Glycobiology

Thioglycoligases: Mutant Glycosidases for Thioglycoside Synthesis**

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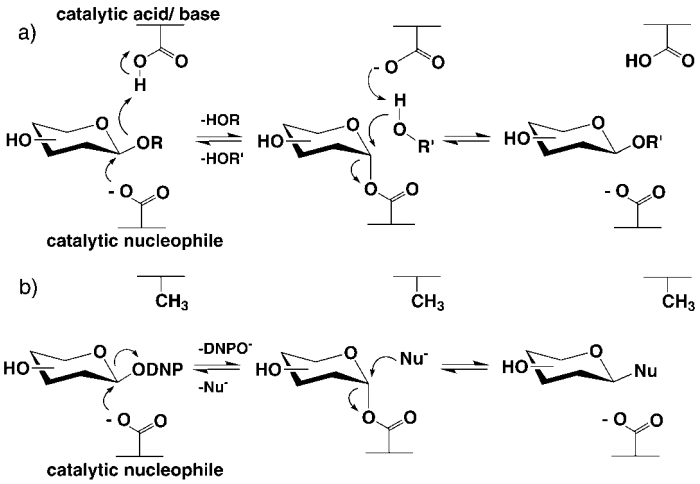
Carbohydrate mimetics that are resistant towards enzymatic hydrolysis have proven to be useful as competitive glycosidase inhibitors and therefore have potential as therapeutics. Thioglycosides, in which the glycosidic oxygen atom has been replaced by a sulfur atom, have been especially valuable as stable glycoside analogues in a range of studies of glycosidases, both as competitive inhibitors, for example, for α-L-fucosidases,[1] pancreatic α-amylase,[2] or for cellulases,[3] and recently in the formation of stable complexes for X-ray crystallographic analysis, for example, with endoglucanase Cel7B from *Fusarium oxysporum*,[4] maize β-glucosidase ZMGlu1,[5] barley β-D-glucan glucohydrolase,[6] endoglucanase Cel5A,[7] or *E. coli* maltodextrin phosphorylase (MalP),[8] and they are gaining increasing interest as targets for the pharmaceutical industry.[9]

Thioglycosides have been synthesized by glycosylation of thioacceptors with activated glycosyl donors,[10–12] by the S_N2 reaction of 1-thio sugars with activated acceptors,[13] and by Michael-like addition of 1-thiosugars to α,β-unsaturated systems;[14] all routes involve numerous protection and deprotection steps and require good control of anomeric stereochemistry.

The few naturally occurring thioglycosides belong to the family of glucosinolates and are found in cruciferous plants.[15] The enzymes that catalyze the formation of the thioglycosidic linkages, *S*-glucosyltransferases, have been purified from plant extracts,[16] as well as cloned into and expressed from *E. coli*.[17] There are a few examples of transfer to simple thiols.[18–20] However, no useful enzymatic syntheses of thioglycosidic linkages in oligosaccharides in vitro have been reported—such approaches would be valuable.

The action of retaining glycosidases on glycosides is in most instances mediated by two key active site amino acid residues, the catalytic nucleophile and the catalytic acid/base. We have previously reported on the efficient synthesis of *O*-glycosidic linkages in oligosaccharides by the use of retaining glycosidases that lack the catalytic nucleophile, glycosynthases, in conjunction with activated donors of the opposite anomeric configuration to that of the natural substrate.[21–23] Herein we present a novel strategy for the synthesis of *S*-glycosidic linkages in oligosaccharides by the use of retaining glycosidases that lack the catalytic acid/base amino acid residue. The choice of appropriate donors and acceptors to be used in such an approach becomes apparent by considering the double displacement mechanism of retaining glycosidases



Scheme 1. Mechanism of a retaining glycosidase: Glycosylation and deglycosylation with wild-type (a) and acid/base mutant (b). DNP = dinitrophenyl, Nu = nucleophile.

first proposed by Koshland (Scheme 1).[24] In the glycosylation step the concerted action of the catalytic acid/base (protonated, acting as an acid) and the catalytic nucleophile (deprotonated) leads to the departure of the aglycon group and to the formation of the covalent glycosyl–enzyme intermediate (a). Use of a mutant glycosidase in which the catalytic acid/base residue has been replaced by a noncatalytically active residue necessitates glycosyl donors with good leaving groups that do not need acid catalysis, for example, dinitrophenyl groups, to allow formation of the glycosyl–enzyme intermediate (b). However, turnover of that intermediate via transglycosylation to an oxygen acceptor is impractically slow, since general base catalysis is required to speed this step (a). The acid/base mutant therefore requires strong nucleophiles as acceptors that do not need general base catalysis (b).

