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Facile formation of β-thioGlcNAc linkages to thiol-containing sugars, peptides, and proteins using a mutant GH20 hexosaminidase.

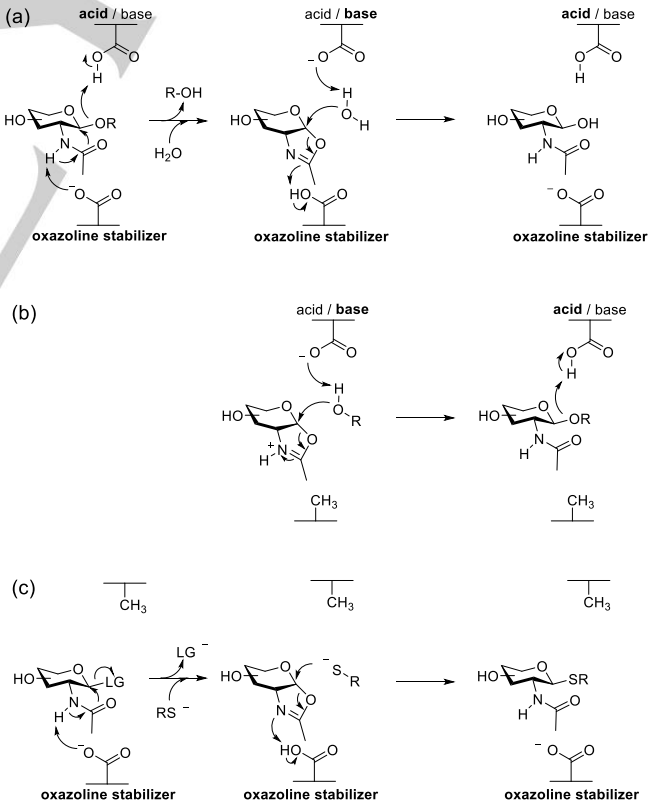
Gregor Tegl[a], John Hanson[b], Hong-Ming Chen[a], David H Kwan[a], Andrés G. Santana[a] and Stephen G. Withers*[a]

Abstract: *Thioglycosides are hydrolase-resistant mimics of O-linked glycosides that can serve as valuable probes for studying the role of glycosides in biological processes. Traditional chemical syntheses of thioglycoside analogues of O-GlcNAc-modified peptides and proteins require lengthy, multi-step approaches. We report the development of efficient, enzyme-mediated syntheses of thioglycosides, including S-GlcNAcylated proteins, using a thioglycoligase derived from a GH20 hexosaminidase from Streptomyces plicatus in which the catalytic acid/base glutamate has been replaced with an alanine (SpHex E314A). This robust, easily-prepared, engineered enzyme uses readily available GlcNAc and GalNAc donors and couples them to a remarkably diverse set of thiol acceptors. Thioglycoligation using 3-, 4-, and 6-thiosugar acceptors from a variety of sugar families produces S-linked disaccharides in nearly quantitative yields. The set of possible thiol acceptors also includes cysteine-containing peptides and proteins, rendering this mutant enzyme a promising catalyst for the production of thio analogs of biologically important GlcNAcylated peptides and proteins.*

Advances in glycobiology are often dependent on the preparation of specific oligosaccharides or complex glycoconjugates. Conventional chemical synthesis of such compounds typically requires time-consuming protecting group manipulations in order to achieve the desired regiochemical and stereochemical control. Thus, enzyme-based approaches have become particularly valuable for the synthesis of oligosaccharides or glycoconjugates.[1] While glycosyltransferases are the primary synthetic enzymes in nature, a variety of glycoside hydrolases (GHs) have been engineered to convert them into effective catalysts for forming glycosidic bonds.[2] Most retaining GHs contain two key active site residues, a catalytic nucleophile and a catalytic acid/base, and catalyze reactions through a glycosyl-enzyme intermediate (Scheme S1a).[3] One common strategy for converting such a retaining GH into a glycosynthase, is to remove the active site nucleophile; the resulting mutant enzyme, with greatly diminished hydrolytic activity, can form glycosidic linkages when used in combination with an activated donor that mimics the glycosyl-enzyme intermediate (Scheme S1b).[4,32]

