Dissertation

Biotransformations from and to methylated flavonoids

Subtitle

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Contents

List of Figures

List of Tables

Notes of Revisors

Preface

1 Abstracts

1.1 English Abstract

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1.2 Deutsche Zusammenfassung

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II

Thesis

2 Introduction

Some introductionary 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

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 $(NH_4)_2SO_4$

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

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	in proportions: 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^- *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, Karlsruhe (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, Karlsruhe (Germany)
Origami(DE3)	Δ(*ara-leu*)7697 Δ*lacX*74 Δ*phoA Pvull 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)

Agrobacterium tumefaciens

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

3.1.5 Plasmids

Table 3.1.: *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 3.2.: *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/*). 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).

3.2 Microbiology

3.3 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 [14].
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 [1] 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.

3.3.1 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 [6, 4]. 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.

3.3.2 Subcloning of genes

All subcloning procedures were performed according to section 3.3 and specifically subsection 3.3.1. Specific steps for the subcloning of any genes discussed can be found in the appendix (p.27). 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.

3.4 Protein biochemistry

Stock solutions of antibiotics, IPTG or sugars were prepared according to the pET System Manual by Novagen [11], unless otherwise noted.

3.4.1 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 lysogeny broth (LB)-medium containing the appropriate antibiotics. The 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 (3.4.2) and analyzed via SDS-polyacrylamide gel electrophoresis (PAGE) (3.4.5).
Exact specifications of growth conditions (e.g. temperature, time, constructs) are discussed in the individual sections.

3.4.2 Protein subfractionation

The protein subfractionation procedure described herein was adapted from the protocol described in the pET Manual [11]. 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 $10000 \times g$, 4 °C for 5 minutes. The protein in the supernatant medium was concentrated by precipitation with trichloro acetic acid (TCA) (3.4.3) for SDS-PAGE analysis. The periplasmic protein was prepared (3.4.4) 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 $10000 \times g$ 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.

3.4.3 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 14000 × *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.

3.4.4 Preparation of periplasmic protein

Target proteins may be directed to the periplasmic space by N-terminal signal sequences like *pelB* or *DsbA/C* [9]. 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 [2]. 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 10000 × *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.

3.4.5 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 [8].
The SDS-PAGE procedure was carried out according to standard protocols described by Sambrook and Russell [14]. Very dilute and/or samples with high ionic strength were concentrated and/or desalted by the TCA precipitation procedure described in subsection 3.4.3. 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).

3.4.6 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.

3.4.7 Production of recombinant protein

Heterologous production of PFOMT

Phenylpropanoid and flavonoid O-methyl transferase (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 150 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 10000 × *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 15000 × *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) (3.4.9). The eluted PFOMT protein was dialyzed (3.4.6) against 25 mM HEPES, 100 mM NaCl, 5 % glycerol pH 7 and stored at −20 °C until use.

Heterologous production of SOMT-2

Soy O-methyl transferase (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} ≈ 0.05. The culture was incubated at 37 °C, 220 rpm in a shaking incubator until OD^{600} ≈ 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 (10000 × *g*, 4 °C, 10 min) and used, or stored at −20 °C until use. SOMT-2 was produced only in inclusion bodies (IBs), which were prepared as laid out in subsection 3.4.8.

BWEIGEL: Überprüfen!

3.4.8 Preparation of IB

When insoluble protein is produced in large amounts 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 prepared [12]

BWEIGEL: complete

3.4.9 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.

3.5 Analytics

3.5.1 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 [15]. 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. The oxidized form of *o*-dianisidine can then be measured photospectrometrically [3].

The methodology employs a coupled photospectrometric assay using GOD and HRP with *o*-dianisidine as reporter substrate. The assay was prepared in micro-titer plate (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).

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.

