Biotransformations from and to methylated flavonoids

Subtitle

Benjamin Weigel
Leibniz-Institute of Plant Biochemistry
Department of Bioorganic Chemistry
Weinberg 3
06120 Halle(Saale)
May 21, 2015

Dissertation

Advisor: Prof. Dr. Ludger A. Wessjohann
wessjohann@ipb-halle.de
+49 (345) 5582-1301

noch nicht bekannt

Contents

List of Figures

List of Tables

Notes of Revisors

1

Preface

1 Abstracts

1.1 English Abstract

Test Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Ut purus elit, vestibulum ut, placerat ac, adipiscing vitae, felis. Curabitur dictum gravida mauris. Nam arcu libero, nonummy eget, consectetuer id, vulputate a, magna. Donec vehicula augue eu neque. Pellentesque habitant morbi tristique senectus et netus et malesuada fames ac turpis egestas. Mauris ut leo. Cras viverra metus rhoncus sem. Nulla et lectus vestibulum urna fringilla ultrices. Phasellus eu tellus sit amet tortor gravida placerat. Integer sapien est, iaculis in, pretium quis, viverra ac, nunc. Praesent eget sem vel leo ultrices bibendum. Aenean faucibus. Morbi dolor nulla, malesuada eu, pulvinar at, mollis ac, nulla. Curabitur auctor semper nulla. Donec varius orci eget risus. Duis nibh mi, congue eu, accumsan eleifend, sagittis quis, diam. Duis eget orci sit amet orci dignissim rutrum.

1.2 Deutsche Zusammenfassung

Nam dui ligula, fringilla a, euismod sodales, sollicitudin vel, wisi. Morbi auctor lorem non justo. Nam lacus libero, pretium at, lobortis vitae, ultricies et, tellus. Donec aliquet, tortor sed accumsan bibendum, erat ligula aliquet magna, vitae ornare odio metus a mi. Morbi ac orci et nisl hendrerit mollis. Suspendisse ut massa. Cras nec ante. Pellentesque a nulla. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Aliquam tincidunt urna. Nulla ullamcorper vestibulum turpis. Pellentesque cursus luctus mauris.

II

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

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 toolbox

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

c

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 aquired 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_2HPO_4, 1 M KH_2PO_4, 0.5 M $(NH_4)_2SO_4$

1 M SSG pH 4 (10×)	14.8 g/l succinic acid, 60.4 g/l $NaH_2PO_4 \cdot H_2O$, 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 $NaH_2PO_4 \cdot H_2O$, 32.8 g/l glycine, 10.3 % 10 M NaOH
5× SDS sample buffer	10 % (w/v) SDS, 10 mM β-mercapto ethanol, 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_2MoO_4, 2 mM Na_2SeO_3, 2 mM H_3BO_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	D-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 yeaxst extract, 0.4 % glycerol, 72 mM K_2HPO_4, 17 mM KH_2PO_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

1 3.1.4 Bacterial strains

2 *E.coli*

BL21(DE3)	F^- *ompT hsdSB*(r_B^-,m_B^-) *gal dcm* λ(DE3) Invitrogen, Karslruhe (Germany)
C41(DE3)	F^- *ompT hsdSB*(r_B^-,m_B^-) *gal dcm* λ(DE3) Lucigen, Wisconsin (USA)
C43(DE3)	F^- *ompT hsdSB*(r_B^-,m_B^-) *gal dcm* λ(DE3) Lucigen, Wisconsin (USA)
DH5α	F^- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17($r_K^-m_K^+$) *phoA supE*44 λ^- *thi*-1 *gyrA*96 *relA*1 Invitrogen, Karslruhe (Germany)
JM110	*rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm glnV*44 Δ(*lac-proAB*) e14- [F' *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *hsdR*17($r_K^-m_K^+$) Martin-Luther-University Halle-Wittenberg
JW1593 (BW25113 derivative)	*rrnB* Δ*lacZ*4787 *HsdR*514 Δ(*araBAD*)568 *rph*-1 Δ*ydgG* (Kan^R) Keio Collection, National Institute of Genetics (Japan)
MG1655	F^- λ^- *ilvG*$^-$ *rfb*-50 *rph*-1 DSMZ, Hamburg (Germany)
One Shot TOP10	F^- Φ80*lacZ*ΔM15 Δ(*mrr-hsdRMS-mcrBC*) *recA*1 *endA*1 *mcrA* Δ*lacX*74 *araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) λ^- *nupG* Invitrogen, Karslruhe (Germany)
Origami(DE3)	Δ(*ara-leu*)7697 Δ*lacX*74 Δ*phoA PvuII phoR araD*139 *ahpC galE galK rpsL* F'[*lac + lacI q pro*] (DE3)*gor*522::*Tn*10 *trxB* (Kan^R, Str^R, Tet^R) Novagen, Wisconsin (USA)
Rosetta(DE3)	F^- *ompT hsdSB*(r_B^-,m_B^-) *gal dcm* λ(DE3) pRARE (Cam^R) Novagen, Wisconsin (USA)
Rosetta(DE3) pLysS	F^- *ompT hsdSB*(r_B^-,m_B^-) *gal dcm* λ(DE3) pLysSRARE (Cam^R) Novagen, Wisconsin (USA)
T7 Express	*fhuA*2 *lacZ*::*T7 gene*1 *[lon] ompT gal sulA*11 *R*(*mcr*-73::*miniTn*10–Tet^S)2 *[dcm]* *R*(*zgb*-210::*Tn*10–Tet^S) *endA*1 Δ(*mcrC-mrr*)114::*IS*10 NEB, Massachusetts (USA)