A desire for carbohydrate mimetics that are resistant to enzymatic hydrolysis *in vivo* has elicited interest in the preparation of thioglycosides (in which the typical glycosidic oxygen atom is replaced with sulfur) for use as GH inhibitors or as stable ligands for structural and functional biology studies.[6,7] Despite advances in the conventional chemical synthesis of thioglycosides, common bottlenecks remain.[8] Enzyme-based methods have the potential to streamline the preparation of thioglycosides. However, due to the paucity of natural thioglycosides, there are relatively few enzymes in nature that normally catalyze the synthesis of thioglycosides.[9,33] One proven strategy for producing enzymes that can form thioglycosidic linkages, is to use mutant GHs in which the catalytic acid/base residue has been replaced by a non-catalytically active residue (Scheme S1c);[6] this approach has resulted in several α and β-thioglycoligases derived from retaining GHs which proceed via a double displacement (Koshland) mechanism.[10–14] The glycosyl-enzyme intermediate is formed by using a donor possessing a good leaving group (LG), avoiding the requirement of acid catalysis for the glycosylation step. De-glycosylation would otherwise be slow due to the absence of a general base to deprotonate the water during nucleophilic attack.

Scheme 1. (a) Wild type neighbouring group-assisted hexosaminidase mechanism, (b) oxazoline stabilizer mutant hexosaminidase acting as a glycosynthase using an oxazoline as the donor, (c) acid/base mutant hexosaminidase acting as a thioligase using a donor with a good leaving group (alternatively the oxazoline may be used as donor).



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However, when using a nucleophile with a low pKa, such as a thiol, activation by the catalytic base is not required, thus allowing the reaction to proceed at a reasonable rate.[6]

Oligosaccharides and glycoconjugates containing beta-linked GlcNAc or GalNAc residues have many important biological roles.[15] Studies on their effects would benefit from the ability to readily prepare hydrolytically stable thioglycoside analogs. For example, O-GlcNAc modification of nuclear and cytosolic proteins is a dynamic process influencing a wide range of cellular processes.[16] Despite advances in analytical methods, the study of the effects of O-GlcNAcylation is still a troublesome endeavor due to omnipresent hexosaminidases capable of removing GlcNAc from proteins and thus dramatically reducing their *in vivo* half-life.[14] Cysteine-based S-GlcNAcylated proteins have been prepared and are reported to mimic O-GlcNAc linkages without altering their biological properties, but were very challenging to synthesize.[17] An attractive alternative strategy to prepare S-GlcNAcylated proteins would be to replace the amino acid that is normally post-translationally modified (serine or threonine) with a cysteine, and then use an appropriate thioglycoligase to attach the GlcNAc to the cysteine. With these objectives in mind we set out to modify a glycoside hydrolase that normally cleaves terminal GlcNAc and GalNAc residues (a hexosaminidase) to produce a thioglycoligase capable of forming S-linked β-GlcNAc (or β-GalNAc) oligosaccharides and glycoconjugates when reacted with the appropriate thiol acceptors.

Hexosaminidases from GH18, GH20, GH25, GH56, GH84, and GH85 operate by a variant of the Koshland mechanism involving neighboring group participation from the 2-acetamido group of the respective sugar substrate, producing an oxazoline intermediate rather than a glycosyl-enzyme intermediate (Scheme 1a).[18,19] While several mutant hexosaminidases have been used as glycosynthases[20], no thioglycoligases from these families have been previously reported. Since the mechanism and charge balance in the active site is substantially different between the double displacement (Koshland) type enzymes and the neighboring group participation type enzymes, it was not clear whether the strategies used to convert the former into thioglcoligases would work for the latter.

