Biocatalysts

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Creation of an α-Mannosynthase from a Broad Glycosidase Scaffold**

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Dedicated to Professor Kunio Ogasawara

Glycosynthases provide a rare and practical source of catalytic synthetic utility for oligosaccharide[1–3] and other glycoside[4–6] synthesis. These biocatalysts, nucleophile-less mutants of retaining glycosidases,[2,7] are typically exquisitely stereoselective (inverting) enzymes and some can also attach monosaccharides with good regioselectivity.[7–10] Many examples now exist,[2] but in most cases β-glycosides are synthesized; α-glycosynthases are rare[11–14] and have not been applied widely to practical synthetic use, and some important linkages in biology, such as α-mannosides, have been inaccessible.[2]

In synthesis glycosynthases advantageously obviate some of[15] the typical need in oligosaccharide chemistry for hydroxy protection.[16] However, when glycosynthases are derived from *exo*-glycosidases (Figure 1 a) their mode of action often leads to repetitive condensation of these unprotected substrates, sometimes resulting uncontrollably in the creation of a mixture of varying oligomers as products[7,8,17–20] (Figure 1 b). This limitation may, at first sight, seem an inherent consequence of 'reversing' the natural *exo*-glycosidase mechanism (Figure 1 a), since an enzyme that evolved to bind the repetitive sugar structure may then readily accommodate product containing this sugar type at its nonreducing terminus. However, we describe herein a strategy for discrete, controlled glycoside synthesis in which a glycosynthase is created that can bind one sugar type as a donor substrate in the −1 (or D) subsite but then bind it less favorably as an acceptor when displayed in any ensuing product (Figure 1 d). This strategy has allowed the discovery of a novel α-mannosynthase that does not uncontrollably catalyze oligomerization.

Our design necessitated the combination of a glycosidase and a monosaccharide with good mutual affinity at the donor binding site (D site) but poor affinity at the acceptor-binding site (A site). This goal could, in principle, be achieved by identifying a nonpreferred substrate for a glycosidase with plasticity in its D site (Figure 1 c,d). Validation for this approach came from the contrasting behavior of the *Agrobacterium sp.* β synthase[7] towards Gal and Glc substrates. Although a priori prediction of enzyme sugar specificity and plasticity is difficult, Nishio et al. have reported an interesting feature of the glycoside hydrolase family 31 (GH31) in the carbohydrate-active enzyme (CAZy)[21] classification: these hydrolases can display their activity toward both α-glucosides and 2-deoxy derivatives.[22] This activity suggested that the 2-OH group of α-D-glucose might be less important for substrate recognition and implied that the epimer of α-D-glucose at the 2-OH position, α-D-mannose, might also be accommodated at the D site of these enzymes. If this were true, and D-mannose is also not favorable as an acceptor, our design concept (Figure 1 d) might be realized.

To test this hypothesis, we selected and expressed GH31 open reading frames (orfs) and discovered previously unknown[23] α-mannosidase activity for the product of GH31 gene *malA*[24,25] from *Sulfolobus solfataricus*. The enzyme's