3 *Agrobacterium tumefaciens*

GV3101	chromosomal background: C58, marker gene: *rif*, Ti-plasmid: cured, opine: nopaline Sylvestre Marillonet, IPB

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)
pQE30	QIAGEN, Hilden (Germany)
pUC19	Invitrogen, Karslruhe (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 AAG ACA AAA TGG CTT CTT CAT TAA ACA ATG GCC G	BpiI
somt2	TTG AAG ACA AGG ACA CCC CAA ATA CTG TGA GAT CTT CC	BpiI
somt3	TTG AAG ACA AGT CCT TAG GAA CAC CTT TCT GGG AC	BpiI
somt4	TTG AAG ACA 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	CAT ATG GAT TTT GCT GTG ATG AAG CAG GTC	NdeI
pfomt2.rv	GAA TTC AAT AAA GAC GCC TGC AGA AAG TG	EcoRI
pRha1.fw	CTC TAG CAG ATC TCG GTG AGC ATC ACA TCA CCA CAA TTC	BglII
pRha1.rv	CAA TTG AGG ATC 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/*) [35]. 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 seperately 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 [41].
Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.
In vitro site-directed mutatgenesis 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 [21, 10]. 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 inbcubated 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.34). The *pfomt* 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. 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. OD^{600} of the culture was measured and adjusted to 0.2 by dilution with infiltration buffer (10 mM MES/NaOH, 10 mM $MgSO_4$ pH 5.5). When multiple *A. tumefaciens* transformed with different contructs/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.

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 (≈6 mg), were weighed exactly and extracted with 500 µl 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 × *g*, 4 °C, 10 min) and the supernatant transferrerd to a new tube, to remove the insoluble plant material. The supernatant was centrifuged again (20 000 × *g*, 4 °C, 10 min) and the resulting supernatant was transferred to a high-performance liquid chromatography (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 [32], 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 [14]. Extinction coefficients of proteins were calculated from the amino acid sequence using the ExpPASy servers's ProtParam tool [13].

Table 3.7.: Calculated extinction coefficients of proteins used in this work.

protein/enzyme	$\varepsilon_{280\mathrm{nm}}^{1\,\mathrm{g/l}}$ in $\mathrm{ml\,mg^{-1}\,cm^{-1}}$
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 antiobiotics 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 OD^{600} 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 OD^{600} 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-polyacrylamide gel electrophoresis (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 [32]. Overall 5 protein subfractions can be obtained, including *total cell protein, culture supernatant (medium) protein, periplasmic protein, solube cytoplasmic protein* and *insoluble protein*.
The OD^{600} 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 ($OD^{600} \times V \times 50$) 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 concetrated 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 × *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* [25]. The periplasma is, other than the cytosol, an oxidizing environment and often used for the production of proteins containing dilsufide linkages. The preparation of periplasmic protein was accomplished by an osmotic shock protocol modified from Current Protocols in Molecular Biology [3]. 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 × *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 $MgSO_4$. 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 Discontinous SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The analysis of samples via SDS-PAGE was realized via the discontinous system first described by Laemmli, which allows separation of proteins based on their electrophoretic mobility, which in turn depends on their size [23].
The SDS-PAGE procedure was carried out according to standard protocols described by Sambrook and Russell [41]. 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 rebuffered 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, *y*east extract, *p*hosphate (ZYP-5052) containing 200 µg/ml kanamycin) was inoculated with the starter culture such that OD^{600} was 0.05. The culture was incubated in a shaking incubator at 37 °C, 220 rpm over night (≈16 h). Due to the autoinducing nature of the ZYP-5052 medium, addition of IPTG was not neccesary. Cells were harvested by centrifugation at 10 000 × *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 ≈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 × *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 (≈ 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 $OD^{600} \approx 0.05$. The culture was incubated at 37 °C, 220 rpm in a shaking incubator until $OD^{600} \approx 0.6$. 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 × *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.