Earlier experiments revealed that small anionic molecules, such as N_3^-, AcO^-, HCO_2^-,[25] and F^-,[26] rescue the reaction by enhancing the rate of the deglycosylation step. In the present study we exploit this concept to allow the synthesis of thiooligosaccharides by use of deoxythio sugars as nucleophilic acceptors that do not need base catalysis in the deglycosylation step. In summary, our approach requires activated donor glycosides, such as dinitrophenyl glycosides, and chemically synthesized deoxythio sugars as acceptors in conjunction with mutant glycosidases modified at the acid/base position.

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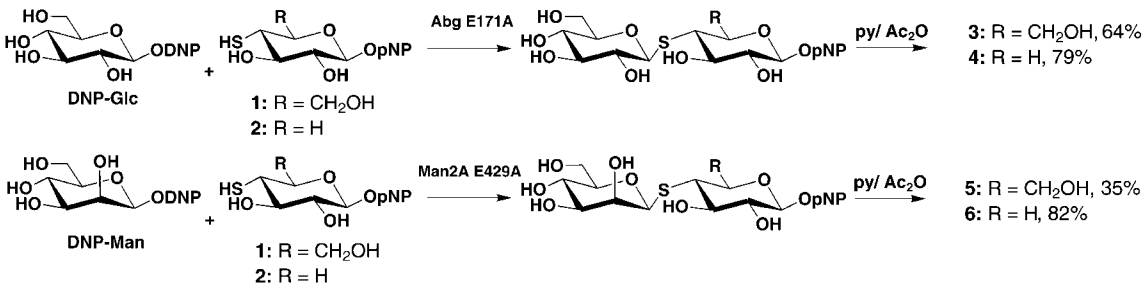
We have probed our strategy using the alanine acid/base mutants of two retaining β-glycosidases, the β-glucosidase from *Agrobacterium sp.* Abg E171A and the β-mannosidase from *Cellulomonas fimi* Man2A E429A. The mutant Abg E171A was generated by site-directed mutagenesis by a "megaprimer" PCR method by using three oligonucleotide primers, one containing the mutation, as described for the Abg E358S mutant.[22] The purified product of the first PCR reaction served as a megaprimer in the second PCR reaction. The purified gene was subcloned into an expression vector, and after expression the protein was purified in a single step by Ni^{2+}-chelation chromatography. The mutant Man2A E429A was the same protein sample described by Zechel et al.[26]

As donors we chose the readily available dinitrophenyl glycosides: 2,4-dinitrophenyl β-D-glucopyranoside (DNP-Glc) for studies with the mutant glucosidase and 2,5-dinitrophenyl β-D-mannopyranoside (DNP-Man) for experiments involving the mutant mannosidase.[26] The low pK_a values of 3.96 for 2,4-dinitrophenol and 5.15 for 2,5-dinitrophenol render acid catalysis for the glycosylation step unnecessary.[27] As an acceptor we initially chose *para*-nitrophenyl 4-deoxy-4-thio-β-D-glucopyranoside (**1**) since the parent sugar *para*-nitrophenyl β-D-glucopyranoside (pNP-Glc) acts as an excellent acceptor for both wild type and nucleophile mutant forms of Abg[21] and Man2A[23] undergoing transfer preferentially to the 4-hydroxy group. The deoxythio sugar **1** was readily synthesized by regioselective protection of the *galacto* sugar, activation of the unprotected axial alcohol by formation of the triflate, nucleophilic substitution with thioacetate with inversion of configuration, and finally Zemplen deprotection under exclusion of oxygen (in the presence of DTT). Anaerobic conditions, even during the enzymatic reactions, are compulsory to prevent oxidation of the thiols to the corresponding disulfides.

Upon incubation of **1** (20 mM) with the appropriate donor (45 mM) and mutant enzyme (~1 mg mL^{-1}) in phosphate buffered solutions at pH 6.8 TLC analysis revealed that a new product with the expected mobility of a disaccharide was formed quite rapidly in each case. These products were stable towards hydrolysis by both the wild-type and mutant enzymes. By contrast, incubation of the wild-type enzymes with the appropriate DNP sugar donors and thiosugar acceptor **1** resulted in no detectable formation of disaccharide. It was therefore suspected that a novel enzymatic coupling reaction was occurring involving specific thiolinkage formation. This was confirmed by isolation of the products of the enzymatic reactions by silica gel chromatography after acetylation. Analysis of the products by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry revealed that they were indeed sulfur-linked disaccharides (Scheme 2).

