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Synthesis with Glycosynthases: Cello-Oligomers of Isofagomine and a Tetrahydrooxazine as Cellulase Inhibitors

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Isofagomine and a carbohydrate-like tetrahydrooxazine, as their *N*-benzyloxycarbonyl derivatives, have been subjected to a glycosynthase in the presence of α-D-glucopyranosyl fluoride as a glucosyl donor. In each case, after protecting group removal, a mixture of 1,4-β-linked di-, tri-, and tetra-'saccharides' was obtained. These novel oligosaccharide derivatives were tested as inhibitors of the endo-glycanase Cex from *Cellulomonas fimi*. Affinities increased progressively as additional D-glucosyl residues were incorporated, which is consistent with the known substrate specificity of this enzyme.

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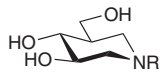
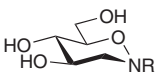
The detailed understanding of the mechanism of action of retaining glycoside hydrolases,[1–3] coupled with the ability to identify[4,5] and subsequently mutate[6] the catalytic nucleophile, greatly contributed to the invention of 'glycosynthases'.[7] Such mutant enzymes, in which the nucleophilic carboxylate has been replaced by a smaller, non-carboxylate residue, are able to glycosylate an acceptor alcohol using a glycosyl fluoride donor of the 'wrong' configuration, but are unable to cause hydrolysis of the product glycoside. Since the initial discovery, a number of other glycosynthases have been developed, of which most are specific for the construction of 1,4-β-D-glycosides.[8–13]

We have recently reported syntheses of isofagomine (1) and the tetrahydrooxazine (2)[14] (Diagram 1). Isofagomine, an unnatural molecule,[15] is a potent inhibitor of several glycoside hydrolases; the tetrahydrooxazine (2), while not nearly as potent as isofagomine, is also a reasonable inhibitor.[16]



(1) R = H

(3) COOBn



(2) R = H

(4) COOBn

Diagram 1

It occurred to us that it might be worthwhile to produce glycosylated versions of both (1) and (2) in the hope of improving the binding to target glycan hydrolases. Although it would be possible to use conventional glycosidation techniques to synthesize such molecules, we decided to use enzyme-assisted procedures. Glycosynthases seemed like ideal candidates.

As with other iminosugars, isofagomine is generally accepted to bind to glycosidases in its protonated form. Therefore, in order to minimize the potential problem of inhibition of the glycosynthase through unwanted binding of the isofagomine moiety in the –1 site, we decided to derivatize it in a form that is not protonated under physiological conditions: as an *N*-acyl derivative. Further, it is well established that aryl glycosides are particularly good acceptors in enzyme-assisted glycoside bond formation as the aromatic ring seems to provide good (hydrophobic) binding in the +2 site of the catalytic domain.[17,18]

We first converted (1) and (2) into the corresponding carbamates (3) and (4) to satisfy both needs. The carbamate (3) and α-D-glucopyranosyl fluoride in aqueous ammonium bicarbonate solution (pH 7.9) were then incubated with a second-generation glycosynthase developed from a β-glucosidase from *Agrobacterium* sp. (Glu358Ser).[11] After a few days, thin-layer chromatography (TLC) analysis showed the formation of three new compounds, ostensibly di-, tri-, and tetra-'saccharides' of carbamate (3).

After workup and acetylation of the reaction mixture, careful chromatography on silica gel provided the expected series of compounds (5)–(7) (Diagram 2). The complexity of the nuclear magnetic resonance (NMR) spectra of these compounds, arising presumably from the partial double-bond character within the carbamate functionality,

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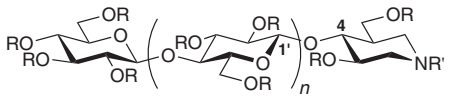
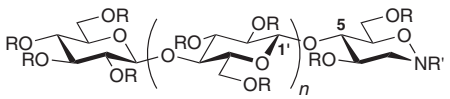
frustrated the acquisition of convincing evidence concerning the 1,4-β-D-linkage that would be expected on the basis of previous observations with this enzyme.[7,11] Each of the products (5)–(7) was therefore selectively deprotected (transfer hydrogenolysis) to provide the acetylated amines (8)–(10). A combination of ^{1}H and ^{13}C NMR spectroscopy then established the various 1,4-β-D-linkages in (8)–(10). For example, two-dimensional ^{1}H NMR spectroscopy of the amine (8) clearly identified H4 as an apparent triplet with an expected greater upfield chemical shift (δ 3.62) relative to all other protons directly adjacent to acetoxy groups. Furthermore, heteronuclear single quantum correlation (HSQC) NMR spectroscopy showed H4 to be coupled to downfield C4 (δ 79.1). Lastly, the $J_{1',2'}$ coupling constant (7.9 Hz) was indicative of a β-D-glycosidic linkage. The amines (8)–(10) were then separately deprotected (sodium methoxide in methanol) to give the putative inhibitors (11)–(13). ^{13}C NMR spectroscopy of these fully deprotected products showed chemical shifts [δ 79.1 for C4′ of (12) and δ 79.0, 79.1 for C4′,4″ of (13)] characteristic for 1,4-β-D-glycosylation.[19]



(5)	R = Ac	R' = COOBn	*n* =	0
(6)	Ac	COOBn		1
(7)	Ac	COOBn		2
(8)	Ac	H		0
(9)	Ac	H		1
(10)	Ac	H		2
(11)	H	H		0
(12)	H	H		1
(13)	H	H		2

Diagram 2

The whole process was then repeated on carbamate (4) to provide the oxazine suite of intermediates (14)–(16) (Diagram 3). No line broadening was observed for these compounds, possibly owing to the reduced basicity of the nitrogen atom, and sound NMR spectral analysis was possible. Therefore, one-pot deprotection by way of basic hydrolysis (sodium hydroxide in aqueous methanol) gave the putative inhibitors (17)–(19).



