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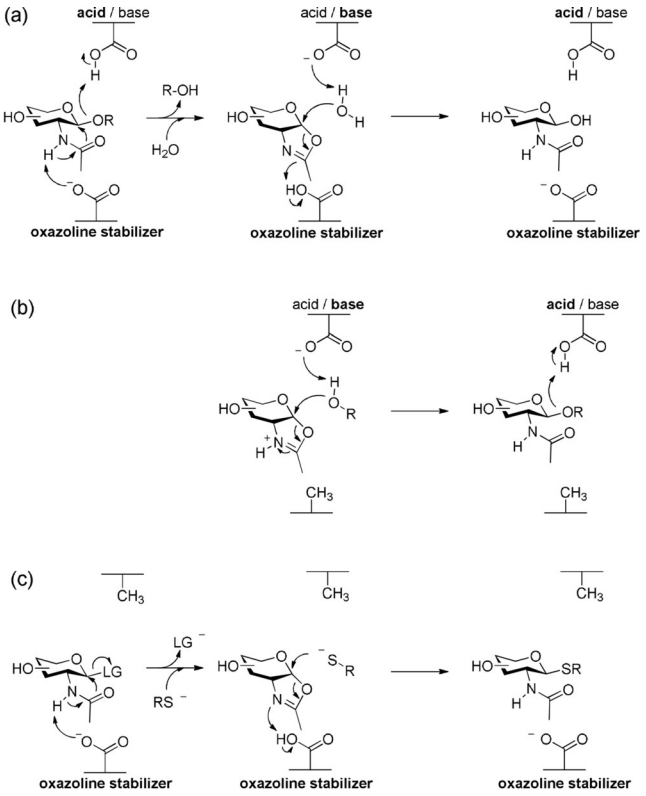
Facile Formation of β-thioGlcNAc Linkages to Thiol-Containing Sugars, Peptides, and Proteins using a Mutant GH20 Hexosaminidase

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

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. The development of an efficient, enzyme-mediated synthesis of thioglycosides, including S-GlcNAcylated proteins, is reported, using a thioglycoligase derived from a GH20 hexosaminidase from Streptomyces plicatus in which the catalytic acid/base glutamate has been mutated to an alanine (SpHex E314A). This robust, easily-prepared, engineered enzyme uses 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 analogues 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 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 S1 a).[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 S1 b).[4,5,32]



Scheme 1. a) Wild-type neighboring-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 (LG; alternatively the oxazoline may be used as donor).

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

[*] Dr. G. Tegl, Dr. H.-M. Chen, Prof. D. H Kwan, Dr. A. G. Santana, Prof. S. G. Withers
Department of Chemistry, University of British Columbia
Vancouver, British Columbia, V6T 1Z1 (Canada)
E-mail: withers@chem.ubc.ca
Prof. J. Hanson
Department of Chemistry, University of Puget Sound
Tacoma, WA 98416 (USA)
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201809928.

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 S1 c);[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. However, when using a nucleophile with a low pK_a, 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 β-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 analogues. For example, O-GlcNAcylation 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 have been 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 GH 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 1 a).[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 thioglycoligases 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 1 a).[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 1 b).[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 the replacement of E314 by an alanine would produce an enzyme capable of catalyzing the thioligation of activated GlcNAc donors to appropriate thiol acceptors (Scheme 1 c).

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 when using pNP–GlcNAc as substrate (Table 1).

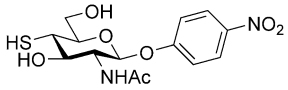
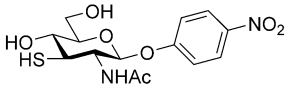
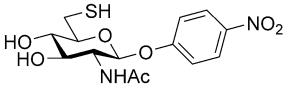
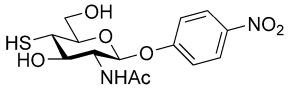
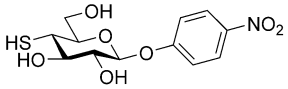
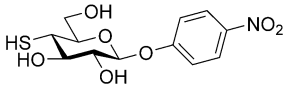
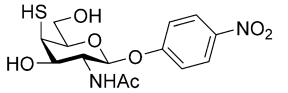
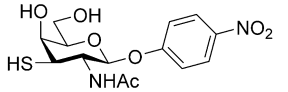
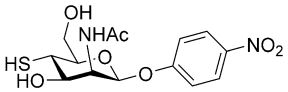
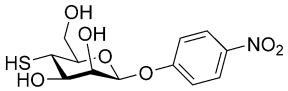
Table 1: Kinetic parameters for SpHex and its mutants at pH 5 and 7 (K_M in μM, k_{cat} in s^{-1}, and k_{cat}/K_M in $s^{-1}\mu M^{-1}$); n.d.: not determined.