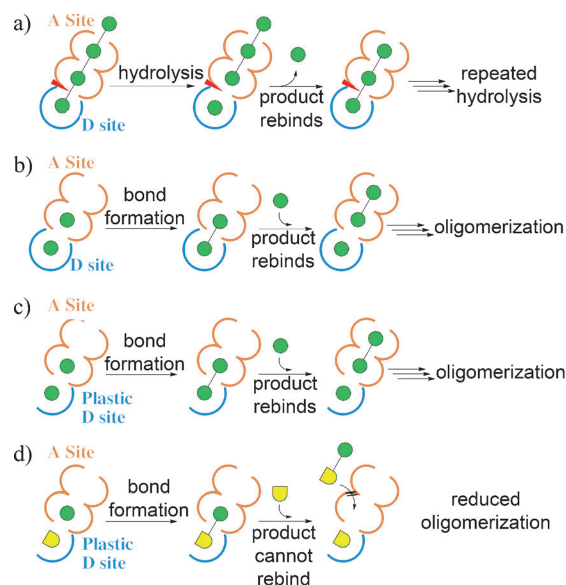

Figure 1. *exo*-Glycosidase trims oligosaccharides sequentially from nonreducing termini (a), and its glycosynthase can catalyze the reverse reaction, which may result in the formation of unwanted oligomers (b). When the enzyme has plasticity at its D site (c, d), the selection of an appropriate donor could, in principle, prevent oligomerization (d). A site and D site denote acceptor binding site(s) (+1 (,2,3...)) and donor binding site (−1), respectively. Green circle = preferred glycan substrate residues; yellow motif = donor substrate that binds to the donor site but less favorably to the acceptor site; red triangle indicates the site of hydolysis between the sites −1 and +1.

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preferred substrate was glucose (*para*-nitrophenyl α-D-glucopyranoside (*p*NP-α-D-Glc); $K_M = (1.7 \pm 0.2)$ mM, Table S1 in the Supporting Information) yet its affinity for *p*NP-α-D-Man was comparable ($K_M = (3.8 \pm 0.3)$ mM, Table S1 in the Supporting Information) as we desired, albeit with lower turnover.[26–29] After this identification of a promising candidate glycosidase scaffold with breadth, a nucleophile-less mutant D320G was prepared (using site-directed mutagenesis, see the Supporting Information), which expressed well (10–14 mg L^{-1} from *E. coli*). Putative α-glucosynthase activity and α-mannosynthase activity were tested using β-D-glucosyl fluoride (**1**) and β-D-mannosyl fluoride (**2**) as donors, respectively. β-Fluorides were chosen since, as for all synthases derived from retaining (double inversion step) GHs, inverting (single step) synthase activity would be logically anticipated[2,7] from removal of the nucleophile D320. Both donors **1** and **2** were readily prepared (see the Supporting Information), are stable as solids at 4° C, and even in aqueous solution have half lives longer than 11 h;[30] their sufficient stability has been previously noted.[31]

para-Nitrophenyl β-D-glucoside (**3a**) was used as an acceptor to allow ready reaction monitoring by UV spectroscopy at 300 nm (alternative methods based on quantitative MS, high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), evaporation light-scattering detection (ELSD), and NMR spectroscopy were less precise), and we were delighted to see effective glycosylation of this acceptor. Under near-identical conditions with stoichiometric amounts of donor and acceptor (20 mM each with 0.5 mg mL^{-1} enzyme in 100 mM sodium phosphate buffer, pH 6.0), similar conversion levels were observed for α-gluco- and α-manno-synthase activities (Figure 2). However, oligomerization was observed in the glucosynthase reaction using donor **1**. In striking contrast, no oligomerized product was observed in the mannosynthase reaction using donor **2** (Figure 2) at similar conversion. This initial result therefore critically demonstrated the validity of our design to develop an enzyme for discrete glycoside synthesis (Figure 1 d). The kinetics of this mannosylation were determined (Table S2 in the Supporting Information) and indicated that this designed, unnatural biocatalytic reaction displays parameters that are comparable[32] or superior to several other synthases[5,18,20,33] (k_{cat}(**3a**) $= (5.3 \pm 0.1)$ min^{-1}, K_M(**3a**) $= (9.1 \pm 0.7)$ mM at $[\mathbf{2}]_0 = 20$ mM; k_{cat}(**2**) $= (3.8 \pm 0.1)$ min^{-1}, K_M(**2**) $= (2.2 \pm 0.1)$ mM at $[\mathbf{3a}]_0 = 20$ mM). Notably, those values determined from hydrolytic kinetic analyses (see above) of the scaffold GH31 were indicative of useful (indeed superior) synthetic parameters in the resulting synthase (K_M(synthesis) $= (2.2 \pm 0.1)$ mM for donor **2** at $[\mathbf{3a}]_0 = 20$ mM confer K_M(hydrolysis) $= (3.8 \pm 0.3)$ mM for *p*NP-α-D-Man). The values of $k_{cat} = [(3.8–5.3) \pm 0.1]$ min^{-1} for mannosylation compare well with those for spontaneous hydrolysis of donor **2** (k_{app} ca. $(0.8–1.1) \times 10^{-3}$ min^{-1}).[30] Glucosylation kinetics were also determined (see the Supporting Information); these showed kinetic profiles (Figure S7 in the Supporting Information) that could not be interpreted by discrete reaction. In contrast, mannosynthase kinetics (Figures S5 and S6 in the Supporting Information) are consistent with a model (see the Supporting Information) of minimal oligomerization as