(14)	R = Ac	R' = COOBn	*n* =	0
(15)	Ac	COOBn		1
(16)	Ac	COOBn		2
(17)	H	H		0
(18)	H	H		1
(19)	H	H		2

Diagram 3

In these glycosylations, mixtures of oligosaccharides of different lengths were obtained. Such were our intentions, since the full series of inhibitors was required for the inhibition studies, and it was anticipated that chromatographic separation of these products would not be too problematic. However, should single products of defined lengths be desired it should be possible to adopt the strategy employed previously on another endo-glycanase in which a temporary protecting group is attached to the C4 hydroxyl of the non-reducing sugar of the glycosyl fluoride donor.[12,20]

Inhibition studies were carried out with a well-analysed endo-glycanase (Cex from *Cellulomonas fimi*) for which many kinetic studies have been performed, and for which a number of three-dimensional structures have been determined, both of the pure enzyme and of complexes with various inhibitors.[21,22] The majority of these inhibitor studies to date have been with xylo-oligosaccharides, thus comparative studies with cello-oligosaccharide derivatives would be of interest.

As can be seen in Figure 1, which depicts inhibition by the trisaccharide analogue cellobiosyl isofagomine (12), these compounds act as good competitive inhibitors, in this case with a K_i of 0.8 μM. Inhibition parameters for the two series of inhibitors with the endo-glycanase Cex from *Cellulomonas fimi* are presented in Table 1. These data reveal some interesting changes in K_i values across the series. The monosaccharide analogue, isofagomine (1) is a very modest (millimolar) inhibitor, but addition of a single β-glucosyl residue to the 4-position improves binding 1000-fold. However, further additions of β-glucosyl moieties provides very little additional binding enhancement, with K_i values dropping only approximately two-fold for the next sugar residue cellobiosyl isofagomine (12), and a further 1.5-fold for the third addition (13). These increases in affinity correlate with kinetic parameters for the cleavage of oligosaccharide substrates. Thus, *p*-nitrophenyl β-D-glucopyranoside is only a slow substrate (k_{cat}/K_m = 0.0041 s^{-1} mM^{-1}) compared with *p*-nitrophenyl cellobioside

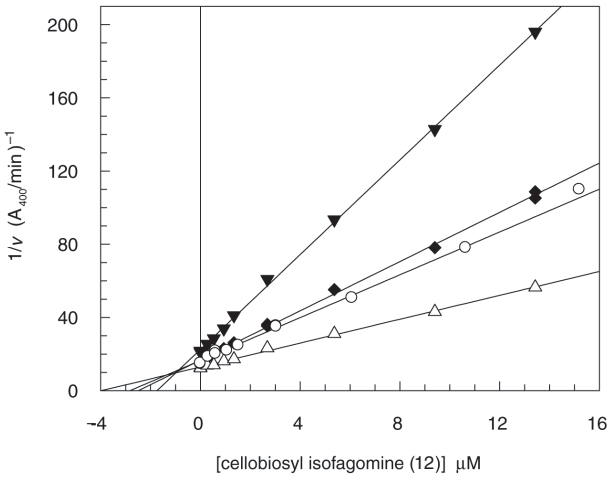
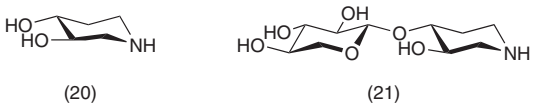


Fig. 1. Dixon plot of inhibition of *C. fimi* endo-glycanase by the cellobiosyl isofagomine (12). The concentrations of the substrate, 2,4-dinitrophenyl β-cellobioside, used were 0.059 (▼), 0.113 (◆), 0.117 (○), and 0.234 (△) mM.

Table 1. Inhibition of the endo-glycanase Cex from *Cellulomonas fimi*

Inhibitor	K_i (μM)
Isofagomine (1)	2000
Glucosyl Isofagomine (11)	2.0
Cellobiosyl Isofagomine (12)	0.8
Cellotriosyl Isofagomine (13)	0.6
Oxazine (2)	No Inhibition
Glucosyl Oxazine (17)	1900
Cellobiosyl Oxazine (18)	1000
Cellotriosyl Oxazine (19)	80

(k_{cat}/K_m = 26 s^{-1} mM^{-1}).[23] Indeed, Cex has been shown to have three glycone sites and two aglycone sites.[24] Thus, cleavage of oligosaccharides by Cex never occurs to release a monosaccharide from the non-reducing end, but rather di- or trisaccharides.[24] Large increases in inhibitor affinity upon the addition of a single sugar residue to a 'monosaccharide' iminosugar have been seen for Cex previously,[21] and three-dimensional structures have been determined.[22] In those cases, addition of a single xylose residue was shown to increase affinity by several orders of magnitude, with the xylosyl *xylo*-isofagomine (21) binding with a K_i value of 0.13 μM, while the 'monosaccharide' iminosugar, *xylo*-isofagomine (20) binds only very weakly (Diagram 4). Clearly, interactions in the –2 site are very important for effective binding and catalysis. It is interesting, however, that the glucosyl isofagomine (11) studied here binds only 15-fold more weakly to Cex than does the *xylo* analogue (21). This stands somewhat in contrast to the approximately thousand-fold faster hydrolysis of *xylo*-oligosaccharide substrates than of the corresponding cello-oligosaccharides.[24]



(20) (21)

Diagram 4

The oxazines bind much more weakly, which is consistent with earlier findings in regards to monosaccharidases.[14] The parent oxazine (2) shows no significant inhibition at 6.6 mM concentrations, while weak inhibition (K_i = 1.9 mM and 1.0 mM, respectively) is seen for the glucosyl (17) and cellobiosyl (18) derivatives. Only when the third glucosyl residue is added is significant additional binding observed, this third glucosyl residue improving binding approximately 12-fold. It is interesting that useful binding is only observed in this case when four sites are apparently filled by the inhibitor. This would suggest that the oxazine moiety, in contrast to the isofagomine, provides no special affinity through binding in the –1 site, and that the increase in affinity upon reaching a 'tetrasaccharide' might be a consequence of binding across the active site. This would be completely consistent with the earlier observation that cleavage of tetrasaccharides is much faster than that of trisaccharides.[24]

Experimental

Syntheses

General experimental procedures for syntheses have been given previously.[25]

*(3*R*,4*R*,5*R*)*-N-*Benzyloxycarbonyl-3,4-dihydroxy-5-(hydroxymethyl)-piperidine (3)*

