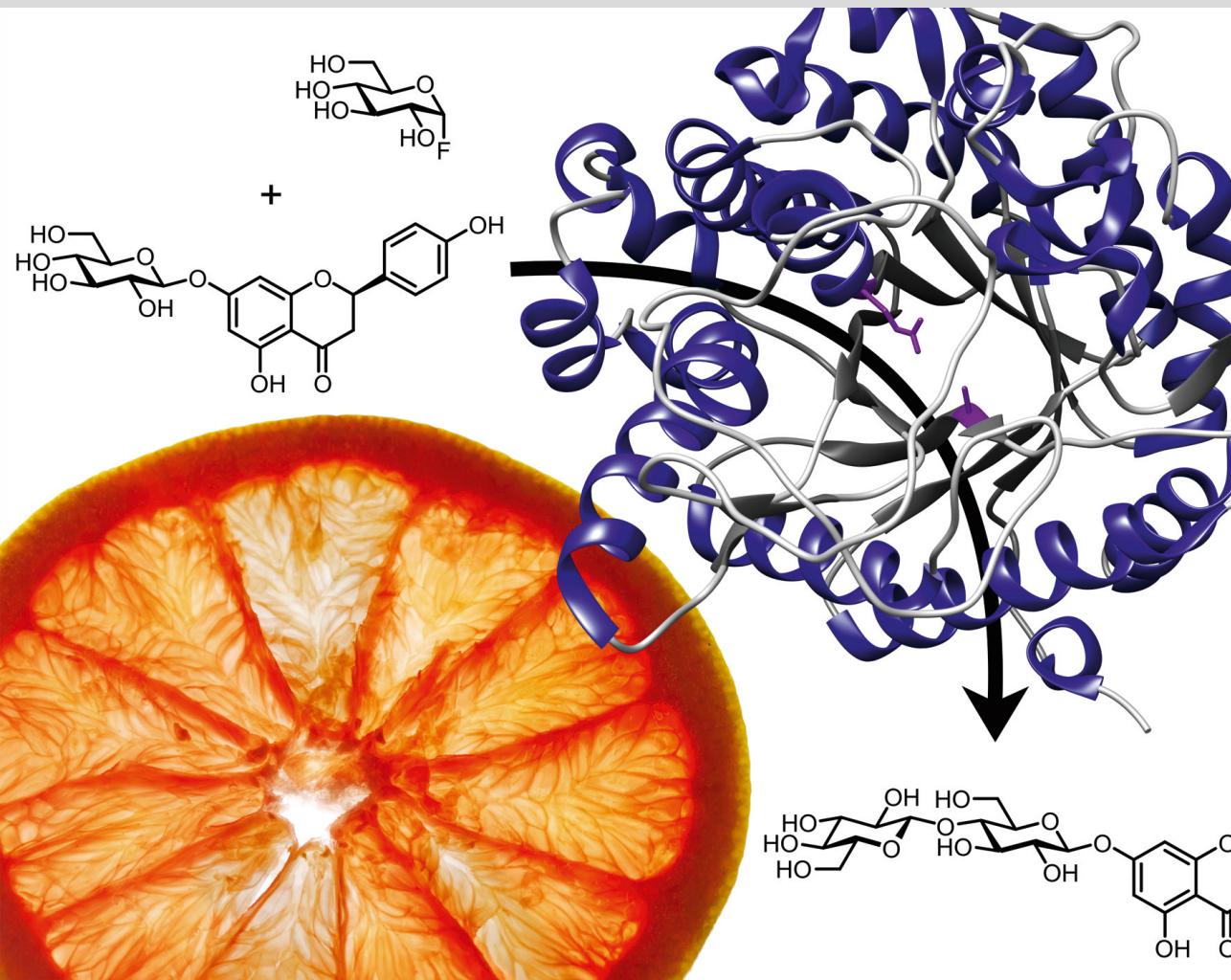


Glycosynthases – tuning glycosidase activity towards glycoside diversification and synthesis

Marc Richard Hayes



Bioorganische Chemie an der Heinrich-Heine-Universität
im Forschungszentrum Jülich

Forschungszentrum Jülich GmbH
Institut für Bio- und Geowissenschaften
IBOC – Bioorganische Chemie

