Glycosynthases: Mutant Glycosidases for Oligosaccharide Synthesis

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Efficient methods for the synthesis of oligosaccharides, particularly on the large scale, are unavailable despite the enormous importance of this class of molecules in a range of biological processes[1] and their potential as new therapeutics.[2] The synthetic problems reside in the control of both the stereochemistry and the regiochemistry of bond formation, requiring complex protection and activation strategies. While significant advances in methodologies for chemical synthesis have been made in recent years,[3] particularly in the use of polymer-supported approaches,[4] these remain complex and unsuitable for large-scale production. The alternative is enzymatic synthesis, and glycosyl transferases have indeed been used for this extensively in recent years, even on a large scale.[5] However, the poor availability of these enzymes and the high substrate cost[6] have limited their application. Retaining glycosidases, run in the transglycosylation mode, are the other choice.[7] These enzymes function through the mechanism shown in Figure 1 (lower path) in which a covalent glycosyl−enzyme intermediate is formed and hydrolyzed with general acid/base catalytic assistance.[8] Synthesis can be achieved, as shown in Figure 1 (upper path), by intercepting the reactive glycosyl−enzyme intermediate with an added acceptor sugar. While this approach offers the use of a cheap donor sugar[9] and relatively easily available enzymes, it has the substantial disadvantage that the product is necessarily a substrate for the enzyme and is subsequently hydrolyzed, resulting in poor yields, unless the equilibrium can somehow be displaced.[10] We report here a new approach to oligosaccharide synthesis involving the use of a specifically mutated glycosidase (Glycosynthase) which, in conjunction with activated glycosyl donors of the *opposite* anomeric configuration to that of the normal substrate and suitable acceptors, can efficiently synthesize oligosaccharides, but does not hydrolyze them.

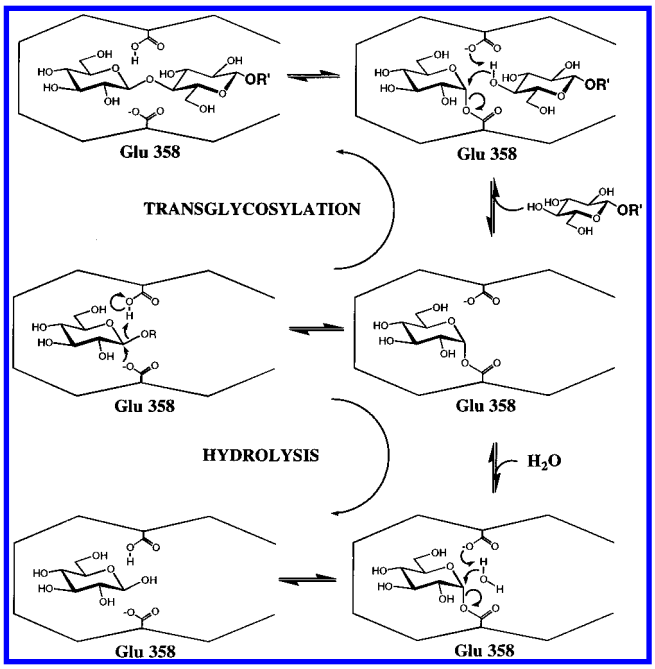

Figure 1. Mechanisms of hydrolysis and transglycosylation catalyzed by *Agrobacterium* sp. β-glucosidase.

Earlier studies on β-glycosidases revealed[11,12] that replacement of the active site carboxylate nucleophile with a nonnucleophilic amino acid side chain results in a correctly folded enzyme, which is catalytically inactive[13] since it cannot form the requisite α-glycosyl−enzyme intermediate. However, since the rest of the active site is intact[14] it might be expected to catalyze the ligation of an activated α-glycosyl derivative, bound at the active site in place of the normal glycosyl−enzyme intermediate, to a suitable acceptor sugar bound in the aglycon pocket. The oligosaccharide, once formed, could not then be hydrolyzed by the enzyme. This process would be assisted by general base catalysis from the deprotonated acid/base residue.[15] The Glu358Ala mutant of the *Agrobacterium* sp. β-glucosidase/galactosidase (AbgGlu358Ala) was chosen to test this approach because the wild-type enzyme ordinarily catalyses efficient transglycosylation[16] and the mutant has no detectable hydrolytic activity.[12] α-Glycosyl fluorides were chosen as readily synthesized[17] activated α-glycosyl donors with

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(1) Kobata, A. *Acc. Chem. Res.* **1993**, *26*, 319. Varki, A. *Glycobiology* **1993**, *3*, 97.

(2) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 521. Sears, P.; Wong, C.-H. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12086. Zopf, D.; Roth, S. *The Lancet* **1996**, *347*, 1017. Simon, P. M. *Drug Discovery Today* **1996**, 1, 522.

(3) Danishefsky, S. J.; Bilodeau, M. *Angew. Chem., Int. Ed.* **1996** *35*, 1380. Boons, G.-J. *Tetrahedron* **1996**, *52*, 1095.