Isofagomine (1) (hydrogen chloride salt)[14] (156 mg, 0.85 mmol) was treated with $NaHCO_3$ (210 mg, 2.5 mmol) and benzyl chloroformate (160 μL, 1.1 mmol) in H_2O/MeOH/THF (2:1:1, 15 mL), and the mixture was stirred (30 min, rt). The mixture was treated with hydrochloric acid (1 mL of 1 M) and then concentrated, co-evaporating with toluene. Flash chromatography (EtOAc/petrol, 1:1 then MeOH/$CHCl_3$, 1:9) of the residue gave the *carbamate* (3) (192 mg, 80%) as a colourless oil, $[a]_D$ +3.8° (MeOH) (Found: C, 59.7; H, 7.1%. $C_{14}H_{19}NO_5$ requires C, 59.8; H, 6.8%). ^{1}H NMR (300 MHz, D_2O) δ 1.40–1.54, m, H5; 2.42–2.64, m, H2,6; 3.13, t, $J_{3,4} \approx J_{4,5}$ 9.7 Hz, H4; 3.20–3.30, m, H3; 3.41, dd, $J_{5,H}$ 6.9, $J_{H,H}$ 11.5 Hz, CH_2O; 3.61, dd, $J_{5,H}$ 3.2 Hz, CH_2O; 3.84–4.09, m, H2,6; 4.94, s, PhC**H**$_2$; 7.15–7.28, m, Ph. ^{13}C NMR (75.5 MHz, D_2O) δ 44.3, C5; 45.8, 48.4, C2,6; 60.8, CH_2O; 68.6, Ph**C**H_2; 71.5, 74.2, C3,4; 128.5, 129.2, 129.5, 137.1, Ph; 157.3, NCO. High-resolution mass spectrum (HRMS) fast-atom bombardment (FAB) *m/z* 282.1354. $C_{14}H_{20}NO_5$ $[M+H]^{+\bullet}$ requires 282.1341.

*(4*R*,5*S*,6*R*)*-N-*Benzyloxycarbonyl-4,5-dihydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H*-1,2-oxazine (4)*

The tetrahydrooxazine (2)[14] (340 mg, 2.3 mmol) was treated with $NaHCO_3$ (370 mg, 4.5 mmol) and benzyl chloroformate (420 μL, 2.9 mmol) in H_2O/MeOH/THF (2:1:1, 20 mL), and the mixture was stirred (30 min, rt). The mixture was treated with hydrochloric acid (3 mL of 1 M) and then concentrated, co-evaporating with toluene. Flash chromatography (EtOAc/petrol, 1:1 then MeOH/$CHCl_3$, 1:9) of the residue gave the *carbamate* (4) (580 mg, 90%) as a colourless oil, $[\alpha]_D$ +71.9° (MeOH) (Found: C, 54.7; H, 5.6; N, 4.7%. $C_{13}H_{17}NO_6$ requires C, 55.1; H, 6.0; N, 4.9%). ^{1}H NMR (300 MHz, D_2O) δ 2.93, dd, $J_{3,3}$ 13.4, $J_{3,4}$ 10.3 Hz, H3; 3.35, t, $J_{4,5} \approx J_{5,6}$ 8.9 Hz, H5; 3.39–3.51, m, H4,6; 3.57, dd, $J_{6,H}$ 5.4, $J_{H,H}$ 10.0 Hz, CH_2O; 3.72, dd, $J_{6,H}$ 1.8 Hz, CH_2O; 4.03, dd, $J_{3,4}$ 5.3 Hz, H3; 4.89, 4.94, ABq, *J* 12.2 Hz, PhC**H**$_2$; 7.09–7.21, m, Ph. ^{13}C NMR (75.5 MHz, D_2O) δ 50.4, C3; 59.9, CH_2O; 69.1, Ph**C**H_2; 69.4, 70.3, C4,5; 85.1, C6; 128.7, 129.3, 129.4, 136.1, Ph; 156.7, NCO. HRMS (FAB) *m/z* 284.1151. $C_{13}H_{18}NO_6$ $[M+H]^{+\bullet}$ requires 284.1134.

*(3*R*,4*R*,5*R*)-3-Acetoxy-5-acetoxymethyl-*N*-benzyloxycarbonyl-4-[(tetra-*O*-acetyl-β-D-glucopyranosyl)oxy]piperidine (5), (3*R*,4*R*,5*R*)-3-acetoxy-5-acetoxymethyl-*N*-benzyloxycarbonyl-4-{[(tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*O*-(tri-*O*-acetyl-β-D-glucosyl)]oxy}piperidine (6) and (3*R*,4*R*,5*R*)-3-acetoxy-5-acetoxymethyl-*N*-benzyloxycarbonyl-4-{[(tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-*O*-(tri-*O*-acetyl-β-D-glucosyl)-(1→4)-*O*-(tri-*O*-acetyl-β-D-glucosyl)]oxy}piperidine (7)*

A mixture of the carbamate (3) (130 mg, 0.47 mmol) and α-D-glucopyranosyl fluoride (140 mg, 0.76 mmol) in aqueous NH_4HCO_3 (10 mL of 0.15 M) was treated with AbgGlu358Ser (2 mg) and the mixture was then incubated (7 days, rt). The mixture was concentrated, co-evaporating with pyridine, and then taken up in pyridine (5 mL) and treated with Ac_2O (2 mL) (20 h, rt). This mixture was quenched upon the addition of MeOH (3 mL), and then concentrated to give a residue. Flash chromatography (EtOAc/petrol, gradient from 3:7 to 3:2) of this residue gave, firstly, the *pseudo-disaccharide* (5) (87 mg, 26%) as a colourless gum, $[\alpha]_D$ –6.4°. ^{1}H NMR (300 MHz) δ 1.87–1.93, m, H5; 1.97, 1.99, 2.01, 2.05, 4 × s, 18H, CH_3; 3.41–3.76, m, 5H; 4.00–4.30, m, 5H; 4.57, d, $J_{1',2'}$ 7.9 Hz, H1′; 4.81–5.23, m, H2′,3,3′,4′,PhC**H**$_2$; 7.26–7.40, m, Ph. ^{13}C NMR (75.5 MHz) δ 20.5, 20.6, 20.7, 6C, CH_3; 39.2, C5; 41.6, 41.9, 43.9, C2,6; 61.8, 62.1, C6′,CH_2O; 67.4, Ph**C**H_2; 68.2, 69.3, 71.4, 71.9, 72.6,

76.3, C2′,3,3′,4,4′,5′; 100.9, C1′; 127.9, 128.1, 128.5, 136.4, Ph; 155.5, NCO; 169.1, 169.3, 169.5, 170.2, 170.6, 6C, CO. HRMS (FAB) *m/z* 696.2524. $C_{32}H_{42}NO_{16}$ $[M+H]^{+\bullet}$ requires 696.2504.

