

A Glycosynthase Catalyst for the Synthesis of Flavonoid Glycosides**

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In memory of Charles W. Rees

Glycosylation of lipophilic small molecules is one of the predominant strategies by which the bioactivity of these compounds is modulated in living organisms.^[1–4] Indeed, even simple plants, such as *Arabidopsis thaliana*, dedicate over 120 open reading frames encoding many activated sugar-dependent glycosyltransferases to this task alone.^[5] Novel synthetic routes to such compounds provide a powerful tool for exploring not only this activity by providing ready access to probes, standards, and inhibitors but also to the many plant natural products such as flavonoids and steroid glycosides that have been implicated as potential therapeutics.^[6–12] Despite the many elegant methods for chemical glycosylation,^[13] methods for direct regioselective glycosylation of acceptors are limited,^[14] which has led to a heavy dependence upon protecting-group regimes that introduce many additional steps. The often exquisite selectivity of biocatalytic methods provides the potential to overcome such problems. Although the use of glycosyltransferases (GTs) is nature's solution to glycoside bond formation, access to suitable GTs and donors limits this approach.^[15] Furthermore, the use of GTs is typically limited to the transfer of only single carbohydrate residues.

The use of glycosidases provides an alternative method for biocatalytic glycosylation.^[16,17] One of the most powerful approaches to the enzymatic synthesis of glycosides is Withers's "glycosynthase" technology.^[18–21] Glycosynthases are genetically engineered nucleophile-less mutant glycosidases that can catalyze the formation of glycosidic linkages, primarily but not exclusively by using glycosyl fluoride donors, yet are incapable of hydrolysing the product. A

number of glycosynthases have been described; yet, strikingly, owing to the inherent preference of the binding subsites of these enzymes for carbohydrate residues, only a single study of non-carbohydrate acceptors has been conducted to date. Logically this elegant work allowed the synthesis of glycosceramides through the construction of an endoglycosceramidase glycosynthase.^[22] In this way the inherent acceptor specificity of this biocatalyst was still the primary determinant of function. However, to our knowledge no glycosynthase with a wild-type preference for carbohydrate acceptors has been used efficiently with non-carbohydrate acceptors. Herein we demonstrate that the substrate tolerance of glycosynthases can be significantly broadened to allow the use of non-carbohydrate acceptors, thereby providing valuable access to lipophilic glycoconjugates. Specifically, the E197S mutant^[23] of the *Humicola insolens* Cel7B enzyme has been identified as a catalyst for the formation of flavonoid glycosides, with sugar transfer directly to the flavonoid scaffold. These represent substrates to which the E197S mutant has no known specificity. Remarkably, rates of glycosyltransfer are comparable with those of naturally occurring uridine diphosphate (UDP)-sugar-dependent glycosyltransferases on the same acceptors.

We have recently developed a high-throughput MS-based screen for glycosyltransfer.^[24,25] By using this method, large panels of substrates may be readily screened allowing the determination of a "green–amber–red" (GAR) virtual microarray of activity that reflects the presence of the glycosylated product. By using this GAR screen with the aim of identifying optimal catalysts for the synthesis of glyco-lipophilic products, we screened 80 acceptors (see Figure S1 in the Supporting Information) with greater than 100 biocatalysts and more than 20 glycosyl donors. We identified not only the expected^[26] GT catalysts capable of such a transfer, but also, surprisingly, a glycosidase mutant, the glycosynthase E197S mutant from the *H. insolens* glycosidase Cel7B.^[27] As can be seen from the GAR array (Figure 1; Cel7B–E197S with disaccharide donor lactosyl fluoride (**LacF**)), this striking non-natural activity is specific. From 80 potential acceptors, only the glycosylation of flavonoids **2** and **20–22** was observed; this suggested the first non-GT-catalyzed biocatalytic methods for access to such glycoflavonoids **81–85**. The screen also, less unusually, revealed carbohydrate acceptors: the known^[23] substrate benzyl- β -glucoside (**52**) and the unknown substrate, the plant cytokinin *trans*-zeatin glucoside **66**. No activity at all was observed against a range of other representative acceptor substrates.