BWEIGEL: Überprüfen!

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). 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 [33].
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 Mg^{2+} or Ni^{2+}, 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 Ni^{2+}- (HisTrap FF crude) or Co^{2+}-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. Unound 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 Enzymatic production of SAM and SAE

S-adenosyl-L-methionine (SAM) and *S*-adenosyl-L-ethionine (SAE) were prepared according to the method described by Dippe, et. al [8].
Preparative reactions (20 ml) were performed in 0.1 M Tris/HCl, 20 mM $MgCl_2$, 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 ($\varepsilon_0 = 15\,400\,\mathrm{M^{-1}\,cm^{-1}}$) after resuspension in water [43].

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. Crystallization screens were set up as described above.

PFOMT was crystallized using the following conditions 2 M $(NH_4)_2SO_4$, 20 %glycerol. The protein solution contained 0.25 mM *S*-adenosyl-L-homocysteine (SAH), 0.25 mM $MgCl_2$, 0.25 mM ferulic acid and 7.53 mg/ml PFOMT.

BWEIGEL: nochma genau guckn

Crystallization of proteins using NADES

BWEIGEL: add DES crystallization

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 was accomplished using *XDS* [19, 18, 20] or *MOSFLM* [34]. *Scala* [11], 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 Fourier transformation two terms are neccessary as coefficients; the *structure factor amplitudes*, $F_{obs}(\mathbf{h})$ 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 [40]. Phases of the structures herein were exclusively determined by MR [37, 38].

MR was performed using the software *Phaser* [26, 27], which is included in the CCP4-Suite [49]. A previously published PFOMT structure (PDB-code: 3C3Y [22]) was used as a template during MR procedure for the PFOMT structure solution. For the MR of the lysozyme structure the PDB-entry XXXX was used.

BWEIGEL: Lysozym struktur

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 [9]. Structure refinement was done using the software REFMAC5 [30, 47] 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/*) [4]. 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/*) [15, 29, 46]. 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, 25.718 (x,y,z) and had size of 22, 20, 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 *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 [44]. 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 [5].

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 3.1). 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.

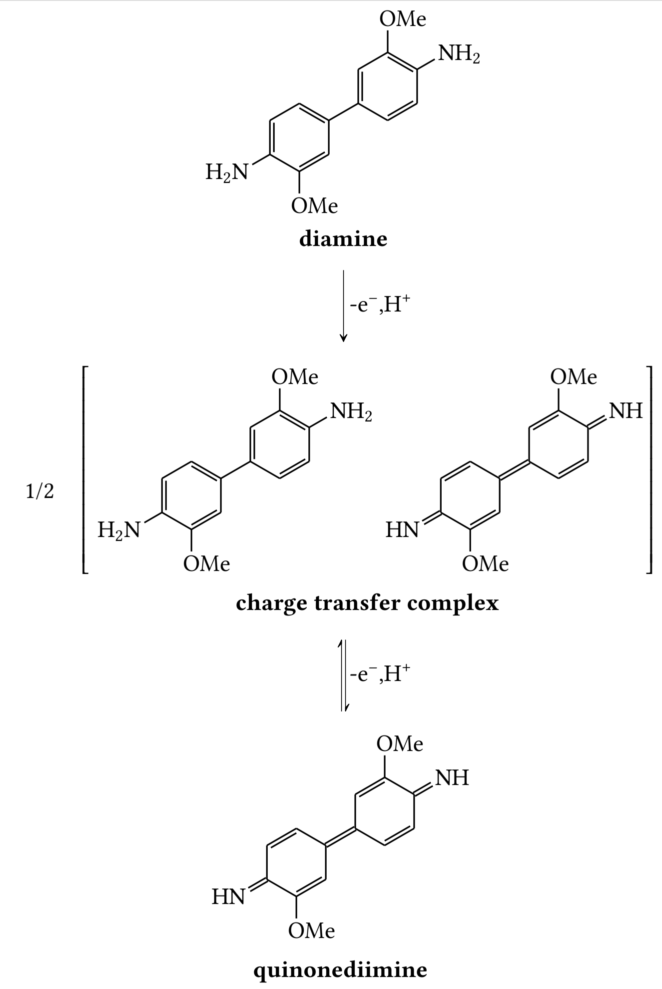

Figure 3.1.: Oxidation of the reporter substrate o-dianisidine. Consecutive one-electron transfers lead to the fully oxidized diimine form of o-dianisidine. The first electron transfer is believed to produce a charge transfer complex intermediate. [17, 5]

3.6.2 *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 L-glutathion (GSH) pH 7.5. 1 mM $MgCl_2$, 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.

