

Biotransformations from and to methylated flavonoids

Subtitle

Benjamin Weigel
Leibniz-Institute of Plant Biochemistry
Department of Bioorganic Chemistry
Weinberg 3
06120 Halle(Saale)
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Advisor: Prof. Dr. Ludger A. Wessjohann
wessjohann@ipb-halle.de
+49 (345) 5582-1301

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Preface

1 Abstracts

1.1 English Abstract

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1.2 Deutsche Zusammenfassung

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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 nature's 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

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 Instruments

circular dichroism (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)
fast protein liquid chromatography (FPLC)	ÄKTA purifier (GE Healthcare, Freiburg, Germany)
gas chromatography coupled mass-spectrometry (GC/MS)	GC-MS-QP2010 Ultra (Shimadzu, Duisburg, Germany)
high-performance liquid chromatography (HPLC)	VWR-Hitachi LaChrom Elite (VWR, Darmstadt, Germany)
Isothermal Titration Calorimetry (ITC)	MicroCal iTC200 (Malvern, Worcestershire, UK)
micro-titer plate (MTP)-reader	SpectraMax M5 (Molecular Devices, Biberach, Germany)
nuclear magnetic resonance (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)

3.1.3 Bacterial strains

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(r_K⁻m_K⁺) phoA supE44</i> λ ⁻ <i>thi-1 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(r_K⁻m_K⁺)</i> Martin-Luther-University Halle-Wittenberg
JW1593 (BW25113 derivative)	<i>rrnB</i> Δ <i>lacZ</i> 4787 <i>HsdR514</i> 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</i> <i>hsdSB</i> (r_B^- , m_B^-) <i>gal dcm</i> λ (DE3) pRARE (Cam ^R) Novagen, Wisconsin (USA)
Rosetta(DE3) pLysS	F^- <i>ompT</i> <i>hsdSB</i> (r_B^- , m_B^-) <i>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 Δ(<i>mcrC-mrr</i>)114::IS10</i> NEB, Massachusetts (USA)

Agrobacterium tumefaciens

GV3101	chromosomal background: C58, marker gene: <i>rif</i> , tumor inducing plasmid (Ti-plasmid): cured, opine: nopaline Sylvestre Marillonet, IPB
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3.1.4 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)
pQU30-PFOMT	Thomas Vogt (IPB, Halle, Germany)

3.1.5 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	

3.1.6 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 [10].

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 [12] 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 [5, 3]. 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 *pfomt*

The *pfomt* gene was subcloned from the pQE-30 vector kindly provided by Thomas Vogt (IPB, Halle, Germany) into the pET-28a(+) vector. First an endogenous NdeI site was removed by site directed mutagenesis using primers *pfomt1.fw* and *pfomt1.rv*. The *pfomt* gene was then amplified using oligonucleotides *pfomt2.fw* and *pfomt2.rv* and cloned into pET-28 employing the NdeI and EcoRI cloning sites.

3.3.3 Subcloning of *somt-2*

3.4 Protein biochemistry

Stock solutions of antibiotics, IPTG or sugars were prepared according to the pET System Manual by Novagen [9], 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 antibiotics used was as follows: 200 $\mu\text{g ml}^{-1}$ ampicillin, 150 $\mu\text{g ml}^{-1}$ kanamycin, 50 $\mu\text{g ml}^{-1}$ chloramphenicol, 20 $\mu\text{g ml}^{-1}$ tetracycline.

The starter culture was allowed to grow at 37 °C and 200 rpm over night. A 5 ml sampling culture of the medium to be studied containing the appropriate antibiotics was prepared. The media tested included LB, terrific broth (TB) and auto-induction media like ZYP-5052. The sampling culture was inoculated to an OD⁶⁰⁰ of 0.075 using the starter culture and incubated at different temperatures and 200 rpm in a shaking incubator. 1 mM isopropyl-D-thiogalactopyranosid (IPTG) was added when the OD⁶⁰⁰ reached 0.6-0.8, if appropriate for the studied construct. 1 ml samples were removed after different times of incubation (e.g. 4, 8, 12 hours), sub-fractionated (3.4.2) and analyzed via sodium dodecylsulfate (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 [9]. Overall 5 protein subfractions can be obtained, including *total cell protein*, *culture supernatant (medium) protein*, *periplasmic protein*, *soluble cytoplasmic protein* and *insoluble protein*.

The OD⁶⁰⁰ 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 (OD⁶⁰⁰ $\times V \times 50$) 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 cyto-

lic protein was omitted. Sample volumes of 10 μ 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 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* [8]. The periplasma is, other than the cytosol, an oxidizing environment and often used for the production of proteins containing disulfide linkages. The preparation of periplasmic protein was accomplished by an osmotic shock protocol modified from Current Protocols in Molecular Biology [1]. 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 $10000 \times g$, 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 [7].

The SDS-PAGE procedure was carried out according to standard protocols described by Sambrook and Russell [10]. 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 Production of recombinant protein

Heterologous production of PFOMT

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 [11]. 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 [2].