The hexosaminidase from *Streptomyces plicatus* (SpHex) is a retaining GH20 exo-hexosaminidase that uses the neighboring group mechanism to cleave terminal β-linked GlcNAc or GalNAc residues from the non-reducing end of oligosaccharides (Scheme 1a).[21] A conserved aspartate (D313) in the active site assists by helping to polarize and position the 2-acetamido group.[18] Mutation of this conserved aspartate to alanine produces a mutant enzyme SpHex D313A that catalyzes formation of glycosidic bonds when synthetically prepared oxazoline is used as the donor (Scheme 1b).[22] The active site of SpHex also contains a conserved glutamate (E314) that acts as the catalytic acid/base, promoting cleavage of the glycosidic linkage by general acid catalysis and subsequent nucleophilic opening of the oxazoline by general base catalysis.[23] In analogy with previous strategies for producing thioglycoligases, we hoped that replacement of glutamate 314 by an alanine would produce an enzyme capable of catalyzing thioligation of activated GlcNAc donors to appropriate thiol acceptors (Scheme 1c).

Site-directed mutagenesis was used to convert the catalytic acid/base glutamate of SpHex to alanine, producing SpHex E314A. The mutant enzyme containing a polyhistidine tag was readily produced in large quantities as a robust, soluble protein. The enzyme retained high activity over several hours at 37 °C at pH values ranging from pH 5 to 9 in solutions containing up to 10% DMSO. Use of this co-solvent was occasionally necessary to help solubilize acceptor or donor saccharides.

As expected, and as previously reported for the E314Q mutant,[22] the removal of the catalytic acid/base results in a significant drop in the hydrolytic efficiency of the enzyme; k_{cat}/K_M (and k_{cat}) dropped to slightly less than 10% of the wild type values using pNP-GlcNAc as substrate (Table 1) The pH profile was fairly flat from pH 5 to 9, with k_{cat}/K_M values staying within a factor of 2 over this range (Figure S1). pNP-GalNAc was used as substrate by the mutant with rate constants approximately 3-fold lower. This substrate and pH-tolerance will facilitate the use of this mutant in synthesis.

To explore the ability of SpHex E314A to act as a thioglycoligase, an excess of the donor GlcNAc oxazoline was incubated at 37 °C and pH 7 with a variety of sugar thiol acceptors (5 mM) in the presence of SpHex E314A (0.5 mg/mL) (Figure S2 + S3, Table 2); formation of the corresponding thioglycosides was monitored by TLC and ESI MS analysis. Ligation to the thiol residue, rather than to sugar hydroxyls, was demonstrated by TLC using a DTNB stain which reacts with free thiols;[24] the products did not react with this stain (Figure S4). Since wildtype SpHex cleaves β-linked glycosides of GlcNAc, we initially tested the 4-thio analogue of pNP-GlcNAc as an acceptor.[24] Using excess oxazoline donor, we observed quantitative formation of the desired disaccharide containing the thioglycosidic linkage. Encouraged by this result, we also tested the 3-thio- and 6-thio- pNP-GlcNAc analogs as acceptors and were pleasantly surprised to again observe rapid conversion to the corresponding disaccharides (Figure S3). Given the observed acceptor promiscuity, several other 3- and 4-thio analogues were tested, comprising representatives of the D-gluco, D-galacto and D-manno series (Table 2). Remarkably, thioglycoside formation was observed for all candidates (Figure S2 and S3). The donor was typically consumed within an hour; if unreacted thiol acceptor was still present, additional oxazoline donor was added as necessary, resulting in complete conversion of the respective thio-acceptor and quantitative product yields. Sub-stoichiometric product formation was observed only for 4-thio ManNAc and 3-thio GalNAc acceptors. No detectable disaccharide formation was observed in control reactions using either wt or SpHex D313A.

Although the GlcNAc oxazoline donor could be synthesized from GlcNAc,[26] we found that the commercially available pNP-GlcNAc was an even more convenient glycosyl donor for these thioligation reactions, especially on a preparative scale; pNP-GlcNAc is presumably converted into the intermediate GlcNAc oxazoline by SpHex E314A (Scheme 1c). We also showed that pNP-GalNAc is an effective donor substrate, as demonstrated by synthesis of β-GalNAc-1,4-S-GlcNAc-pNP in 98% isolated yield (SI 1.4 NMR Data). Further confirmation of the identity of the products from preparative scale (approximately 10 mg (20 μmol) of product) reactions using pNP-GlcNAc and pNP-GalNAc as donors (Table 2, Entries 1-4) was obtained by NMR analysis of HPLC purified products (SI 1.1-1.4 NMR Data). Interestingly self-condensation of the thio-pNP-GlcNAc sugars listed in Table 2 was not observed,