Next to elute was the *pseudo-trisaccharide* (6) (127 mg, 27%), which was isolated as a colourless gum, $[\alpha]_D$ –19°. ^{1}H NMR (500 MHz) δ 1.86–1.92, m, H5; 1.96, 1.99, 1.99, 2.01, 2.07, 2.10, 6 × s, 27H, CH_3; 3.34, m, 8H; 3.98–4.22, m, 4H; 4.35, dd, *J* 4.3, 12.4 Hz, 1H; 4.42–4.49, m, 2H; 4.55, d, *J* 7.9 Hz, 1H; 4.83–5.20, m, H2′,2″,3,3′,3″,4″,PhC**H**$_2$; 7.26–7.37, m, Ph. ^{13}C NMR (125.5 MHz) δ 20.4, 20.5, 20.6, 20.6, 20.7, 20.8, 9C, CH_3; 39.1, C5; 41.4, 41.7, 43.6, 43.8, C2,6; 61.5, 61.8, 62.0, 62.1, C6′,6″,CH_2O; 67.3, 67.4, Ph**C**H_2; 67.6–76.3, 10C; 100.7, 100.8, C1′,1″; 127.9, 128.1, 128.5, 136.4, Ph; 155.5, NCO; 169.0–170.6, 9C, CO. HRMS (FAB) *m/z* 984.3396. $C_{44}H_{58}NO_{24}$ $[M+H]^{+\bullet}$ requires 984.3349.

Last to elute was the *pseudo-tetrasaccharide* (7) (45 mg, 8%), which was isolated as a colourless gum, $[\alpha]_D$ –12.6°. ^{1}H NMR (500 MHz) δ 1.86–1.92, m, H5; 1.96–2.13, 10 × s, 36H, CH_3; 3.25–3.85, m, 9H; 3.27–3.98, m, 6H; 4.31–4.53, m, 5H; 4.54, d, *J* 7.9 Hz, 1H; 4.75–5.21, m, 10H; 7.27–7.38, m, Ph. ^{13}C NMR (125.5 MHz) d 20.4–20.7, 12C, CH_3; 39.1, C5; 41.4, 41.8, 43.6, 43.8, C2,6; 61.4, 61.7, 62.1, C6′,6″,6‴,CH_2O; 67.7, Ph**C**H_2; 67.6–76.3, 14C; 100.5, 100.6, 100.7, C1′,1″,1‴; 127.9, 128.1, 128.5, 136.4, Ph; 155.5, NCO; 169.0–171.2, 12C, CO. HRMS (FAB) *m/z* 1272.4184. $C_{56}H_{74}NO_{32}$ $[M+H]^{+\bullet}$ requires 1272.4194.

*(3*R*,4*R*,5*R*)-3-Acetoxy-5-acetoxymethyl-4-[(tetra-*O*-acetyl-β-*D*-glucopyranosyl)oxy]piperidine (8)*

A mixture of cyclohexene (200 μL), Et_2NH (10 μL) and $Pd(OH)_2$/C (18 mg) in EtOH (4 mL) was heated under reflux (10 min) and then allowed to cool. The carbamate (5) (61 mg, 88 μmol) in EtOAc (4 mL) was added and then this mixture was heated under reflux (15 min) and allowed to cool. The mixture was filtered, washing with EtOAc, and the combined filtrate and washings were concentrated to give a residue. Flash chromatography (EtOAc/petrol/Et_3N, 9:9:2 then 9:0:1) gave the *amine* (8) (45 mg, 91%) as a colourless glass, $[\alpha]_D$ +3.3°. ^{1}H NMR (500 MHz) δ 1.88–1.96, m, H5; 1.99, 2.01, 2.04, 2.07, 2.08, 5 × s, 18H, CH_3; 2.48, dd, $J_{5,6}$ 9.4, $J_{6,6}$ 13.1 Hz, H6; 2.52, dd, $J_{2,2}$ 12.8, $J_{2,3}$ 8.6 Hz, H2; 3.07, dd, $J_{5,6}$ 3.8 Hz, H6; 3.25, dd, $J_{2,3}$ 4.4 Hz, H2; 3.62, t, $J_{3,4} \approx J_{4,5}$ 8.4 Hz, H4; 3.66, ddd, $J_{4',5'}$ 9.5, $J_{5',6'}$ 2.4, 4.6 Hz, H5′; 4.04, dd, $J_{6',6'}$ 12.3 Hz, H6′; 4.07, dd, $J_{5,H}$ 7.1, $J_{H,H}$ 11.1 Hz, CH_2O; 4.29, dd, $J_{5,H}$ 3.8 Hz, CH_2O; 4.33, dd, H6′; 4.60, d, $J_{1',2'}$ 7.9 Hz, H1′; 4.79, dt, H3; 4.96, dd, $J_{2',3'}$ 9.4 Hz, H2′; 5.08, t, $J_{3',4'}$ 9.5 Hz, H4′; 5.17, t, H3′. ^{13}C NMR (125.8 MHz) δ 20.6, 20.6, 20.8, 21.0, 6C, CH_3; 42.3, C5; 46.4, C6; 48.0, C2; 61.8, C6′; 62.3, CH_2O; 68.1, C4′; 71.71, 71.74, C2′,5′; 72.0, C3; 72.9, C3′; 79.1, C4; 100.8, C1′; 169.2, 169.3, 169.8, 170.3, 170.6, 170.8, 6C, CO. HRMS (FAB) *m/z* 562.2172. $C_{24}H_{36}NO_{14}$ $[M+H]^{+\bullet}$ requires 562.2136.