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^{-1} *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 $100 \mu\text{g ml}^{-1}$), that was always part of the experiments, was used to quantify the sample measurements.

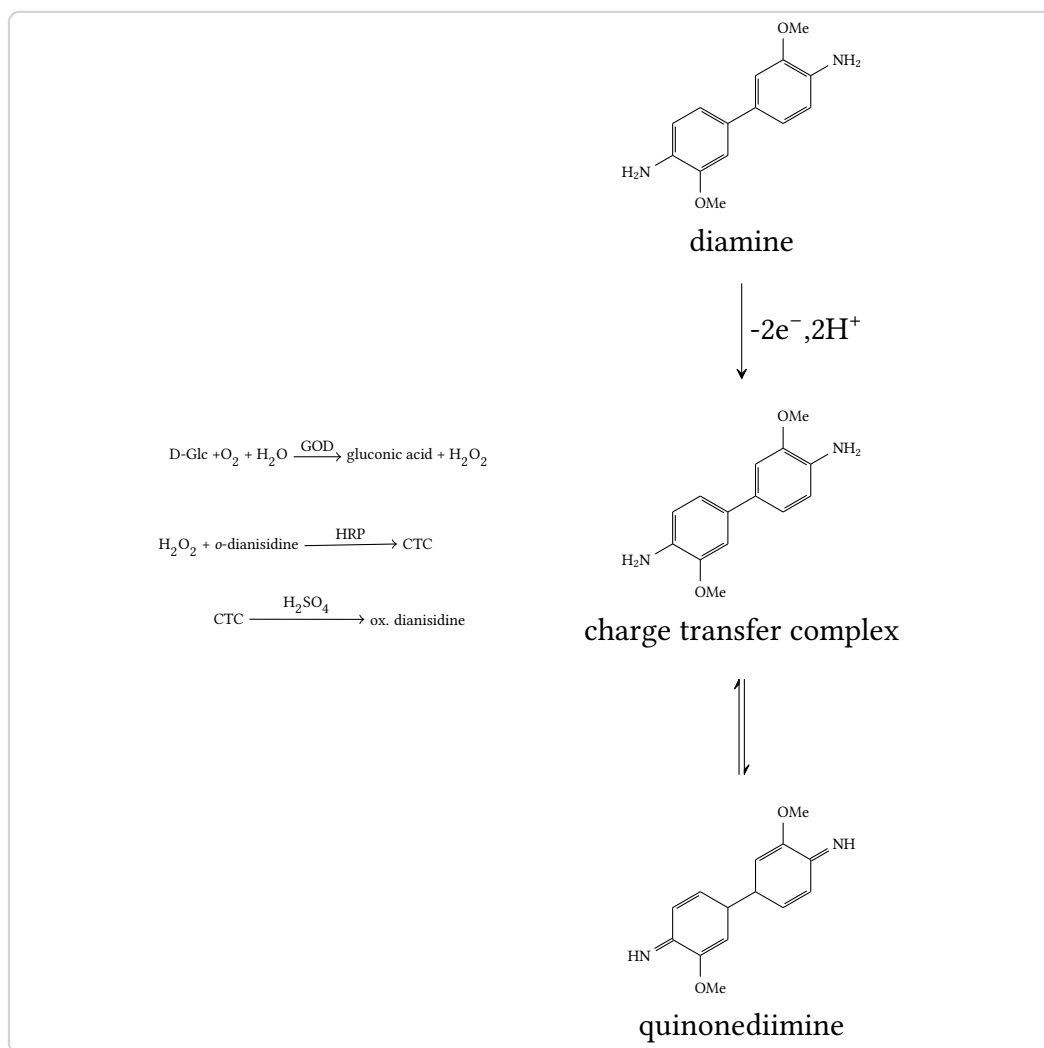


Figure 3.1.: GOD assay

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-L-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