BWEIGEL: nochmal auseinanderklamüsern wegen den konzentrationen und eingesetzten enzymmengen...

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 extraced 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 (3.6.5).

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 3.6.5).

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 [31].

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 enyzme. $MgCl_2$ was either omitted or added at 10 mM to assess influences of divalent cations. Assays were stopped as described in 3.6.2 and analyzed accordingly.

3.6.3 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 [42, 28]. Hence, caffeic acid can complex ferric (Fe^{3+}) ions and form a colored complex with λ_{max} = 595 nm [7]. Since the complex formation is specific for caffeic acid and methylated derivatives (i.e. ferulic and iso-ferulic acid) cannot complex Fe^{3+}, this can be used as a measure for methylation reactions. *O*-MT assays were prepared as before (3.6.2). 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 $FeCl_3$ 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.

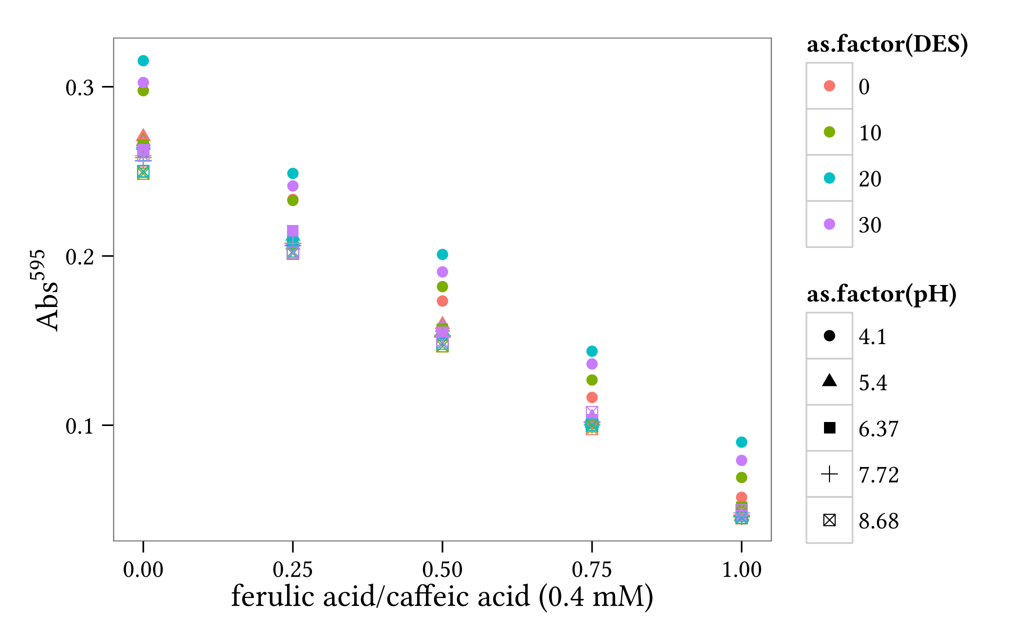

Figure 3.2.: This is a test.

3.6.4 Binding experiments using Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry (ITC) can be used to directly characterize the thermodynamics of an observed process, be this a binding interaction or an enzymatic reaction [12].

ITC measurements to describe the interaction between PFOMT and its substrates/-effector where 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 susequently centrifuged (14 000 × 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.

3.6.5 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 [24]. 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 (3.6.2) 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.

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

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

4.1 Introduction

4.2 Substrate binding studies using ITC

4.3 Determination of the structure of *apo*-PFOMT

4.3.1 PFOMT activity in deep eutectic solvents (DES) / Solubility-enhancing effects of DES

vielleicht eigenes kapitel DES?

4.4 Study of variants for long-chain alkylations

4.4.1 PFOMT-Paper (DIM)

4.4.2 Dockings???

4.5 Colclusion/Discussion

5 Enzymatic methylation of Non-catechols

5.1 Introduction

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

5.2 SOMT-2

5.2.1 In vivo methylation studies using *N. benthamiana*

5.2.2 In vivo studies in *E. coli*

5.2.3 In vitro studies using recombinantly produced SOMT-2

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 Consensus or Bioinformatic points-of-view (COMT)???

