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towards bifunctional catalysts of tunable basicity

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Novel triple mutant of an extremophilic glycosyl hydrolase enables the rapid synthesis of thioglycosides

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In order to expand the toolbox of enzymes available for thioglycoside synthesis, we describe here the first example of an extremophilic glycosyl hydrolase from *Halothermothrix orenii* (*Hor*GH1) engineered towards thioglycosynthase activity with a novel combination of mutations. Using the triple mutant, *Hor*GH1 M299R/E166A/E354G, a range of thioglycosides from glycosyl fluoride donors and aromatic thiols could be synthesised with exquisite stereoselectivity and good to excellent conversions (61-93%).

Over a number of years, the ever-growing interest in biocatalysis for the production of diverse natural products and pharmaceutical ingredients has been firmly established.[1] These renewable catalysts not only possess an excellent regio/stereoselectivity, but they also often provide high catalytic efficiencies while operating under much milder conditions.[2] In the last decade alone, a myriad of reactions have been reported due to major advances in enzyme engineering, high-throughput screening and process design, which have dramatically expanded the synthetic chemists' toolbox.[1-4] Consequently, biocatalysis can offer several advantages over conventional chemical synthetic methods.[1-4]

The enzymatic synthesis of thioglycosides is especially underdeveloped despite their presence in many commercial products and pharmaceuticals.[5, 6] These stable glycosidic analogues confer additional stability to drugs due to their low susceptibility to acid/base and enzyme-mediated hydrolysis.[7] Moreover, they are tolerated by many biological systems and have been used as enzyme inhibitors in a range of biochemical studies.[6-9] Because of the ubiquity and the importance of glycosidic linkages in biology, thioglycosides have found many applications in carbohydrate-based therapeutics,[9] for instance, in the reduction of inflammatory leukocyte accumulation,[10] and have even shown promising anti-tumor effects.[11]

A plethora of chemical syntheses to access thioglycosides have been reported,[6,13-16] however, these procedures require multi-step protecting group strategies and offer limited stereochemical control.[8] Moreover, they often require pre-activation of the donor, toxic reagents and/or catalysts and are mostly performed in organic solvents.[6] An interesting strategy to synthesize aryl 1-thioglycosides from unprotected sugars and corresponding thiols was developed by Tanaka *et al.* using 2-chloro-1,3-diemthylimidazolinium chloride (DMC) as dehydrative condensing agent. Although this protecting-group-free synthesis is performed in water, complete stereoselectivity was not guaranteed and the triethylammonium by-products complicated downstream processing.[17] Similar methods have been recently reported to give access to aryl-thioglycosides with excellent yields, however they either require strong bases,[18] fluorine sources in the presence of organic solvents,[19] or thiyl-radicals.[20]

Enzymatic *S*-glycoside synthesis offers an attractive alternative to chemical approaches. While natural *S*-glycosyltransferases have been described in the literature, they require expensive NDP-α-D-sugars as substrates.[21-22] A cheaper and attractive method to access these products is therefore desired. In order to address these challenges, enzymatic syntheses using mutant forms of glycosidases have been developed.[23-34] Mutant glycosidases which are functional in the synthetic direction– aptly termed glycosynthases or glycoligases- are derived from glycosyl hydrolases which have had their catalytic nucleophile and/or acid/base residue replaced with non-nucleophilic residues, such as glycine or alanine. Glycosynthases are inherently unable of hydrolyzing their natural substrates. Nevertheless, when supplemented with an activated donor, such as a glycosyl fluoride, transglycosylation may occur without subsequent hydrolysis of the product due to the nature of the introduced mutation. While thiooligosaccharides have been synthetized with thioglycoligases[23-25] (single acid/base mutant) and with thioglycosynthases[26-27] (double acid/base and nucleophilic mutant), enzymatic syntheses of thioglycoconjugates are scarce.[28-30] Moreover, attempts to translate these elegant proof-of-concepts to industrial processes are often plagued by very low concentration of reactants[1-4] and enzymes being poorly resistant to the organic co-solvents that are often required to dissolve thiols.[1-3]

In order to expand the range of reaction conditions suitable for biocatalytic preparations of thioglycosides, we selected a β-glycosyl hydrolase (GH1; EC 3.2.1.21) from *Halothermothrix orenii* (*Hor*GH1) for further investigation.[31] This enzyme has been shown to perform well under non-physiological conditions.[32,33] Enzymes from extremophilic organisms indeed possess a significantly higher tolerance to extreme temperatures, pHs and organic cosolvents

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Table 1: Summary of the different variants of the β-glycosidase from *Halothermothrix orenii* (*Hor*GH1) investigated in this study.