Excited by this unusual, non-natural but specific flavonoid activity, we investigated it in further detail. Remarkably, initial conversion levels suggested rates of glycoside forma-

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

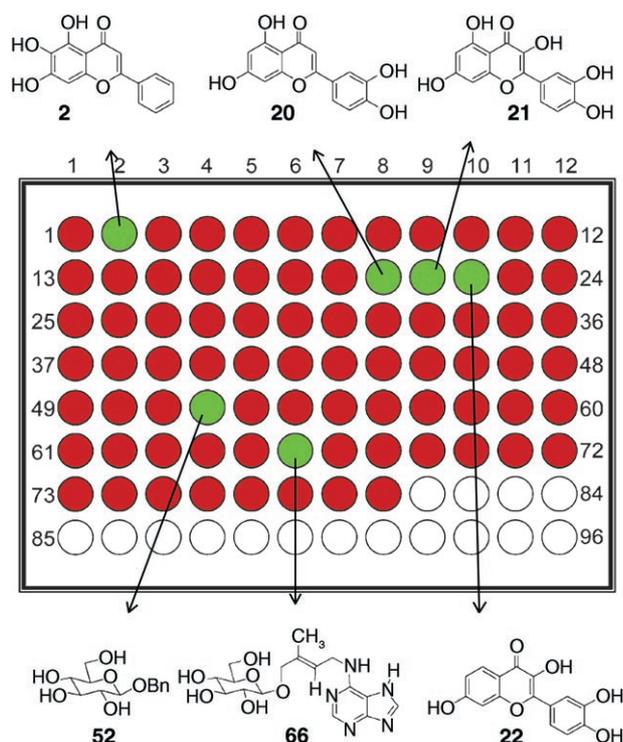


Figure 1. GAR acceptor screen of Cel7B–E197S with LacF as donor. The virtual microarray of activity reflects the presence of glycoconjugated product detected by MS and, hence, glycosylating activity of the biocatalyst with the given acceptor (green = clear activity, signal-to-noise ratio $S/N > 10$; red = complete absence, $S/N = 1$). Bn = benzyl.

tion comparable to those catalyzed by GTs. This very high level of activity for such a non-natural catalyst was confirmed through detailed kinetic analysis by using MS methods under a pseudo-single-substrate method.^[24] Under these conditions,

apparent Michaelis–Menten kinetics for transfer of disaccharides to compounds **2** and **20–22** were observed (Scheme 1, Table 1, Figure 2, and Figure S3 in the Supporting Information).^[*] The resulting parameters highlight specific activities

Table 1: Steady-state kinetic parameters for the *H. insolens* Cel7B–E197S catalyzed synthesis of flavonoid lactosides.

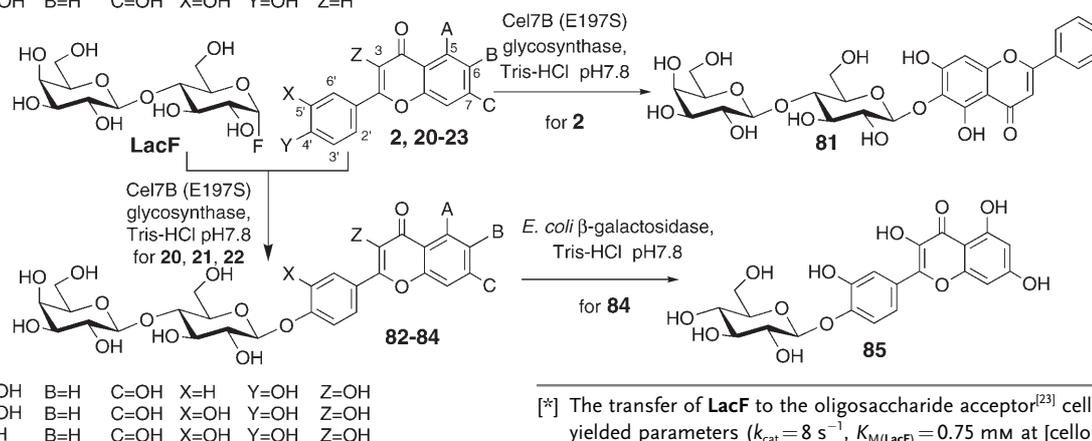
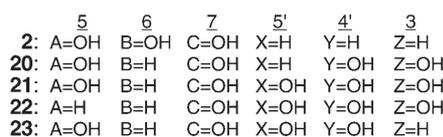
Substrate ^[a]	k_{cat} [s^{-1}]	K_{M} [μM]	$k_{\text{cat}}/K_{\text{M}}$ [$\text{M}^{-1} \text{s}^{-1}$]
baicalein (2)	0.24 ± 0.03	92 ± 12	2597 ± 338
luteolin (20)	0.44 ± 0.04	113 ± 17	3879 ± 581
quercetin (21)	0.14 ± 0.02	106 ± 11	1282 ± 133
fisetin (22)	0.31 ± 0.07	141 ± 31	2199 ± 483

[a] [LacF] fixed at $2000 \mu\text{M}$. Flavonoids varied from $20\text{--}150/180 \mu\text{M}$ depending on solubility.

