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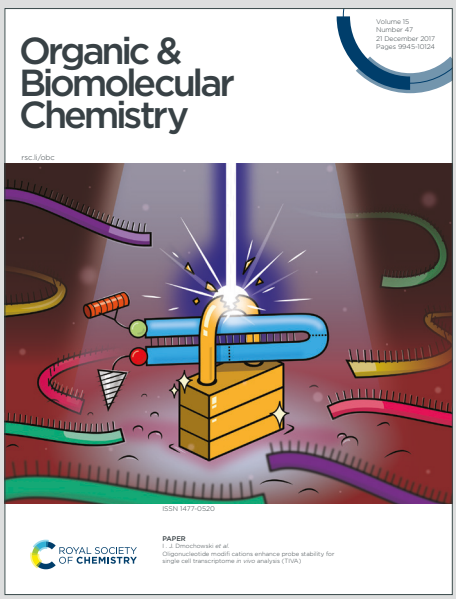

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COMMUNICATION

Thioglycoligation of aromatic thiols using natural glucuronide donor.

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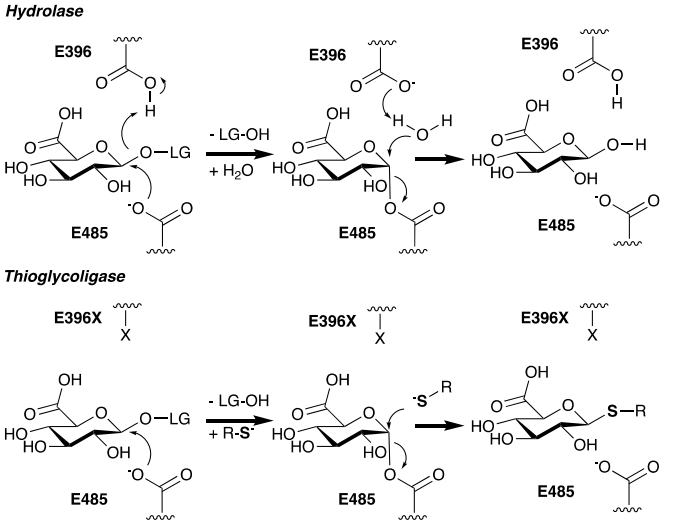
β-D-glucuronidase *Dt*GlcA from *Dictyoglomus thermophilum* was engineered to generate an active thioglycoligase able to catalyse the formation of numerous *S*-glucuronides. Its X-ray structure analysis indicated the ability of the biocatalyst to bind aromatic thiols acceptors for S-glycosylation. Noteworthingly, DtGlcA mutant was found to be the first thioligase able to use a natural sugar donor different from the widely used synthetic paranitrophenyl glycosides.

Thioglycosides *(S*-glycosides) represent highly valuable chemical tools for the study of biological processes, because of their higher resistance to enzymatic or chemical hydrolysis, when compared to their *O*- or *N*- counterparts[1,2]. Since their chemical synthesis remain tedious as many protection/deprotection steps are required, chemoenzymatic or enzymatic synthesis of these compounds has emerged as an attractive alternative in the context of sustainable development[3]. Along with the few glycosyltransferases that have been demonstrated to able to form the thioglycosidic linkage[4–7], thioglycoligases have proven to be an excellent alternative to synthesize diverse thioglycosides[8]. Obtained by the engineering of retaining glycosides hydrolases (GH) where the acid/base catalytic residue has been mutated into a non-carboxylate aminoacid, the nature of the transferred sugar is correlated to the parent GH substrate specificity (Scheme 1).

Among the thioligases that have been reported in literature, only one example able to catalyse the synthesis of thioglucuronides is available[9]. In this study, the authors engineered a β-D-glucuronidase from *Thermotoga maritima* (*Tm*GUA) and used this catalyst to generate several thioglucuronides linked to another carbohydrate. Like all other thioligase examples, an activated synthetic sugar donor was used because the nitrophenyl aglycon is a good leaving group for the first step of the mechanism. Non hydrolysable *S*-glucuronides are of particular interest, because they can be used as biochemical tools to study xenobiotics glucuronidation, or as a targeting approach to cancer cells, since glucuronidase is overexpressed on the surface of solid tumours[10,11].

In this context, we have identified and characterized the β-D-glucuronidase *Dt*GlcA from *Dictyoglomus thermophilum* as a potent candidate to be turned into a thioligase. This thermophilic bacterium has been used by us and others as the source of production of several GH for biocatalysis[12–18]. After cloning of the corresponding gene in an expression vector, the recombinant protein fused with a polyhistidine tag was purified and enzymatically characterized (Figures S3a to S3d). Unlike *T. maritima* glucuronidase, *Dt*GlcA was only able to hydrolyze β-D-glucuronide among 21 glycosidic substrates. As other GH, it exhibits an optimal pH around 5.0, and has an optimal catalysis temperature at 80-90°C. This thermostability, coupled to a good resistance to high organic solvent contents (>50% residual activity in 40% methanol, acetonitrile or DMSO) makes *Dt*GlcA attractive for synthetic purposes.



