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The α-Thioglycoligase Derived from a GH89 α-*N*-Acetylglucosaminidase Synthesises α-*N*-Acetylglucosamine-Based Glycosides of Biomedical Interest

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Abstract: We report here on the preparation of a novel α-thioglycoligase that can be used for the fast and efficient synthesis of α-*N*-acetylglucosamine-based glycosides. Using the α-*N*-acetyl-glucosaminidase from *Clostridium perfringens* of family GH89 (according to the Carbohydrate Active Enzymes classification) as starting point, we prepared mutants in the acid/base residue glutamic acid 483 that were found to have different synthetic efficiencies (maximal yields >80% after 24 h) in the presence of an activated donor and suitable acceptors. The synthetic potential of the Glu483 alanine mutant as an α-thioglycoligase – only the third biocatalyst with this stereospecificity reported to date, and the first selective for the *N*-acetylglucosamine moiety – was demonstrated by producing for the first time *N*-acetyl-α-D-glucosaminyl azide and *N*-acetylglucosamine α-thioglycosides in high yields. To showcase the application of such compounds, we show that they offer the wild-type CpGH89 protection from thermal unfolding, demonstrating their potential as pharmacological chaperones for the treatment of mucopolysaccharidosis IIIB (Sanfilippo syndrome).

Keywords: carbohydrate active enzymes; chemo-enzymatic synthesis; glycoside hydrolases; pharmacological chaperones; reaction mechanism of glycoside hydrolases

Introduction

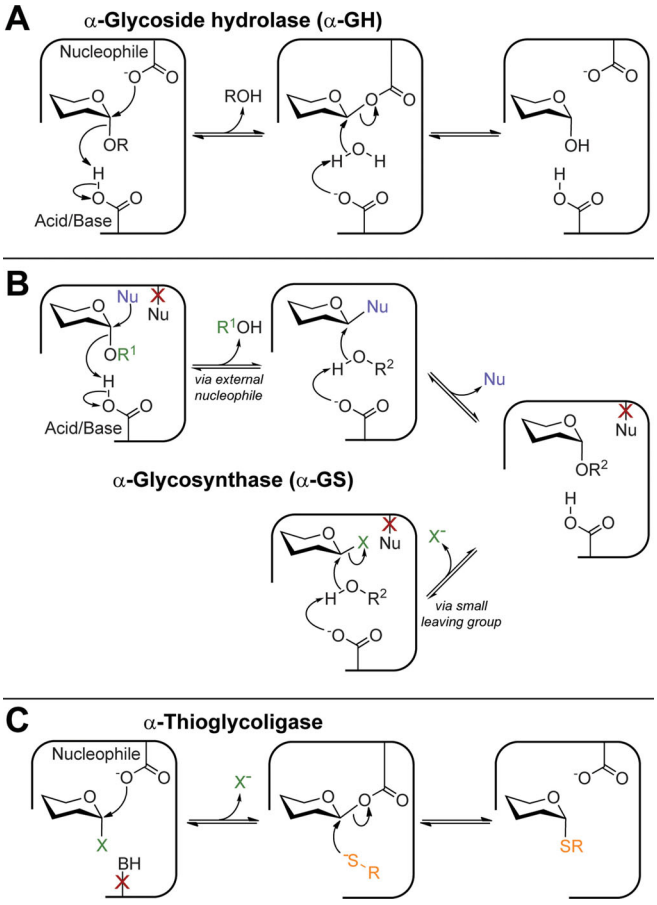
The inhibition of glycoside hydrolases (GHs) is a proven strategy for the study of these interesting enzymes, the discovery of GH-binding molecules, and treatment or study of a variety of metabolic, genetic, and infective diseases.[1] For example, the drugs miglitol and voglibose are used to treat type 2 diabetes based on their competitive inhibition of the α-glucosidase enzyme responsible for the breakdown of starch and smaller oligosaccharides to glucose in the small intestine.[2] However, GH inhibitors can also play a positive role in pharmacological chaperone therapy (PCT) used in lysosomal storage disorders (LSDs) alone or in combination with the enzyme replacement therapy (ERT), helping to maintain the target enzyme in its folded, active form.[3]

The use of GH inhibitors as pharmacological chaperones has become the main treatment option for LSDs where ERT is not possible. A case in point is mucopolysaccharidosis (MPS), a very serious autosomal recessive lysosomal storage disorder caused by the impaired function of enzymes involved in the degradation of heparan sulphate, leading to accumulation

of undegraded glycosaminoglycans in lysosomes. In particular, defective mutations of the human gene encoding for an α-*N*-acetyl-glucosaminidase (NAGLU) causes MPS IIIB, or Sanfilippo syndrome, a tragic rare disease leading to severe neurological disorders.[4] There are no current treatments for MPS IIIB, as gene therapy is in its infancy, and ERT cannot be adopted because the pharmacological enzyme cannot cross the blood–brain barrier to replace the defective enzyme in the nervous system. However, small-molecule chaperones can interact with mutant enzymes to favour their correct conformation and enhance their stability.[5] In this manner pharmacological chaperone therapy (PCT) becomes a viable alternative to ERT in cases where suitable molecules with high affinity for the target enzyme can be identified.

Whether for use as inhibitors or in PCT, the ability of small molecules to selectively bind to their respective targets in the presence of other enzymes with similar substrates would be highly beneficial from a therapeutic perspective. One strategy whereby this can be achieved is by using compounds that have the same anomeric configuration as the natural substrates of the targeted enzymes. However, in the case of α-GHs, this is not easily accomplished. Remarkably, the chemical synthesis of α-linked glycosides of especially *N*-acetyl-glucosamine (GlcNAc) is generally less tractable than that of their β-counterparts. This has severely hampered the discovery and the production in good yields of inhibitors that are selective for α-*N*-acetyl-glucosaminidases, and that can serve as leads for the development of molecules with the potential for use in a clinical setting. Consequently, those compounds that have been shown to inhibit α-*N*-acetyl-glucosaminidase enzymes are in fact repurposed inhibitors of β-*N*-acetyl-glucosaminidases, and do not show selectivity at the anomeric position.[6] Since such a lack of specificity is an undesirable pharmacological feature, the search for α-specific *N*-acetyl-glucosaminidase inhibitors continues. In this regard the discovery of more efficient methods for the reliable synthesis of α-linked glycosides would be extremely useful, and would enable the production of new compounds for testing in such a context.

To address some of the constraints associated with the chemical synthesis of oligosaccharides, such as protection/deprotection strategies and selective activation of the anomeric centre, the use of engineered *retaining* GH enzymes (referring to the anomeric configuration of the product relative to the substrate) was pioneered.[7] This is achieved by mutating either the active site nucleophile or acid/base residues that are required for normal GH catalysis (Scheme 1A) to non-nucleophilic residues, thereby removing the enzymes' ability to hydrolyse glycosidic bonds. To convert the mutant enzymes into biocatalysts, two approaches are followed: in the first, which leads to glycosynthases (GSs), mutation of the active site nucleophile is countered by the use of an activated donor substrate with an appropriate leaving group at the anomeric position (Scheme 1B). If the leaving group is in the same anomeric configuration as the substrate



Scheme 1. ***Mechanisms of α-glycoside hydrolase enzymes and of the biocatalysts that are derived from them.*** **A**: Mechanism of a retaining α-GH showing the active site residues acting as nucleophile and acid/base respectively. R can be a sugar or an alcohol. **B**: Mechanism of an α-glycosynthase (α-GS) derived from the α-GH shown in panel **A** after exchange of its active site nucleophile for a non-nucleophilic residue. Such enzymes can use two types of substrates: if the leaving group is in the α-configuration (–OR^1 in green; with R^1 usually 2-, 4-nitrophenyl, or 2,4-dinitrophenyl), a small external nucleophile (Nu in blue) such as azide or formate is added to allow the reaction with an acceptor (R^2 can be a sugar or an alcohol) to take place, while substrates with small leaving groups (green X = fluoride or azide) in the β-configuration can react directly. In both cases the product is an α-glycoside. **C**: Mechanism of an α-thioglycoligase (α-TGL) derived from the α-GH shown in panel **A** by exchange of its active site acid/base residue for a non-protic/non-nucleophilic residue. In this case the leaving group (X in green) can be 2-, 4-nitrophenyl, 2,4-dinitrophenyl, fluoride or azide, and a thiolate (in orange) is used as acceptor, which acts as nucleophile to form an α-thioglycoside product.

of the wild-type enzyme an external nucleophile such as sodium azide or sodium formate is added to chemically rescue the loss of the active site residue.[8] With some small leaving groups (e.g., fluoride and azide) in a configuration opposite to that of the substrate, chemical rescue is not required (for reviews see ref.[9]). In either case, the product formed accumulates due to the impaired hydrolytic activity of the mutated GH enzyme. In the second approach, which was developed specifically to produce non-hydrolysable thioglycosides, the exchange of the acid/base residue of GHs is again countered by the use of an activated donor with the same anomeric configuration as the substrate but, in this instance, also by an acceptor containing a thiol serving as nucleophile (Scheme 1C). The thioglycoside product accumulates in the reaction, as it is resistant toward enzymatic hydrolysis.[10] Such enzymes are therefore dubbed thioglycoligases (TGL), and their thioglycoside products usually serve as GH inhibitors.