7.1 Introduction

7.2 Solubility enhancement of hydrophobic substances by addition of DES

7.3 Enzymatic *O*-methylation in DES

7.4 DES as precipitants in protein crystallization

7.5 Conclusion/Discussion

8 Acknowledgements

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Appendix

A Figures

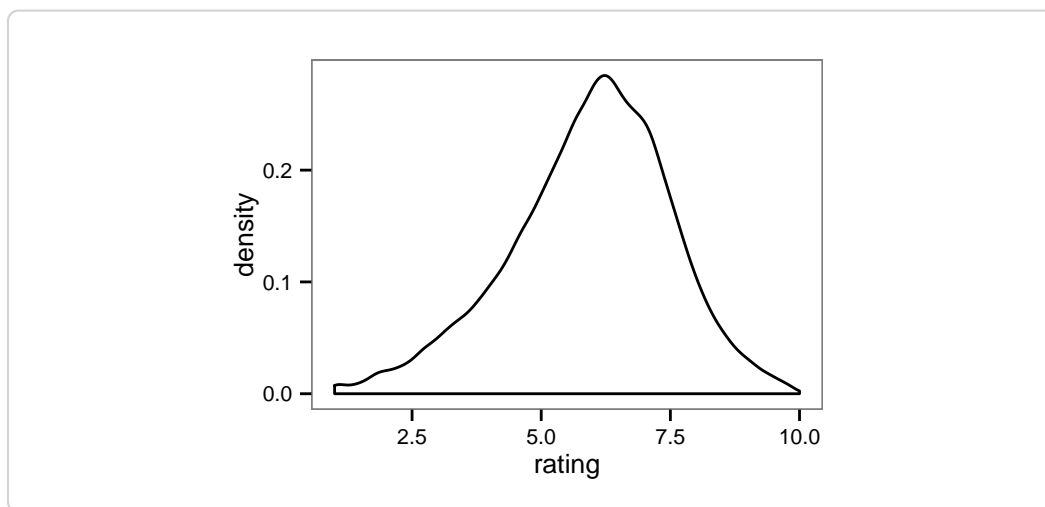


Figure A.1.: Lorem ipsum dolor sit amet, consectetur adipiscing elit. Aenean commodo ligula eget dolor. Aenean massa. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec quam felis, ultricies nec, pellentesque eu, pretium quis, sem.

B Tables

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

name	description	entry constructs	destination	workflow steps (primers/cloning sites)
pBEW101	lsrA promoter			
pBEW102	pBEW102 with BamHI cloning site	pBEW102	pBEW103	amplification (<i>pRha1.fw/rv</i>), cloning (BglII, BamHI)
pBEW103	rhaP _{BAD} promoter	pBEW4b		
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 (Bsal)
pBEW1a				
pBEW1b				
pBEW2a				
pBEW2b				
pBEW3a				
pBEW3b				
pBEW4a				
pBEW4b				
pET28-pfomt	<i>pfomt</i> gene in pET-28a(+), endogenous NdeI site removed	pQE30-pfomt	pET-28a(+)	mutagenesis (<i>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				
pET32-somt	N-terminal Trx-tag fusion		pET-32a(+)	
pET41-somt	N-terminal GST-tag fusion		pET-41a(+)	
pUC19*	added BglII site	pUC19	-	mutagenesis (<i>pUC1.fw/rv</i>)
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] F.M. Ausubel et al. “Current Protocols in Molecular Biology”. In: (2008).
- [2] 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.
- [3] 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.
- [4] Mwafaq Ibdah et al. “A novel Mg(2+)-dependent O-methyltransferase in the phenylpropanoid metabolism of *Mesembryanthemum crystallinum*.” In: *The Journal of biological chemistry* 278.45 (Nov. 2003), pp. 43961–72.
- [5] Youichi Kondou et al. “cDNA Libraries”. In: *Methods in Molecular Biology* 729 (2011), pp. 183–197.
- [6] 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. 154–64.
- [7] U K Laemmli. “Cleavage of structural proteins during the assembly of the head of bacteriophage T4.” In: *Nature* 227.5259 (1970), pp. 680–685.
- [8] Savvas C Makrides. “Strategies for achieving high-level expression of genes in *Escherichia coli*.” In: *Microbiological Reviews* 60.3 (1996), pp. 512–538.
- [9] Novagen. *pET System Manual*. 11th ed. Darmstadt: EMD Biosciences, 2005.
- [10] J Sambrook and D W Russell. *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor (NY, USA): Cold Spring Harbor Laboratory Press, 2001.
- [11] Sigma-Aldrich. *Glucose (GO) Assay Kit - Technical Bulletin GAGO-20*.
- [12] Agilent Technologies. *QuikChange Site-Directed Mutagenesis Kit Instruction Manual*. 2013.

- [13] 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. 159–62.

Acronyms

B-PER bacterial protein extraction reagent

CD circular dichroism 6

EDTA ethylenediaminetetraacetic acid 12

FPLC fast protein liquid chromatography 6

GC/MS gas chromatography coupled mass-spectrometry 6

GOD glucose oxidase 13, 30

HPLC high-performance liquid chromatography 6

HRP horseradish peroxidase 13

IPB Leibniz-Institute of Plant Biochemistry

IPTG isopropyl-D-thiogalactopyranosid 11

ITC Isothermal Titration Calorimetry 6, 30

LB lysogeny broth 11

MTP micro-titer plate 6, 13, 30

MW molecular weight 13

NMR nuclear magnetic resonance 6

PAGE polyacrylamide gel electrophoresis 11–13

PBS phosphate buffered saline 12

PCR polymerase chain reaction 9, 10

RT room temperature 11–13

SAM S-adenosyl-L-methionine 18

SDS sodium dodecylsulfate 11–13

TB terrific broth 11

TCA trichloro acetic acid 11–13

Ti-plasmid tumor inducing plasmid 8, 30

V volume

Glossary

GOD Glucose oxidase is an enzyme.... 28

Isothermal Titration Calorimetry (ITC) Fill in description here 28

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 28

Ti-plasmid Commonly found plasmids in *A. tumefaciens* and *A. rhizogenes* that confer virulence 29