These extremely encouraging results led us to question whether the regiochemical outcome of the transglycosylation reaction could be controlled by virtue of the location of the thiol group within the acceptor sugar. We therefore synthesized and tested *para*-nitrophenyl 4-deoxy-4-thio-β-D-xylopyranoside (**2**) as an acceptor for the two enzymes. Previous studies with both the wild-type Abg and its nucleophile mutant had revealed that *para*-nitrophenyl β-D-xylopyranoside (pNP-Xyl) is an excellent acceptor substrate, but that transfer, surprisingly, occurred exclusively to the 3-position.[21] Use of **2** as an acceptor would reveal whether the greater nucleophilicity of the thiol group controls the reaction outcome. Indeed, incubation of **2** with Abg E171A and DNP-Glc resulted in an excellent yield (79%) of the sulfur-linked disaccharide, as revealed by ^{1}H and ^{13}C NMR spectroscopy and ESI-MS. Similarly, incubation of **2** with Man2A E429A and DNP-Man produced, in 82% yield, the β(1–4)-linked thiodisaccharide product (Scheme 2).

Interestingly, control reactions, in which the parent sugars *para*-nitrophenyl β-D-xylopyranoside (pNP-Xyl) and *para*-nitrophenyl β-D-glucopyranoside (pNP-Glc) were used as acceptors for Man2A E429A in the presence of DNP-Man, showed the formation of disaccharide products, but only very slowly. This is in contrast to Abg E171A where no formation of disaccharides was observed when using pNP-Glc or pNP-Xyl in the presence of DNP-Glc as donor. To assess the relative rates of transfer to the oxygen and sulfur nucleophiles in the case of Man 2A E429A we established a competition reaction in which equimolar amounts of pNP-Xyl and its 4-thio analogue **2** were incubated together with DNP-Man as donor and Man2A E429A (Figure 1). When the concentration of donor was limiting (one third of the concentration of acceptors) we saw exclusive formation of the S-linked disaccharide **6** (a). However, upon addition of further donor to a final concentration twice that of the acceptors the O-linked disaccharides (indicated by arrows) were formed (b). Isolation and characterization after acetylation showed that the β(1→3)- and β(1→4)-linked disaccharides were formed.[28] Integration of HPLC peaks indicates that transfer



Scheme 2. Enzymatic synthesis of thioglycosides **3**–**6**. Experimental details and spectroscopic data are provided in the Supporting Information. DNP = dinitrophenyl, pNP = *para*-nitrophenyl.

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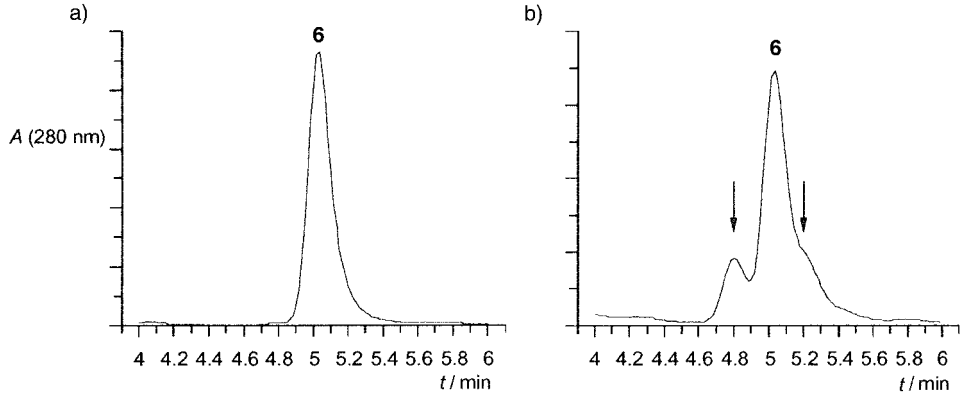

Figure 1. HPLC traces of the Man2A E429A-catalyzed reaction of the two competing acceptors pNP-Xyl (5.5 mM) and its thio analogue **2** (5.5 mM) with varying concentrations of DNP-Man: a) 3.75 mM, b) 22 mM. Arrows indicate the elution of the β(1→3)- and β(1→4)-O-linked disaccharides, **6** is the S-linked disaccharide. Experimental details are provided in the Supporting Information.

to the thiosugar acceptor is at least 100-fold faster than transfer to its oxygen analogue. These results were confirmed by ESI-MS analysis.

The glycosynthases have proven to be powerful tools for the synthesis of O-linked oligosaccharides. Herein we have shown again that the concept of engineering glycosidases on mechanistic grounds in conjunction with use of appropriately modified substrates can lead to novel synthetic methodologies. We anticipate that the outlined approach is general, as demonstrated with two different enzymes from two separate glycosidase families, and will prove to be of considerable value in the specific assembly of thiooligosaccharides for mechanistic and possibly therapeutic use.

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[27] We used 2,5-dinitrophenyl β-D-mannopyranoside instead of 2,4-dinitrophenyl β-D-mannopyranoside as the latter is impractically unstable due to aryl migration. We also tested the appropriate pNP-glycosides as donors, but prolonged reaction times make those less attractive.
[28] The physical data of the compounds were identical to those described by Nashiru et al.[23]

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