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 hydrophopbic 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

Quisque ullamcorper placerat ipsum. Cras nibh. Morbi vel justo vitae lacus tincidunt ultrices. Lorem ipsum dolor sit amet, consectetuer adipiscing elit. In hac habitasse platea dictumst. Integer tempus convallis augue. Etiam facilisis. Nunc elementum fermentum wisi. Aenean placerat. Ut imperdiet, enim sed gravida sollicitudin, felis odio placerat quam, ac pulvinar elit purus eget enim. Nunc vitae tortor. Proin tempus nibh sit amet nisl. Vivamus quis tortor vitae risus porta vehicula.

Fusce mauris. Vestibulum luctus nibh at lectus. Sed bibendum, nulla a faucibus semper, leo velit ultricies tellus, ac venenatis arcu wisi vel nisl. Vestibulum diam. Aliquam pellentesque, augue quis sagittis posuere, turpis lacus congue quam, in hendrerit risus eros eget felis. Maecenas eget erat in sapien mattis porttitor. Vestibulum porttitor. Nulla facilisi. Sed a turpis eu lacus commodo facilisis. Morbi fringilla, wisi in dignissim interdum, justo lectus sagittis dui, et vehicula libero dui cursus dui. Mauris tempor ligula sed lacus. Duis cursus enim ut augue. Cras ac magna. Cras nulla. Nulla egestas. Curabitur a leo. Quisque egestas wisi eget nunc. Nam feugiat lacus vel est. Curabitur consectetuer.

III

Appendix

A Figures

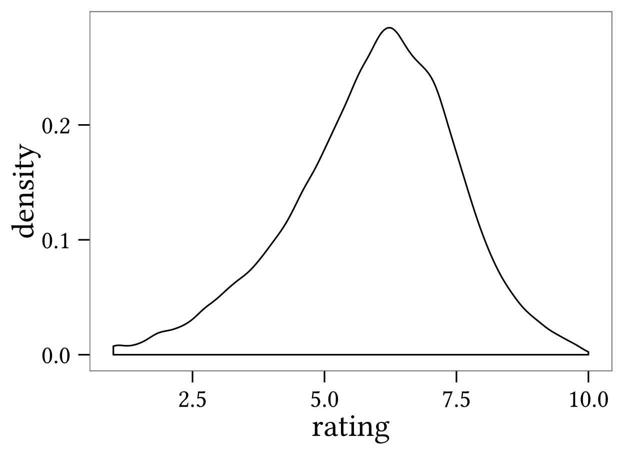

Figure A.1.: Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Aenean commodo ligula eget dolor. Aenean massa. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec quam felis, ultricies nec, pellentesque eu, pretium quis, sem.

B Tables

Table B.1.: Overview over the contructs 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		
pBEW104	rhaP$_{BAD}$ promoter	pBEW4b	pBEW103	amplification (*pRha1.fw/rv*), cloning (BglII, BamHI)
pBEW106	pICH413038-somt	pET28MC-somt	pICH413038	amplification (*somt1/2/3/4*), golden gate cloning (BpiI)
pBEW107		pICH51266, pBEW106, pICH41421	pICH75044	golden gate cloning (BsaI)
pBEW1a				
pBEW1b				
pBEW2a				
pBEW2b				
pBEW3a				
pBEW3b				
pBEW4a				
pBEW4b				
pET28-pfomt	*pfomt* gene in pET-28a(+), endogenous NdeI site removed	pQE30-pfomt	pET-28a(+)	mutagenesis (*pfomt1.fw/rv*), amplification (*pfomt2.fw/rv*), cloning (NdeI, EcoRI)
pET20-somt	N-terminal pelB-tag fusion for periplasmic expression		pET20-b(+)	
pET28-somt			pET28-a(+)	
pET28MC-somt				
pET32-somt	N-terminal TrX-tag fusion		pET-32a(+)	
pET41-somt	N-terminal GST-tag fusion		pET-41a(+)	
pUC19*	added BglII site	pUC19	-	mutagenesis (*pUC1.fw/rv*)
pUCB1	pUC19 derivative with lsrA promoter	lsr-XX-DAS	pUC19*	cloning (NdeI, BglII)
pUCB1-sfGFP-DAS+4				

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:................

