

Biotransformations from and to methylated flavonoids

Subtitle

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May 12, 2015

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noch nicht bekannt

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Preface

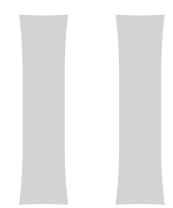
1 Abstracts

1.1 English Abstract

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1.2 Deutsche Zusammenfassung

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Thesis

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¹ 2 Introduction

² Some introductory text

³ 2.1 Natural products and secondary metabolites

⁴ 2.1.1 General

⁵ 2.1.2 Classes of natural products

⁶ Terpenoids and Steroids

⁷ ... here is some text

⁸ Polyketides and non-ribosomal peptides

⁹ ... here is some text

¹⁰ Alkaloids

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⁷ 2.3.4 Other important enzymes in biotech research

⁸ BMVOs

⁹ Esterases/Lipases

¹⁰ Oxidases

¹¹ Lyases

¹² Transaminases

¹³ 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 ₂ HPO ₄ , 1 M KH ₂ PO ₄ , 0.5 M (NH ₄) ₂ SO 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 ₂ MoO ₄ , 2 mM Na ₂ SeO ₃ , 2 mM H ₃ BO ₃ |

1 **Preparation of deep eutectic solvents (DES)**

2 Deep eutectic solvents (DES) were prepared by adding each component in a round-
3 bottom flask with a stirrer and stirring the mixture at 50 °C with intermittent
4 sonication treatments until a clear solution was obtained.

Table 3.1.: NADES-mixtures

| 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 |

5 **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 ₂ HPO ₄ , 17 mM KH ₂ PO 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 |

6 **3.1.4 Bacterial strains**

7 ***E.coli***

| | |
|--------------------------------|---|
| BL21(DE3) | F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm</i> λ(DE3) Invitrogen, Karlsruhe (Germany) |
| C41(DE3) | F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm</i> λ(DE3) Lucigen, Wisconsin (USA) |
| C43(DE3) | F ⁻ <i>ompT hsdSB(r_B⁻,m_B⁻) gal dcm</i> λ(DE3) Lucigen, Wisconsin (USA) |
| DH5α | F ⁻ Φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1</i> <i>gyrA96 relA1</i> Invitrogen, Karlsruhe (Germany) |
| JM110 | <i>rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm glnV44</i> Δ(<i>lac-proAB</i>) e14- [F' <i>traD36 proAB⁺ lacI^q lacZ</i> ΔM15] <i>hsdR17</i> (r _K ⁻ m _K ⁺) Martin-Luther-University Halle-Wittenberg |
| JW1593 (BW25113 derivative) | <i>rrnB</i> Δ <i>lacZ</i> 4787 <i>HsdR</i> 514 Δ(<i>araBAD</i>)568 <i>rph-1</i> Δ <i>ydgG</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 ⁻ Φ80 <i>lacZ</i> ΔM15 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</i> Δ <i>phoA</i> <i>Pvull phoR araD139 ahpC galE galK rpsL</i> F'[<i>lac + lacI q pro</i>] (DE3) <i>gor522::Tn10 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(mcr-73::miniTn10-Tet^S)2 [dcm] R(zgb-210::Tn10-Tet^S) endA1</i> Δ(<i>mcrC-mrr</i>)114::IS10 NEB, Massachusetts (USA) |

¹ *Agrobacterium tumefaciens*

| | |
|--------|--|
| GV3101 | chromosomal background: C58, marker gene: <i>rif</i> , 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, 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> <u>TCG</u> GTG AGC ATC ACA TCA CCA CAA TTC | BglII |
| pRha1.rv | CAA TTG <u>AGG ATC</u> <u>CCC</u> 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 | |

1 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) |

2 3.1.8 Software

3 All mathematical and statistical computations and graphics were done with the R
4 software (versions 3.1.X, <http://cran.r-project.org/>) [27]. Visualizations of macro-
5 molecules were arranged using the PyMol Molecular Graphics System, version
6 1.7.0.0 (Schrödinger, New York, USA).
7 Physicochemical calculations and calculations of different molecular descriptors
8 were performed using Marvin Beans 15.4.13.0 (ChemAxon, Budapest, Hungary) and
9 Molecular Operating Environment 2008.10 (Chemical Computing Group, Montreal,
10 Canada). Special software used for X-ray crystal structure solution is discussed
11 separately in the corresponding section (3.5).