Scheme 1: *Dt*GlcA Hydrolase and DtGlcA-E396Q thioligase reaction mechanism. Catalytic residues identification is described *vide infra.*

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Table 1: *Dt*GlcA WT and E396Q hydrolytic kinetic constants of *p*NP-GlcA compared with *Tm*GUA[19]. Data are expressed as mean± SD from 3 independent experiments.

	k_{cat} (s^{-1})	K_M (µM)	$kcat/K_M$ ($s^{-1}.M^{-1}$)
*Dt*GlcA-WT	9.3 ± 0.8	125 ± 20	74.4 x 10^3
*Dt*GlcA-E396Q	0.12 ± 0.01	148 ± 14	0.8 x 10^3
TmGUA-WT	68 ± 2	150 ± 10	453 x 10^3

Using the commercially available 4-nitrophenyl-β-D-glucuronide (*p*NP-GlcA) as a substrate, kinetic constants of hydrolysis catalysed by *Dt*GlcA were determined (Table 1). When compared those described for *Tm*GUA[19], Michaelis constant K_M was similar whereas the turnover rate k_{cat} was found lower.

*Dt*GlcA readily crystallized in 20% w/v 2-methyl-2,4-pentanediol; 100 mM Sodium HEPES pH 7.5; 100mM Sodium citrate and its structure at 1.8 Å was solved (PDB 6XXW). The closest characterized homologous structure is the human β-glucuronidase[20], sharing 48% (64%) peptidic sequence identity (homology). Structural alignment of *Dt*GlcA structure with human glucuronidase enable the identification of the 2 catalytic residues, namely E396 as the acid/base residue, and E485 as the nucleophile residue. Loop 349-363 differs from human glucuronidase structure, as it is longer, and surrounds the pocket that binds aglycon part of glucuronides hydrolysed by DtGlcA.

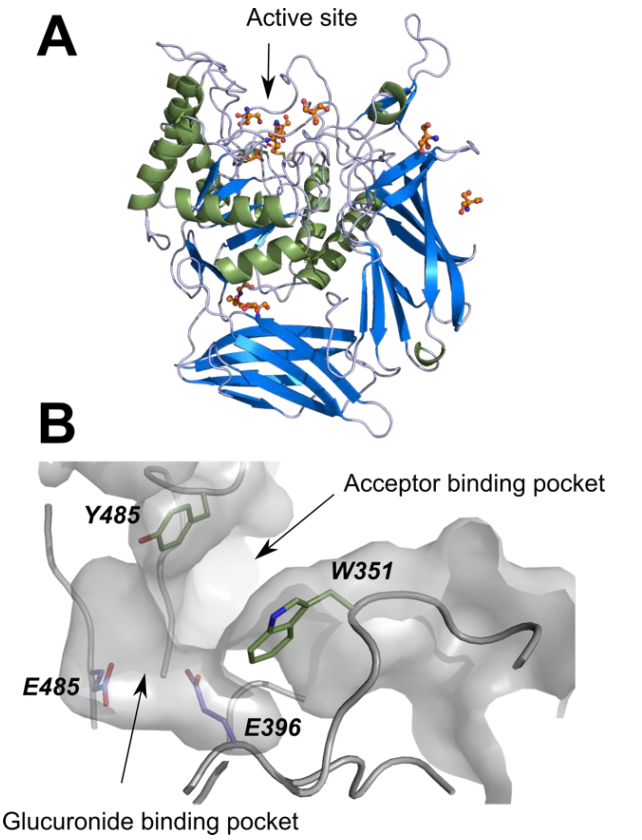


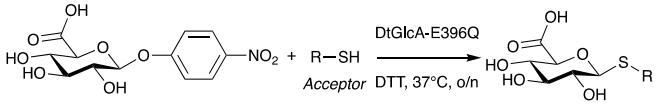
Figure 1: (A) Overall structure of DtGlcA WT (PDB 6XXW). Tris and 2-methylpentane-2,4-diol molecules crystallized with the protein are coloured in orange (B) Active site residues surrounding the acceptor pocket. Catalytic glutamates E396 (acid/base) and E485 (nucleophile) and aromatic residue bordering the acceptor binding site (W351 and Y485) are depicted as sticks and highlighted.

In particular, W351 and Y453 side chains are located along the acceptor binding site, facing each other, making this cavity hydrophobic and prone to π-stacking interactions. In the context of potential thioglycoligation, this is an indication of further possibility to use aromatic thiols as acceptors that would bind into this pocket.