*(3*R*,4*R*,5*R*)-3-Acetoxy-5-acetoxymethyl-4-{[(tetra-*O*-acetyl-β-*D*-glucopyranosyl)-(1→4)-*O*-(tri-*O*-acetyl-β-*D*-glucosyl)]oxy} piperidine (9)*

The carbamate (6) (91 mg, 93 μmol) was treated as (5) above to give, after flash chromatography (EtOAc/petrol/Et_3N, 9:9:2 then 9:0:1), the *amine* (9) (75 mg, 96%) as a colourless glass, $[\alpha]_D$ –7.9°. ^{1}H NMR (500 MHz) δ 1.85–1.94, m, H5; 1.96–2.12, 9 × s, 27H, CH_3; 2.47, dd, $J_{5,6}$ 9.1, $J_{6,6}$ 13.3 Hz, H6; 2.51, dd, $J_{2,2}$ 13.0, $J_{2,3}$ 8.6 Hz, H2; 3.04, dd, $J_{5,6}$ 4.0 Hz, H6; 3.22, dd, $J_{2,3}$ 4.8 Hz, H2; 3.55–3.65, m, H4,5′,5″; 3.76, t, $J_{3',4'} \approx J_{4',5'}$ 9.5 Hz, H4′; 4.02, dd, *J* 2.1, 12.4 Hz, 1H, CH_2; 4.05, dd, *J* 7.1, 11.2 Hz, 1H, CH_2; 4.10, dd, *J* 5.2, 12.0 Hz, 1H, CH_2; 4.28, dd, *J* 3.9, 11.2 Hz, 1H, CH_2; 4.34, dd, *J* 4.3, 12.4 Hz, 1H, CH_2; 4.39, dd, *J* 1.9, 12.0 Hz, 1H, CH_2; 4.48, 4.57, 2 × d, *J* 7.9, 7.9 Hz, H1′,1″; 4.76, dt, $J_{3,4}$ 8.1 Hz, H3; 4.87, 4.90, 2 × dd, *J* 9.5, 9.3 Hz, H2′,2″; 5.04, 5.11, 5.14, 3 × t, *J* 9.3, 9.3, 9.5 Hz, H3′,3″,4″. ^{13}C NMR (125.8 MHz) δ 20.5–20.9, 9C, CH_3; 42.0, C5; 46.2, C6; 47.8, C2; 61.5, 62.2, 62.3, C6′,6″,CH_2O; 67.7, C4″; 71.5–72.9, 7C; 76.3, C4′; 78.8, C4; 100.5, 100.8, C1′,1″; 169.0–170.6, 9C, CO. HRMS (FAB) *m/z* 850.3004. $C_{36}H_{52}NO_{22}$ $[M+H]^{+\bullet}$ requires 850.2981.

*(3*R*,4*R*,5*R*)-3-Acetoxy-5-acetoxymethyl-4-{[(tetra-*O*-acetyl-β-*D*-glucopyranosyl)-(1→4)-*O*-(tri-*O*-acetyl-β-*D*-glucosyl)-(1→4)-*O*-(tri-*O*-acetyl-β-*D*-glucosyl)]oxy}piperidine (10)*

The carbamate (7) (44 mg, 34 μmol) was treated as (5) previously to give, after flash chromatography (EtOAc/petrol/Et_3N, 9:9:2 then 9:0:1), the *amine* (10) (38 mg, 97%) as a colourless glass, $[\alpha]_D$ –12.2°. ^{1}H NMR (500 MHz) δ 1.83–1.92, m, H5; 1.94–2.12, 11 × s, 36H, CH_3; 2.46, dd, $J_{5,6}$ 9.1, $J_{6,6}$ 13.2 Hz, H6; 2.50, dd, $J_{2,2}$ 13.0, $J_{2,3}$ 8.5 Hz, H2; 3.03, dd, $J_{5,6}$ 3.9 Hz, H6; 3.20, dd, $J_{2,3}$ 4.4 Hz, H2; 3.52–3.63, m, 4H; 3.70–3.76, m, H4′,4″; 3.98–4.10, m, 4H, CH_2; 4.25, dd, *J* 3.8, 11.1 Hz, 1H, CH_2; 4.33, dd, *J* 4.3, 12.5 Hz, 1H, CH_2; 4.35–4.40, m, CH_2; 4.44, 4.45, 4.54, 3 × d, *J* 7.8, 7.9, 7.9 Hz, H1′,1″,1‴; 4.75, dt, $J_{3,4}$ 8.2 Hz, H3; 4.78–4.90, m, H2′,2″,2‴; 5.02, t, *J* 9.7 Hz, 1H; 5.05–5.13, m, 3H. ^{13}C NMR (125.8 MHz) δ 20.4–20.9, 12C, CH_3; 42.0, C5; 46.2, C6; 47.8, C2; 61.4, 62.0, 62.2, C6′,6″,6‴,CH_2O; 67.6, C4‴; 71.5–72.8, 10C; 76.0, 76.3, C4′,4″; 78.8, C4; 100.5, 100.7, C1′,1″,1‴; 169.0–170.6, 12C, CO. HRMS (FAB) *m/z* 1138.3864. $C_{48}H_{68}NO_{30}$ $[M+H]^{+\bullet}$ requires 1138.3826.

*(3*R,4R,5R*)-4-(β-D-Glucopyranosyl)oxy-3-hydroxy-5-(hydroxymethyl) piperidine (11)*

The *per-O*-acetylated amine (8) (34 mg) in MeOH (5 mL) was treated with sodium metal (5 mg) (2 h, rt). The reaction mixture was concentrated, taken up in H_2O (5 mL) and brought to pH 5 by the addition of hydrochloric acid (0.1 M). The mixture was applied to a column of cation-exchange resin (Dowex 50W-X2, H^+ form), washed with water and then eluted with aqueous NH_3 (3 M). The eluate was concentrated, taken up in H_2O (1 mL), applied to an anion-exchange column (Sephadex-DEAE A-25) and eluted with H_2O. Freeze-drying of the eluate afforded the glucosyl isofagomine (11) (17 mg, 91%) as a colourless foam, $[\alpha]_D$ –3.3° (H_2O). ^{1}H NMR (500 MHz, D_2O) α 1.79–1.87, m, H5; 2.39, dd, $J_{2,2}$ 12.4, $J_{2,3}$ 10.7 Hz, H2; 2.43, t, $J_{5,6} \approx J_{6,6}$ 12.3 Hz, H6; 3.05–3.11, m, H6; 3.16–3.22, m, H2; 3.34, dd, $J_{1',2'}$ 8.0, $J_{2',3'}$ 9.4 Hz, H2′; 3.43, dd, $J_{3',4'}$ 9.7 Hz, H3′; 3.48–3.55, m, H4,4′,5′; 3.59, ddd, $J_{2,3}$ 5.0, $J_{3,4}$ 8.7 Hz, H3; 3.73, dd, $J_{5,H}$ 6.0, $J_{H,H}$ 11.4 Hz, CH_2O; 3.72, dd, $J_{5',6'}$ 6.0, $J_{6',6'}$ 12.4 Hz, H6′; 3.85, dd, $J_{5,H}$ 3.4 Hz, CH_2O; 3.91, dd, $J_{5',6'}$ 2.2 Hz, H6′; 4.52, d, H1′. ^{13}C NMR (125.8 MHz, D_2O) δ 44.6, C5; 47.0, C6; 49.7, C2; 60.7, CH_2O; 61.3, C6′; 70.2, C3′; 71.5, C3; 74.0, C2′; 76.3, 76.6, C4′,5′; 85.4, C4; 103.5, C1′. HRMS (FAB) *m/z* 310.1486. $C_{12}H_{24}NO_8$ $[M+H]^{+\bullet}$ requires 310.1502.