Despite the potency of these approaches – which recently led to an engineered β-hexosaminidase with improved transglycosidase activity[11] – so far α-GSs are limited to α-fucosynthases [from families GH29 and GH95 according to the classification of carbohydrate active enzymes (CAZy, www.cazy.org[12]], α-glucosynthases (GH31), and α-galactosynthases (GH36).[8a,9a,13] In striking contrast, thioglycoligases are even less common. To the best of our knowledge, only two α-TGLs have been produced so far, a thioglucoligase and a thioxyloligase, both belonging to family GH31.[14]

Considering the need for selective inhibitors and molecular chaperones of the medicinally relevant α-*N*-acetyl-glucosaminidase enzymes, we decided to embark on the modification of the catalytic residues of an α-*N*-acetyl-glucosaminidase to produce a novel α-GS and α-TGL able to synthesise α-*N*-acetyl-glucosaminide-containing glycosides. In CAZy, α-*N*-acetyl-glucosaminidases (EC 3.2.1.50) are reported only in family GH89,[12] in which the enzyme from the bacterium *Clostridium perfringens* (CpGH89) has been identified as a useful model system of the human NAGLU. Studies on the 3D-structure of CpGH89 demonstrated that the enzymes belonging to this family followed the classical *retaining* reaction mechanism as opposed to β-hexosaminidases from families GH18, 20, 25, 56, 84, and 85, which hydrolyse substrates containing an *N*-acetyl (acetamido) or *N*-glycolyl group at the 2-position acting as nucleophile and forming an oxazolinium ion intermediate.[15] In addition, mutations giving rise to MPS IIIB were also identified from a homology model of NAGLU.[6,16] We show here that CpGH89 mutants in the nucleophile of the reaction were recalcitrant to act as α-glycosynthases, but that modification of the acid/base with non-nucleophilic amino acids led to efficient α-thioglycoligases capable of producing glycoside analogues when used with suitable acceptors. The potential of such compounds as pharmacological chaperones was subsequently explored by determining the effects of these novel products on parental CpGH89. Taken together, these observations demonstrated that the first α-TGL is capable of efficiently producing α-*N*-acetyl-glucosaminide-containing thioglycosides and *N*-acetyl-α-D-glucosaminyl azide (α-GlcNAc-N_3), a compound that is not commercially available, yet holds promise for the use in bio-orthogonal click chemistry reactions. Such compounds can find broad application in biomedicine for the synthesis of inhibitors and lead compounds of α-GlcNAc-active enzymes.

Results and Discussion

Preparation of α-*N*-Acetyl-glucosaminosynthases

CpGH89 was selected for investigation as potential chassis to produce mutant enzymes able to synthesise *N*-acetyl-α-D-glucosaminide (α-GlcNAc) derived products. The gene encoding for CpGH89 was produced as a truncated version of the wild type, which comprised residues 26–916 as reported previously.[6] The enzyme purified to homogeneity by a single-step purification by immobilised metal affinity chromatography showed the steady state kinetic constants listed in Table 1. Previous studies demonstrated that the enzyme followed a double-displacement retaining mechanism and that the residues glutamic acid 601 (Glu601) and 483 (Glu483) were the nucleophile and the acid/base of the reaction, respectively, with no evidence of the participation in catalysis of the *N*-acetyl group of the substrate (Figure 1A and B).[6] To test if CpGH89 could be converted into an α-GS by following the approach that was demonstrated to be successful for GHs from several families[7] the nucleophile Glu601 was exchanged for non-nucleophilic residues producing the alanine, glycine, and serine mutants (E601A/G/S). These mutant enzymes had no detectable activity on 2-nitrophenyl-*N*-acetyl-α-D-glucosaminide (2NP-α-GlcNAc) and 2,4-dinitrophenyl-*N*-acetyl-α-D-glucosaminide (2,4DNP-α-GlcNAc) as donor substrates. This is in agreement with previous studies on CpGH89[6] and with the 10^3-fold reduction of specific activity observed in other *retaining* glycoside hydrolases mutated in the nucleophile of the reaction.[17] However, remarkably, attempts to chemically rescue the enzymatic activity of the mutants acting on 2- and 2,4DNP-α-GlcNAc at 25 and 37 °C by using high concentrations (up to 1 M) of external nucleophiles (sodium azide, sodium formate, sodium chloride and sodium fluoride) were all unsuccessful and remained so even with the use of excess of enzyme, different reaction temperatures, buffer/pH

Table 1. Steady-state kinetic parameters of wild type CpGH89 and its Glu483 mutants.

CpGH89 mutant (*substrate*)	K_M [μM]		k_{cat} [s^{-1}]		k_{cat}/K_M [$s^{-1}mM^{-1}$]	
2NP-α-GlcNAc	*25 °C*	*37 °C*	*25 °C*	*37 °C*	*25 °C*	*37 °C*
wild type	416 ± 56	190 ± 10	2.31 ± 0.11	23 ± 0.3	5.6	121
E483Q	11 ± 5	10 ± 1	0.033 ± 0.001	0.029 ± 0.001	3.1	2.90
E483A	46 ± 5	50 ± 3	0.076 ± 0.001	0.051 ± 0.001	1.7	1.02
E483S	34 ± 5	62 ± 4	0.064 ± 0.014	0.093 ± 0.002	1.9	1.49
E483G	79 ± 14	68 ± 5	0.022 ± 0.001	0.046 ± 0.001	0.3	0.67
4NP-α-GlcNAc						
wild type	1049 ± 79	1800 ± 300	0.17 ± 0.01	0.35 ± 0.01	0.16	0.19
E483Q	237 ± 19	200 ± 20	0.0062 ± 0.0001	0.0065 ± 0.0001	0.026	0.028
E483A	459 ± 43	800 ± 40	0.0130 ± 0.0004	0.0146 ± 0.0002	0.028	0.017
E483S	443 ± 25	700 ± 70	0.0158 ± 0.0004	0.0240 ± 0.0078	0.036	0.035
E483G	820 ± 220	600 ± 70	0.0027 ± 0.0002	0.0482 ± 0.0163	0.003	0.080
2,4DNP-α-GlcNAc						
wild type	3.2 ± 0.3	6.2 ± 0.6	5.5 ± 0.1	9.9 ± 0.2	1718	1596
E483Q	nd[a]	nd	nd	nd	nd	nd
E483A	nd	nd	nd	nd	nd	nd
E483S	nd	nd	nd	nd	nd	nd
E483G	nd	nd	nd	nd	nd	nd
2,4DNP-α-GlcNAc + 250 mM NaN_3						
E483Q	2.4 ± 0.3	1.3 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	616	1289
E483A	0.7 ± 0.1	1.8 ± 0.3	1.1 ± 0.1	2.3 ± 1.2	1642	1307
E483S	1.1 ± 0.2	1.8 ± 0.2	2.1 ± 0.1	2.4 ± 0.7	2191	1326
E483G	1.7 ± 0.4	3.2 ± 0.4	1.0 ± 0.1	1.9 ± 0.9	582	591

[a] nd = not detectable.

systems, substrate acceptors, and donor/acceptor ratios (data not shown). In addition, no synthesis was observed by using *N*-acetyl-β-D-glucosaminyl azide (β-GlcNAc-N_3) as donor, in an attempt to apply the same strategy that was successful for other α-GSs (Scheme 1B).[8a,9a] We confirmed that this lack of activity was not due to changes in the stability (Figure 2A) or structure (Supporting Information, Figure S1A) of the E601A/G/S mutants. More likely, the shape of the CpGH89 active site prevents entry and/or placement of the acceptor substrates in catalytically relevant positions (Figure 1B). This renders the enzyme resistant to conversion to an α-GS, as has been reported for similar cases.[18] We therefore turned our attention to the conversion of CpGH89 into a novel α-TGL.