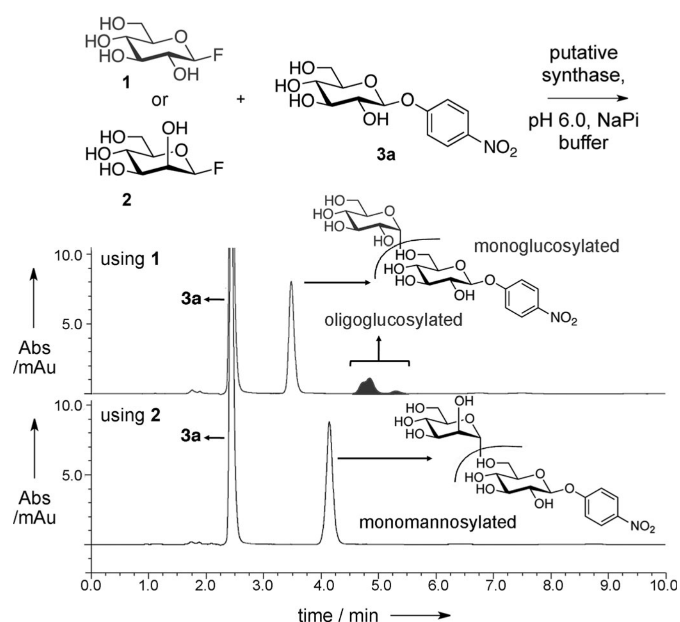

Figure 2. Chromatograms of the gluco- or mannosynthase reactions (both shown at the same ca. 30% conversion; 20 mM of both donor and acceptor with 0.5 mg mL^{-1} enzyme MalA D320G in 100 mM sodium phosphate buffer, pH 6.0). In the glucosynthase reaction using donor **1** (top), oligomerization was observed (highlighted) even at this partial level of conversion, while none was observed in the mannosynthase reaction using donor **2** (bottom). The hemispheres in the structures of the products indicate that mixtures of regioisomers were formed.

a result of weak affinity of terminal mannosides to the acceptor site. Similar relative turnovers also showed that reduced oligomerization for mannosylation was not simply due to lower activity.

After having established this promising utility, we investigated next the potential substrate scope of the α-mannosynthase-catalyzed reaction using **2** as a donor. A representative range of *p*NP glycosides was screened as acceptors and revealed sufficient tolerance within the A site to allow the processing (α-mannosylation) of a variety of biologically important sugars (Glc, Man, Xyl, L-Fuc, L-Rha, and glucuronic acid (GlcA)) whilst others (Gal, galacturonic acid (GalA), *N*-acetyl-glucosamine (GlcNAc)) were not efficient acceptor substrates (Figure S4 in the Supporting Information).

After optimization for synthetic use, the mannosynthase allowed successful preparation (Scheme 1) of a variety of discretely α-mannosylated products in overall yields of up to 77% with effectively absolute stereoselectivity at carbon atom C-1 ($de > 98\%$ α). In most cases good regioselectivities (> 90%) were also observed, typically for α(1,3)-linked product. Moreover, consistent with the intended design, typical oligomerization ratios were low (OF < 20% for several of these products) despite the use of an excess of donor. Notably the mannosynthase catalyst is highly robust and could be readily recovered after separation from small molecules using ultrafiltration and dialysis and was readily recyclable for other reactions without any significant deterioration in its activity (Figure S10 in the Supporting Informa-