Type of glycosidase	Catalytic residues maintained	Acid/base residue removed	Nucleophilic residue removed	Nucleophilic and acid/base residue removed
β-glycosidase	Wild type	E166A	E354G	E166A/E354G
β-thioglycosidase	M299R	M299R/E166A	M299R/E354G	M299R/E166A/E354G

compared to their mesophilic counterparts.[34-35]

We previously reported on the engineering of a β-glycosidase into a β-thioglycosidase *via* rational design and highlighted the key role of an arginine residue in the recognition of thioglycosides. The *Hor*GH1 M299R mutant exhibited a 3-fold increase in specificity for the hydrolysis of β-thioglycosides without any loss in turnover rate compared to the wild type (WT) enzymes.[36]

The depletion of catalytic residues [26, 27] combined with *Hor*GH1 M299R mutant (Supplementary information, **Figure S1**) could yield a very powerful catalyst for the synthesis of still elusive thioglycosides. To date, single acid/base mutants of a GH20 hexosaminidase,[28] a GH3 β-glycosidase[29] and of a thermophilic glucosidase[30] have shown to be able to access thioglycoconjugates, but a double deletion in any enzyme for this reaction has not been reported. Here, the mutations E166A (acid/base residue) and E354G (nucleophilic residue) were introduced into the catalytic site of *Hor*GH1 WT and *Hor*GH1 M299R as single, double and triple mutants (generating 8 *Hor*GH1 variants, **Table 1**) in order to understand which combination leads to the most efficient thioglycosynthase. Herein, we present the first example of an extremophilic glycosyl hydrolase from the GH1 family engineered towards thioglycosynthase activity and shed light on the differences between the thioglycoligase and the thioglycosynthase strategies in a same enzyme.

All mutants were successfully obtained in pure form (Supplementary information, **Figure S2**) and tested for thioglycoside

synthesis. Reactions with the variants depleted of the acid/base residue (E166A or E166A/M299R) were performed with *p*-nitrophenyl-β-D-glucopyranoside (**1**) as the sugar donor bearing a standard leaving group in the β position (*p*-nitrophenol (*p*NP), **Table 2** entries 1-4, **1a**) and *p*-nitrothiophenol (**2a**) as the thiol acceptor. Reactions with the variants lacking the nucleophilic residue (E354G and E354G/M299R) or both acid/base and nucleophilic amino acids (E166A/E354G and E166A/E354G/M299R) were performed using α-D-glucopyranosyl fluoride as the donor instead (F in α, **Table 2** entries 5-8, **1b**). A good leaving group in the α position is needed with these latter variants to compensate for the absence of the glycosyl-enzyme intermediate normally formed with the nucleophilic residue. In order to alleviate thiol oxidation, the buffers were degassed and various reducing agents were screened. While tris(2-carboxyethyl)phosphine (TCEP) completely inactivates the enzyme at concentrations above 2 mM, and 1,4-dithiothreitol (DTT) reduced conversion by half at a concentration of 20 mM, β-mercaptoethanol was found to be tolerated by the enzyme and was used for further experiments (Supplementary information, **Figure S4**). No formation of thioglycosides was observed upon incubation of WT enzyme and M299R mutant (**Table 2**, entries 1-2). Moreover, no spontaneous reactions (*i.e.* direct S_N2 displacement of the anomeric group) were observed in the absence of enzyme under our working conditions. The neutralization of the acid/base residue in E166A and

Table 2: Thioglycoside synthesis with the different *Hor*GH1 mutants.