Preparation of α-*N*-Acetyl-thioglycosaminoligases

α-TGLs are mutants in which the general acid/base catalytic residue of the enzyme has been replaced by a non-nucleophilic amino acid.[10] The mutant, in the presence of a sugar donor with an excellent leaving group and suitable acceptors with a thiol goup, catalyses the formation of *S*-glycosidic linkages in high yields. The replacement of the general acid/base of CpGH89 with non-acidic residues (E483A/S/Q/G) produced mutants with very low enzymatic activity on 2NP-α-GlcNAc (up to 780-fold less than the wild type), on 4-nitrophenyl-*N*-acetyl-α-D-glucosaminide (4NP-α-GlcNAc, up to 70-fold less) and on 2,4DNP-α-GlcNAc (up to 30-fold less) (Table 1), confirming what was previously reported for the CpGH89–E483A mutant on 4NP-α-GlcNAc as substrate.[6] We confirmed that this lack of activity is not due to structure-based defects introduced by the mutation through circular dichroism (CD) analysis of the protein's secondary structure elements (Supporting Information, Figure S1B) which produced mutants with very low enzymatic activity on 2NP-α-GlcNAc (up to 780-fold less than the wild type), 4-nitrophenyl-*N*-acetyl-α-D-glucosaminide and by comparative analysis of their temperature melting curves (Figure 2B). No significant differences could be observed, indicating that the loss of activity can be directly attributed to the mutation.

Chemical Rescue of the Acid/Base Mutants with NaN_3

To test if the inactivation caused by mutation of the general acid/base residue could be chemically rescued, we used sodium azide (NaN_3) as external nucleophile. In an initial test, the rate of the release of 2,4-dinitrophenolate from the more reactive donor 2,4DNP-α-GlcNAc at 37 °C was measured in the presence of increasing concentrations of sodium azide as catalysed by the Glu483 mutants (Figure 3A). The re-

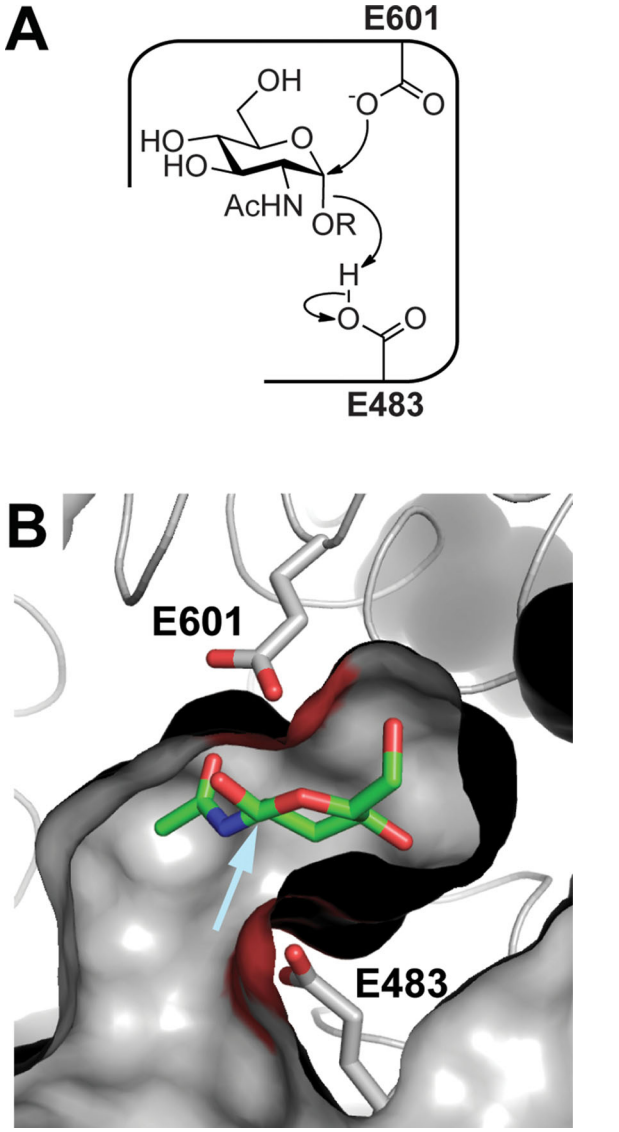


Figure 1. ***CpGH89 catalytic mechanism and active site structure.*** **A**: Mechanism of the initial hydrolytic step catalyced by CpGH89, showing the roles of the active site nucleophile (Glu601) and acid/base (Glu483) residues. **B**: Substrate binding pocket of CpGH89 shown as a section through the solvent-accessible surface with GlcNAc (stick structure with carbon atoms in green) bound. The anomeric carbon that is the point of initial attack by Glu601 (nucleophile) is indicated by an arrow. Both Glu601 and Glu483 are shown as stick structures with carbon atoms in grey.

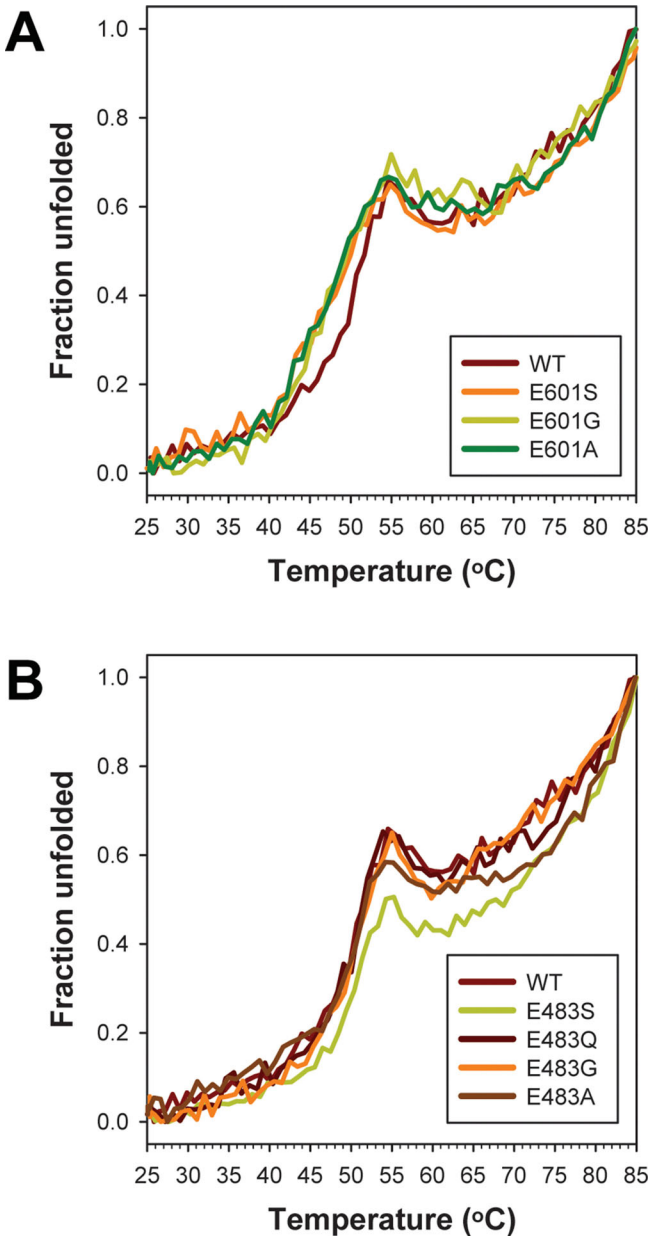


Figure 2. ***CpGH89 structure-based stability.*** **A**: Melting curves of CpGH89 and its Glu601 mutants measured by following the change in CD absorption at 220 nm by heating from 25 °C to 85 °C in intervals of 1 °C each minute. **B**: As for **A**, but for the Glu483 mutants.

sults clearly show that addition of azide restored the mutants' activity, with the CpGH89–E483A and E483S mutants showing the best performance under these conditions. This finding was confirmed by determining the steady-state kinetic constants of the mutants acting on 2,4DNP-α-GlcNAc in the presence of 250 mM sodium azide (Table 1). TLC analysis of these reactions incubated for 16 h showed that the donor was converted to a glycoside with a retention factor (R_f) very similar to that of the control β-GlcNAc-N_3, the closest commercially available analogue of the expected product α-*N*-acetyl-D-glucosamide azide (α-GlcNAc-N_3) (data not shown). These same conditions were subsequently used to determine the synthetic efficiency of all the mutants (calculated as the % products formed from the donor assuming the amount of GlcNAc available in 2,4DNP-α-GlcNAc as 100%) by analysis with high performance anionic exchange chromatography using pulsed amperometric detection (HPAEC-PAD). The efficiency was found to be remarkably high for all the mutants,