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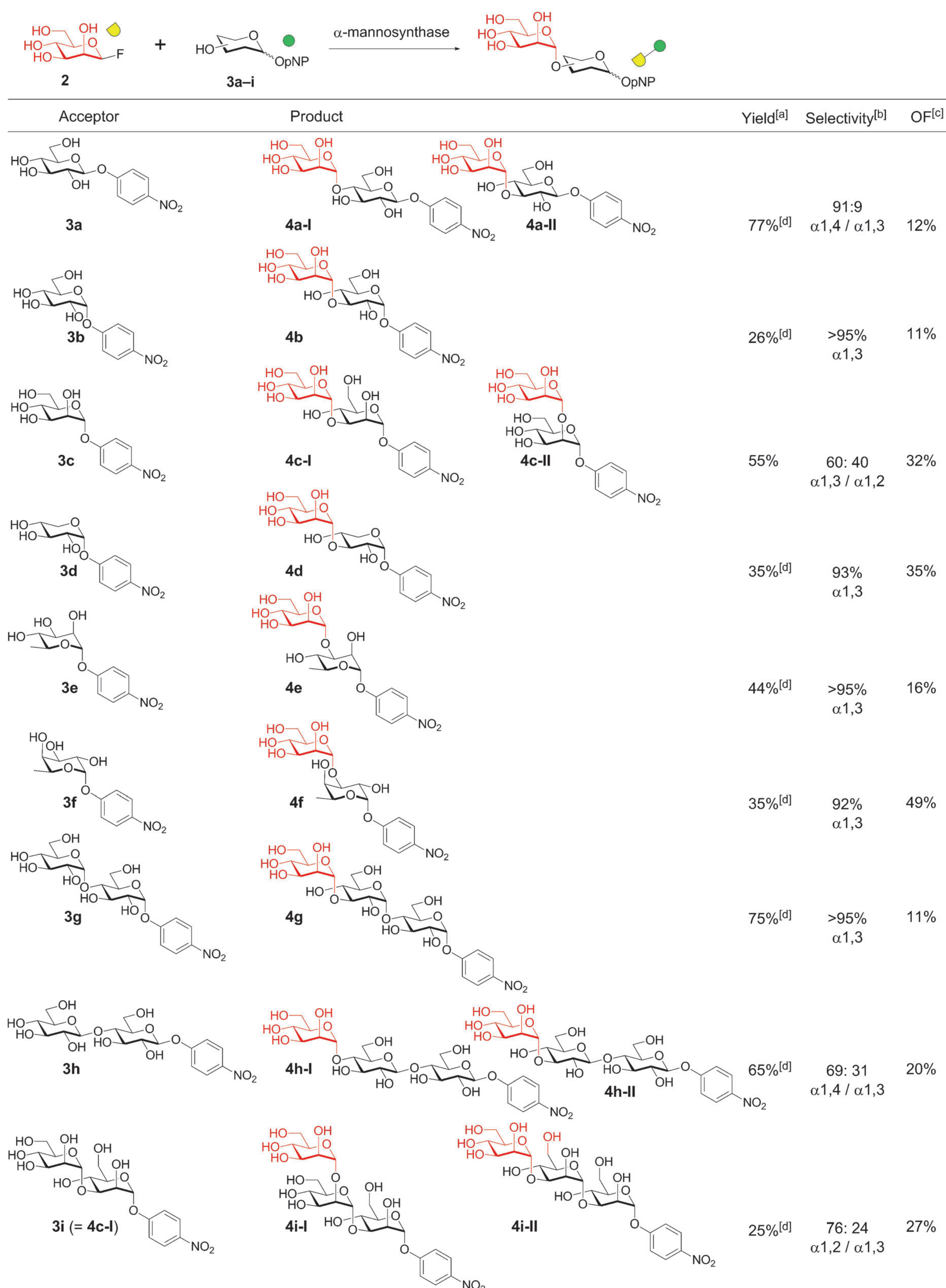