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Table 1. Kinetic parameters for SpHex and its mutants at pH5 and 7 (K_M in µM, k_{cat} in s^{-1} and k_{cat}/K_M in s^{-1}*$µM^{-1}$); n.d.: not determined

	pH 5 SpHex wt	pH 5 SpHex E314A	pH 7 SpHex wt	pH 7 SpHex E314A
pNP-GlcNAc				
K_M	36	20	13	16
k_{cat}	237	9.1	64	5.9
k_{cat}/K_M	6.6	0.45	4.92	0.37
pNP-GalNAc				
K_M	n.d.	34	n.d.	11
k_{cat}	n.d.	2.7	n.d.	1.2
k_{cat}/K_M	n.d.	0.08	n.d.	0.11

presumably because the bulkier thiosugar is not accepted in the donor (-1) subsite.

Table 2. SpHex E314A catalyzed thioligation using thiosugar acceptors. Reactions using pNP-GlcNAc or pNP-GalNAc as donor were performed on a preparative scale (~20 µmol); products were purified by HPLC and characterized by NMR spectroscopy. Yields were essentially stoichiometric with respect to acceptor. Reactions using GlcNAc oxa as donor were performed on an analytical scale; products were characterized by TLC and MS.

Acceptor	Product	Nr
β-pNP-4-S-GlcNAc	GlcNAc-(β-1,4)-4-S-GlcNAc-β-pNP	1
β-pNP-3-S-GlcNAc	GlcNAc-(β-1,3)-3-S-GlcNAc-β-pNP	2
β-pNP-6-S-GlcNAc	GlcNAc-(β-1,6)-6-S-GlcNAc-β-pNP	3
β-pNP-4-S-GlcNAc	GalNAc-(β-1,4)-4-S-GlcNAc-β-pNP	4
β-pNP-4-S-Glc	GlcNAc-(β-1,4)-4-S-Glc-β-pNP	5
β-pNP-3-S-Glc	GlcNAc-(β-1,3)-3-S-Glc-β-pNP	6
β-pNP-4-S-GalNAc	GlcNAc-(β-1,4)-4-S-GalNAc-β-pNP	7
β-pNP-3-S-GalNAc	GlcNAc-(β-1,3)-3-S-GalNAc-β-pNP	8
β-pNP-4-S-ManNAc	GlcNAc-(β-1,4)-4-S-ManNAc-β-pNP	9
β-pNP-4-S-Man	GlcNAc-(β-1,4)-4-S-Man-β-pNP	10

Given the relatively narrow substrate specificities of most enzymatic systems, the broad acceptor specificity observed in this system is somewhat surprising. Similar acceptor promiscuity has been reported for a thioglycoligase derived from the β-glucosidase from *Agrobacterium sp.*, Abg E171A.[6,27] Apparently the high nucleophilicity of the thiolate, more so than specific binding interactions of the acceptor, is the major determinant for formation of the thioglycosides in these thioligations. Inspection of *in-silico* models confirms that, as expected, mutation of glutamate 314 to alanine (in SpHex) results in a significant increase in space within the +1 subsite (Figure 1). In the wt enzyme, the glutamate 314 sidechain interacts with the glycosidic oxygen (2.5 Å H-bond). Replacing the glutamate by alanine widens the binding pocket by 3.5 Å, presumably making steric hindrance much less of an issue and enabling nucleophilic attack by a wide array of thiol acceptors. Thioglycoligases formed by mutation of the catalytic acid/base residue of GHs do not normally act as O-glycoligases since there is no base present to help deprotonate the otherwise poorly nucleophilic sugar hydroxyl (pKa ~14-20) on the acceptor.[28] However, thioglycoligases can catalyze the formation of O-linked glycosides if acceptors with more acidic hydroxyl groups (e.g., phenols) are utilized, as previously reported for α-thioglycoligases.[11] The ability of SpHex E314A to catalyze the synthesis of O-aryl glycosides was investigated using a variety of substituted phenols covering a pKa range of 4.1-10 (Scheme 3). Reactions were performed at pH 8.0 using GlcNAc oxazoline as donor; TLC analysis revealed O-aryl glycoside formation for all acceptor candidates within short reaction times (< 1 h). The absence of any significant difference in the synthesis efficiency between the phenols suggested that neither the pKa value nor the substitution patterns influenced the reaction significantly. By contrast, steric hindrance was a previously reported issue in the use of ortho- substituted phenols by an α-xylosidase thioglycoligase in O-aryl glycoside synthesis[28]; our results suggest that no such steric issues occur in the +1 subsite of SpHex E314A.