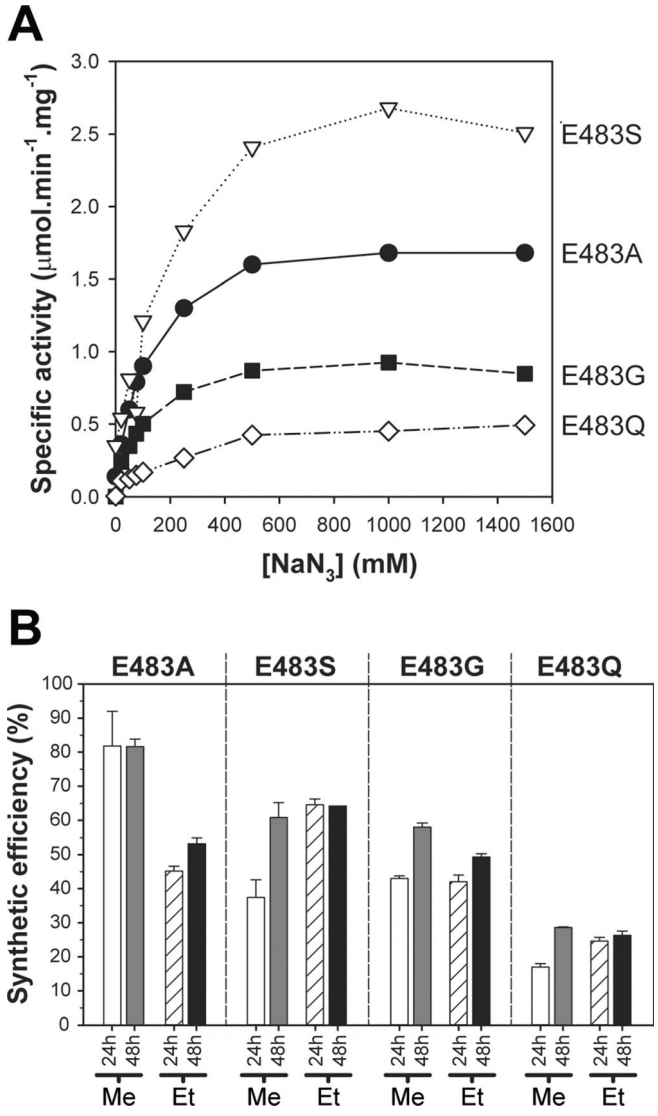


Figure 3. ***Thioglycoligase activity of CpGH89-derived acid/base mutants.*** **A**: Chemical rescue of the activity of the CpGH89–Glu483 mutants in the presence of increasing amounts of sodium azide. The activity was measured at 37 °C using 2,4DNP-α-GlcNAc as donor under standard conditions. **B**: Synthetic efficiency of the CpGH89–Glu483 mutants using 2NP-α-GlcNAc as donor and either methyl thioglycolate (Me) or ethyl thioglycolate (Et) as acceptors. The reaction mixtures were analysed by HPAEC-PAD after 24 and 48 h to quantify the amount of product formed. The synthetic efficiency of the mutants was determined assuming the amount of GlcNAc available in 2NP-α-GlcNAc as 100%. Bars indicate the average value obtained from duplicate experiments; the error bars indicate the standard deviation.

being 70.3±0.1%, 78.6±0.1%, 75.0±1.5% and 81.4±0.8% for E483A/S/Q/G, respectively, while the blank mixture, containing all the reagents but the enzyme, revealed only GlcNAc resulting from the spontaneous hydrolysis of the donor (Supporting Information, Figure S2).

These high yields prompted us to reinvestigate the activity of the mutants at either 25 or 37 °C with all three donors and either low (5 mM) or high (100 mM) concentrations of NaN_3. TLC analysis showed spots corresponding to α-GlcNAc-N_3 for all the mutants but only when 100 mM NaN_3 was used with either 2NP-α-GlcNAc or 2,4DNP-α-GlcNAc as donors; we found that no reaction took place with 4NP-α-GlcNAc (Figure 4). Interestingly, the most productive reactions were found to be those using either 2NP-α-GlcNAc as donor at 37 °C or 2,4DNP-α-GlcNAc at 25 °C. The results also show that when 2NP-α-GlcNAc was used as donor at 37 °C or 2,4DNP-α-GlcNAc at either temperature, hydrolysis (i.e., formation of GlcNAc) was the predominant reaction being catalysed. These findings show that donor reactivity (as mediated by the nature of the leaving group or the reaction temperature) is the primary consideration for activity. However, when reactivity is too high, or in the absence of sufficient acceptor (NaN_3), donor hydrolysis becomes a significant problem. Optimised product formation would therefore entail finding a balance between donor reactivity in the presence of a specific acceptor, and hydrolysis.

Taking these results together, we concluded that the Glu483 mutants were promising candidates for application as α-TGLs and set out to demonstrate this using a range of thiol acceptors.

Evaluating α-TGL Activity using Selected Thiol Acceptors

Encouraged by the chemical rescue of the activity of the CpGH89 acid/base mutants using sodium azide, we decided to perform a comparative evaluation of their α-TGL activity. This was achieved by measuring their synthetic efficiencies when incubated at 37 °C using 2NP-α-GlcNAc as donor (the preferred choice, since it is commercially available and showed high levels of product formation in the reactions with NaN_3). As acceptors, the thiolated esters methyl or ethyl thioglycolate were used, since the pK_a values of their thiol groups (~8.08 at 25 °C for methyl thioglycolate – from the National Center for Biotechnology Information. PubChem Compound Database; CID = 16907, https://pubchem.ncbi.nlm.nih.gov/compound/16907, accessed on August 5, 2016) would ensure that at least half of the molecules in the reaction mixture would be in the more nucleophilic thiolate form at pH 8.0, i.e., the conditions under which the tests were performed. Mutants were incubated using >20-fold molar excess of the donor, and the amount of thioglycoside product formed was measured by HPAEC-PAD analysis of the reaction mixtures after 24 h and 48 h. The yield of converted product in each case was calculated based on the amount of GlcNAc available