*Dt*GlcA-E396Q mutant, lacking the possibility to deprotonate water in the deglycosylation step during hydrolysis (see scheme 1) was generated by site-directed mutagenesis, and purified under the same conditions as wild-type *Dt*GlcA. As expected with similar mutations, the turnover rate dramatically decreases by a factor of around 90 when compared to the wild-type enzyme (table 1), whereas the Michaelis constant K_M remained almost unchanged. This indicates that *p*NP-GlcA is able to bind in the active site, whereas the rate-limiting nucleophilic attack of water is decreased after mutation.

As the crystal structure analysis was indicating that aromatic acceptor would bind in the aglycon cavity, thiophenol and 4-chlorothiophenol were used as model acceptors, and were efficiently glycosylated by DtGlcA-E396Q when using *p*NP-GlcA as glucuronide donor (resp. 72 and 90 % conversion rates as calculated from HPLC chromatograms) (Scheme 2). Identification of products was confirmed by HRMS analysis of crude reactions that gave atomic composition of each product.

Having demonstrated that DtGlcA-E396Q was able to efficiently catalyse the thioglycoligation reaction using simple aromatic thiols, we used the enzyme to catalyse the formation of two other glucuronides *S*-linked either to *p*-nitrothiophenol or 7-mercapto-4-methylcoumarine. Both thiols were efficiently *S*-glycosylated, with respective conversion rates of 91 and 77 %, pKa values of acceptors can be correlated to their nucleophilicity and to the reaction rate[7]. For thiophenol derivatives, the pKa is in agreement with the observed rates. 7-mercapto-4-methylcoumarine has a low pKa of 5, yet its low conversion rate might be attributed to its bigger size, that would increase steric hindrance.



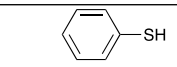
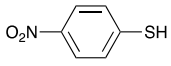
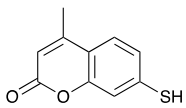
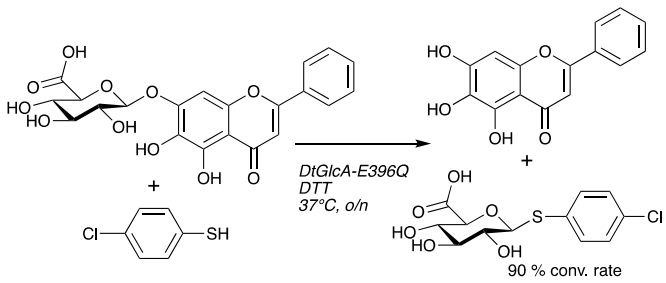
Acceptor	pKa	Conversion rate (%)	Yield (%)
	6.61	72	14
	6.12	90	85
	4.68	91	54
	5.03	77	51

Table 2 Thioglycoligation reactions catalysed by *Dt*GlcA-E396Q using *p*NP-GlcA as sugar donor and aromatic thiol acceptors.



Scheme 2: Thioglycoligation of 4-chlorothiophenol using baicalin as glucuronide donor.

Taken these 4 thiols as model acceptors, this is the first example of using a thioligase to generate *S*-glucuronides bearing aromatic aglycon moiety.

In addition, we also probed the ability of DtGlcA-E396Q to use a natural glucuronide as sugar donor, namely baicalin. This natural compound is the glucuronide of flavone baicalein, which is found in roots of *Scutellaria* species[21] and was demonstrated to exhibit various pharmacological activities, from antitumor to antimicrobial properties[22].

We thus have used baicalin as a glucuronide donor in thioligation reaction by DtGlcA-E396Q, instead of the synthetic *p*NP-GlcA. Using 4-chlorothiophenol as a model acceptor, we showed that the glucuronide product can be efficiently formed with conversion rate similar to those observed when using pNP-GlcA (90%). As for other thioligases, the rate-limiting step during thioglycoligation remains the deglycosylation step (*ie* nucleophilic attack of the thiol acceptor), thus the overall catalysis is unchanged because baicalein has similar leaving group ability to *p*-nitrophenol. Most interestingly, baicalin doesn't need to be chemically prepared, is commercially available in large quantity and is really cheap, making it a promising source of glucuronyl donor in enzymatic synthesis of natural and unnatural O- and S-glycosides owing biological properties.

Conclusions

In the search of original biocatalyst promoting thioligation, *Dt*GlcA has been identified as an interesting target. Mutation of the acid/base catalytic E396 residue into the glutamine aminoacid led to an efficient catalyst, whose X-ray structure gave evidence of potential binding and reactivity using aromatic thiols. Successful *in vitro* thioglycosylation reactions using a set of 4 aromatic thiols confirmed this hypothesis, and DtGlcA represents thus one of the first thioglycoligases able to *S*-glycosylate non-carbohydrate like acceptors (the first when considering *S*-glucuronide formation)[23,24]. Moreover, baicalin, a natural glucuronide donor can be used as an attractive alternative to synthetic donors for thioglycosylation reaction, in the context of enzymatic sustainability. Exemplification on a wider range of acceptors and other natural donors, as well as structure based engineering are currently under investigations, and this study paves the way in *S*-glycoside enzymatic synthesis, as it broadens the potentiality of this approach.