	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

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 (Supporting Information, Figure S1). pNP–GalNAc was used as substrate by the mutant with rate constants approximately three-fold lower than the wild type values. 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^{-1}) (Supporting Information, Figures S2 and S3; Table 2); the formation of the corresponding thioglycosides was monitored by TLC and ESI-MS analysis. Ligation to the

Table 2: SpHex-E314A-catalyzed thioligation using thiosugar acceptors. Reactions using pNP–GlcNAc or pNP–GalNAc as donors (entries 1–4) were performed on a preparative scale; products were purified by HPLC and characterized by NMR spectroscopy. Yields were essentially stoichiometric with respect to acceptor. Reactions using GlcNAc-oxazoline as a donor (entries 1–3, 5–10) 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

thiol residue, rather than to sugar hydroxyls, was demonstrated by TLC using a DTNB stain that reacts with free thiols;[24] the products did not react with this stain (Supporting Information, Figure S4). Since wildtype SpHex cleaves β-1,4-linked glycosides of GlcNAc, the 4-thio analogue of pNP–GlcNAc was initially tested as an acceptor.[24] Using excess oxazoline donor, quantitative formation of the desired disaccharide containing the thioglycosidic linkage was observed. Encouraged by this result, the 3-thio- and 6-thio-pNP–GlcNAc analogues were also tested as acceptors and rapid conversion to the corresponding disaccharides was observed (Supporting Information, 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 (Supporting Information, 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 wild type or SpHex D313A.

Although the GlcNAc-oxazoline donor could be synthesized from GlcNAc,[26] 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). It was also shown that pNP–GalNAc is an effective donor substrate, as demonstrated by the synthesis of β-GalNAc-1,4-S-GlcNAc-pNP in 98% yield of isolated product (see the NMR data in section 1.4 in the Results and Discussion in the Supporting Information). 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 (see the NMR data in sections 1.1–1.4 in the Results and Discussion in the Supporting Information). Interestingly self-condensation of the thio-pNP–GlcNAc sugars listed in Table 2 was not observed, presumably because the bulkier thiosugar is not accepted in the donor (−1) subsite.

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, the E314A mutation (in SpHex) results in a significant increase in space within the +1 subsite (Figure 1). In the wild type enzyme, the E314 sidechain interacts with the glycosidic oxygen (2.5 Å H-bond). The E314A mutation 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 the 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 ($pK_a \approx 14$–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 pK_a range of 4.1–10 (Scheme 2).

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

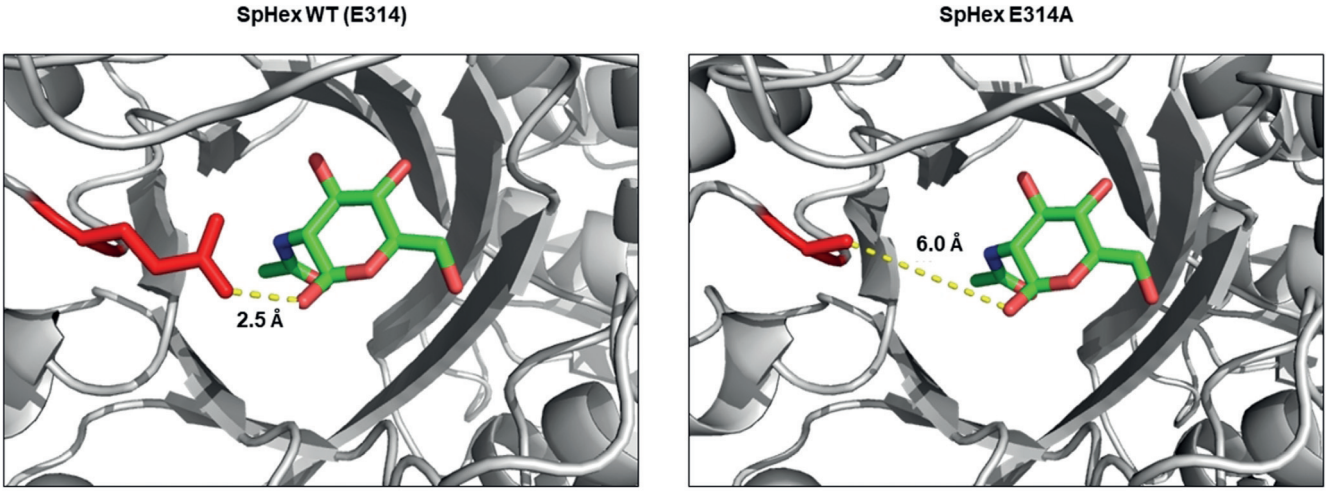
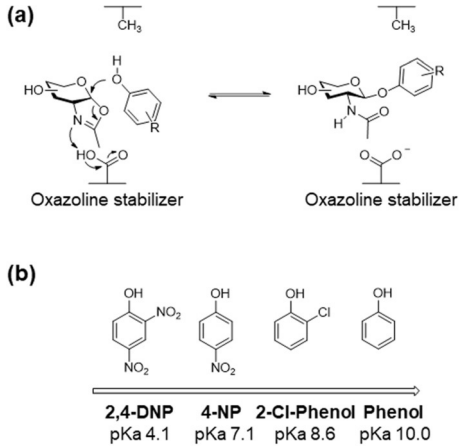