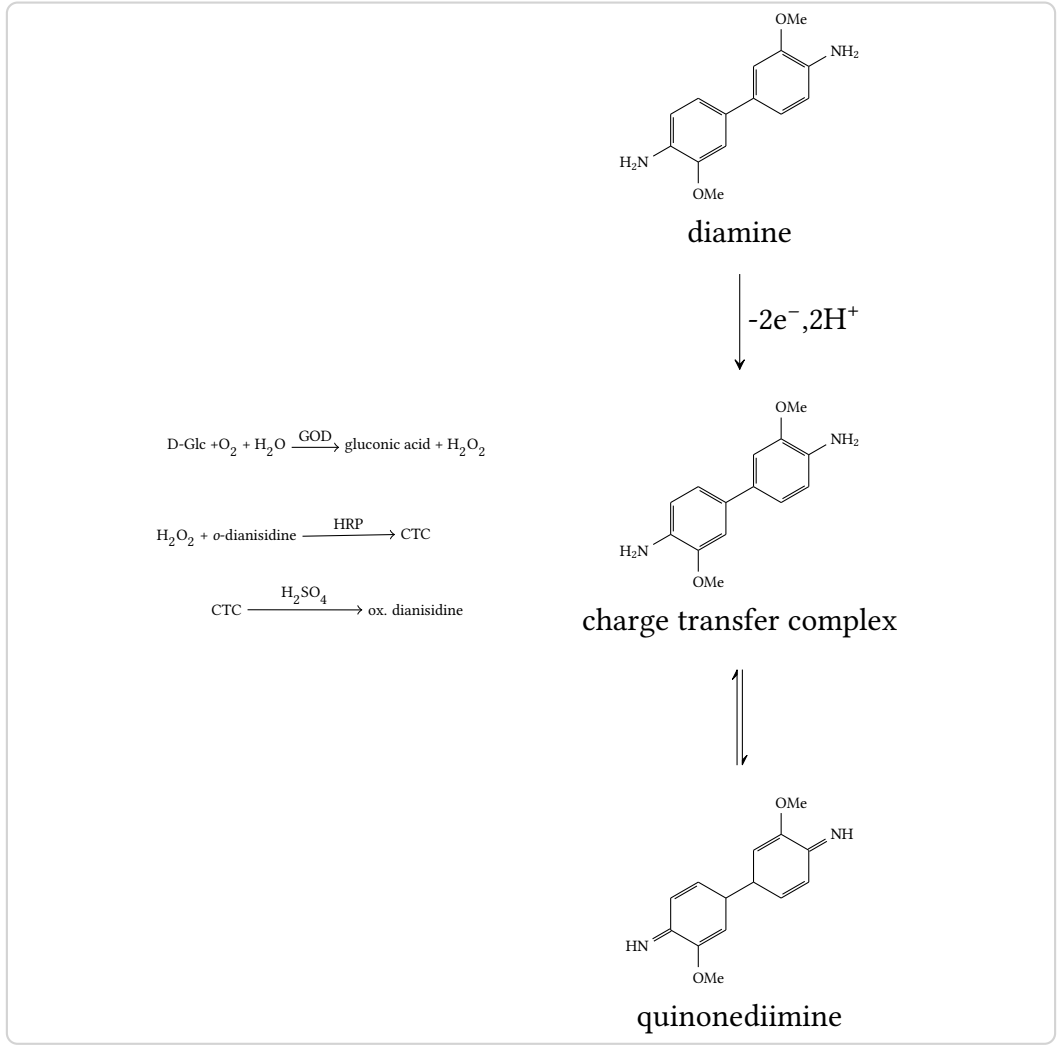
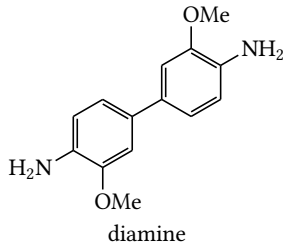
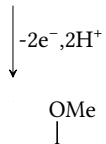
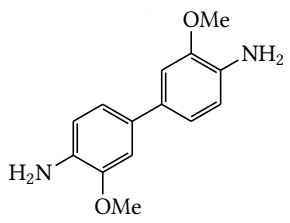