Bibliography

[1] Paul D. Adams et al. “PHENIX: A comprehensive Python-based system for macromolecular structure solution”. en. In: *Acta Crystallographica Section D: Biological Crystallography* 66.2 (Feb. 2010), pp. 213–221.

[2] Agilent Technologies. *QuikChange II Site-Directed Mutagenesis Kit: Instruction Manual.* 2011.

[3] Frederick M Ausubel et al. “Current Protocols in Molecular Biology”. In: (2008), p. 23.

[4] Vincent B Chen et al. “MolProbity: all-atom structure validation for macromolecular crystallography.” In: *Acta crystallographica. Section D, Biological crystallography* 66.Pt 1 (Jan. 2010), pp. 12–21.

[5] Al Claiborne and Irwin Fridovich. “Chemical and Enzymatic Intermediates in the Peroxidation of o-Dianisidine by Horseradish Peroxidase. 1. Spectral Properties of the Products of Dianisidine Oxidation”. In: *Biochemistry* 18 (1979), pp. 2324–2329.

[6] Martin Dippe, Lars Dressler, and Renate Ulbrich-Hofmann. “Fe(III)-resorcylate as a spectrophotometric probe for phospholipid-cation interactions.” In: *Analytical biochemistry* 445 (Jan. 2014), pp. 54–9.

[7] Martin Dippe et al. “Engineering of a Mg2+-dependent O-methyltransferase towards novel regiospecificity”. In: *manuscript submitted* (2015).

[8] M Dippe et al. “Rationally engineered variants of S-adenosylmethionine (SAM) synthase: reduced product inhibition and synthesis of artificial cofactor homologues.” en. In: *Chemical communications (Cambridge, England)* 51.17 (Feb. 2015), pp. 3637–40.

[9] P. Emsley et al. “Features and development of Coot”. In: *Acta Crystallographica Section D: Biological Crystallography* 66.4 (2010), pp. 486–501.

[10] Carola Engler, Romy Kandzia, and Sylvestre Marillonnet. “A one pot, one step, precision cloning method with high throughput capability”. In: *PLoS ONE* 3.11 (Jan. 2008), e3647.

[11] Philip Evans. “Scaling and assessment of data quality”. In: *Acta Crystallographica Section D: Biological Crystallography* 62.1 (Jan. 2006), pp. 72–82. arXiv: *S0907444905036693* [doi:10.1107].

[12] Matthew W Freyer and Edwin a Lewis. “Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions.” In: *Methods in cell biology* 84.07 (Jan. 2008), pp. 79–113.

[13] E. Gasteiger et al. “Protein Identification and Analysis Tools on the ExPASy Server”. In: *The Proteomics Protocols Handbook*. Ed. by John M. Walker. Humana Press, 2005, pp. 571–607.

[14] S C Gill and P H von Hippel. “Calculation of protein extinction coefficients from amino acid sequence data.” In: *Analytical biochemistry* 182.2 (Nov. 1989), pp. 319–26.

[15] Ruth Huey et al. “A semiempirical free energy force field with charge-based desolvation.” In: *Journal of computational chemistry* 28.6 (Apr. 2007), pp. 1145–52.

[16] Mwafaq Ibdah et al. “A Novel Mg2+-dependent O-Methyltransferase in the Phenylpropanoid Metabolism of Mesembryanthemum crystallinum”. In: *Journal of Biological Chemistry* 278.45 (Nov. 2003), pp. 43961–43972.

[17] PD Josephy, T Eling, and RP Mason. “The horseradish peroxidase-catalyzed oxidation of 3, 5, 3’, 5’-tetramethylbenzidine. Free radical and charge-transfer complex intermediates.” In: *Journal of Biological Chemistry* 257 (1982), pp. 3669–3675.

[18] Wolfgang Kabsch. “Automatic processing of rotation diffraction data from crystals of initially unknown symmetry land cell constants”. In: *Journal of Applied Crystallography* 26.pt 6 (Dec. 1993), pp. 795–800.

[19] Wolfgang Kabsch. “Integration, scaling, space-group assignment and post-refinement”. In: *Acta Crystallographica Section D: Biological Crystallography* 66.2 (Feb. 2010), pp. 133–144.

[20] Wolfgang Kabsch. “Xds”. In: *Acta Crystallographica Section D: Biological Crystallography* 66.2 (Feb. 2010), pp. 125–132.

[21] Youichi Kondou et al. “cDNA Libraries”. In: *Methods in Molecular Biology* 729 (2011), pp. 183–197.