Conflicts of interest

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The authors state that there are no conflicts to declare.

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References

1 H. Driguez, *ChemBioChem*, 2001, **2**, 311–318.
2 L. Guillotin, P. Lafite and R. Daniellou, in *Carbohydrate Chemistry: Volume 40*, The Royal Society of Chemistry, 2014, vol. 40, pp. 178–194.
3 J. Ati, P. Lafite and R. Daniellou, *Beilstein J. Org. Chem.*, 2017, **13**, 1857–1865.
4 H. Wang and W. A. van der Donk, *J Am Chem Soc*, 2011, **133**, 16394–16397.
5 T. J. Oman, J. M. Boettcher, H. Wang, X. N. Okalibe and W. A. van der Donk, *Nat. Chem. Biol.*, 2011, **7**, 78–80.
6 S. Marroun, S. Montaut, S. Marquès, P. Lafite, G. Coadou, P. Rollin, G. Jousset, M. Schuler, A. Tatibouët, H. Oulyadi and R. Daniellou, *Org. Biomol. Chem.*, 2016, **14**, 6252–6261.
7 P. Lafite, S. Marroun, G. Coadou, S. Montaut, S. Marquès, M. Schuler, P. Rollin, A. Tatibouët, R. Daniellou and H. Oulyadi, *Mol. Catal.*, 2019, **479**, 110631.
8 F. A. Shaikh and S. G. Withers, *Biochem. cell Biol.*, 2008, **86**, 169–177.
9 J. Müllegger, H.-M. Chen, W. Y. Chan, S. P. Reid, M. Jahn, R. A. J. Warren, H. M. Salleh and S. G. Withers, *Chembiochem*, 2006, **7**, 1028–1030.
10 W. H. Fishman and A. J. Anlyan, *Science (80-.).*, 1947, **106**, 66–67.
11 W. H. Fishman and A. J. Anlyan, *Cancer Res.*, 1947, **7**, 808–817.
12 D. D. Morris, M. D. Gibbs, C. W. J. Chin, M.-H. Koh, K. K. Y. Wong, R. W. Allison, P. J. Nelson and P. L. Bergquist, *Appl. Environ. Microbiol.*, 1998, **64**, 1759–1765.
13 R. Shi, Z. Li, Q. Ye, J. Xu and Y. Liu, *Bioresour. Technol.*, 2013, **142**, 338–344.
14 K. Hu, C.-X. Li, J. Pan, Y. Ni, X.-Y. Zhang and J.-H. Xu, *Appl. Biochem. Biotechnol.*, 2014, **172**, 1215–1226.
15 C. Schröder, V.-A. Janzer, G. Schirrmacher, J. Claren and G. Antranikian, *Extremophiles*, 2017, **21**, 85–94.
16 L. Guillotin, N. Richet, P. Lafite and R. Daniellou, *Biochimie*, 2017, **137**, 190–196.
17 L. Guillotin, Z. Assaf, S. G. Pistorio, P. Lafite, A. V. Demchenko and R. Daniellou, *Catalysts*, 2019, **9**, 826.
18 L. Guillotin, H. Kim, Y. Traore, P. Moreau, P. Lafite, V. Coquoin, S. Nuccio, R. de Vaumas and R. Daniellou, *ACS Omega*, 2019, **4**, 1916–1922.

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DOI: 10.1039/D0OB00226G

19 H. M. Salleh, J. Müllegger, S. P. Reid, W. Y. Chan, J. Hwang, R. A. J. Warren and S. G. Withers, *Carbohydr. Res.*, 2006, **341**, 49–59.

20 S. Jain, W. B. Drendel, Z. W. Chen, F. S. Mathews, W. S. Sly and J. H. Grubb, *Nat. Struct. Biol.*, 1996, **3**, 375–81.

21 W. T. Seo, Y. H. Park and T. B. Choe, *Plant Cell Rep.*, 1993, **12**, 414–417.

22 T. Huang, Y. Liu and C. Zhang, *Eur. J. Drug Metab. Pharmacokinet.*, 2019, 44, 159–168.

23 M. Almendros, D. Danalev, M. François-Heude, P. Loyer, L. Legentil, C. Nugier-Chauvin, R. Daniellou and V. Ferrières, *Org. Biomol. Chem.*, 2011, **9**, 8371–8378.

24 G. Tegl, J. Hanson, H. M. Chen, D. H. Kwan, A. G. Santana and S. G. Withers, *Angew. Chemie - Int. Ed.*, 2019, **58**, 1632–1637.