Unsurprisingly, after further incubation overnight, the more activated O-aryl glycosides formed (2,4-DNP and 4-NP), which do not require acid catalysis for cleavage, were hydrolysed (Figure S5). No such hydrolysis of the other glycosides (2-chlorophenol, pKa 8.5; phenol, pKa 10.0) was seen, indicating that a phenol pKa of less than approximately 8 is required for efficient hydrolysis by SpHex E314A. The effect of the pH on the rate of the synthetic reaction was measured using GlcNAc-oxa as donor and 4-NP as acceptor. Only a narrow pH range (7-9) could be studied due to the instability of GlcNAc-oxa at a pH < 7 and the previously determined complete loss of enzyme activity at pH 10. The ligase activity dropped only gradually from pH 7>8>9, presumably due to a decreasing ability to activate the oxazoline by protonation.

Having shown that *Sp*Hex E314A has relatively broad acceptor specificity, we decided to try other thiols with biological significance, such as cysteine; thioglycosides of cysteine would be stable analogs of O-GlcNAc post-translationally modified serines and threonines. When 4-NP-GlcNAc donor was mixed with cysteine ethyl ester (Scheme 4) as acceptor in the presence of SpHex E314A, thioligation proceeded in a manner similar to that observed with the sugar acceptors and resulted in the GlcNAc-cysteine conjugate whose structure was confirmed by NMR (SI 1.5 NMR Data, Figure S6). Inspection of the *in-silico* model structure of SpHex E314A suggested that the active site

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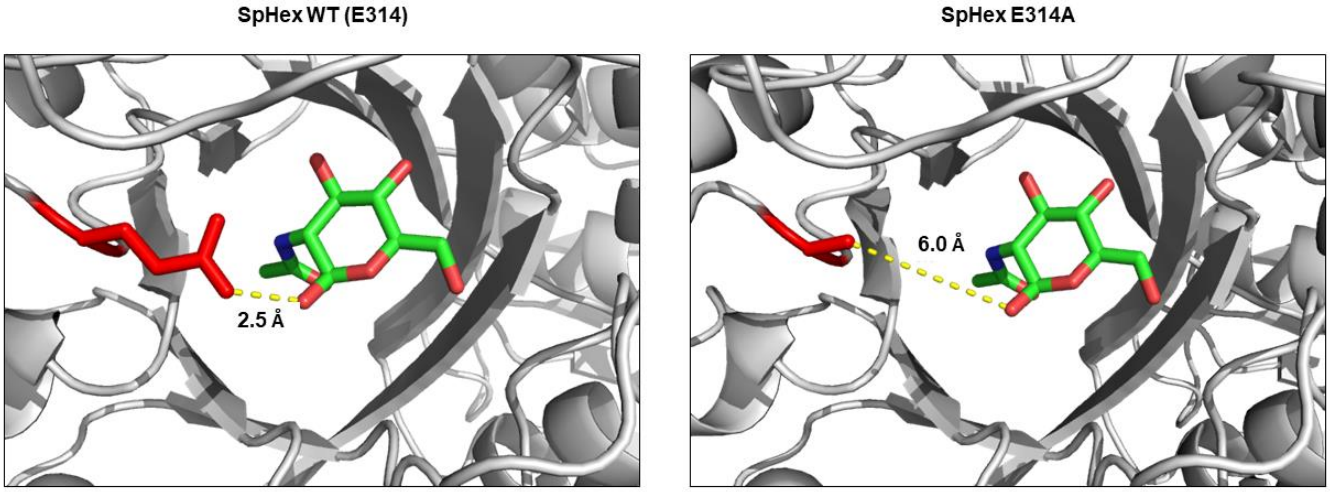
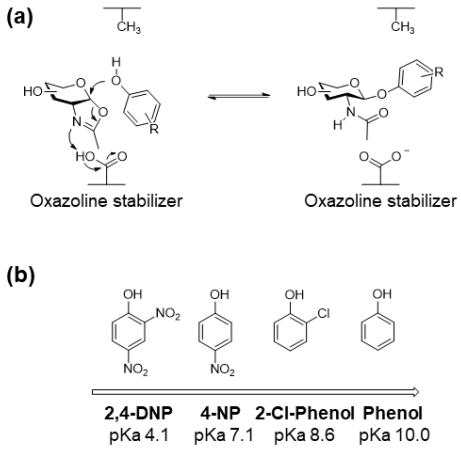