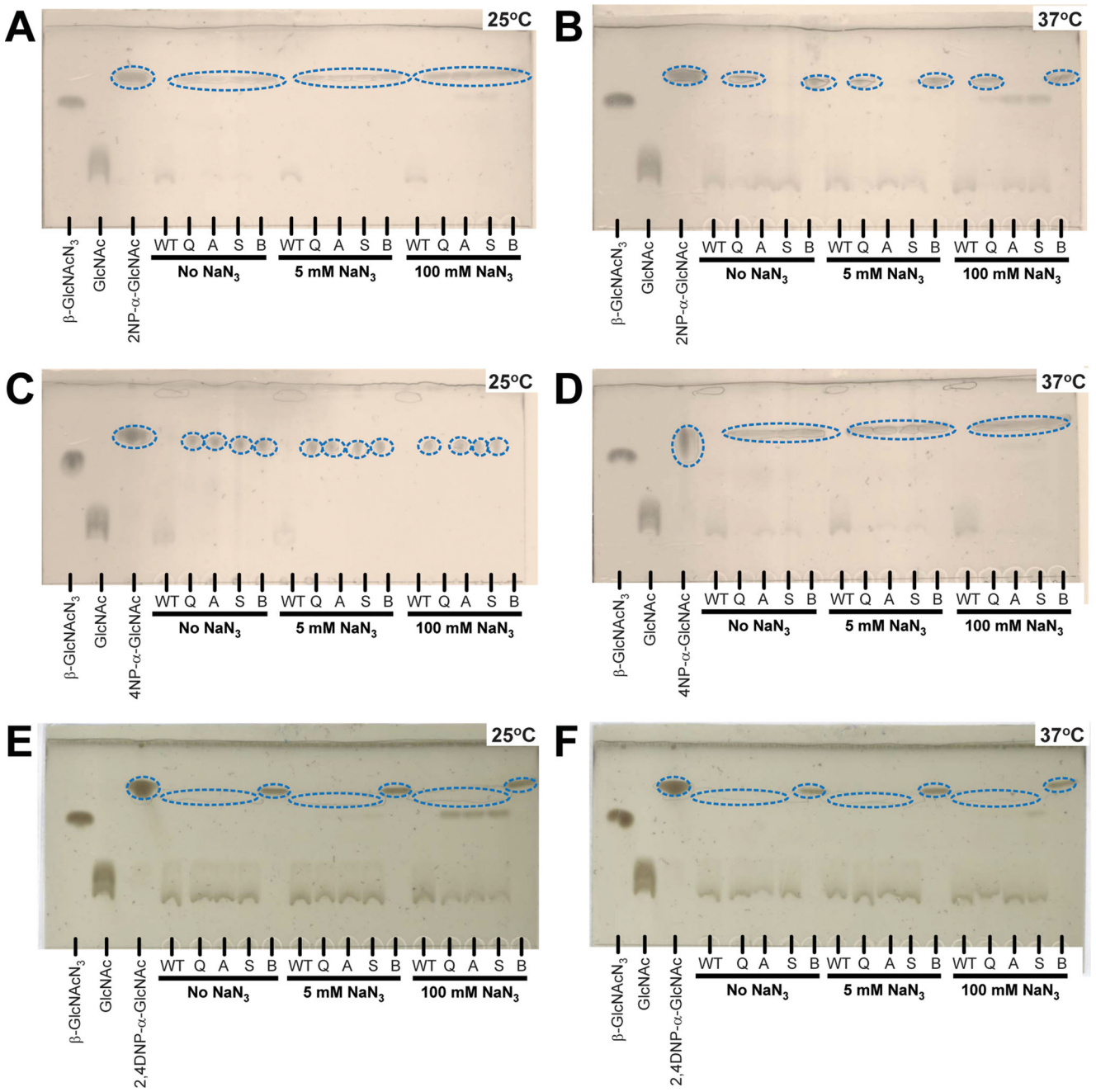


Figure 4. ***Products of the chemical rescue of CpGH89–E483 mutants from different donors as analysed by thin layer chromatography (TLC)***. β-GlcNAc-N_3, GlcNAc, and the respective donor are loaded in the first three lanes of each TLC plate as references. Three sets of reaction mixtures were analysed in each case, with either no NaN_3, or 5 mM or 100 mM NaN_3 present, and with each set containing wild-type enzyme (WT), E483Q (Q), E483A (A), E483S (S) mutant, and a blank reaction without enzyme (B). Reaction mixtures were incubated at either 25 °C or 37 °C, as indicated. Spots that were UV-visible are indicated by dotted circles; other spots were visualised by staining with 10% sulphuric acid in methanol, followed by incubation at 150 °C for 15 min. **A**: Using 2NP-α-GlcNAc as donor at 25 °C. **B**: Using 2NP-α-GlcNAc as donor at 37 °C. **C**: Using 4NP-α-GlcNAc as donor at 25 °C. **D**: Using 4NP-α-GlcNAc as donor at 37 °C. **E**: Using 2,4DNP-α-GlcNAc as donor at 25 °C. **F**: Using 2,4DNP-α-GlcNAc as donor at 37 °C.

in 2NP-α-GlcNAc set as 100%. Blanks showed only unreacted donor (Supporting Information, Figure S3). The results show that in the case of methyl thioglycolate, the CpGH89–E483A mutant is by far the most efficient α-TGL, with a yield of 81.8 ± 10.2% after only 24 h (Figure 3B). With the larger ethyl thioglycolate acceptor the efficiency of the mutants was reduced, and in this case the E483S mutant gave the

best results (64.6 ± 1.70%, also after only 24 h). The E483Q mutant was found to show the least efficient activity, whereas the activity of the E483G mutant corresponded to those of the alanine and serine mutants with ethyl and methyl thioglycolate, respectively.

In the light of these results, we chose the CpGH89–E483A and E483S mutants to investigate their activity as α-TGLs using a variety of thiol acceptors at concentrations of between 2.5 mM and 100 mM. Reactions were performed at 37 °C using 5 mM 2NP-α-GlcNAc as donor in 100 mM sodium phosphate buffer. Based on previous reports that the pK_a of the thiol group is an important consideration in its ability to act as acceptor in a thioglycoligase reaction,[19] we chose the thiols to span a range of pK_a values, and also performed the reactions at various pHs to evaluate if the predominance of the thiolate anion is a prerequisite for the TGL reaction to proceed. Reactions were evaluated by TLC and scored for product formation and hydrolysis as a side-reaction (Table 2). The results show that α-TGL activity is usually observed with thiols that have pK_a values of 8 and lower, i.e., thiols that would be at least 50% ionised at pH 8.0. We found that promoting the ionisation of thiols with higher pK_a values was not practically possible, since the hydrolysis of the donor becomes a significant side reaction at higher pH values. In fact, although the 2NP-α-GlcNAc donor (as an acetal) is expected to show enhanced stability at increased pH, the observed side reaction likely is the result of the higher concentration of HO^- by acting as nucleophile on the enzyme-GlcNAc intermediate. Indeed, we observed that for the thiosugar methyl 4-deoxy-4-thioglucoside and for cyclohexanethiol, both of which have pK_a values of 9.5 and above, GlcNAc was the major product formed. These findings indicated that the CpGH89–E483A and E483S mutants have excellent α-TGL activity, but only if the thiol acceptor is carefully chosen.

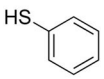
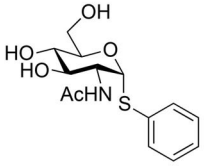
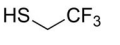
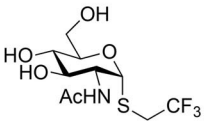
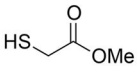
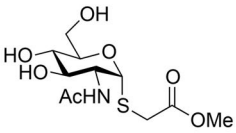
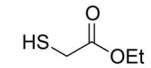
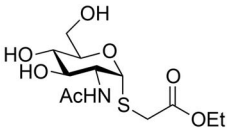
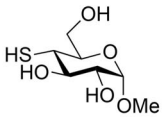
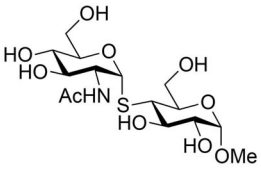
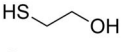
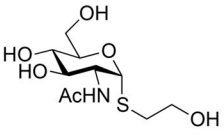
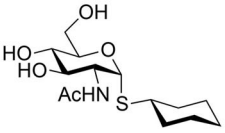
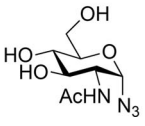
CpGH89–E483A/S Mutants as Preparative Scale α-TGL Biocatalysts

The excellent synthetic efficiency of the CpGH89–E483A mutant motivated us to determine if its α-TGL activity would also translate to reactions on a preparative (multimilligram) scale, and allow the synthesis and purification of α-GlcNAc-N_3 (using NaN_3 as external nucleophile) or α-GlcNAc thioglycosides (using thiol acceptors with pK_a values of 8 or lower). Towards this end, we performed biocatalysis reactions using CpGH89–E483A and/or E483S mutants using ~0.08 mmol 2NP-α-GlcNAc as donor and a 20-fold molar excess of acceptor at 37 °C. Reaction progress was monitored by TLC, and once being judged as complete, the reaction product was purified by means of four sequential steps that removed the protein, buffer and nitrophenol side-product, respectively, before the final product was purified by flash chromatography on silica. The purified products were analysed by ^1H and ^{13}C NMR and HR-MS analysis to confirm their structures, including the expected α-configuration of the newly-formed glycosidic bond. The preparative scale yields were also determined based on the amount of donor used. These values, summarised in Table 2, show that the small scale synthetic efficiencies were excellent predictors of the mutants preparative scale abilities, with the CpGH89–E483A mutant also giving preparative yields in excess of 80%. The lower synthetic yields observed in some cases are due to less favourable transglycosylation/hydrolysis ratios (compare the columns showing donor hydrolysis and product formation for each enzyme and substrate in Table 2) and, in some cases to difficulties encountered in the purification of the products. However, it is worth mentioning that the overall efficiency of the chemo-enzymatic approach shown here is much better than the classical chemical synthesis strategy that needs many more steps. Moreover, the yields obtained for the compounds prepared in this study are comparable to the overall yield of 35% reported for one of these compounds (entry 1, Table 2) prepared in six steps by traditional synthetic methods.[21] These findings confirm that the CpGH89–E483A mutant is an excellent α-TGL biocatalyst. In addition, this mutant also provides a means for the preparation of α-GlcNAc-N_3, a compound that is currently not commercially available and, according to the chemical database SciFinder, has never been described before. Nevertheless, it yet has multiple potential uses – including as azide substrate in the exceedingly versatile "click" reactions such as copper(I)-catalysed- and strain-promoted azide–alkyne cycloadditions (CuAAC and SPAAC).[22] Taken together, the CpGH89–E483A mutant opens routes to the facile preparation of a variety of α-linked *S*-glycosides and triazoles of high potential value that were previously not synthetically tractable.