($k_{\text{cat}}/K_{\text{M}} = 1282\text{--}3879 \text{M}^{-1} \text{s}^{-1}$ at [LacF] = 2mM) comparable with those for natural glycosyltransferases that transfer monosaccharides to the same compound (**21**; e.g. *V. vinifera* UDP-Glc dependent glucosyltransferase (UGT)^[26] $k_{\text{cat}}/K_{\text{M}} = 2437 \text{M}^{-1} \text{s}^{-1}$ at [UDPGlc] = 2mM).

Some key structural substrate specificity features emerge. Interestingly, although broad tolerance of the position of hydroxylation in the chromene moiety at C6, C7, and C8 is observed in the flavonoid acceptor substrates, a requirement for dihydroxylation in the C2 aryl moiety appears important as monohydroxylated kaempferol (**23**) showed no detectable activity as an acceptor.

The full synthetic utility of Cel7B–E197S was assessed through preparative-scale syntheses of flavonoid glycosides **81–85** in yields of 72–95%. This highlighted an exquisite stereoselectivity (only β -glycosides) and regioselectivity for the glycosylation of the hydroxy group at O4' to generate the flavonoid with the lactoside at O4' (Scheme 1). When O4' is absent, as in baicalein (**2**), O6 is glycosylated instead. This transfer to O4', coupled with the requirement for polyhy-



Scheme 1. Sequential biocatalysis: stereo- and regioselective transfer of lactosyl from lactosyl fluoride (LacF) to flavonoids, catalyzed by Cel7B–E197S glycosynthase followed by selective hydrolytic cleavage allows ready access to unusual regioisomers of natural glycoflavonoids.

[*] The transfer of LacF to the oligosaccharide acceptor^[23] cellobiose yielded parameters ($k_{\text{cat}} = 8 \text{s}^{-1}$, $K_{\text{M(LacF)}} = 0.75 \text{mM}$ at [cellobiose] = 5mM) consistent with those determined previously for the (better) acceptor pNP–cellobiose ($k_{\text{cat}} = 18 \text{s}^{-1}$, $K_{\text{M(LacF)}} = 1.1 \text{mM}$ at [pNP–cellobiose] = 20.6mM) by using a fluoride electrode.

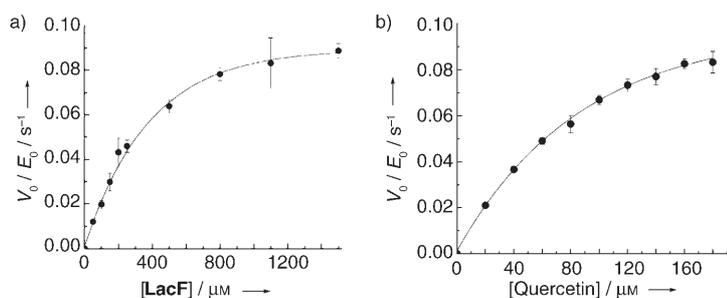


Figure 2. Observed Michaelis–Menten kinetics for Cel7B–E197S for the transfer of lactosyl from **LacF** to quercetin (**21**), under pseudo-single-substrate conditions of fixed [**21**] (a) and fixed [**LacF**] (b).

droxylation in the C2 aryl substituent (O4', O5'), suggests that the aryl ring of **2** effectively mimics a monosaccharide residue in the +1 subsite of Cel7B–E197S.^[28] This substrate mimicry is consistent with a mode of recognition often observed^[23,27] in such subsites that utilizes a hydrophobic “sandwich” coupled with equatorial hydrogen bonding. Here, this motif simultaneously engages O5, O6, and O7 and π faces. Alternatively, the more electron-rich aryl phenols may have more suitable pK_a values and/or nucleophilicities. Consistent with this possibility, variation of pH from 7 to 9 led to a threefold increase in the rate of glycosylation of **21** by Cel7B–E197S by using **LacF** as a donor (see the Supporting Information; “baseless” mutants of Cel7B–E197S are currently also being investigated). Regardless, this functional “glycomimicry” in the biocatalysis shown here by the polyhydroxylated-2-phenyl of flavonoids gives rise to an unusual activity and otherwise inaccessible regioselectivity (O4' and O6) in biocatalytic flavonoid glycosylation. Furthermore, the catalyst used here allows the unusual transfer of a disaccharide rather than monosaccharide motif.