Entry	Sugar donor	Enzyme variant	Molar conversion [%]
1	**1a,** X= pNP in β	WT	0
2	**1a,** X= pNP in β	M299R	0
3	**1a,** X= pNP in β	E166A	23
4	**1a,** X= pNP in β	M299R/E166A	26
5	**1b,** X= F in α	E354G	0
6	**1b,** X= F in α	M299R/E354G	0
7	**1b,** X= F in α	E166A/E354G	61
8	**1b,** X= F in α	M299R/E166A /E354G	92

Reactions were performed in the presence of 1 mM sugar donor, 10 mM thiol acceptor at 25 °C in bicarbonate buffer pH 9.4 containing 30% DMSO and the enzyme (1 mg/mL). Reactions were all run for 24 h. Conversions were determined by HPLC analysis based on product formation.

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M299R/E166A variants (**Table 2**, entries 3-4) induced some thioglycosynthetic capability, but yields were low (20%). This inefficient transglycosylation is likely due to the poor leaving group ability of the *p*-nitrophenyl substituent in the absence of acid catalysis (Supplementary information, **Figure S5**). Interestingly, no thioglycoside activity was observed with the mutants E354G and M299R/E3354G lacking the nucleophilic residue (**Table 2**, entries 5-6). The variants E166A/E354G and M299R/E166A/E354G depleted of both catalytic residues (**Table 2**, entries 7-8), gave very good (61%) to excellent (92%) conversions with *p*-nitrothiophenol as acceptor, respectively (Supplementary information, **Figure S6**). The synthetic capability of the variants was significantly enhanced by the M299R mutation and confirms the key role of arginine in the recognition of sulfur atom. The high nucleophilicity of the thiolate tested with the double mutant scaffold stabilizing the transition states,[26,27] is indeed sufficient to achieve a significant product yield, but the active site is clearly positively impacted by the M299R mutation, as it was reported for the hydrolysis of thioglycosides.[36] Whereas K_M towards α-D-glucopyranosyl fluoride was found to be similar for the M299R/E166A/E354G and the E166A/E354G variants (2.26 and 2.12 mM, respectively), K_M towards *p*NT was lower for M299R/E166A/E354G (3.82 mM) than for E166A/E354G (7.46 mM) (**Figure S7**). Analysis of the product by ^{1}H NMR confirmed the presence of a β-thioglycosidic linkage, thereby showing the absolute stereoselectivity of the enzyme (Supplementary information, **Figure S8**). Synthesis of thioglycoside products was also confirmed by HRMS (**Table S2**). As expected, none of the mutants showed any significant hydrolytic activity towards *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-thioglucopyranoside substrates (Supplementary information, **Figure S9**).

The encouraging results obtained with *Hor*GH1 M299R/E166A/E354G variant triggered the exploration of additional thiols that could be accepted by this mutant (Supplementary information, **Figure S10**). The triple mutant (1mg/mL) was successfully tested with a range of substituted thiophenols at 1 mM scale. We also observed a pH dependence of the reaction, with pH 9.4 furnishing the highest conversion for all respective substrates (Supplementary information, **Figure S11**). While most of the tested aromatic thiols yielded the corresponding thioglycosides in good to excellent conversions (61 to 93%, **Table 3, entries 1-4 and 6**), no conversion was observed with *p*-aminothiophenol (**2e**), as well as with N,N'-dibenzoyl-L-cystine and diphenyl diselenide as acceptors (**Table 3**, entries 7 and 8, respectively). Two additional substrates, *p*-methoxythiophenol and 2-naphtalenethiol were tried but gave inconsistent results due to poor solubility.

Besides being easy to handle and cofactor-independent, this robust catalyst remained active for 48 hours despite the presence of 30% DMSO. With an excess of thiol acceptor, cheap starting materials were converted into valuable chemical products with high added value. Moreover, the products formed are interesting intermediates in the synthesis of pharmaceuticals; **3b**, for example, in its peracetylated version was shown to efficiently reduce inflammatory leukocyte accumulation by functioning as metabolic decoy,[10] while the nitrosylated **3f** showed an antitumor effect which was significantly enhanced in combination with insulin.[11] Similarly, the bromide substituted **3d** could be further derivatized *via* cross-coupling methodologies for the formation of valuable thioglycoconjuguates.[28,37,38] More importantly, the triple mutant approach could be extended to other enzymes that have a different acceptor tolerance.