Effect of the α-TGL Products on CpGH89

Since GH enzymes cannot hydrolyse the *S*-glycosidic bond, we next sought to demonstrate the potential of the biocatalytically-prepared GlcNAc α-thioglycosides as inhibitors of *N*-acyl-glucosaminidases. This was done by evaluating the ability of the compounds to bind to and inhibit the GH activity of wild-type CpGH89. We included in these tests commercially obtained β-GlcNAc-N_3 to demonstrate the importance of the configuration of the glycosidic bond, as well as GlcNAc-α(1→4)-(methyl 4-deoxy-4-thioglucoside) [GlcNAc-*S*-(Me4-thioGlc)], the expected product of

Table 2. The α-thioglycoligase activity of CpGH89–E483A and E483S with various thiol acceptors or sodium azide, and 2NP-α-GlcNAc as donor.

Entry	CpGH89 mutant	Acceptor	pK_a[a]	Expected product	Donor hydrolysis[b]	Product formation[c]	Preparative scale yield [%][d]
1	E483A	 thiophenol	6.6		pH 6.5 + pH 7.3 + pH 8.0 +	pH 6.5 ++ pH 7.3 ++ pH 8.0 ++	18
2	E483S				pH 6.5 + pH 7.3 + pH 8.0 +	pH 6.5 +++ pH 7.3 +++ pH 8.0 +++	
3	E483A	 2,2,2-trifluoro-ethanethiol	7.6		pH 7.3 + pH 8.0 +	pH 7.3 + pH 8.0 +	15
4	E483S				pH 7.3 + pH 8.0 +	pH 7.3 ++ pH 8.0 ++	
5	E483A	 methyl thioglycolate	8.1		pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	83
6	E483S				pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	49
7	E483A	 ethyl thioglycolate	8.1		pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	46
8	E483S				pH 7.3 + pH 8.0 +	pH 7.3 +++ pH 8.0 +++	33
9	E483A	 methyl 4-deoxy-4-thioglucoside	9.5		pH 7.3 + pH 8.0 ++	pH 7.3 - pH 8.0 -	
10	E483S				pH 7.3 + pH 8.0 ++	pH 7.3 - pH 8.0 -	
11	E483A	 β-mercapto-ethanol	9.7		pH 7.3 ++ pH 8.0 ++	pH 7.3 - pH 8.0 +	
12	E483S				pH 7.3 + pH 8.0 +	pH 7.3 - pH 8.0 +	
13	E483A	 cyclohexanethiol	10.7		pH 7.3 ++ pH 8.0 ++	pH 7.3 - pH 8.0 -	
14	E483S				pH 7.3 + pH 8.0 +	pH 7.3 - pH 8.0 -	
15	E483A	NaN_3 sodium azide					79

[a] Values taken from the National Center for Biotechnology Information (NCBI) PubChem Compound Database (https://pubchem.ncbi.nlm.nih.gov/), or from ref.[20] The pK_a value for methyl 4-deoxy-4-thioglucoside was calculated using ChemAxon's pK_a predictor.

[b] Relative extent of donor hydrolysis based on the intensity of the spots representing GlcNAc and 2NP-α-GlcNAc as visualised from the TLC analyses of the reaction mixtures.

[c] Relative extent of product formation based on the intensity of the anticipated product spot (identified by comparison with a standard, if available) as visualised from the TLC analyses of the reaction mixtures.

[d] Yield of purified product prepared from preparative scale biocatalytic transformations, based on the amount of 2NP-α-GlcNAc donor used as starting material.

the TGL reaction with methyl 4-deoxy-4-thioglucoside as acceptor. While we were not able to prepare this compound through biocatalysis, we prepared it by chemical synthesis to act as standard for the TGL evaluation tests, and it was therefore available for testing.

To quantify the inhibitory effect we measured the specific activity at standard conditions of wild-type CpGH89 at 600 μM of each compound. This concentration was chosen based on this amount of *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc), a known competitive inhibitor of CpGH89.[6] leading to 20% residual activity. Among the α-TGL products tested, the products of the reactions with methyl and ethyl thioglycolate (GlcNAc-*S*-CH_2CO_2Me and GlcNAc-*S*-CH_2CO_2Et, respectively) showed the highest inhibition with 79% and 62% of residual activity, respectively (Figure 5A).

In order to evaluate if the products obtained by chemical rescue and by the TGL approach could protect wild-type CpGH89 from unfolding by binding to the enzyme, we performed differential scanning fluorimetry (DSF) experiments in the 25–94 °C temperature range and in the presence of 10 mM of each product, the highest concentration used for allosteric pharmaceutical chaperones (PCs).[23] As positive controls we used PUGNAc (0.2 mM) and two other known *N*-acetylglucosaminidase inhibitors: 2-acetamido-1,2-dideoxynojirimycin (2AcDNJ, 0.1 μM) and 6-acetamido-6-deoxycastanospermine (6AcCAS, 2.8 μM), i.e., at concentrations 30-fold higher than their respective K_i or K_d values.[6] The thermal stability analysis (Figure 5B) revealed that the three inhibitors 6AcCAS, PUGNAc and 2AcDNJ increased the melting temperature (T_m) of wild-type CpGH89 by 6.21 °C, 3.76 °C, and 2.11 °C, respectively (Figure 5C). Among the products of the α-TGL, the best stabilising effect was produced by α-GlcNAc-N_3 (ΔT_m = 4.02 °C), while β-GlcNAc-N_3 did not show any stabilisation of the wild-type CpGH89. This finding demonstrates the importance of the configuration of the glycosidic bond for binding, and as a potential factor in

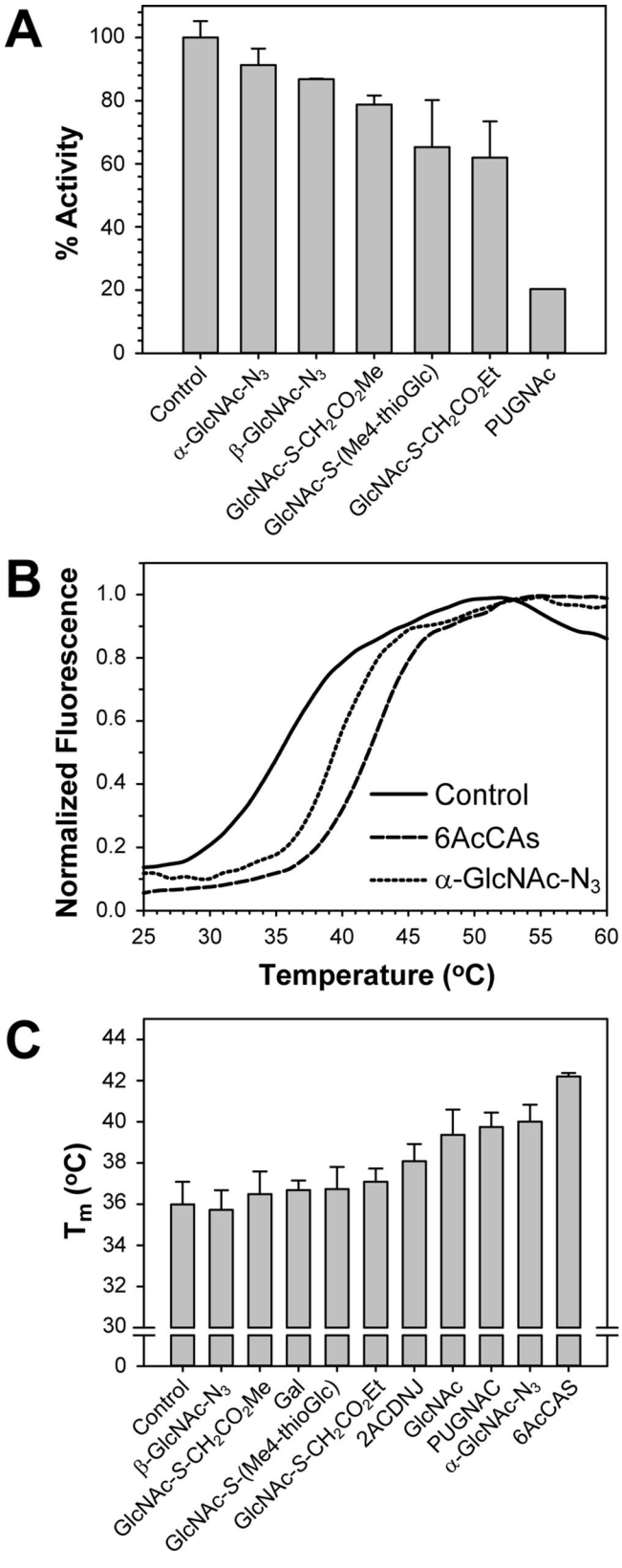


Figure 5. ***Effect of the α-thioglycoligase products on CpGH89 activity and stability.*** **A**: Effect by known inhibitors and α-TGL products on the activity of wild type CpGH89. Bars indicate the average value obtained from duplicate experiments; the error bars indicate the standard deviation. **B**: Denaturation curves followed by differential scanning fluorimetry (DSF) of wild type CpGH89 in the absence of ligands (control) and in the presence of the 6AcCAS inhibitor and α-GlcNAc-N_3 are shown as case examples. **C**: The increasing melting temperatures (T_m) of wild type CpGH89 in the presence of the indicated ligands as determined by DSF. Bars indicate the average value obtained from triplicate experiments; the error bars indicate the standard deviation.