*(3*R,4R,5R*)-4-[(β-D-Glucopyranosyl)-(1→4)-O-(β-D-glucosyl)]oxy-3-hydroxy-5-(hydroxymethyl)piperidine (12)*

The *per-O*-acetylated amine (9) (70 mg) was treated as (8) above to give, after freeze-drying, the *cellobiosyl isofagomine* (12) (33 mg, 85%) as a colourless, amorphous solid, $[\alpha]_D$ –10.4° (H_2O). ^{1}H NMR (500 MHz, D_2O) δ 1.85–1.93, m, H5; 2.49, t, $J_{2,2} \approx J_{2,3}$ 11.8 Hz, H2; 2.54, t, $J_{5,6} \approx J_{6,6}$ 12.6 Hz, H6; 3.16, dd, $J_{5,6}$ 3.6 Hz, H6; 3.26, dd, $J_{2,3}$ 4.6 Hz, H2; 3.33, dd, $J_{1',2'}$ 8.0, $J_{2',3'}$ 9.3 Hz, H2′; 3.37–3.45, m, 2H; 3.48–3.54, m, 2H; 3.58, dd, *J* 8.7, 9.2 Hz, 1H; 3.62–3.74, m, 6H; 3.80–3.88, m, 3H; 3.93, dd, *J* 2.2, 12.4 Hz, 1H, CH_2; 3.98, dd, *J* 2.1, 12.4 Hz, 1H, CH_2; 4.52, 5.57, 2 × d, *J* 7.9, 8.0 Hz, H1′,1″. ^{13}C NMR (125.8 MHz, D_2O) δ 44.1, C5; 46.7, C6; 49.3, C2; 60.4, 60.6, 61.3, C6′,6″,CH_2O; 70.1, 70.9, C3,3″; 73.8–76.7, 6C; 79.1, C4′; 84.6, C4; 103.26, 103.32, C1′,1″. HRMS (FAB) *m/z* 472.2008. $C_{18}H_{34}NO_{13}$ $[M+H]^{+\bullet}$ requires 472.2030.

*(3*R,4R,5R*)-4-[(β-D-Glucopyranosyl)-(1→4)-O-(β-D-glucosyl)-(1→4)-O-(β-D-glucosyl)]oxy-3-hydroxy-5-(hydroxymethyl)piperidine (13)*

The *per-O*-acetylated amine (10) (33 mg) was treated as (8) previously to give, after freeze-drying, the *cellotriosyl isofagomine* (13) (15 mg, 82%) as a colourless, amorphous solid, $[\alpha]_D$ –1.6° (H_2O). ^{1}H NMR (500 MHz, D_2O) δ 1.81–1.91, m, H5; 2.43, t, $J_{2,2} \approx J_{2,3}$ 11.6 Hz, H2; 2.47, t, $J_{5,6} \approx J_{6,6}$ 12.4 Hz, H6; 3.11, dd, $J_{5,6}$ 3.9 Hz, H6; 3.22, dd, $J_{2,3}$ 4.7 Hz, H2; 3.31–3.46, m, 4H; 3.48–3.59, m, 3H; 3.60–3.77, m, 9H; 3.81–3.89, m, 3H; 3.93, dd, *J* 1.8, 12.2 Hz, 1H; 3.96–4.02, m, 2H; 4.51–4.59, m, H1′,1″,1‴. ^{13}C NMR (125.8 MHz, D_2O) δ 44.5, C5; 46.9, C6; 49.7, C2; 60.6, 61.3, C6′,6″,6‴,CH_2O; 70.1, 71.3, C3,3‴; 73.6–76.7, 9C; 79.0, 79.1, C4′,4″; 85.1, C4; 103.0, 103.3, 103.4, C1′,1″,1‴. HRMS (FAB) *m/z* 634.2609. $C_{24}H_{44}NO_{18}$ $[M+H]^{+\bullet}$ requires 634.2558.

*(4*R*,5*S*,6*R*)-4-Acetoxy-6-acetoxymethyl*-N-*benzyloxycarbonyl-5-(tetra*-O-*acetyl*-β-D-*glucopyranosyl)oxy-3,4,5,6-tetrahydro-2*H-*1,2-oxazine (14), (4*R*,5*S*,6*R*)-4-acetoxy-6-acetoxymethyl*-N-*benzyloxycarbonyl- 5-[(tetra*-O-*acetyl*-β-D-*glucopyranosyl)-(1→4)*-O-*(tri*-O-*acetyl*-β-D-*glucosyl)]oxy-3,4,5,6-tetrahydro-2*H-*1,2-oxazine (15) and (4*R*,5*S*,6*R*)- 4-acetoxy-6-acetoxymethyl*-N-*benzyloxycarbonyl-5-[(tetra*-O-*acetyl*-β-D-*glucopyranosyl)-(1→4)*-O-*(tri*-O-*acetyl*-β-D-*glucosyl)-(1→4)*-O-*(tri*-O-*acetyl*-β-D-*glucosyl)]oxy-3,4,5,6-tetrahydro-2*H-*1,2-oxazine (16)*