[22] Jakub G. Kopycki et al. "Biochemical and Structural Analysis of Substrate Promiscuity in Plant Mg2+-Dependent O-Methyltransferases". In: *Journal of Molecular Biology* 378.1 (Apr. 2008), pp. 154–164.

[23] Ulrich K Laemmli. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." In: *Nature* 227.5259 (1970), pp. 680–685.

[24] Tom J. Mabry, K. R. Markham, and M. B. Thomas. *The Systematic Identification of Flavonoids.* Berlin, Heidelberg: Springer Berlin Heidelberg, 1970.

[25] Savvas C Makrides and Savvas C Makrides. "Strategies for Achieving High-Level Expression of Genes in Escherichia coli". In: *Microbiological reviews* 60.3 (1996), pp. 512–538.

[26] Airlie J. McCoy. "Solving structures of protein complexes by molecular replacement with Phaser". In: *Acta Crystallographica Section D: Biological Crystallography* 63.1 (Jan. 2006), pp. 32–41.

[27] Airlie J. McCoy et al. "Phaser crystallographic software". In: *Journal of Applied Crystallography* 40.4 (Aug. 2007), pp. 658–674.

[28] Edoardo Mentasti and Ezio Pelizzetti. "Reactions between iron(III) and catechol (o-dihydroxybenzene). part I. Equilibria and kinetics of complex formation in aqueous acid solution". en. In: *Journal of the Chemical Society, Dalton Transactions* 23 (Jan. 1973), p. 2605.

[29] Garrett M Morris et al. "AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility." In: *Journal of computational chemistry* 30.16 (Dec. 2009), pp. 2785–91.

[30] Garib N. Murshudov, Alexei a. Vagin, and Eleanor J. Dodson. "Refinement of macromolecular structures by the maximum-likelihood method". In: *Acta Crystallographica Section D: Biological Crystallography* 53.3 (May 1997), pp. 240–255.

[31] Janet Newman. "Novel buffer systems for macromolecular crystallization". In: *Acta Crystallographica Section D: Biological Crystallography* 60.3 (2004), pp. 610–612.

[32] Novagen. *pET System Manual.* 11th ed. Darmstadt: EMD Chemicals, 2010.

[33] Ira Palmer and Paul T. Wingfield. "Preparation and extraction of insoluble (Inclusion-body) proteins from Escherichia coli". In: *Current Protocols in Protein Science* 1.SUPPL.70 (Nov. 2012), Unit6.3.

[34] Harold R. Powell. "The Rossmann Fourier autoindexing algorithm in MOSFLM". In: *Acta Crystallographica Section D: Biological Crystallography* 55.10 (1999), pp. 1690–1695.

[35] R Core Team. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing. Vienna, Austria, 2015.

[36] Randy J. Read. “Pushing the boundaries of molecular replacement with maximum likelihood.” en. In: *Acta Crystallographica. Section D: Biological crystallography* 57.Pt 10 (Jan. 2001), pp. 1373–1382.

[37] M. G. Rossmann and D. M. Blow. “The detection of sub-units within the crystallographic asymmetric unit”. In: *Acta Crystallographica* 15.1 (Jan. 1962), pp. 24–31.

[38] Michael G. Rossmann. “Molecular replacement - Historical background”. In: *Acta Crystallographica - Section D Biological Crystallography* 57.10 (Sept. 2001), pp. 1360–1366.

[39] Rainer Rudolph and Hauke Lilie. “In vitro folding of inlusion body proteins”. In: *FASEB Journal* 10 (1996), pp. 49–56.

[40] Bernhard Rupp. *Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology.* 1st ed. New York: Garland Science, 2009, p. 800.

[41] J Sambrook and D W Russell. *Molecular Cloning: A Laboratory Manual.* 3rd ed. Cold Spring Harbor (NY, USA): Cold Spring Harbor Laboratory Press, 2001.

[42] N. Schweigert, a. J B Zehnder, and R. I L Eggen. “Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals”. In: *Environmental Microbiology* 3.2 (2001), pp. 81–91.

[43] Stanley K. Shapiro and Dimis J. Ehninger. “Methods for the analysis and preparation of adenosylmethionine and adenosylhomocysteine”. In: *Analytical Biochemistry* 15.2 (May 1966), pp. 323–333.

[44] Sigma-Aldrich. *Technical Bulletin no. 2003-03: freezing of microbial samples prior to testing. Parenteral Drug Association.* 2003.

[45] F William Studier. “Protein production by auto-induction in high density shaking cultures.” In: *Protein expression and purification* 41.1 (May 2005), pp. 207–234. arXiv: *NIHMS150003*.