establishing selectivity. GlcNAc, the product of the hydrolysis reaction, also significantly improved the thermal stability of the enzyme ($\Delta T_m = +3.38\,°C$) compared to galactose ($\Delta T_m = +0.70\,°C$), indicating that the stabilising effect of a carbohydrate-derivative molecule could be directly related to the donor −1 subsite.[16] It is worth noting that α-GlcNAc-N_3, which is the second-best PC after 6AcCAS, showed no inhibition of CpGH89 at 0.6 mM concentration (Figure 5A), a characteristic highly recommended in second generation PCs as they can promote the correct folding of the defective target enzyme with no detrimental effect on its activity in the lysosome.[3,24] In addition, as already stated above, α-GlcNAc-N_3 can be used as azide substrate in "click" reactions, thus, showing interesting potential as lead for novel α-linked PC molecules to test in pharmacological chaperone therapies for MPS IIIB.

Conclusions

Stable α-linked analogues of GlcNAc glycosides hold great potential for the development of new selective therapies for a range of diseases, for example, compounds that can act as inhibitors of the enzymes involved in the biosynthesis of essential cofactors in pathogenic organisms,.[25] or by stabilising the structures of defective mutant variants of key metabolic enzymes whose deficiency lead to genetic diseases.[4,5] However, such compounds are not easily obtained through chemical means due to the requirement for multistep synthesis with several protection/deprotection protocols. Here we have demonstrated that α-GlcNAc-N_3 and several α-GlcNAc thioglycosides can be obtained by preparative scale biocatalysis using an engineered mutant of the α-*N*-acetyl-glucosaminidase from *Clostridium perfringens* (CpGH89) in which its catalytically essential acid/base residue (Glu483) has been replaced by alanine. The development of this α-*N*-acetyl-thioglycosaminoligase lowers the barrier to the access of α-linked GlcNAc glycoside analogues significantly, and should facilitate the investigation of such compounds as therapeutic agents. In this study we highlight the potential scope for such compounds by showing their ability to inhibit or stabilise the wild-type form of CpGH89, a proxy for the NAGLU enzyme whose deficiency causes mucopolysaccharidosis IIIB, or Sanfilippo syndrome.

Experimental Section

General Materials and Methods

The donor substrates 2- and 4-NP-α-GlcNAc were obtained from Carbosynth (Compton, U.K.) while 2,4DNP-α-GlcNAc was prepared by chemical synthesis using a previously published protocol.[26] Methyl 4-deoxy-4-thioglucoside and GlcNAc-*S*-(Me4-thioGlc) were prepared by chemical synthesis as outlined in the Supporting Information. General chemicals, reagents, and media were purchased from Sigma–Aldrich or Merck Chemicals (Darmstadt, Germany) and were of the highest purity. Solvents used for reactions were Chromasolv HPLC grade solvents (Sigma–Aldrich), and the ethyl acetate (EtOAc), methanol (MeOH) and water used for purification were purchased from Merck Chemicals. Protein purifications were conducted on an Äkta Prime system (GE Healthcare). Spectrophotometric assays were performed on a Cary 100 Scan spectrophotometer (Varian, Australia), coupled with a thermally controlled Peltier system. High performance anionic exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed on a Thermofisher Scientific system equipped with a CarboPac PA200 Analytical (2×205 mm) column. Solid phase extraction (SPE) columns (Phenomenex Strata®) were obtained from Separations (Cape Town, South Africa).

Cloning and Mutagenesis of CpGH89 Wild Type and Mutants

The synthetic *CpGH89* wild type and E601A mutant genes were custom-made by GeneArt Gene Synthesis (Thermofisher Scientific) in pET28a vector, with N-terminal 6×His tag. The mutants E601G/S and E483G were prepared from the pET28a-CpGH89wt by using the GeneTailor Site-Directed Mutagenesis system (Invitrogen) with the following synthetic oligonucleotides (PRIMM, Milan, Italy).

E601G_fwd: 5′-TATTGGTATTACACCGGGCGCCATTAATACCAATCCGC-3′
E601G_rev: 5′-GCGGATTGGTATTAATGGCGCCCGGTGTAATACCAATA-3′
E601S_fwd: 5′-TGGGTATTGGTATTACACCGTCAGCCATTAATAC-3′
E601S_rev: 5′-CGGTGTAATACCAATACCCACCATATGTTC-3′
E483G_fwd: 5′-ATGGTGTGGATCCGTTTCATGGTGGTGGTAATACCGGTGATCT-3′
E483G_rev: 5′-AGATCACCGGTATTACCACCACCATGAAACGGATCCACACCAT-3′
E483A_fwd: 5′-GGATCCGTTTCATGCAGGTGGTAATACCGG-3′
E483A_rev: 5′-CCGGTATTACCACCTGCATGAAACGGATCC-3′
E483S_fwd: 5′-GGATCCGTTTCATTCAGGTGGTAATACCGG-3′
E483S_rev: 5′-CCGGTATTACCACCTGAATGAAACGGATCC-3′
E483Q_fwd: 5′-GGATCCGTTTCATCAAGGTGGTAATACCGG-3′
E483Q _rev: 5′-CCGGTATTACCACCTTGATGAAACGGATCC-3′

PCR-generated constructs were verified by sequencing.

Expression and Purification of CpGH89 Wild Type and Its mutants

CpGH89 wild type and mutants were expressed in *E. coli* strain JM109 (DE3) or BL21*(DE3). The cells transformed with the appropriate construct were grown at 37 °C in 2 L of Luria-Bertani (LB) broth supplemented with kanamycin (50 μg.mL^{-1}). Gene expression was induced by the addition of 1 mM IPTG when the culture reached an OD_{600} of 1.0. Growth was allowed to proceed for 16 h, and cells were harvested by centrifugation at 5000 *g*. The resulting cell pellet

was resuspended in 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl and 1% TRITON-X100 with a ratio of 5 mL g^{-1} cells, followed by incubation at 37 °C for 1 h with 20 mg of lysozyme (Fluka) and 25 U g^{-1} cell of DNAseI (SIGMA). Cells were lysed by 5 sonication cycles and cell debris was removed by centrifugation at 12000 *g* for 30 min. The cell free extract (CFE) was loaded on a HisTrap FF column (GE-Healthcare) equilibrated with 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl (Buffer A). After an initial wash-step (20 column volumes) with Buffer A, the protein was eluted at 250 mM imidazole in Buffer A. The active fractions were pooled, dialysed against 20 mM sodium phosphate buffer, pH 7.3, 150 mM NaCl (PBS buffer) and stored at 4 °C. The protein concentration was determined with the Bradford assay (Bio-Rad). After this procedure the enzymes were more than 95% pure by SDS-PAGE, with an approximate final yield of 20 mg L^{-1} of culture.

Comparative Structural Analysis

CD spectroscopy and thermal analysis were conducted using a Photophysics Chirascan-plus spectrometer at 25 °C. The samples contained 0.5 mg mL^{-1} protein in 100 mM sodium phosphate buffer (pH 7.3). The optimum wavelength for analysis of thermal stability was determined by a CD absorption scan from 200–300 nm at 25 °C. The thermal stability experiments were conducted by heating the samples from 25 °C to 100 °C and measuring the changes in CD absorption at 220 nm.