Acceptor	Product	Yield[a]	Selectivity[b]	OF[c]
3a	4a-I, 4a-II	77%[d]	91:9 α1,4 / α1,3	12%
3b	4b	26%[d]	>95% α1,3	11%
3c	4c-I, 4c-II	55%	60: 40 α1,3 / α1,2	32%
3d	4d	35%[d]	93% α1,3	35%
3e	4e	44%[d]	>95% α1,3	16%
3f	4f	35%[d]	92% α1,3	49%
3g	4g	75%[d]	>95% α1,3	11%
3h	4h-I, 4h-II	65%[d]	69: 31 α1,4 / α1,3	20%
3i (= 4c-I)	4i-I, 4i-II	25%[d]	76: 24 α1,2 / α1,3	27%

Scheme 1. α-Mannosynthase-catalyzed reactions (scale: from ca. 2.5 up to ca. 20 mg product). [a] Yields after purification or isolation. These yields are not based on recovered starting material, and starting material was typically also recovered in the acceptor mass balance. [b] Determined by HPLC or Man(1-H) ^{1}H NMR integration. [c] Oligomerization factor (OF) defined as % of oligomannosylated products in total mannosylated products. [d] Isolated as peracetate. Reaction conditions: acceptor (**3a**–**i**; 20 mM, 1 equiv), donor (**2**) 2 equiv × 3, sodium phosphate buffer (1 mL, 300 mM, pH 7), room temperature, synthase (2.0 mg mL^{-1}); DMF (5–15%, v/v) used as cosolvent as needed to aid solubility.

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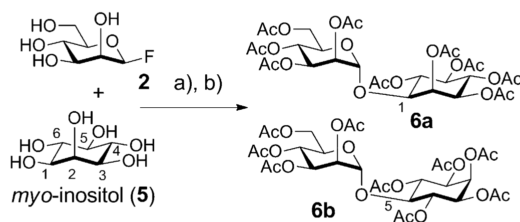
tion). Moreover, the catalyst could be used in the presence of up to 15% DMF (v/v), as an organic cosolvent for starting materials that were sparingly soluble in water, again with little effect upon overall efficiency (Figure S11 in the Supporting Information) over several cycles.

Together these syntheses allowed the ready preparation of a number of naturally occurring α-mannoside motifs: Man-α(1,4)-Glc[34] (**4a-I**), Man-α(1,3)-Glc[35] (**4b**), Man-α(1,3)-L-Rha[36] (**4e**), Man-α(1,3)-L-Fuc[37] (**4f**), and Man-α(1,3)-Cel[38] (**4h-II**) found in bacterial polysaccharides. These motifs also included the dimannosides Man-α(1,2)-Man (**4c-II**, **4i-I**) and Man-α(1,3)-Man (**4c-I**, **4i-II**) found in N-glycans. In this way a strategy designed to select against uncontrolled oligomannoside formation can nonetheless be used for discrete dimannosides (and even higher) through suitable control of substrate concentrations. Notably, the preparation of **4c-I** also allowed its use as a starting material in a subsequent discrete mannosylation reaction, which afforded easy access to the core trisaccharide (**4i-I**) of the D1-arm of high-mannose N-linked protein glycans. Although the yield was lower than for some of the other mannosynthase-catalyzed reactions, use of minimally protected building blocks (and avoidance of associated protection/deprotection steps) makes this a competitive overall route. This product is a key ligand motif in the binding of neutralizing human antibodies (e.g., 2G12) to HIV-1[39] and, as such has been used by us[40] and others as a building block in anti-HIV vaccine design.[41]

Finally, the utility of the α-mannosynthase was tested in highly crowded substrates: the target synthesis of α-mannosylated inositols. Mannosylated *myo*-inositols are components of the cell wall glycolipids found in *Mycobacterium tuberculosis* and, as such, have been the targets of traditional glycoside syntheses.[42,43] Reaction of more hindered acceptor *myo*-inositol (**5**) was more sluggish than those for some of the other acceptors. Nonetheless, reaction provided a 41% yield of mono-α-mannosylated inositols from which Man-α(1,5)-*myo*-inositol (**6b**)[44,45] and, intriguingly, Man-α(1,1)-*myo*-inositol (**6a**)[46] were isolated (Scheme 2) in approximately 3:2 ratio. The latter was formed from a rare example of simultaneous glycosylation and desymmetrization of such a complex *meso* polyol acceptor.[47]