(4) Douglas, S. P.; Whitfield, D. M.; Krepinsky, J. J. *J. Am. Chem. Soc.* **1991**, *113*, 3, 5095. Roberge, J. Y.; Beebe, X.; Danishefsky, S. J. *Science* **1995**, *269*, 202. Liang, R.; Yan, L.; Loebach, J.; Ge, M.; Uozumi, Y.; Sekanina, K.; Horan, N.; Gildersleeve, J.; Thompson, C.; Smith, A.; Biswas, K.; Still, W. C.; Kahne, D. *Science* **1996**, *274*, 1520.

(5) Nilsson, J. G. I. *TIBTECH* **1988**, *6*, 256. Thiem, J. *FEMS Microbiol. Rev.* **1995**, *16*, 193.

(6) Complex, but in many cases effective, recycling schemes can be used to regenerate NDPsugars in some cases (Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G.-J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 9283).

(7) Retaining glycosidases hydrolyze glycosides with net retention of anomeric configuration. Synthesis can also be carried out under equilibrium conditions, using high concentrations of the free sugars.

(8) McCarter, J. D.; Withers, S. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885−892. Sinnott, M. L. *Chem. Rev.* **1990**, *90*, 1171.

(9) Typical donors are nitrophenyl glycosides or glycosyl fluorides. Having high k_{cat}/K_m values they are used by the enzyme in preference to the accumulating products.

(10) Equilibria have been displaced using a coupled enzyme to selectively convert product formed by adsorption of product onto charcoal and by selective crystallization of product or use of organic cosolvents.

(11) Wakarchuk, W. W.; Campbell, R. L.; Sung, W. L.; Davoodi, J.; Yaguchi, M. *Protein Sci.* **1994**, *3*, 467. Yuan, J.; Martinez-Bilbao, M.; Huber, R. E. *Biochem. J.* **1994**, *299*, 527. Withers, S. G.; Rupitz, K.; Trimbur, D.; Warren, R. A. J. *Biochemistry* **1992**, *31*, 9979.

(12) Wang, Q.; Graham, R. W.; Trimbur, D.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc.* **1994**, *116*, 11594.

(13) Utmost care must be exercised to avoid contamination with wild-type enzyme including use of new column packings for mutant purifications. A Glu358Gly mutant of the *Agrobacterium* sp. β-glucosidase was contaminated (1 in 10 000) due to translational misincorporation.[12]

(14) Additional evidence for correct folding of this mutant resides in its substantial activity as an inverting glycosidase if azide is added as an alternate nucleophile.[12]

(15) Conversion of the nucleophilic Glu to Gln in the mechanistically analogous *Bacillus circulans* xylanase dropped the pK_a of the acid/base catalyst 2.5 units, to a value below the optimum pH (McIntosh, L. P.; Hand, G.; Johnson, P. E.; Joshi, M. D.; Korner, M.; Plesniak, L. A.; Ziser, L.; Wakarchuk, W. W.; Withers, S. G. *Biochemistry* **1996**, *35*, 9958).

(16) Kempton, J. B.; Withers, S. G. *Biochemistry* **1992**, *31*, 9961. Prade, H.; Mackenzie, L. F.; Withers, S. G. *Carbohydr. Res.* **1998**. In press.

(17) α-Glycosyl fluorides are synthesised by reaction of the per-O-acetylated sugar with HF/pyridine followed by deprotection with methoxide in methanol.

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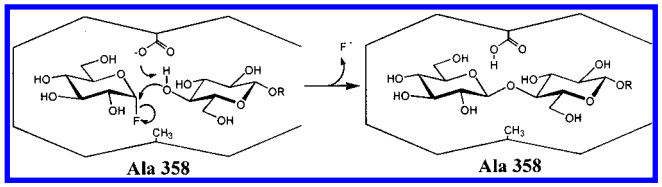
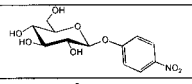
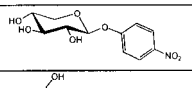
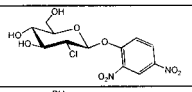
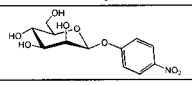
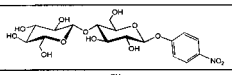
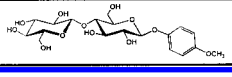


Figure 2. Proposed mechanism of Glycosynthase AbgGlu358Ala.

Table 1. Glycosynthase-Catalyzed Transglycosylation Reactions Using α-Galactosyl Fluoride as Donor

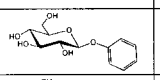
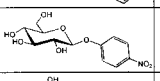
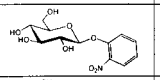
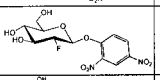
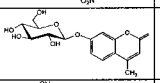
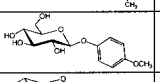
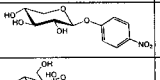
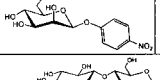
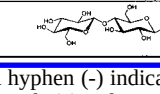
#	ACCEPTOR	PRODUCTS (% YIELD) β-1,4 linked(unless otherwise stated)
		Disaccharide
1		**84%**
2		**81%** (β-1,3 linked)
3		**64%**
4		**66%**
		Trisaccharide
5		**92%**
6		**88%**

a small aglycon/leaving group.[18] Acceptors chosen initially were aryl glycosides, which are known to bind well to the aglycon site[16,19] but cannot be cleaved by the mutant. The proposed reaction mechanism is shown in Figure 2.