Glycosynthases – tuning glycosidase activity towards glycoside diversification and synthesis

Marc Richard Hayes

Bioorganische Chemie an der Heinrich-Heine-Universität
im Forschungszentrum Jülich

Band 37

ISBN 978-3-95806-441-6

TABLE OF CONTENTS

1	Abbreviations.....	1
2	Abstract	1
3	Kurzzusammenfassung.....	3
4	Introduction.....	5
4.1	Importance of glycosides.....	5
4.2	Thesis objective.....	7
5	State of knowledge	9
5.1	Glycosynthases — from hydrolysis to synthesis	9
5.2	Biocatalysis towards β -linked glycosides	12
5.2.1	Synthesis of β -glycosides	12
5.2.2	Glycosidase candidates — a versatile toolbox for β -glycoside synthesis	21
5.3	Biocatalysis towards α -linked glycosides	27
5.3.1	Synthesis with α -glycosynthases	27
5.3.2	Biocatalysis towards rhamnosides	31
5.4	Transferring glycans by <i>endo</i> - β -N-acetylglucosaminidases.....	34
5.4.1	Glycosynthases derived from <i>endo</i> - β -N-acetylglucosaminidases.....	34
5.4.2	Endo-CC — an ENGase with high potential	36
6	Results.....	38
6.1	From hot to cold — searching for optimal glycosynthase conditions by varying temperature optima	38
6.1.1	Glucosidase gene isolation	38
6.1.2	Characterisation of the wild type glycosidases	43
6.1.3	Structural analysis and mutagenesis of the β -glucosidases	49
6.1.4	Detecting glycosynthase activity	57
6.1.5	Development of a high-throughput assay for glycosynthase characterisation .	66
6.1.6	Glycosynthase kinetics and acceptor screening	71
6.2	A substrate based approach for glycosynthase development.....	77
6.2.1	Choice and isolation of β -glucosidase Cbg1	77
6.2.2	Characterisation of Cbg1	80
6.2.3	Mutagenesis and screening for synthetic activity.....	83

6.2.4	Expanding the mutant library of Cbg1 for synthetic application	88
6.2.5	Cbg1 for transglycosylation	97
6.3	Creating a rhamnosynthase	101
6.3.1	Synthesis of rhamnosyl substrates.....	101
6.3.2	Characterisation of the <i>wt</i> α -L-rhamnosidase	104
6.3.3	Mutagenesis of α -L-rhamnosidase RhaB	109
6.3.4	Screening for azide release.....	111
6.3.5	Attempts to find a α -L-rhamnosynthase.....	114
6.4	From monosaccharides to <i>en bloc</i> glycan transfer.....	125
6.4.1	<i>En bloc</i> glycosylation.....	125
6.4.2	Glycosylating RNase B-GlcNAc.....	126
6.4.3	Transferring glycans to low molecular weight acceptors.....	127
6.4.4	Heterologous expression of Endo-CC N180H.....	130
7	Summary and Outlook	133
8	Experimental Section	144
8.1	General	144
8.1.1	Devices.....	144
8.1.2	Consumables	145
8.1.3	Chemicals and enzymes	146
8.1.4	Oligonucleotides and plasmids.....	146
8.1.5	Bacterial strains	151
8.1.6	Software	151
8.2	Molecular biological methods	152
8.2.1	Isolation of genomic DNA from <i>R. radiobacter</i>	152
8.2.2	Plasmid isolation	152
8.2.3	DNA concentration determination	153
8.2.4	Agarose gel electrophoresis	153
8.2.5	DNA gel elution	154
8.2.6	DNA ligation and restriction	154
8.2.7	DNA amplification by PCR	155
8.2.8	Mutagenesis by inverse-PCR.....	156