In conclusion, we have created a rationally designed α-mannosynthase using an α-glucosidase as a scaffold for discrete mono-mannoside synthesis. This is the first example of a glycosynthase for this biologically important linkage and a rare example of a glycosynthase that constructs α-anomers. It has been highlighted that the use of protecting groups necessitates an average of up to seven additional manipulation steps for each corresponding formation of a glycosidic bond in total oligosaccharide synthesis;[48] this reduces average yields by more than 50% even if individual yields for these steps are more than 90%. Therefore, the use of methods, such as those explored here, that minimize or avoid the use of protecting groups are valuable and give rise to alternative and competitive overall synthetic strategies. For comparative balance, it should also be noted that, if regioselectivity can be achieved, the α-mannoside linkage is more readily synthesized than several others using chemical synthesis.



Scheme 2. Synthesis of mannoinositols. Reagents and conditions: a) α-mannosynthase, sodium phosphate buffer (pH 7, 300 mM), **2**; b) Ac_2O, pyridine (1:1 v/v).

In nature glycosidic bonds are made, without protection, by specialized glycosyltranferases that are typically selective for one bond type. That is, they sometimes lack the plasticity/breadth needed for pragmatic synthetic application. The specialized glycosyltranferases also usually employ rare and difficult to access sugar phosphate donors (nucleotides e.g., mannose guanosine diphosphate (ManGDP), or lipids) as the source of glycosylation. This requirement, as well as lack of enzyme availability (to our knowledge no α-mannosyltransferases are commercially available), has prevented their widespread use in synthetic chemistry. One of the most common biologically relevant glycoside motifs is the α-mannoside bond. Therefore, it is notable and striking that this bond is rarely made through biocatalysis[49–53] in current synthetic routes. Herein we have presented a catalyst that will now efficiently and selectively form α-mannosides in a broad manner using a simple mannosyl fluoride reagent. Recently, other strategies for engineering enzymes (e.g. glycoligases[54]) have allowed efficient xylosylation. The logic that we have shown here, of conversion (through rational engineering) of a glycosidase that preferentially cleaves a linkage of one type to a glycosynthase that catalyzes the formation of another, may be a broadly useful strategy that we are exploring in other systems.

Experimental Section

Representative procedure for α-mannosynthase reaction: β-D-Man-F (**2**; 200 μL of 200 mM) in sodium phosphate buffer (400 mM, pH 7.0) and *p*NP-sugar (**3**; 500 μL of 40 mM) in sodium phosphate buffer (400 mM, pH 7.0) were mixed and then synthase solution in sodium phosphate buffer (100 mM, pH 6.0), sodium phosphate buffer (400 mM, pH 7.0), and water were added so that the final concentrations of the enzyme and the buffer became 2.0 $mg\,mL^{-1}$ and 300 mM, respectively (the final volume was 1 mL). When the acceptor substrate was sparingly soluble in the aqueous buffer, it was added as a solution in DMF, giving the same substrate concentration. The reaction solution was incubated at room temperature and, if needed, additional aliquots of **2** were added (200 μL of 200 mM β-D-Man-F (**2**) in 300 mM sodium phosphate buffer, pH 7.0). After completion, the reaction solution was loaded onto an ultrafiltration device (Vivaspin 6, MWCO 10000, Sartorius, pretreated with 0.1% Triton X-100) to separate the protein fraction and the small-molecule fraction. The recovered protein fraction was dialyzed against sodium phosphate buffer (100 mM, pH 6.0) for recycled use. The small-molecule fraction was purified by HPLC (Synergi 4u Fusion-RP 80A, 100 × 21.20 mm (Phenomenex) then Luna 5u NH2, 250 × 21.20 mm, 100 A (Phenomenex)) and lyophilized prior to analysis or, when necessary, additionally peracetylated and analyzed as their peracetates.

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