Figure 3.1.: *GOD assay*

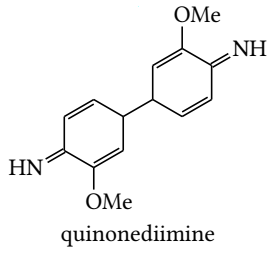


diamine





charge transfer complex



quinonediimine

3.5.2 HPLC 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 solvent (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-textscL-homocysteine (SAH)

S-adenosyl-L-methionine (SAM)

6.4 Usage of the lsr-promoter for true autoinduction

6.5 Conclusion/Discussion

7 DES in protein crystallography

8 Acknowledgements

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III

Appendix

A Figures

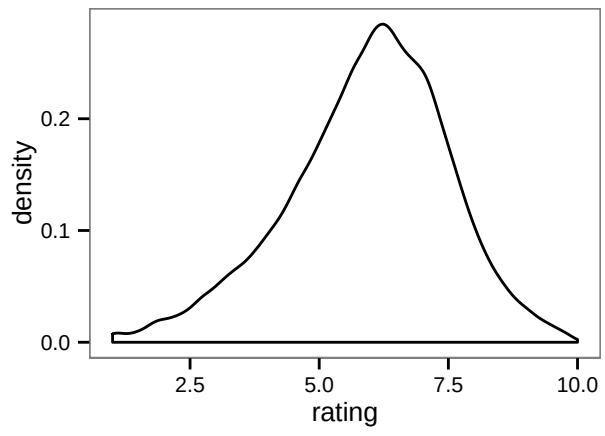

Figure A.1.: *Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Aenean commodo ligula eget dolor. Aenean massa. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec quam felis, ultricies nec, pellentesque eu, pretium quis, sem.*

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: . . . . . .

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Acronyms

B-PER bacterial protein extraction reagent

CD circulary dichroism 9
CV column volumes

EDTA ethylenediaminetetraacetic acid 13

FPLC fast protein liquid chromatography 15

GOD glucose oxidase 16, 33

HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HRP horseradish peroxidase 16

IB inclusion body 15
IMAC immobilized metal affinity chromatography
IPB Leibniz-Institute of Plant Biochemistry
IPTG isopropyl-D-thiogalactopyranosid 12, 14, 15

LB lysogeny broth 11, 12, 14, 15

MES 2-(*N*-morpholino)ethanesulfonic acid
MMT L-malic acid/MES/Tris 6
MTP micro-titer plate 16, 33
MW molecular weight 13

NPS nitrogen, phosphate, sulfate buffer
NTA nitrilo triacetic acid 15

PAGE polyacrylamide gel electrophoresis 12, 13
PBS phosphate buffered saline 13
PCR polymerase chain reaction 10, 11
PFOMT phenylpropanoid and flavonoid O-methyl transferase 14, 33

RT room temperature 12–14

SAM *S*-adenosyl-L-methionine 21
SDS sodium dodecylsulfate 7, 12, 13
SOMT-2 soy O-methyl transferase 15

TB terrific broth 12
TCA trichloro acetic acid 12, 13
Ti-plasmid tumor inducing plasmid 8, 33
Tris tris(hydroxymethyl)-aminomethane

V volume

ZYP N-*Z*-amine, *y*east extract, *p*hosphate 14, 33

Glossary

GOD Glucose oxidase is an enzyme.... 31

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 31

PFOMT Phenylpropanoid and flavonoid O-methyl transferase from *Mesembryanthemum crystallinum*, which was first described by Ibdah et al. in 2003 [5] 31

Ti-plasmid Commonly found plasmids in *A. tumefaciens* and *A. rhizogenes* that confer virulence 32

ZYP-5052 Autoinduction medium developed by Studier [16]. 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. 32