8.2.9	Gibson Assembly cloning.....	157
8.2.10	DNA sequencing	158
8.3	Microbiological methods.....	158
8.3.1	Chemically competent cells.....	158
8.3.2	Transformation of chemically competent <i>E. coli</i> strains	159
8.3.3	Cultivation of <i>E. coli</i> strains	160
8.3.4	Heterologous expression of enzymes	160
8.3.5	Esculin-agar plate assay	161
8.4	Protein biochemical methods	162
8.4.1	Cell lysis	162
8.4.2	Protein isolation.....	162
8.4.3	Buffer exchange, desalting, and concentrating protein samples.....	163
8.4.4	Protein concentration determination	164
8.4.5	Lyophilisation	164
8.4.6	SDS-PAGE.....	165
8.5	Biocatalytic assays.....	166
8.5.1	Hydrolytic activity	166
8.5.2	Coniferin hydrolysis.....	167
8.5.3	Glycosynthase activity assay	167
8.5.4	Chemical recovery experiments.....	168
8.5.5	Glycosylation modification of RNase B	169
8.5.6	Naringinase deactivation and activity assay	169
8.6	Chemical synthesis	171
8.6.1	Chromatography	171
8.6.2	LC-MS analysis.....	171
8.6.3	Mass spectrometry.....	172
8.6.4	NMR-Spectroscopy	172
8.6.5	IR-Spectroscopy.....	173
8.6.6	Measurement of rotatory power ($[\alpha]_D^{20}$).....	173
8.6.7	Melting points	173
8.6.8	Peracetylation of monosaccharides	173

8.6.9	Fluorination of peracetylated glycosides	174
8.6.10	O-Glycosylation	176
8.6.11	One-pot peracetylation and bromination of α -L-rhamnose (11e).....	178
8.6.12	Synthesis of 2,3,4-tri-O-acetyl β -L-rhamnopyranosyl azide (22v)	179
8.6.13	Deprotection of acetylated compounds	180
8.6.14	Synthesis of triisopropyl-(4-nitrophenoxy)-silane (TIPSpNP, 23)	183
8.6.15	Enzymatic synthesis of prunin (1b) from naringin (1c).....	184
8.6.16	Biocatalytic synthesis of β -D-glucosides.....	185
8.6.17	Glycosylation of glycoside molecules by Endo-CC N180H	189
9	Appendix.....	192
9.1	Gene and protein sequences	192
9.1.1	Standard expression vectors	192
9.1.2	<i>abg</i> — β -Glucosidase of <i>R. radiobacter</i>	193
9.1.3	<i>bglU</i> — β -Glucosidase of <i>M. antarcticus</i>	195
9.1.4	<i>bglC</i> — β -Glycosidase of <i>P. furiosus</i>	196
9.1.5	<i>cbg1</i> — β -Glucosidase of <i>R. radiobacter</i>	198
9.1.6	<i>rhaB</i> — α -L-Rhamnosidase of <i>Bacillus</i> sp. GL1	200
9.1.7	<i>endo-CC N180H</i> — <i>Endo-N-acetylglucosaminidase</i> variant of <i>C. cinerea</i>	202
9.2	Codon harmonisation of <i>bglU</i>	204
9.3	Content of own contribution to the published publications during the work of this thesis	206
10	References	207
11	List of Synthesised Molecules	219
12	Acknowledgements.....	222
13	Declaration.....	225

**Bioorganische Chemie an der Heinrich-Heine-Universität Düsseldorf
im Forschungszentrum Jülich**

Herausgegeben von Jörg Pietruszka

The most abundant biological molecules on earth are carbohydrates. They cover the surface of all cellular organisms and are added to the structure of numerous molecules produced by these cells. Glycosides are essential for the given biological and physicochemical properties of a specific compound, and exert a major influence on cell recognition, health and immunity, the functionality and stability of peptides, and many other processes throughout biology.

Due to the large impact of glycosylation, the demand for simple methods for the synthesis of defined glycosides is constantly rising. The use of enzymes such as glycosyltransferases, glycosidases, and glycosynthases capable of transferring or hydrolysing glycosidic structures has gained much interest by organic chemists. This thesis focussed on the development of new glycosynthases with the aim of creating a versatile biocatalytic toolbox for glycosylation and glycodiversification in organic synthesis.

The objective was approached in four projects, each focussing on a different aspect of the glycosylation reaction carried out by glycosynthases. These encompassed the influence of temperature on the glycosylation reaction by applying mutated glycosidases with extremophilic properties; a substrate based approach to glycosynthase development in order to enable glycosylation of phenolic compounds; studies toward the development of an α -L-rhamnosynthase; and the transfer of large glycoside structures to small molecules by *endo*-N-acetylglucosaminidases.

Band 37

ISBN 978-3-95806-441-6