CpGH89 Hydrolytic Activity Assay

Hydrolytic activity assays for CpGH89 wild type and mutants at 25 °C were conducted using a microplate-based continuous spectrophotometric assay measuring the release of the nitrophenolate from the respective donor that was used. For assays conducted at 25 °C, reactions were performed in triplicate in a final reaction volume of 250 µL. The assays contained 2 µM enzyme, 100 mM sodium phosphate buffer (pH 7.3), and substrate concentrations in a range varying between 0–2.5 mM. The liberation of the nitrophenolate ion was monitored at 400 nm ($\varepsilon = 1.8$ $mM^{-1}cm^{-1}$ and 13.3 $mM^{-1}cm^{-1}$ for 2- and 4-nitrophenolate, respectively) for 10 min. Assays conducted at 37 °C were performed in a final reaction volume of 1 mL. The assays typically contained 220 nM of wild type and 1.3–4.11 µM of mutant enzymes, in 100 mM sodium phosphate buffer (pH 7.3), and substrate concentrations in a range varying between 0.5 µM and 5 mM. The liberation of the nitrophenolate ion was monitored at 405 nm ($\varepsilon = 3.63$ $mM^{-1}cm^{-1}$, 16.39 $mM^{-1}cm^{-1}$and 11.92 $mM^{-1}cm^{-1}$ for 2-, 4- and 2,4-dinitrophenol, respectively) for 10 min. The rates of product formation were calculated using linear regression analysis of the initial linear portions of the progress curves; these were plotted against the substrate concentration to obtain the activity profiles. The kinetic parameters k_{cat} and K_M were obtained by performing a least squares fitting of the data to the Michealis–Menten equation. One unit of enzyme activity was defined as the amount of enzyme catalysing the hydrolysis of 1 µmol of substrate in 1 min under the conditions described. Blank mixtures, containing all the reagents but the enzyme(s) were incubated at the same conditions and used to subtract the spontaneous hydrolysis of the donor(s).

Chemical Rescue with Sodium Azide

The chemically rescued hydrolase activity of the four CpGH89 acid/base mutants (E483A/S/Q/G) was measured in 100 mM sodium phosphate buffer at pH 7.3 using 5 mM 2,4DNP-α-GlcNAc and increasing the concentrations of sodium azide (between 50 mM and 1.5 M) at 37 °C. The specific activity was measured as described for the hydrolytic activity assay above. Blank mixtures, containing all the reagents but the enzyme(s) were incubated at the same conditions and used to subtract the spontaneous hydrolysis of the donor(s). Aliquots of the reaction mixtures (20 µL) incubated for 16 h were analysed by thin layer chromatography (TLC) as described previously.[27] Briefly, reaction mixtures were loaded on a silica gel 60 F_{254} plate by using EtOAc/MeOH/H_2O (70:20:10 v/v) as eluent and were detected by exposure to 10% sulphuric acid in methanol, followed by charring at 170 °C. The efficiency of each mutant was evaluated by HPAEC-PAD analysis at 35 °C using a flow rate 0.4 mL min^{-1} and 16 mM sodium hydroxide (NaOH) as eluent. For quantification, a calibration curve was created with three different amounts (0.25, 0.75 and 1.5 nmol) of the α-GlcNAc-N_3, and cellobiose (0.75 nmol) was included as internal standard. The synthetic efficiency of the mutants was determined assuming the amount of GlcNAc available in 2,4DNP-α-GlcNAc as 100%. All the samples were analysed in duplicate.

Evaluating the α-Thioglycoligase Activity of CpGH89–E483A/S with Selected Thiols

The α-thioglycoligase activities of the CpGH89–E483A/S mutants were evaluated using 2NP-α-GlcNAc as the donor. All thioglycoligase reactions were performed using 100 mM sodium phosphate buffer, with pH ranging from 5.8–8.0 (values indicated in Table 2), 1.33 µM enzyme and 5 mM 2NP-α-GlcNAc in a final volume of 200 µL. The final thiol concentration ranged from 2.5 mM to 100 mM. The reactions were incubated at 37 °C, and the mixtures were analysed by TLC with ethyl acetate/methanol/water (EtOAc/MeOH/H_2O) (70:20:10 v/v) using UV light to detect the nitrophenol group, and staining with 10% sulphuric acid in methanol, followed by incubation at 150 °C for 15 min, to visualise all other components. For each reaction 4 × 5 µL of the solution were spotted on the TLC.

Synthetic Efficiency of CpGH89–E483 Mutants with Methyl and Ethyl Thioglycolate as Acceptor

To determine the synthetic efficiency of the four CpGH89 acid/base mutants (CpGH89–E483A/S/Q/G), reactions were performed at 37 °C using 100 mM sodium phosphate buffer (pH 8.0), 1.33 µM enzyme, 5 mM 2NP-α-GlcNAc as donor and 106 mM methyl or ethyl thioglycolate as acceptor (supplied with 17.9 mM DTT) in a final volume of 200 µL. At time intervals (0–48 h) aliquots (10 µL) were withdrawn, diluted 1:100 with water and analysed by HPAEC-PAD at 35 °C using a flow rate of 0.4 mL. min^{-1} and the following elution program: [segment 1] 0–12′ isocratic 16 mM NaOH, [segment 2] 12–42′ up to 96 mM NaOH, [segment 3] 42–52′

step to 160 mM NaOH. The total amount of the expected thioglycoside product was determined by using a calibration curve with three different amounts (0.53, 1.06 and 2.12 nmol of α-GlcNAc-*S*-CH_2CO_2Me and 0.25, 0.75 and 1.5 nmol of α-GlcNAc-*S*-CH_2CO_2Et, respectively) of the product, and cellobiose (0.73 nmol) as internal standard. The authentic compounds used to obtain the calibration curves were obtained by preparative scale biocatalysis as outlined below. The synthetic efficiency of the mutants was determined assuming the amount of GlcNAc available in 2NP-α-GlcNAc as 100%. All the samples were analysed in duplicate.

Preparative Scale Biocatalysis

Preparative scale α-TGL reactions were carried out in 15 mL of 100 mM sodium phosphate buffer (pH 8.0). 2NP-α-GlcNAc (0.026 g, 0.076 mmol) was added as the donor, followed by addition of thiol (20 equiv.) or 100 mM sodium azide as the acceptor. DTT (0.160 mM, 0.370 mg, 0.0024 mmol,) was added as reducing agent to prevent disulphide bond formation in the case of the thiol acceptors. The reaction was initiated by addition of the thioglycoligase catalyst (1.33 μM of either CpGH89–E483A or CpGH89–E483S) to the reaction mixture followed by incubation at 37 °C. Reaction progress was monitored and analysed by TLC (EtOAc/MeOH/H_2O; 70:20:10 v/v). Upon completion (usually after overnight incubation), the reaction was filtered through a syringe filter (13 mm GHP Acrodisc® filter with 0.2 μM GHP membrane) to remove the protein. The filtered mixture was loaded onto an SPE column (Phenomenex Strata C18-U, 55 μm, 70 A, 10 g/60 mL) and the crude product was eluted with methanol. The fractions with the crude product were pooled together and concentrated by rotary evaporation. In the case of reactions with acceptors that do not proceed close to completion (as in the case of thiophenol), the crude product was treated with wild-type CpGH89 for 72 h under standard conditions to hydrolyse any residual donor that may be present. The enzyme was subsequently removed as described above. The crude product was purified by flash column chromatography on silica (EtOAc/MeOH/H_2O; 70:20:10 v/v). Any residual nitrophenol was removed by passing the purified product through Phenomenex Strata® Phenyl (55μm 70 Å) SPE column, eluting with water. The final purified products were obtained following by freeze drying for three days to remove any water, and were fully characterised by ^{1}H NMR, ^{13}C NMR and MS-ESI or HR-MS (see the Supporting Information for data).

CpGH98 Wild Type Inhibition assay and Thermal Stability

The inhibitory effects of the different compounds (0.6 mM each) were assayed by measuring the specific activity of CpGH89 wild type on 2NP-α-GlcNAc in 100 mM phosphate buffer (pH 7.3) at 37 °C as reported above. Thermal stability of CpGH89 wild type was measured by DSF as previously reported[23] with some modifications. Briefly, CpGH89 wild type (2.5 μg) was incubated in citrate phosphate buffer and SYPRO Orange dye 5X, in a total volume of 25 μL in the presence of the different compounds under test. Samples were heated from 25 to 94 °C in a Real-Time Light Cycler (Bio-Rad, Milan, Italy). Thermal stability scans were performed at 0.2 °C min^{-1} and the SYPRO Orange fluorescence was normalised to maximum fluorescence value within each scan to obtain relative fluorescence. All the measurements were performed in triplicate. The T_m value represents the flection point of the transition curve, as described by the Boltzmann equation.[28]

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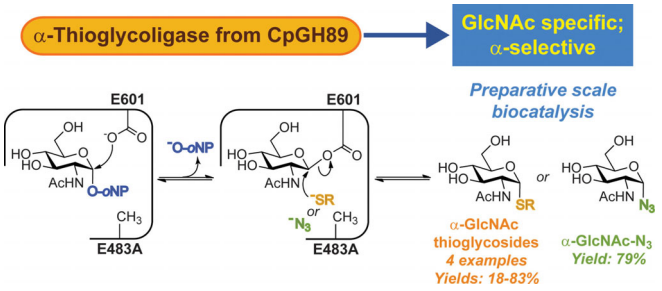

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