Figure 1. The in silico mutation of glutamate 314 to alanine (right) in SpHex opens up space in the +1 subsite compared to the wild type (left).

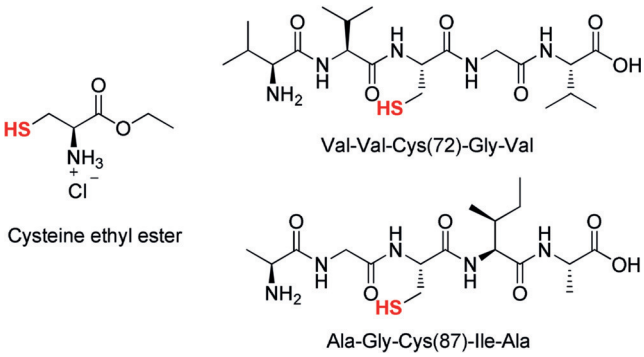


Scheme 2. a) Mechanism of O-aryl glycoside synthesis by SpHex E314A; b) Substituted phenols used for O-aryl glycoside synthesis by SpHex E314A.

neither the pK_a value nor the substitution patterns significantly influenced the reaction. In 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 (2,4-dinitrophenol and 4-nitrophenol; 2,4-DNP and 4-NP), which do not require acid catalysis for cleavage, were hydrolysed (Supporting Information, Figure S5). No such hydrolysis of the other glycosides (2-chlorophenol, pK_a 8.5; phenol, pK_a 10.0) was seen, indicating that a phenol pK_a 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-oxazoline as a donor and 4-NP as an acceptor. Only a narrow pH range (7–9) could be studied due to the instability of GlcNAc-oxazoline at a pH <7 and the previously determined complete loss of enzyme activity at pH 10. The ligase activity dropped only gradually as the pH was increased from 7 to 9, presumably due to a decreasing ability to activate the oxazoline by protonation.

Having shown that SpHex E314A has relatively broad acceptor specificity, other thiols with biological significance were tested, such as cysteine; thioglycosides of cysteine would be stable analogues of O-GlcNAcylated serines and threonines. When the 4-NP–GlcNAc donor was mixed with cysteine ethyl ester (Scheme 3) as acceptor, in the presence



Scheme 3. Investigated peptide thiol acceptors; cysteine ethyl ester as well as synuclein model peptides containing a known O-GlcNAcylation site (72 and 87) that was replaced by a cysteine.

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 (Figure S6 and NMR Data in section 1.5 of the Results and Discussion in the Supporting Information). Inspection of the in silico model structure of SpHex E314A suggested that the active site pocket is oriented in a manner that would accommodate peptide chains attached to the cysteine.