Table 3: Range of thioglycosides synthesized with the mutant, *Hor*GH1 M299R E166A E354G.
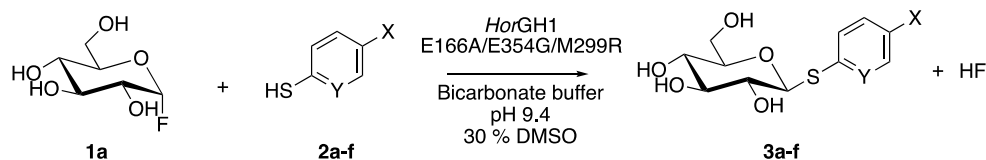
Entry	Sugar donor	Acceptor	Reaction time [h]	Molar conversion [%]
1	α-D-glucopyranosyl fluoride (**1a**)	*p*-nitrothiophenol (Y=C, X=NO$_2$, **2a**)	2	92
2	α-D-glucopyranosyl fluoride (**1a**)	thiophenol (Y=C, X=H, **2b**)	48	61
3	α-D-glucopyranosyl fluoride (**1a**)	*p*-thiotoluene (Y=C, X=CH$_3$, **2c**)	48	81
4	α-D-glucopyranosyl fluoride (**1a**)	*p*-bromothiophenol (Y=C, X=Br, **2d**)	2	93
5	α-D-glucopyranosyl fluoride (**1a**)	*p*-aminothiophenol (Y=C, X=NH$_2$, **2e**)	72	0
6	α-D-glucopyranosyl fluoride (**1a**)	2-mercaptopyridine (Y=N, X=H, **2f**)	72	70
7	α-D-glucopyranosyl fluoride (**1a**)	N,N'-dibenzoyl-L-cystine	48	0
8	α-D-glucopyranosyl fluoride (**1a**)	Diphenyl diselenide	48	0

Reactions were performed in the presence of 1 mM sugar donor, 10 mM thiol acceptor at 25 °C in 30% DMSO, 50 mM bicarbonate buffer pH 9.4, 20 mM of β-mercaptoethanol and the enzyme (1 mg/mL) for products **3a-d**. Conversions were determined by HPLC based on product formation. Reactions were performed in the presence of 10 mM sugar donor, 100 mM thiol acceptor at 25 °C in 30% deuterated DMSO, 50 mM bicarbonate buffer pH 9.4 in D$_2$O, 200 mM β-mercaptoethanol and the enzyme (1 mg/mL in D$_2$O) for products **3e-f**. Conversion was determined by NMR.

In conclusion, this work provides critical insights on the differences between the use of thioglycoligases and the thioglycosynthases for the synthesis of thioglycosides in a same thermophilic β-glycosyl hydrolase from the GH1 family. For the first time, mutations of the acid/base (E166A) and the nucleophilic catalytic residues (E354G) into neutral amino acids were combined with a thiol-specific mutation (M299R) aiming to increase specificity towards β-thioglycoside synthesis. The repertoire of mutant glycosidases available for the biosynthesis of thioglycosides was expanded by constructing an evolved enzyme, *Hor*GH1 M299R/E166A/E354G, which proved to be an efficient thioglycosynthase as highlighted by the range of accepted aromatic thiols. The exquisite stereoselectivity

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and the good yields obtained demonstrated the catalytic efficiency of this enzyme. Our engineered enzyme provides an innovative, safe, green and profitable synthetic route for the construction of *S*-glycosidic linkages. By endowing robust and efficient enzymes with new functions, we can pave the way to the effective implementation of biocatalytic processes at the industrial scale while alleviating their ecological imbalance.

Contributions

F.P. conceptualized the idea and supervised the project. L.P. performed most of the experimental work and wrote the initial draft. D.L. guided the biotransformations and synthesized all standards. N.A. developed the double mutated versions of the enzyme. A.I.B.M. designed and supervised the genetic experiments. All authors discussed and agreed to the final version of the manuscript.

Acknowledgements

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