Figure 1. The *(in-silico)* mutation of glutamate 314 to alanine (right) in SpHex opens up space in the +1 subsite, compared to the wiltype (left)

pocket is oriented in a manner that would accommodate peptide chains attached to the cysteine.

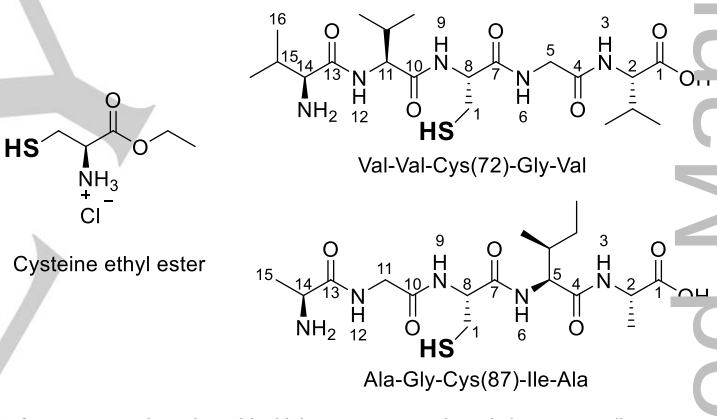
Consequently, we tested the S-GlcNAcylation ability of SpHex E314A on two model peptides derived from synuclein. The aggregation of α-synuclein, a protein found in presynaptic neurons, is associated with Parkinson's disease; interestingly, O-GlcNAcylation of α-synuclein has an inhibitory effect on this toxic aggregation.[29]



Scheme 3. (a) Mechanism of aryl glycoside synthesis by SpHex E314A and (b) Substituted phenols used for aryl glycoside synthesis by SpHex E314A.

Pentapeptides were synthesized corresponding to residues 70-74 and 85-89 of α-synuclein, in which threonine 72 and serine 87 had been replaced by cysteine (Scheme 4). MALDI-TOF (Figure 2a) and HPLC analysis (Figure S9) indicated GlcNAcylation of the cysteine residue after short incubation times for both pentapeptides; control reactions using the native pentapeptides (Thr/Ser) in the presence of SpHex E314A did not result in GlcNAcylated reaction products. Product yields of 44% for VVC(-GlcNAc)GV and 30% for AGC(-GlcNAc)IA were obtained, as determined by HPLC.

To our knowledge, this is the first report of the direct enzymatic S-GlcNAcylation of a peptide using an engineered glycoside hydrolase. However enzymatic S-GlcNAcylation of peptides had been previously demonstrated using native O-GlcNAc transferase (OGT).[33]



Scheme 4. Investigated peptide thiol acceptors; cysteine ethyl ester as well as synuclein model peptides containing a known GlcNAcylation site (72 and 87) which was replaced by a cysteine.