A mixture of the carbamate (4) (138 mg, 0.49 mmol) and α-D-glucopyranosyl fluoride (142 mg, 0.78 mmol) in aqueous NH_4HCO_3 (6 mL of 0.15 M) was treated with AbgGlu358Ser (2 mg) and was then incubated (8 days, rt). The mixture was concentrated, co-evaporating with pyridine, and then taken up in pyridine (5 mL) and treated with Ac_2O (2 mL) (20 h, rt). This reaction mixture was quenched upon the addition of MeOH (3 mL) and then concentrated to give a residue. Flash chromatography (EtOAc/petrol, gradient from 3 : 7 to 3 : 2) of this residue gave, firstly, the *pseudo-disaccharide* (14) (135 mg, 40%) as a colourless solid. A small portion was recrystallized to give colourless, fine needles, m.p. 126–131°C (CH_2Cl_2/Et_2O), $[\alpha]_D$ +18.0° (Found: C, 53.2; H, 5.6; N, 1.8%. $C_{31}H_{39}NO_{17}$ requires C, 53.4; H, 5.6; N, 2.0%). ^{1}H NMR (500 MHz) δ 1.96, 1.99, 2.00, 2.00, 2.03, 2.06, 6 × s, 18H, CH_3; 3.36, dd, $J_{3,3}$ 13.5, $J_{3,4}$ 8.6 Hz, H3; 3.65, ddd, $J_{4',5'}$ 9.9, $J_{5',6'}$ 2.4, 4.6 Hz, H5′; 3.77, t, $J_{4,5} \approx J_{5,6}$ 7.7 Hz, H5; 3.96–4.01, m, H6; 4.03, dd, $J_{6',6'}$ 12.4 Hz, H6′; 4.21, dd, $J_{6,H}$ 6.5, $J_{H,H}$ 12.5 Hz, CH_2O; 4.25, dd, $J_{3,4}$ 5.0 Hz, H3; 4.29, dd, H6′; 4.38, dd, $J_{6,H}$ 2.5 Hz, CH_2O; 4.56, d, $J_{1',2'}$ 7.9 Hz, H1′; 4.92, dd, $J_{2',3'}$ 9.5 Hz, H2′; 5.01, ddd, H4; 5.05, t, $J_{3',4'}$ 9.7 Hz, H4′; 5.11–5.21, m, 3H, H3′,PhC**H**$_2$, 7.28–7.35, m, Ph. ^{13}C NMR (125.8 MHz) δ 20.4, 20.4, 20.5, 20.5, 20.6, 6C, CH_3; 47.1, C3; 60.8, CH_2O; 61.6, C6′; 67.9, 68.0, C4,4′; 68.2, Ph**C**H_2; 71.3, C2′; 71.9, C5′; 72.6, C3′; 75.1, C5; 79.9, C6; 100.4, C1′; 128.1, 128.4, 128.5, 135.4, Ph; 154.8, NCO; 169.0, 169.19, 169.23, 170.1, 170.3, 170.4, 6C, CO. HRMS (FAB) *m/z* 698.2305. $C_{31}H_{40}NO_{17}$ $[M+H]^{+\bullet}$ requires 698.2296.

Next to elute was the *pseudo-trisaccharide* (15) (131 mg, 27%), which was isolated as a colourless solid. A small portion was recrystallized to give colourless, fine needles, m.p. 153–162°C (CH_2Cl_2/Et_2O), $[\alpha]_D$ +2.1° (Found: C, 52.3; H, 5.4; N, 1.2%. $C_{43}H_{55}NO_{25}$ requires C, 52.4; H, 5.6; N, 1.4%). ^{1}H NMR (300 MHz) δ 1.94–2.08, 5 × s, 27H, CH_3; 3.36, dd, $J_{3,3}$ 13.4, $J_{3,4}$ 8.1 Hz, 1H, H3; 3.50–3.65, m, 2H; 3.68–3.79, m, 2H; 3.92–4.09, m, 3H; 4.12–4.24, m, 2H; 4.26–4.42, m, 3H; 4.45, 4.51, 2 × d, *J* 7.8, 7.9 Hz, H1′,1″; 4.78–4.90, m, 2H; 4.91–5.20, m, 6H; 7.27–7.38, m, Ph. ^{13}C NMR (75.5 MHz) δ 20.5, 20.6, 20.7, 9C, CH_3; 47.0, C3; 60.8, CH_2O; 61.4, 61.9, C6′,6″; 67.7, 67.9, C4,4″; 68.1, Ph**C**H_2; 71.5, 71.9, 72.4, 72.8, 6C; 75.0, C5; 76.1, C4′; 79.9, C6; 100.2, 100.7, C1′,1″; 128.1, 128.4, 128.5, 135.4, Ph; 154.8, NCO; 169.0–170.4, 9C, CO. HRMS (FAB) *m/z* 986.3165. $C_{43}H_{56}NO_{25}$ $[M+H]^{+\bullet}$ requires 986.3141.

Last to elute was the *pseudo-tetrasaccharide* (16) (46 mg, 7.5%), which was isolated as a colourless solid. A small portion was recrystallized to give colourless, fine needles, m.p. 195–199°C (CH_2Cl_2/Et_2O), $[\alpha]_D$ −4.0° (Found: C, 51.7; H, 5.3; N, 0.8%. $C_{55}H_{71}NO_{33}$ requires C, 51.8; H, 5.6; N, 1.1%). ^{1}H NMR (300 MHz) δ 1.93–2.11, 10 × s, 36H, CH_3; 3.37, dd, $J_{3,3}$ 13.4, $J_{3,4}$ 8.1 Hz, H3; 3.50–3.63, m, 3H; 3.67–3.77, m, 3H; 3.93–4.10, m, 4H; 4.14–4.23, m, 2H; 4.29–4.40, m, 4H; 4.42, 4.43, 4.50, 3 × d, *J* 7.7, 8.0, 7.8 Hz, H1′,1″1‴; 4.76–4.90, m, 3H; 4.92–5.20, m, 7H; 7.27–7.36, m, Ph. ^{13}C NMR (75.5 MHz) δ 20.5, 20.6, 20.7, 12C, CH_3; 47.1, C3; 60.8, CH_2O; 61.5, 61.9, 62.0, C6′,6″,6‴; 67.7, 67.9, C4,4‴; 68.2, Ph**C**H_2; 71.5–72.8, 9C; 75.0, C5; 76.0, 76.1, C4′,4″; 80.0, C6; 100.2, 100.5, 100.7, C1′,1″,1‴; 128.1, 128.4, 128.6, 135.5, Ph; 154.9, NCO; 169.0–170.4, 12C, CO. HRMS (FAB) *m/z* 1274.4037. $C_{55}H_{72}NO_{33}$ $[M+H]^{+\bullet}$ requires 1274.3987.