Finally, highly selective representative hydrolysis of **83** using *E. coli* β -galactosidase allowed access to the monoglycosylated glucoflavonoid natural product **85** in 90% yield. Cel7B–E197S showed no activity with the monosaccharide donor, α -glucosyl fluoride (**GlcF**). Therefore, this sequential two-step biocatalytic procedure allowed unique and ready access to unusual regioisomers of natural glucoflavonoids in a striking overall yield (65%) without the need for protecting groups. Spiraeoside (**85**), synthesized before in only 6% yield,^[29] shows powerful cytotoxic activity in oral cancers^[30] and in human trials showed clear inhibition of platelet cell signaling and thrombus formation.^[31] This highly selective one-pot tandem biocatalytic method also outstrips the yields of other glucoflavonoid syntheses such as that of the quercetin glucoside isoquercitrin obtained from chemical syntheses^[32–34] (<37%) and even those of fermentor and whole-cell glycosylations (<41%) with GTs.^[35]

In summary, a non-natural glycosidase mutant (Cel7B–E197S glycosynthase) has been identified that has novel regioselectivity (O4', O6) and activity (disaccharide transfer to flavonoids) with catalytic efficiencies comparable with those of natural glycosyltransferase counterparts. The mutant can be used as a component in sequential biocatalysis and

strikingly allows efficient, high-yielding, one-step access to glycolipophilic compounds, such as the glycoflavonoids **81–85** described herein, and includes unique regioisomers of natural glycoflavonoids in a manner not previously possible.

Experimental Section

GAR screening was performed as described by Yang et al.^[24] Briefly, in a 96-well plate, each well was filled with Tris-HCl buffer solution (Tris = Tris(hydroxymethyl) aminomethane; 1.0 mM, pH 7.8, 150 μ L), donor (10 mM, 2 μ L), acceptor (10 mM, 2 μ L), and enzyme (1 mg mL⁻¹, 2 μ L). The solutions and the plate were incubated at 37°C for 16 h, and the solutions were analyzed for formation of product by LCMS monitoring. Single-substrate concentration kinetics were also performed by MS at 25°C with both **LacF** fixed at 2 mM and the acceptors fixed at 100 μ M. LCMS was used to monitor the formation of product through the appearance of corresponding ions and relating this to the total ion count with concentration determined through internal calibration. Pseudo-single-substrate kinetics were also performed by MS at 25°C with the acceptor fixed at 100 μ M and the donor varied at different concentrations from 50–1500 μ M (i.e., up to 3.1 times K_M); or **LacF** was fixed at 2000 μ M and the acceptor typically varied from 20–150 μ M. Product formation was monitored by LCMS through the appearance of corresponding ions and relating this to the total ion count with concentration determined through the internal standard glucose (500 μ M).