12 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 [33].

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.

3.3.1 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 [15, 7]. 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.

3.3.2 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.30). 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

¹ from the pQE-30 vector kindly provided by Martin Dippe (IPB, Halle, Germany)
² into the pET-28-MC vector.

³ 3.4 Protein biochemistry

⁴ Stock solutions of antibiotics, IPTG or sugars were prepared according to the pET
⁵ System Manual by Novagen [Novagen2011], unless otherwise noted.

⁶ 3.4.1 Protein production test (expression test)

⁷ The heterologous production of proteins in *E. coli* was assessed in a small scale
⁸ protein production test, henceforth called expression test. Single colonies of *E. coli*
⁹ transformed with the constructs to be studied were used to inoculate a 2 ml starter
¹⁰ culture in lysogeny broth (LB)-medium containing the appropriate antibiotics. The
¹¹ concentrations of antibiotics used was as follows: 200 µg/ml ampicillin, 150 µg/ml
¹² kanamycin, 50 µg/ml chloramphenicol, 20 µg/ml tetracycline.

¹³ The starter culture was allowed to grow at 37 °C and 200 rpm over night. A 5 ml
¹⁴ sampling culture of the medium to be studied containing the appropriate antibiotics
¹⁵ was prepared. The media tested included LB, terrific broth (TB) and auto-induction
¹⁶ media like ZYP-5052. The sampling culture was inoculated to an OD⁶⁰⁰ of 0.075
¹⁷ using the starter culture and incubated at different temperatures and 200 rpm in
¹⁸ a shaking incubator. 1 mM isopropyl-D-thiogalactopyranosid (IPTG) was added
¹⁹ when the OD⁶⁰⁰ reached 0.6-0.8, if appropriate for the studied construct. 1 ml
²⁰ samples were removed after different times of incubation (e.g. 4, 8, 12 hours),
²¹ subfractionated (3.4.2) and analyzed via SDS-polyacrylamide gel electrophoresis
²² (PAGE) (3.4.5).

²³ Exact specifications of growth conditions (e.g. temperature, time, constructs) are
²⁴ discussed in the individual sections.

²⁵ 3.4.2 Protein subfractionation

²⁶ The protein subfractionation procedure described herein was adapted from the pro-
²⁷ tocol described in the pET Manual [Novagen2011]. Overall 5 protein subfractions
²⁸ can be obtained, including *total cell protein*, *culture supernatant (medium) protein*,
²⁹ *periplasmic protein*, *soluble cytoplasmic protein* and *insoluble protein*.

³⁰ The OD⁶⁰⁰ of the culture sample was measured and the cells harvested by centrif-
³¹ gation at 10000 × *g*, 4 °C for 5 minutes. The protein in the supernatant medium was
³² concentrated by precipitation with trichloro acetic acid (TCA) (3.4.3) for SDS-PAGE
³³ 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 $(OD^{600} \times V \times 50) \mu\text{l}$ 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 $10 \mu\text{l}$ of each fraction were used for SDS-PAGE analysis.

3.4.3 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 $14000 \times g$, 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 $1 \times \text{SDS-sample buffer}$ by heating to 85°C and vigorous vortexing, to achieve a $10 \times$ 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* [19]. The periplasm 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 [3]. The cell pellet was resuspended in the same volume as the culture sample of 30 mM tris-HCl , 20% (w/v) sucrose, $\text{pH } 8$ and $1 \text{ mM ethylenediaminetetraacetic acid (EDTA)}$ was added. The suspension was stirred for 10 min at RT and the cells were collected by centrifugation at $10000 \times g$, 4°C for 10 min. The supernatant was discarded and the cell pellet was resuspended in the same volume of ice-cold 5 mM 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 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 [17]. The SDS-PAGE procedure was carried out according to standard protocols described by Sambrook and Russell [33]. 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 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.

3.4.7 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, yeast extract, phosphate (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 necessary. Cells were harvested by centrifugation at $10000 \times g$, 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 ≈ 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 $15000 \times g$, 4 °C for 15 minutes followed by filtration through a 0.45 μ m 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 μ g/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 μ g/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 ($10000 \times g$, 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 3.4.8.