*(4*R*,5*S*,6*R*)-5-(*β-D-*Glucopyranosyl)oxy-4-hydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H-*1,2-oxazine (17)*

A mixture of the *per-O*-acetylated carbamate (14) (64 mg) and NaOH (300 mg) in MeOH/H_2O (2 : 1, 4.5 mL) was heated under reflux (1 h) and then allowed to cool. The reaction mixture was concentrated, taken up in H_2O (2 mL) and brought to pH 5 by the addition of hydrochloric acid (2.5 M). The mixture was applied to a column of cation-exchange resin (Dowex 50W-X2, H^+ form), washed with water and then eluted with aqueous NH_3 (3 M). The eluate was concentrated, taken up in H_2O (1 mL), applied to an anion-exchange column (Sephadex-DEAE A-25) and eluted with H_2O. Freeze-drying of the eluate afforded the *glucosyl oxazine* (17) (24 mg, 85%) as a colourless, amorphous solid, $[\alpha]_D$ −14.2° (H_2O). ^{1}H NMR (500 MHz, D_2O) δ 2.94, dd, $J_{3,3}$ 13.2, $J_{3,4}$ 10.7 Hz, H3; 3.29–3.37, m, H2′,3; 3.41, dd, $J_{3',4'}$ 9.2, $J_{4',5'}$ 9.8 Hz, H4′; 3.47–3.54, m, H3′,5′; 3.64, t, H5; 3.68, ddd, $J_{5,6}$ 9.7, $J_{6,H}$ 1.8, 5.1 Hz, H6; 3.72, dd, $J_{5',6'}$ 6.1, $J_{6',6'}$ 12.3 Hz, H6′; 3.79, dd, $J_{H,H}$ 12.6 Hz, CH_2O; 3.83, ddd, $J_{3,4}$ 5.5, $J_{4,5}$ 8.0 Hz, H4; 3.92, dd, $J_{5',6'}$ 2.2 Hz, H6′; 3.96, dd, CH_2O; 4.51, d, $J_{1',2'}$ 8.0 Hz, H1′. ^{13}C NMR (125.8 MHz, D_2O) δ 52.6, C3; 59.8, CH_2O; 61.3, C6′; 69.7, C4; 70.2, C4′; 73.8, C2′; 76.1, 76.6, C3′,5′; 80.3, C5; 82.6, C6; 103.3, C1′. HRMS (FAB) *m/z* 312.1294. $C_{11}H_{22}NO_9$ $[M+H]^{+\bullet}$ requires 312.1295.

*(4*R*,5*S*,6*R*)-5-[(*β-D-*Glucopyranosyl)-(1→4)-O-(*β-D-*glucosyl)]oxy-4-hydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H-*1,2-oxazine (18)*

The *per-O*-acetylated carbamate (15) (90 mg) was treated as (14) above to give, after freeze-drying, the *cellobiosyl oxazine* (18) (28 mg, 65%) as a colourless glass, $[\alpha]_D$ −10.4° (H_2O). ^{1}H NMR (500 MHz, D_2O) δ 3.00, dd, $J_{3,3}$ 13.2, $J_{3,4}$ 10.8 Hz, H3; 3.31, dd, *J* 5.6, 8.0 Hz, 1H; 3.34–3.45, m, 3H; 3.46–3.54, m, 2H; 3.59–3.78, m, 6H; 3.79–3.89, m, 3H; 3.91, dd, *J* 2.0, 12.1 Hz, 1H; 3.95–4.01, m, 2H; 4.50, 4.55, 2 × d, *J* 7.9, 8.0 Hz, H1′,1″. ^{13}C NMR (125.8 MHz, D_2O) δ 52.1, C3; 59.6, CH_2O; 60.6, C6′; 61.2, C6″; 69.0, 70.1, C4,4″; 73.6–76.6, 6C; 79.1, 79.6, C4′,5; 82.7, C6; 103.0, 103.2, C1′,1″. HRMS (FAB) *m/z* 474.1841. $C_{17}H_{32}NO_{14}$ $[M+H]^{+\bullet}$ requires 474.1823.

*(4*R*,5*S*,6*R*)-5-[(*β-D-*Glucopyranosyl)-(1→4)-O-(*β-D-*glucosyl)-(1→4)-O-(*β-D-*glucosyl)]oxy-4-hydroxy-6-hydroxymethyl-3,4,5,6-tetrahydro-2*H-*1,2-oxazine (19)*

The *per-O*-acetylated carbamate (16) (33 mg) was treated as (14) previously to give, after freeze-drying, the *cellotriosyl oxazine* (19) (9.5 mg, 58%) as a colourless glass, $[\alpha]_D$ +5.4° (H_2O). ^{1}H NMR (500 MHz, D_2O) δ 2.93, dd, $J_{3,3}$ 13.2, $J_{3,4}$ 10.7 Hz, H3; 3.23–3.44, m, 5H; 3.46–3.53, m, 2H; 3.58–3.70, m, 9H; 3.73, dd, *J* 5.8, 12.5 Hz, 1H; 3.76–3.85, m, 3H; 3.91, dd, *J* 2.2, 12.4 Hz, 1H; 3.93–4.00, m, 3H; 4.50, 4.53, 4.54, 3 × d, *J* 7.9, 7.9, 8.0 Hz, H1′,1″,1‴. ^{13}C NMR (125.8 MHz, D_2O) δ 52.6, C3; 59.8, CH_2O; 60.5, 60.6, C6′,6″; 61.2, C6‴; 69.7, 70.1, C4,4‴; 73.6–76.6, 9C; 78.98, 79.02, C4′,4″; 80.2, C5; 82.7, C6; 103.0, 103.1, 103.2, C1′,1″,1‴. HRMS (FAB) *m/z* 636.2349. $C_{23}H_{42}NO_{19}$ $[M+H]^{+\bullet}$ requires 636.2351.

Enzymology

The serine nucleophile mutant of the *Agrobacterium* sp. β-glucosidase (Abg E358S) was created and purified as described previously.[11] α-D-Glucopyranosyl fluoride was synthesized according to literature protocols.[7,26] Glycosynthase reactions were carried out as described above. Inhibition constants for Cex were determined as described previously[21] using 2,4-dinitrophenyl β-cellobioside as substrate and a range of concentrations of the inhibitors from approximately one fifth of the K_i value ultimately determined to five times that value.

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