[46] Oleg Trott and Arthur J Olson. “AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading.” In: *Journal of computational chemistry* 31.2 (Jan. 2010), pp. 455–61.

[47] Alexei a. Vagin et al. “REFMAC5 dictionary: Organization of prior chemical knowledge and guidelines for its use”. en. In: *Acta Crystallographica Section D: Biological Crystallography* 60.12 I (Nov. 2004), pp. 2184–2195.

[48] Thomas Vogt. “Regiospecificity and kinetic properties of a plant natural product O-methyltransferase are determined by its N-terminal domain”. In: *FEBS Letters* 561.1-3 (Mar. 2004), pp. 159–162.

[49] Martyn D. Winn et al. “Overview of the CCP4 suite and current developments”. In: *Acta Crystallographica Section D: Biological Crystallography* 67.4 (Apr. 2011), pp. 235–242.

Acronyms

Å Ångstrom, 0.1 nm

ATP adenosine triphosphate 18

B-PER bacterial protein extraction reagent

CCP4 Collaborative Computational Project No. 4 20, 21
CD circulary dichroism 10
COMT catechol O-methyl transferase 14
Coot Crystallographic Object-Oriented Toolkit 21
CV column volumes

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

EDTA ethylenediaminetetraacetic acid 15, 17, 18, 23

FPLC fast protein liquid chromatography 18

GdmCl guanidinium hydrochloride
GOD glucose oxidase 21, 44
GSH L-glutathion 23

HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPLC high-performance liquid chromatography 13, 23, 25
HRP horseradish peroxidase 21

IB inclusion body 17, 18
IEX ion exchange chromatography 19
IMAC immobilized metal affinity chromatography
IPB Leibniz-Institute of Plant Biochemistry
IPTG isopropyl-D-thiogalactopyranosid 14, 17
ITC Isothermal Titration Calorimetry 24, 25, 44

LB lysogeny broth 12–14, 16, 17

MES 2-(*N*-morpholino)ethanesulfonic acid
MLU Martin-Luther-Universität
MMT L-malic acid/MES/Tris 6, 25
MR molecular replacement
MTP micro-titer plate 19, 21, 22, 44
MW molecular weight 16
MWCO molecular weight cut-off

NADES natural deep eutectic solvent vii, 7
NPS nitrogen, phosphate, sulfate buffer
NTA nitrilo triacetic acid 18

***O*-MT** *O*-methyl transferase 23–25

PAGE polyacrylamide gel electrophoresis 14–16
PBS phosphate buffered saline 15
PCR polymerase chain reaction 11
PDA photo diode array 25
PDB Protein Data Base 20, 21
PFOMT phenylpropanoid and flavonoid O-methyl transferase 14, 16, 17, 19–21, 23, 25, 44
PHENIX Phyton-based Hierarchial Environment for Integrated Xtallography 21
PMSF phenylmethylsulfonylfluoride

RT room temperature

SAE *S*-adenosyl-L-ethionine, (2*S*)-2-amino-4-[[(2*S*,3*S*,4*R*,5*R*)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methyl-ethylsulfonio]butanoat 18, 19
SAH *S*-adenosyl-L-homocysteine 20, 23
SAM *S*-adenosyl-L-methionine 18, 19, 23, 28
SAMS *S*-adenosylmethionine synthase 19
SDS sodium dodecylsulfate 7, 14–16
SOMT-2 soy O-methyl transferase 14, 17
SSG succinate/sodium phosphate/glycine 7

TB terrific broth 14
TCA trichloro acetic acid 14–16, 23
Ti-plasmid tumor inducing plasmid 9, 44
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) 19, 25

1 **V** volume

2 **ZYP** N-Z-amine, *y*east extract, *p*hosphate 17, 44

Glossary

GOD Glucose oxidase is an enzyme.... 41

Isothermal Titration Calorimetry (ITC) Fill in description here 41

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 42

PFOMT Phenylpropanoid and flavonoid O-methyl transferase from *Mesembryanthemum crystallinum*, which was first described by Ibdah et al. in 2003 [16] 42

Ti-plasmid Commonly found plasmids in *A. tumefaciens* and *A. rhizogenes* that confer virulence 42

ZYP-5052 Autoinduction medium developed by Studier [45]. The naming stems from the components N-*Z*-amine, *y*east extract and *p*hosphate. The numbering designates the composition; e.g. 5052 refers to 0.5 % glycerol, 0.05 % glucose and 0.2 % lactose. 43