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Biotransformations from and to methylated flavonoids

Hpw all went to shit

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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

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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 Thermo Scientific (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

circulary 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$^-$ *ompT hsdSB*(r_B^-,m_B^-) *gal dcm* λ(DE3) Invitrogen, Karslruhe
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*, tumor inducing plasmid (Ti-plasmid): cured, opine: nopaline Sylvestre Marillonet, IPB

3.1.4 Plasmids

Table 3.1.: Plasmids used in this work.

name	description
pACYC Duet-1	..
pCDF Duet-1	..
pET20b(+)	...
pET28a(+)	..
pET32a(+)	..
pET41a(+)	..
pQE30	..
pUC19	..

3.1.5 Oligonucleotides

Oligonucleotides and primers were ordered from Eurofins Genomics (Ebersberg, Germany). The purity grade was *high purity salt free* (HPSF).

Table 3.2.: Primers used in this work. Recognition sites for endonucleases are underlined.

name	sequence (5'→3')	cloning site
somt1	TTGAAGACAAAATGGCTTCTTCATTAAACAATGGCCG	BpiI
somt2	TTGAAGACAAGGACACCCCAAATACTGTGAGATCTTCC	BpiI
somt3	TTGAAGACAAGTCCTTAGGAACACCTTTCTGGGAC	BpiI
somt4	TTGAAGACAAAAGCTCAAGGATAGATCTCAATAAGAGAC	BpiI

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 [6].
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 [8] 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 [4, 2]. 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.

3.4 Protein biochemistry

3.4.1 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 [7]. 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 [1].
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 µg ml^{-1}), that was always part of the experiments, was used to quantify the sample measurements.