The natural extension of this peptide work was to investigate whether SpHex E314A could be used to modify a cysteine in a folded protein. Tau is a microtubule-associated protein suggested to be a key player in Alzheimer´s disease (AD) pathogenesis. O-GlcNAcylation of Tau commonly prevents phosphorylation of the glycosylation site that was found to be the key factor of Tau pathogenesis.[30] Tau naturally contains two cysteine residues (Cys301 and Cys322). In the version we employed, Tau(244-441), those two Cys residues had been replaced by Ser. We further replaced the critical O-GlcNAcylation site, Ser 400, with a cysteine designating the triply modified protein as (Tau S400C). After 3 h incubation of Tau S400C (mass = 23,377) with pNP-GlcNAc and SpHex E314A, ESI-TOF MS analysis of the protein showed the formation of a GlcNAcylated version (mass = 23,580), heavier by the expected 203 mass units (Figure 2b). GlcNAcylation of the thiol of Cys 400 was further confirmed by thiol titration using

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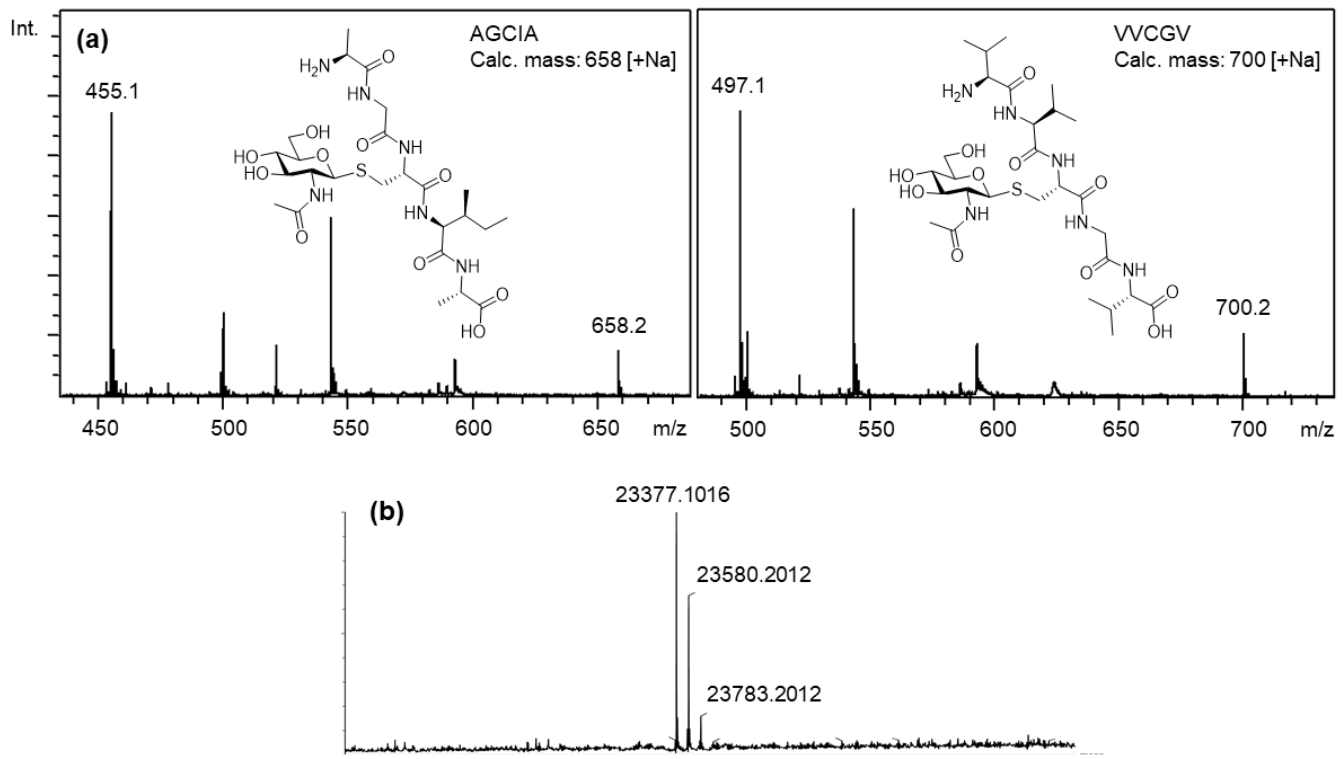

DTNB, revealing 44% modification of the thiol (Figure S8), in agreement with the ESI-TOF data shown in Figure 2b.

