)
pET20-somt	N-terminal pelB-tag fusion for periplasmic expression			pET20-b(+) pET28-a(+)
pET28-somt				
pET28MC-somt				
pET32-somt				
pET41-somt	N-terminal TrX-tag fusion			pET-32a(+) pET-41a(+)
pUC19*	N-terminal GST-tag fusion added BglII site	pUC19 lsr-XX-DAS	-	mutagenesis (<i>pUC1.fw/rv</i>) cloning (NdeI, BglII)
pUCB1	pUC19 derivative with lsrA promoter			
pUCB1-sfGFP-DAS+4				

Appendix B. Tables

Table B.4.: Crystallographic data, phasing and refinement statistics.

	140519_PFOMT	MC001413-G10.1
<i>data collection</i>		
wavelength (Å)		
resolution (Å)	1.95	
total reflections	392 368	
unique reflections	125 822	
completeness (%)	99.12	
$I/\sigma(I)$	9.9	
R_{sym}^a		
redundancy		
space group	$P2_12_12_1$	
cell dimensions (Å)		
<i>a</i>	86.16	48.88
<i>b</i>	128	71.36
<i>c</i>	129.3	127.80
<i>refinement</i>		
R_{work}/R_{free}	0.21369 / 0.24700	
rmsd bond lengths (Å)	0.0199	
rmsd bond angles (°)	2.0568	
B-values (Å ²)	21.593	
water		
ions		
<i>Ramachandran plot (%)</i>		
favoured	96.82	
allowed	2.38	
outliers	0.8	

C Affidavit

I hereby declare that this document has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this document other than those indicated in the thesis itself.

Date:....., Location:....., Signature:.....

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Acronyms

Å Ångström, 0.1 nm

ABPP activity based protein profiling 38

AC-9 anthracene-9-carboxylic acid 23

ATP adenosine triphosphate 24

BisTris 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol

B-PER bacterial protein extraction reagent

CCP4 Collaborative Computational Project No. 4 26, 27

CD circulary dichroism 10

CHS chalcone synthase 51

CID collsion induced dissociation 55, 57

C-MT C-methyl transferase 36

COMT catechol O-methyl transferase 15

Coot Crystallographic Object-Oriented Toolkit 27

CV column volumes

DMSO dimethyl sulfoxide 25, 44

DNA desoxyribonucleic acid

DNA MT DNA methyl transferase vii, 36, 37

DoE design of experiments 21

DTT dithiothreitol; (2*S*,3*S*)-1,4-bis(sulfanyl)butane-2,3-diol

EDTA ethylenediaminetetraacetic acid 17, 20, 21, 30

EEC enthalpy-entropy compensation 39

ESI electrospray ionization 55

- FFD** fractional factorial design xi, 21, 22
FPLC fast protein liquid chromatography 21, 33
FT Fourier transformation 26
- GdmCl** guanidinium hydrochloride
GFP green fluorescent protein 28
GOD glucose oxidase 28, 88
GSH glutathione, γ -L-glutamyl-L-cysteinylglycine 22, 30, 31
GSSG glutathione disulfide 22
GST Glutathion S-transferase 54
- HEPES** 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIC hydrophobic interaction chromatography 31
HPLC high-performance liquid chromatography 14, 23, 30, 34, 53
HRP horseradish peroxidase 28
- IB** inclusion body 19, 20, 22, 60
IEX ion exchange chromatography 24
IMAC immobilized metal affinity chromatography
IPB Leibniz-Institute of Plant Biochemistry
IPTG isopropyl-D-thiogalactopyranosid 16, 19, 60
ITC Isothermal Titration Calorimetry viii, xi, 33, 39, 40, 45, 47, 88
- LB** lysogeny broth 13, 15, 19, 59
LC/MS liquid chromatography coupled mass-spectrometry 42, 55, 57, 58
- m/z** mass-to-charge ratio
MES 2-(*N*-morpholino)ethanesulfonic acid
MLU Martin-Luther-Universität
MMT L-malic acid/MES/Tris 7, 33
MR molecular replacement
MT methyl transferase vii, xi, 35–38, 69
MTP micro-titer plate 24–26, 28, 29, 88
MW molecular weight 18
MWCO molecular weight cut-off