BWEIGEL: Überprüfen!

3.4.8 Preparation of inclusion bodies (IBs)

Often, when recombinant protein is produced in high levels in *E. coli* it is accumulated in so-called inclusion 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 [25].

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 μ g/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 μ g/ml) was

added and the solution was incubated on ice for 10 min. The lysate was clarified by centrifuging for 1 h at $20000 \times g$, 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 $20000 \times g$, 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.

3.4.9 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 imidazole 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. 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.

3.4.10 Enzymatic production of SAM and derivatives

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 $14000 \times g$, 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 $(\text{NH}_4)_2\text{SO}_4$, 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

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/MSD, Tokyo, 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/MSD, Tokyo, Japan).

Indexing and integration of the reflexes via Fourier transformation was accomplished using XDS [13, 12, 14] or MOSFLM [26]. Scala [8], 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 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 [32]. Phases of the structures herein were exclusively determined by MR [29, 30].

MR was performed using the software *Phaser* [20, 21], which is included in the CCP4-Suite [39]. A previously published PFOMT structure (PDB-code: 3C3Y [16]) 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 [6]. Structure refinement was done using the software REFMAC5 [23, 37] as part of the CCP4-suite or the Python-based Hierarchical 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/>) [9, 22, 36]. 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 [34]. 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 $\mu\text{g/ml}$), that was always part of the experiments, was used to quantify the sample measurements.

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 mM L-glutathion 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. To start the reactions enzyme was added; 20 μg , PFOMT. 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 ($10000 \times g$, 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 $10000 \times g$ for 10 min to remove unsoluble matter. The supernatant was