Figure 2. (a) S-GlcNAcylation of α-synuclein model peptides representing cysteine mutations of the GlcNAcyation sites 72 (AGCIA) and 87 (VVCGV); (b) Whole protein ESI-TOF spectrum of TauS400C that has been S-GlcNAcylated by SpHex E314A. Mass of TauS400C = 23377.10, GlcNAcylated TauS400C = 23580.20 and double GlcNAcylated TauS400C = 23783.

This yield can likely be improved by using more appropriate hexosaminidases.

A small amount of a double GlcNAcylated product (+406) was also detected which might be caused by non-enzymatic glycation, a process previously observed by Parsons et al. in performing transglycosylations of N-linked glycans using a large excess of oxazoline donor substrates and a mutant Endo-S.[31] To test this possibility, blank reactions were performed using different donor substrates (GlcNAc, pNP-GlcNAc or GlcNAc oxazoline) in the absence of the thioglycoligase. ESI-TOF analysis indicated traces of glycation product when using GlcNAc oxazoline as donor, while no reaction product was observed with just GlcNAc or pNP-GlcNAc (Figure S7). The non-enzymatic formation of glycation product in the presence of GlcNAc oxazoline concurs with the observations of Parsons et al.;[31] our observation that pNP-GlcNAc produces small amounts of similar non-enzymatic glycation, but only in the presence of SpHex E314A, seems to suggest that GlcNAc oxazoline may be liberated during incubation of pNP-GlcNAc with SpHex E314A, although efforts at detecting released GlcNAc oxazoline under these conditions were not successful.

In conclusion, the strategy of producing thioglycoligases by removing the catalytic acid/base sidechain in a glycoside hydrolase has been extended to a new mechanistic class of glycoside hydrolases (GH20 hexosaminidase) that utilizes neighboring group participation in the initial cleavage step. The mutant enzyme, SpHex E314A, catalyzes the attachment of terminal GlcNAc or GalNAc residues onto a range of thiol acceptors, including peptides and proteins, through β linked-S-linkages. These thioglycosides are stable mimics of biologically important O-GlcNAc and O-GalNAc oligosaccharides and glycoconjugates. State of the art approaches such as the semisynthesis of O/S-GlcNAcylated proteins have required multiple preparation steps, including chemical synthesis.[17,32] In contrast, the direct enzymatic GlcNAcylation of proteins in a single step embodies a greatly simplified concept, providing a fast and easy-to-handle tool for protein S-GlcNAcylation. It is noteworthy that this thioglycoligase utilizes an inexpensive, commercially available glycosyl donor (pNP-GlcNAc) which greatly reduces the workload and increases the practicability of this S-GlcNAcylation system. Whether this strategy can be applied to proteins displaying more than one cysteine is the subject of ongoing studies.

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Keywords: Glycoprotein synthesis • Glycosynthase • Thioglycoligase • Thioglycosides • Hexosaminidase

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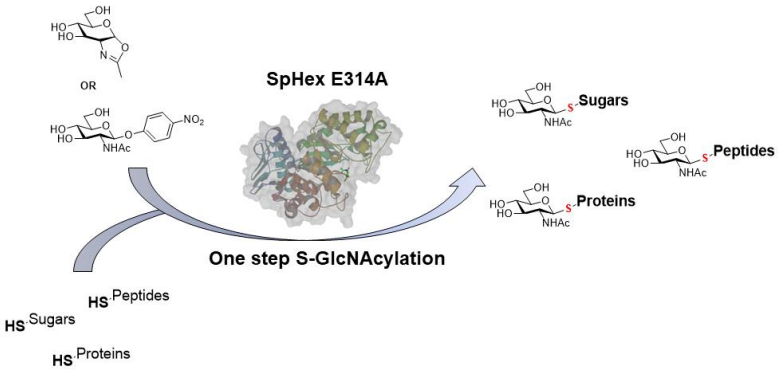

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Title

Thioglycosides are stable carbohydrate mimetics frequently used for structural and functional biology studies. A thioglycoligase was developed from a GH 20 hexosaminidase enabling direct S-GlcNAcylation of a diverse set of thio-acceptors. Thio-sugars of various sugar families were GlcNAcylated in quantitative yields and the acceptor pool was successfully expanded to peptides and proteins.

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