Consequently, the S-GlcNAcylation ability of SpHex E314A was tested 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]

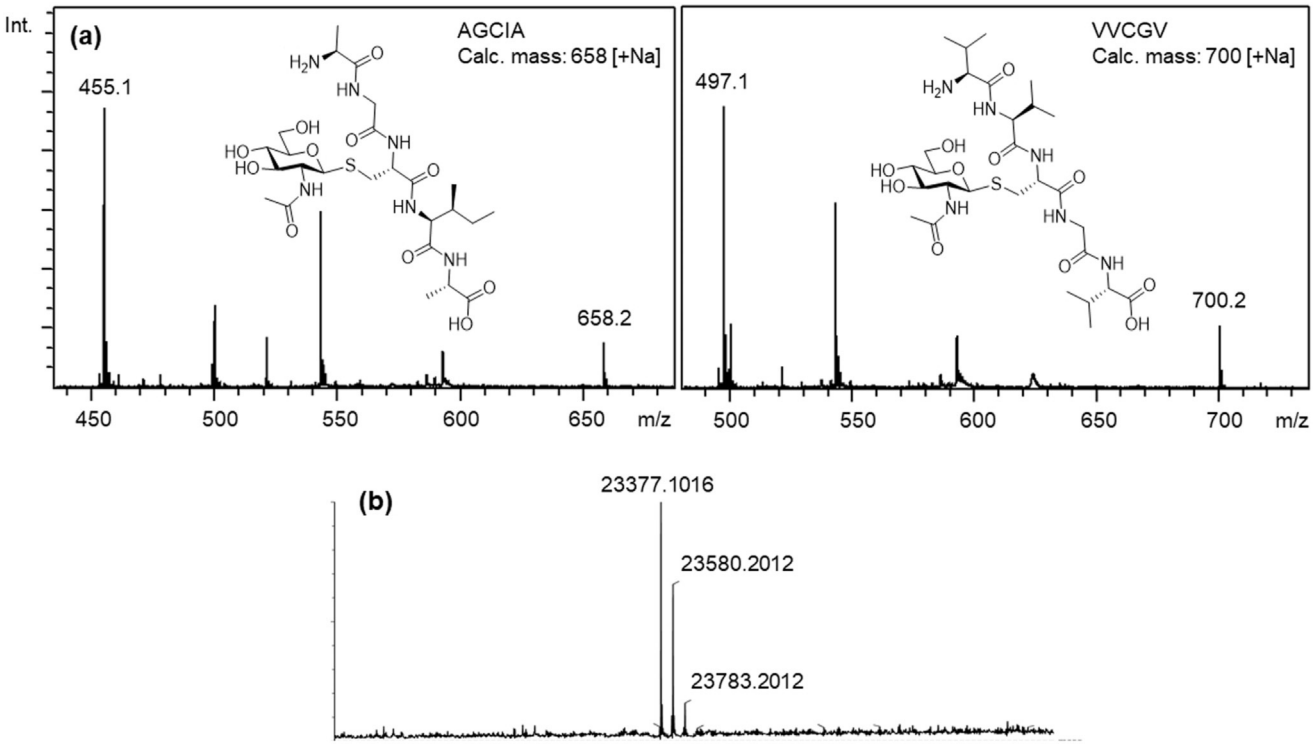


Figure 2. (a) S-GlcNAcylation of α-synuclein model peptides representing cysteine mutations of the GlcNAcyation sites 72 (AGCIA) and 87 (VVCGV); (b) Whole protein ESI-TOF MS spectrum of TauS400C that has been S-GlcNAcylated by SpHex E314A. Mass of TauS400C = 23377.10, S-GlcNAcylated TauS400C = 23580.20,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

Pentapeptides were synthesized corresponding to residues 70–74 and 85–89 of α-synuclein, in which threonine 72 and serine 87 were replaced by cysteine (Scheme 3). MALDI-TOF (Figure 2a) and HPLC analysis (Supporting Information, Figure S9) indicated that GlcNAcylation of the cysteine residue occurred 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 a native O-GlcNAc transferase (OGT).[33]

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 construct used here, Tau(244–441), those two Cys residues had been replaced by Ser. We further replaced the critical O-GlcNAcylation site, Ser400, 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 product (mass = 23,580), heavier by the expected 203 mass units (Figure 2b). GlcNAcylation of the thiol of Cys400 was further confirmed by thiol titration using DTNB, revealing 44% modification of the thiol (Supporting Information, Figure S8), in agreement with the ESI-TOF MS data shown in Figure 2b. This yield can likely be improved by using more appropriate hexosaminidases.

A small amount of a double GlcNAcylated product (+406) was also detected that might result from non-enzymatic glycation, a process previously observed by Parsons et al. when performing transglycosylation 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 MS analysis indicated the presence of traces of glycation product when using GlcNAc-oxazoline as donor, while no reaction product was observed with just GlcNAc or pNP–GlcNAc (Supporting Information, 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 the 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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Conflict of interest

The authors declare no conflict of interest.

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