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

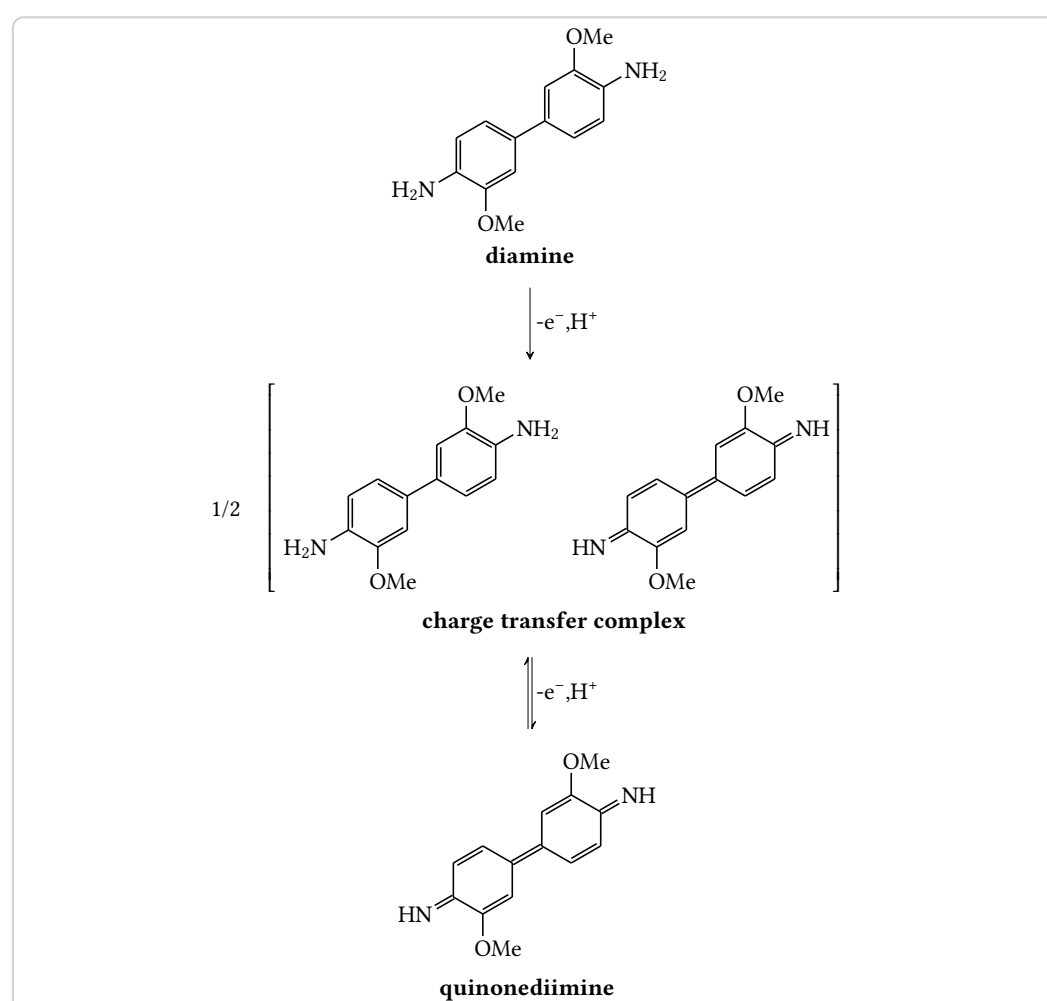


Figure 3.1.: Oxidation of the reporter substrate o-dianisidine. Consecutive one-electron transfers lead to the fully oxidized diimine form of o-dianisidine. The first electron transfer is believed to produce a charge transfer complex intermediate. [11, 5]

transferred into a HPLC vial and analyzed by high-performance liquid chromatography (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 ($10000 \times g$, 4 °C, 10 min) the supernatant was transferred into HPLC-sample vials and analyzed (see 3.6.5).

3.6.3 Measurement of activity/pH profiles

Assays to measure activity over larger pH ranges were set up in 100 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.

3.6.4 Photospectrometric assay for the methylation of catecholic moieties

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 [18]. 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. *O*-MT enzyme assay extracts (3.6.2) were analyzed using an acetonitrile gradient starting with 5 % acetonitrile (1 min). The acetonitrile content was increased to 100 % in 29 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.

¹4 **Evaluation of PFOMT towards** ²**the acceptance of long-chain SAM** ³**analogues**

⁴4.1 **Introduction**

⁵4.2 **Substrate binding studies using ITC**

⁶4.3 **Determination of the structure of *apo*-PFOMT**

⁷4.3.1 **PFOMT activity in deep eutectic solvents (DES) / Solubility- ⁸enhancing effects of DES**

⁹vielleicht eigenes kapitel DES?

¹⁰4.4 **Study of variants for long-chain alkylations**

¹¹4.4.1 **PFOMT-Paper (DIM)**

¹²4.4.2 **Dockings???**

¹³4.5 **Colclusion/Discussion**

¹ 5 Enzymatic methylation of Non- ² catechols

³ 5.1 Introduction

⁴ Non-catechols in nature (biosynthesis, mode of action?), chemical methylation???

⁵ 5.2 SOMT-2

⁶ 5.2.1 In vivo methylation studies using *N. benthamiana*

⁷ 5.2.2 In vivo studies in *E. coli*

⁸ 5.2.3 In vitro studies using recombinantly produced SOMT-2

⁹ 5.3 PFOMT

¹⁰ 5.3.1 Acidity and Nucleophilicity of phenolic hydroxyl-groups

¹¹ 5.3.2 pH-Profiles of PFOMT-catalysis

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

¹³ 5.4 Consensus or Bioinformatic points-of-view
¹⁴ (COMT)???

¹⁵ 5.5 Conclusion/Discussion

¹ **6 Development of an whole cell** ² **methyl transferase screening sys-** ³ **tem**

⁴ **6.1 Introduction**

⁵ **6.2 Theoretical considerations / design of system**

⁶ **6.3 Detectability of *S*-adenosyl-L-homocysteine** ⁷ **(SAH)**

⁸ *S*-adenosyl-L-methionine (SAM)