- NADES** natural deep eutectic solvent xi, 7, 25, 86
- nos** nopaline synthase 51
- NPS** nitrogen, phosphate, sulfate buffer
- NRPS** non-ribosomal peptide synthase 35
- NTA** nitrilo triacetic acid 20
- O-MT** *O*-methyl transferase 30, 31, 34, 38, 58
- PAGE** polyacrylamide gel electrophoresis ix, x, 16–18, 23, 33, 54, 55, 60, 68
- PAL** phenylalanine ammonia-lyase 51
- PBS** phosphate buffered saline 17, 23, 28
- PCA** principal component analysis ix, x, 53, 54, 67
- PCH** propane-1,2-diol/choline chloride,NADES-mixture 25
- PCR** polymerase chain reaction 12
- PDA** photo diode array 34
- PDB** Protein Data Base 27
- PFOMT** phenylpropanoid and flavonoid *O*-methyl transferase viii, 15, 19, 25, 27, 30, 33, 38–48, 88
- PHENIX** Phyton-based Hierachial Environment for Integrated Xtallography 27
- PKS** poly ketide synthase 35
- PMSF** phenylmethylsulfonylfluoride
- P-MT** protein methyl transferase vii, 36, 37
- QSAR** quantitative structure activity relationship 35
- rmsd** root mean squared deviation 73
- rna** ribonucleic acid vii, 37
- RT** room temperature
- SAE** *S*-adenosyl-L-ethionine, (2*S*)-2-amino-4-[[*(2S,3S,4R,5R)*-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl-ethylsulfonio]butanoat viii, 24, 25, 36, 39–43, 45, 47, 48
- SAH** *S*-adenosyl-L-homocysteine viii–x, 22, 30, 39–41, 43–48, 66
- SAM** *S*-adenosyl-L-methionine vii, viii, xi, 24, 30, 31, 36–42, 45, 47, 62, 69
- SAMS** *S*-adenosylmethionine synthase 24

SAR structure activity relationship 35

SDS sodium dodecylsulfate ix, x, 7, 16–18, 23, 33, 54, 55, 60, 68

SeAM *Se*-adenosyl selenomethionine vii, 36, 37, 69

SOMT-2 soy O-methyl transferase ix, xi, 15, 19, 21–23, 31, 50–52, 54–60

SSG succinate/sodium phosphate/glycine 7

TB terrific broth 15, 59

TCA trichloro acetic acid 16–18, 23, 30, 33

Ti-plasmid tumor inducing plasmid 9, 88

Tris tris(hydroxymethyl)-aminomethane

U enzyme unit; measure for enzymatic activity (1 U = 1 µmole/min = 1/60 µkat)

UV/VIS ultra violet/visible (light spectrum) 24, 34

V volume

ZYP N-Z-amino, yeast extract, phosphate 19, 54, 88

Glossary

GOD Glucose oxidase is an enzyme.... 85

His₆-tag Hexa-histidine tag commonly used for recombinant protein production.
59

Isothermal Titration Calorimetry (ITC) Fill in description here 85

MTP Micro-titer plate. Small format rectangular plastic plate containing wells to allow for storage of multiple small samples or the containment multiple simultaneous reactions. Typical sizes include 24, 96 and 384-wells 85

PFOMT Phenylpropanoid and flavonoid O-methyl transferase from *Mesembryanthemum crystallinum*, which was first described by Ibdah et al. in 2003 [35]
86

T7-tag Initial 11 amino acids of the T7 gene 10 protein. 59

Ti-plasmid Commonly found plasmids in *A. tumefaciens* and *A. rhizogenes* that confer virulence 87

Trx-tag Thioredoxin tag used to increase solubility and stability of recombinantly expressed proteins. 59

ZYP-5052 Autoinduction medium developed by Studier [85]. The naming stems from the components N-Z-amino, yeast extract and phosphate. The numbering designates the composition; e.g. 5052 refers to 0.5 % glycerol, 0.05 % glucose and 0.2 % lactose. 87