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- [1] C. E. Ruefer, C. Gerhaeuser, N. Frank, H. Becker, S. E. Kulling, *Mol. Nutr. Food Res.* **2005**, *49*, 851.
- [2] H. Steinkellner, S. Rabot, F. Kassie, S. Knasmüller, *Adv. Exp. Med. Biol.* **2001**, *500*, 629.
- [3] C. M. M. Gachon, M. Langlois-Meurinne, P. Saindrenan, *Trends Plant Sci.* **2005**, *10*, 542.
- [4] E. K. Lim, D. J. Bowles, *EMBO J.* **2004**, *23*, 2915.
- [5] B. Henrissat, P. M. Coutinho, G. J. Davies, *Plant Mol. Biol.* **2001**, *47*, 55.
- [6] H. Pellissier, *Tetrahedron* **2004**, *60*, 5123.
- [7] A. Furstner, *Eur. J. Org. Chem.* **2004**, 943.
- [8] A. Kohara, C. Nakajima, K. Hashimoto, T. Ikenaga, H. Tanaka, Y. Shoyama, S. Yoshida, T. Muranaka, *Plant Mol. Biol.* **2005**, *57*, 225.
- [9] S. Walker, M. J. Sofia, R. Kakarla, N. A. Kogan, L. Wierichs, C. B. Longley, K. Bruker, H. R. Axelrod, S. Midha, S. Babu, D. Kahne, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 1585.
- [10] H. Matsuda, T. Wang, H. Managi, M. Yoshikawa, *Bioorg. Med. Chem.* **2003**, *11*, 5317.
- [11] B. Mishra, K. I. Priyadarsini, M. S. Kumar, M. K. Unnikrishnan, H. Mohan, *Bioorg. Med. Chem.* **2003**, *11*, 2677.
- [12] X. L. Yang, Y. Sun, Q. Xu, Z. J. Guo, *Org. Biomol. Chem.* **2006**, *4*, 2483.
- [13] B. G. Davis, *J. Chem. Soc. Perkin Trans. 1* **2000**, 2137.
- [14] F. Z. Kong, *Curr. Org. Chem.* **2003**, *7*, 841.
- [15] A. M. Daines, B. A. Maltman, S. L. Flitsch, *Curr. Opin. Chem. Biol.* **2004**, *8*, 106.
- [16] D. H. G. Crout, G. Vic, *Curr. Opin. Chem. Biol.* **1998**, *2*, 98.
- [17] H. D. Ly, S. G. Withers, *Annu. Rev. Biochem.* **1999**, *68*, 487.

- [18] S. J. Williams, S. G. Withers, *Aust. J. Chem.* **2002**, *55*, 3.
- [19] D. L. Jakeman, S. G. Withers, *Trends Glycosci. Glycotechnol.* **2002**, *14*, 13.
- [20] G. Perugino, B. Cobucci-Ponzano, M. Rossi, M. Moracci, *Adv. Synth. Catal.* **2005**, *347*, 941.
- [21] G. Perugino, A. Trincone, M. Rossi, M. Moracci, *Trends Biotechnol.* **2004**, *22*, 31.
- [22] M. D. Vaughan, K. Johnson, S. DeFrees, X. P. Tang, R. A. J. Warren, S. G. Withers, *J. Am. Chem. Soc.* **2006**, *128*, 6300.
- [23] V. M.-A. Ducros, C. A. Tarling, D. L. Zechel, A. M. Brzozowski, T. P. Frandsen, I. von Ossowski, M. Schulein, S. G. Withers, G. J. Davies, *Chem. Biol.* **2003**, *10*, 619.
- [24] M. Yang, M. Brazier, R. Edwards, B. G. Davis, *ChemBioChem* **2005**, *6*, 346.
- [25] M. Yang, M. R. Proctor, D. N. Bolam, J. C. Errey, R. A. Field, H. J. Gilbert, B. G. Davis, *J. Am. Chem. Soc.* **2005**, *127*, 9336.
- [26] W. Offen, C. Martinez-Fleites, M. Yang, E. Kiat-Lim, B. G. Davis, C. A. Tarling, C. M. Ford, D. J. Bowles, G. J. Davies, *EMBO J.* **2006**, *25*, 1396.
- [27] S. Fort, V. Boyer, L. Greffe, G. J. Davies, O. Moroz, L. Christiansen, M. Schulein, S. Cottaz, H. Driguez, *J. Am. Chem. Soc.* **2000**, *122*, 5429.
- [28] No other regioisomers were observed in the analyses (HPLC, NMR spectroscopy, LCMS) of any of the crude products, therefore suggesting a regioselectivity of greater than 95% in all cases.
- [29] L. Farkas, B. Vermes, M. Nogradi, *Chem. Ber.* **1972**, *105*, 3505.
- [30] A. M. Browning, U. K. Walle, T. Walle, *J. Pharm. Pharmacol.* **2005**, *57*, 1037.
- [31] G. P. Hubbard, S. Wolfram, J. A. Lovegrove, J. M. Gibbins, *J. Thromb. Haemostasis* **2004**, *2*, 2138.
- [32] L. Hoerhammer, H. Wagner, H. G. Arndt, R. Dirscherl, L. Farkas, *Chem. Ber.* **1968**, *101*, 450.
- [33] Z. Chen, Y. Hu, H. Wu, H. Jiang, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3949.
- [34] M. Bouktaib, A. Atmani, C. Rolando, *Tetrahedron Lett.* **2002**, *43*, 6263.
- [35] E.-K. Lim, D. A. Ashford, B. Hou, R. G. Jackson, D. J. Bowles, *Biotechnol. Bioeng.* **2004**, *87*, 623.