⁹ **6.4 Usage of the *lsr*-promoter for true autoinduc-** ¹⁰ **tion**

¹¹ **6.5 Conclusion/Discussion**

¹ **7 DES in protein crystallography**

² **7.1 Introduction**

³ **7.2 Solubility enhancement of hydrophobic substances by addition of DES** ⁴

⁵ **7.3 Enzymatic *O*-methylation in DES**

⁶ **7.4 DES as precipitants in protein crystallization**

⁷ **7.5 Conclusion/Discussion**

8 Acknowledgements

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Appendix

1

2

A Figures

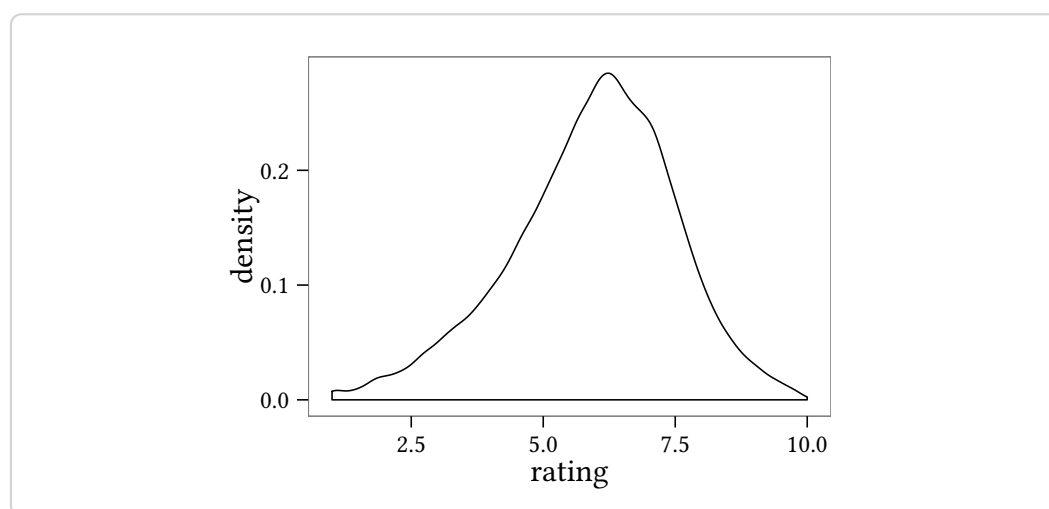


Figure A.1.: Lorem ipsum dolor sit amet, consectetur adipiscing elit. Aenean commodo ligula eget dolor. Aenean massa. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec quam felis, ultricies nec, pellentesque eu, pretium quis, sem.

B Tables

Table B.1.: Overview over the 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 | lsrA promoter pBEW102 with BamHI cloning site rhaP _{BAD} promoter | pBEW102 pBEW4b | pBEW103 | amplification (<i>pRha1.fw/rv</i>), cloning (BglII, BamHI) |
| pBEW102 | | | | |
| pBEW103 | | | | |
| pBEW104 | | | | |
| pBEW106 | pICH413038-somt | pET28MC-somt | pICH413038 | amplification (<i>somt1/2/3/4</i>), golden gate cloning (Bpil) |
| pBEW107 | | pICH51266, pBEW106, pICH41421 | pICH75044 | golden gate cloning (BsaI) |
| 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>pfomt1.fw/rv</i>), amplification (<i>pfomt2.fw/rv</i>), cloning (NdeI, EcoRI) |
| pET20-somt | N-terminal pelB-tag fusion for periplasmic expression | | pET20-b(+) | |
| pET28-somt | | | pET28-a(+) | |
| pET28MC-somt | N-terminal Trx-tag fusion N-terminal GST-tag fusion added BglII site pUC19 derivative with lsrA promoter | pUC19 lsr-XX-DAS | pET-32a(+) | mutagenesis (<i>pUC1.fw/rv</i>) cloning (NdeI, BglII) |
| pET32-somt | | | pET-41a(+) | |
| pET41-somt | | | - | |
| pUC19* | | | pUC19* | |
| pUCB1 | | | | |
| pUCB1-sfGFP-DAS+4 | | | | |

¹ **C Affidavit**

² I hereby declare that this document has been written only by the undersigned and
³ without any assistance from third parties. Furthermore, I confirm that no sources
⁴ have been used in the preparation of this document other than those indicated in
⁵ the thesis itself.

⁶ Date:....., Location:....., Signature:.....