The approach is illustrated with the synthesis of a trisaccharide Gal-β(1,4)-Glc-β(1,4)-Glc-*p*-nitrophenyl. A 2 mL reaction mixture containing α-galactosyl fluoride (14 mg, 40 mM) and *p*-nitrophenyl β-cellobioside (28 mg, 30 mM) plus AbgGlu358Ala (0.01 mg) in 150 mM ammonium bicarbonate buffer (to neutralize released HF) was incubated for 8 h at room temperature, when thin-layer chromatographic analysis (silica; ethyl acetate/methanol/water, 7:2:1) indicated that reaction was complete. Workup involved removal of the enzyme by ultrafiltration followed by lyophilization of the volatile buffer and purification of the product by HPLC to yield 34 mg (92%) of the trisaccharide product which was characterized by NMR, MS, and elemental analysis.

The scope of this new enzymatic reaction was explored using both α-galactosyl fluoride and α-glucosyl fluoride as donors in the presence of a range of acceptors. Products from galactose transfer are summarized in Table 1. The linkage formed was β(1,4) in all cases shown with the exception of transfer to β-xylosides, which occurred via a β(1,3) linkage [#2, #13].[20] Transfer to an acceptor with an axial 4-hydroxyl group was very inefficient, thus ensuring that only a single galactosyl transfer occurred, the product itself being a poor acceptor. By contrast, transfer of glucose yielded a product which was itself an excellent acceptor, resulting in formation of longer oligosaccharides. As is shown in Table 2, use of approximately 1 equiv of α-glucosyl fluoride yielded a mixture of di- and trisaccharide products since disaccharides, once formed, are preferred acceptors for this enzyme.[21] However, when a disaccharide acceptor such as *p*-nitrophenyl cellobioside was employed initially, reaction was essentially stoicheometric, 1 equiv of α-glucosyl fluoride giving a 79% yield of trisaccharide, while 2 equiv gave a 64% yield of the tetrasaccharide directly [#15].

Table 2. Glycosynthase-Catalyzed Transglycosylation Reactions Using α-Glucosyl Fluoride as Donor[a]

#	ACCEPTOR	PRODUCTS (% YIELD) β-1,4 linked			
		Disaccharide	Trisaccharide	Tetrasaccharide	Total Yield†
7		48%	34%	-	**82%**
8		38%	24%	10%	**72%**
9		41%	29%	6%	**76%**
10		38%	42%	4%	**84%**
11a b		-	75%* 23%*	8%* 54%*	**83%*** **77%***
12a b		44% 12%*	29% 66%*	- 8%*	**73%** **86%***
13		12%	51%	3%	**66%** (β-1,3 linked)
14		31%	42%	6%	**79%**
15a b			79% 8%*	13% 64%*	**92%** **72%***

[a] A hyphen (-) indicates reactions performed with 1−1.4 equiv and an asterisk (*) indicates those with 2.2−3.0 equiv of glycosyl donor.

The utility of this Glycosynthase approach was well illustrated by the gram-scale syntheses of several valuable substrates and inactivators for cellulases as shown in Table 2. Methylumbelliferyl cellotrioside and cellotetraoside [#11a,b], chromogenic substrates for cellulases, were readily synthesized from the easily accessible monosaccharide glycosides by successive transfers from α-glucosyl fluoride. Similarly, two mechanism-based inactivators for cellulases, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside and -cellotrioside [#10, see also #3] were made, as an intentional mixture, by reaction of 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-glucoside with 1.5 equiv of α-glucosyl fluoride in the presence of the mutant. The latter synthesis would be impossible using conventional glycosidase-catalyzed transglycosylation since the 2-deoxy-2-fluoro glycoside rapidly inactivates the wild-type enzyme by trapping of the intermediate.

This new Glycosynthase approach to oligosaccharide synthesis has the twin advantages of utilizing inexpensive glycosyl donors and of obviating hydrolysis of product. We believe this approach has considerable potential as an alternative strategy for oligosaccharide synthesis, particularly on a large scale.

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(18) α-Glucosyl fluoride does not function as a substrate for, or even bind to, the wild-type enzyme. The axial fluorine should also disfavor binding to the aglycon site, decreasing competition between the donor and acceptor sugars.

(19) Day, A. G.; Withers, S. G. *Can. J. Biochem.* **1986**, *64*, 914.

(20) Such β(1,3) transfer to xylose has been seen with other glycosidases (Lopez, R.; Fernandez-Mayoralas A. *J. Org. Chem.* **1994**, *59*, 737.).

(21) The yield of the disaccharide was maximized by adding only 0.5−0.75 equiv of the glycosyl donor.