3.5.2 HPLC measurements

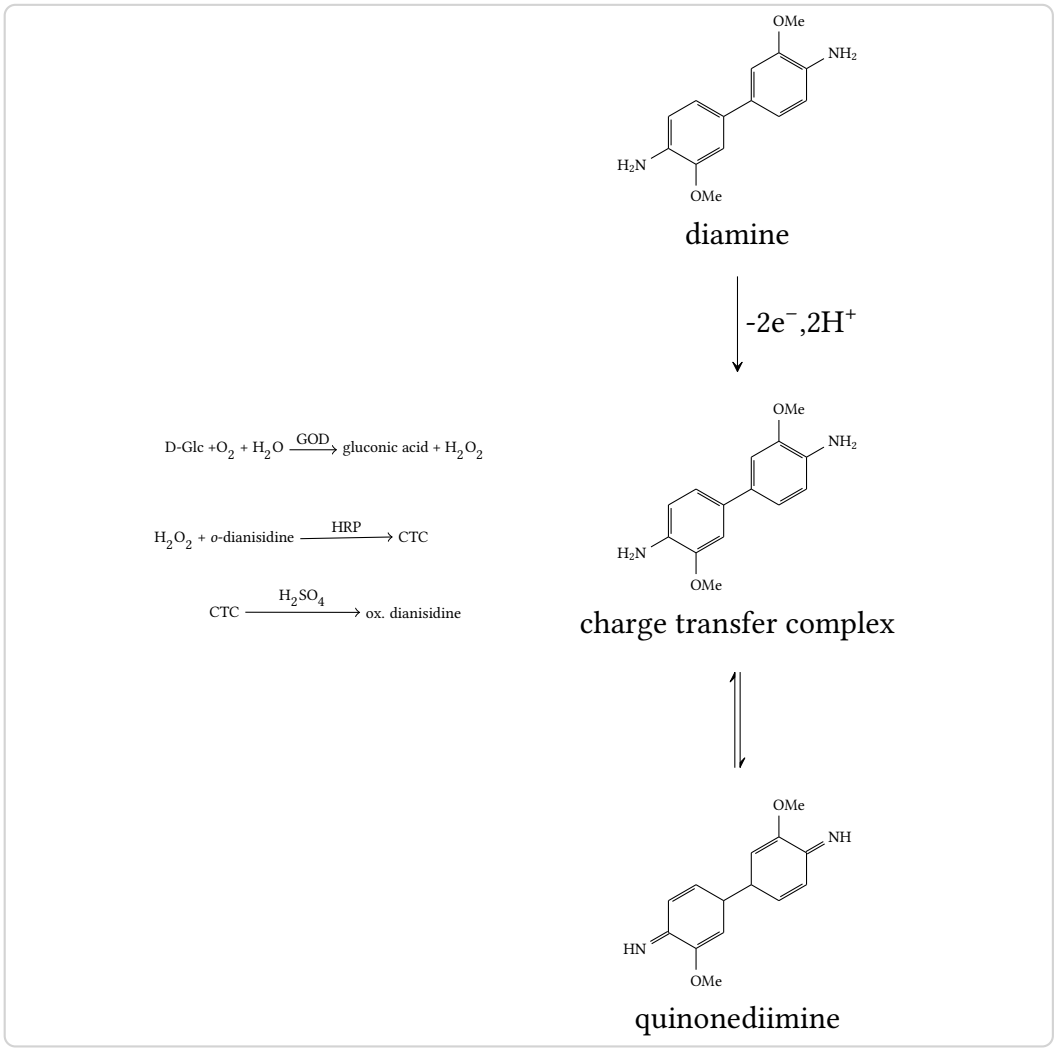

Figure 3.1.: GOD assay

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

5 Enzymatic methylation of Non-catechols

testing the HPLC and again the HPLC.

Blöalala phenylpropanoid and flavonoid O-methyl transferase (PFOMT) and PFOMT

6 Development of an whole cell methyl transferase screening system

7 Acknowledgements

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III

Appendix

A Figures

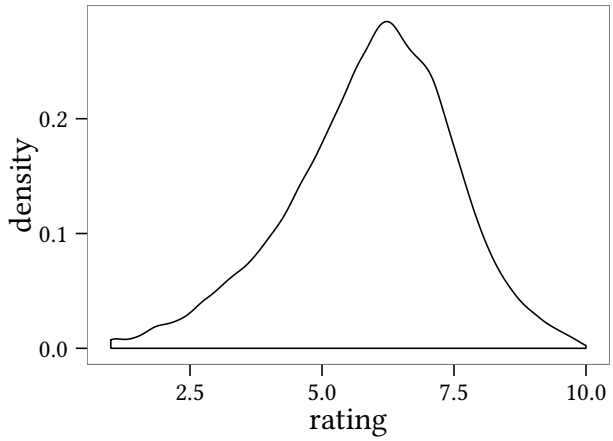

Figure A.1.: *Lorem ipsum dolor sit amet, consectetuer adipiscing elit. Aenean commodo ligula eget dolor. Aenean massa. Cum sociis natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec quam felis, ultricies nec, pellentesque eu, pretium quis, sem.*

B Tables

Table B.1.: *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.*

A	B	C	D	E	F	G	H	I
1	2	3	4	5	6	7	8	9
1	2	3	4	5	6	7	8	9
1	2	3	4	5	6	7	8	9

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

CD circulary dichroism. 10

FPLC fast protein liquid chromatography. 10

GC/MS gas chromatography coupled mass-spectrometry. 10
GOD glucose oxidase. 14, 37

HPLC high-performance liquid chromatography. 10, 19
HRP horseradish peroxidase. 14

ITC Isothermal Titration Calorimetry. 10, 37

MTP micro-titer plate. 10, 14, 37

NMR nuclear magnetic resonance. 10

PCR polymerase chain reaction. 13
PFOMT phenylpropanoid and flavonoid O-methyl transferase. 19, 37

Ti-plasmid tumor inducing plasmid. 12, 37

Glossary

GOD Glucose oxidase is an enzyme.... . 35

Isothermal Titration Calorimetry (ITC) Fill in description here. 35

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. 35

PFOMT Phenylpropanoid and flavonoid O-methyl transferase from *Mesembryanthemum crystallinum*, which was first described by Ibdah et al. in 2003 [**Ibdah2003b**]. 35

Ti-plasmid Commonly found plasmids in *A. tumefaciens* and *A. rhizogenes* that confer virulence. 35