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¹ Acronyms

² **Å** Ångstrom, 0.1 nm

³ **B-PER** bacterial protein extraction reagent

⁴ **CCP4** Collaborative Computational Project No. 4 17, 18

⁵ **CD** circular dichroism 10

⁶ **Coot** Crystallographic Object-Oriented Toolkit 18

⁷ **CV** column volumes

⁸ **DES** deep eutectic solvent, see also NADES 7

⁹ **DTT** dithiothreitol; (2S,3S)-1,4-bis(sulfanyl)butane-2,3-diol

¹⁰ **EDTA** ethylenediaminetetraacetic acid 13, 15, 16

¹¹ **FPLC** fast protein liquid chromatography 16

¹² **GdmCl** guanidinium hydrochloride

¹³ **GOD** glucose oxidase 19, 38

¹⁴ **HEPES** 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

¹⁵ **HPLC** high-performance liquid chromatography 21

¹⁶ **HRP** horseradish peroxidase 19

¹⁷ **IB** inclusion body 15, 16

¹⁸ **IMAC** immobilized metal affinity chromatography

¹⁹ **IPB** Leibniz-Institute of Plant Biochemistry

²⁰ **IPTG** isopropyl-D-thiogalactopyranosid 12, 15

²¹ **LB** lysogeny broth 12, 14, 15

²² **MES** 2-(N-morpholino)ethanesulfonic acid

²³ **MLU** Martin-Luther-Universität

²⁴ **MMT** L-malic acid/MES/Tris 6

-
- ¹ **MR** molecular replacement
- ² **MTP** micro-titer plate 17, 19, 38
- ³ **MW** molecular weight 14
- ⁴ **MWCO** molecular weight cut-off
- ⁵ **NADES** natural deep eutectic solvent vii, 7, 36
- ⁶ **NPS** nitrogen, phosphate, sulfate buffer
- ⁷ **NTA** nitrilo triacetic acid 16
- ⁸ **O-MT** O-methyl transferase 19, 21
- ⁹ **PAGE** polyacrylamide gel electrophoresis 12–14
- ¹⁰ **PBS** phosphate buffered saline 13
- ¹¹ **PCR** polymerase chain reaction 11
- ¹² **PDA** photo diode array 21
- ¹³ **PDB** Protein Data Base 18
- ¹⁴ **PFOMT** phenylpropanoid and flavonoid O-methyl transferase 14, 15, 17–19, 38
- ¹⁵ **PHENIX** Phyton-based Hierarchial Environment for Integrated Xtallography 18
- ¹⁶ **PMSF** phenylmethanesulfonylfluoride
- ¹⁷ **RT** room temperature 13, 14
- ¹⁸ **SAH** S-adenosyl-L-homocysteine 17
- ¹⁹ **SAM** S-adenosyl-L-methionine 24
- ²⁰ **SDS** sodium dodecylsulfate 7, 12–14
- ²¹ **SOMT-2** soy O-methyl transferase 15
- ²² **TB** terrific broth 12
- ²³ **TCA** trichloro acetic acid 12–14, 21
- ²⁴ **Ti-plasmid** tumor inducing plasmid 8, 38
- ²⁵ **Tris** tris(hydroxymethyl)-aminomethane
- ²⁶ **UV/VIS** ultra violet/visible (light spectrum) 21
- ²⁷ **V** volume
- ²⁸ **ZYP** N-Z-amine, yeast extract, phosphate 14, 15, 38

¹ Glossary

² **GOD** Glucose oxidase is an enzyme.... 36

³ **MTP** Micro-titer plate. Small format rectangular plastic plate containing wells
⁴ to allow for storage of multiple small samples or the containment multiple
⁵ simultaneous reactions. Typical sizes include 24, 96 and 384-wells 37

⁶ **PFOMT** Phenylpropanoid and flavonoid O-methyl transferase from *Mesembryan-*
⁷ *themum crystallinum*, which was first described by Ibdah et al. in 2003 [10]
⁸ 37

⁹ **Ti-plasmid** Commonly found plasmids in *A. tumefaciens* and *A. rhizogenes* that
¹⁰ confer virulence 37

¹¹ **ZYP-5052** Autoinduction medium developed by Studier [35]. The naming stems
¹² from the components N-Z-amine, yeast extract and phosphate. The numbering
¹³ designates the composition; e.g. 5052 refers to 0.5 % glycerol, 0.05 % glucose and
¹⁴ 0.2 % lactose. 37