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Gaining insight into the catalysis by GH20 lacto-*N*-biosidase using small molecule inhibitors and structural analysis†

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The synthesis of potent inhibitors for lacto-*N*-biosidases and X-ray structural characterization of these compounds in complex with BbLNBBase is described.

The role that the microbiome plays in human health and disease is being shown to be extremely important and as such is receiving considerable attention.¹ Bacteria of the genus *Bifidobacterium* are especially critical to the health of the GI tract as they constitute a large proportion of the GI microbiome² and they have been shown to be important in influencing the distribution of other GI microbiota.³ Colonization of the GI tract by these bacteria occurs soon after birth and it is believed that they play a beneficial role in stimulating the immune response, preventing colonization of pathogenic bacteria and suppressing inflammatory responses.^{4,5}

To establish and maintain colonization bifidobacteria express a wide range of carbohydrate-processing enzymes² which allows them to utilize carbohydrates that are not digestible by the host, or other microbes. This trait offers a competitive advantage especially in breast-fed infants.⁶ One specific class of carbohydrates that is acted upon by bifidobacterial enzymes are the human milk oligosaccharides (HMOs),⁷ that include over 130 different glycans and are found in concentrations of up to 20 g per litre in human milk.^{8,9} Due to the importance of these compounds for the life of bifidobacteria,¹⁰ unique biochemical pathways have evolved in these bacteria to breakdown these compounds. One such pathway is termed the lacto-*N*-biose (LNB) pathway^{11,12} which allows for

metabolic utilization of LNB (Gal-(β1,3)-GlcNAc),¹³ a common structural motif found in HMOs.^{9,14} Consequently, an enzyme important to the LNB pathway is lacto-*N*-biosidase (LNBBase), a β-*N*-acetyl-hexosaminidase that liberates LNB from HMOs.

LNBases are classified currently as members of family 20 of the glycoside hydrolases (GHs)† and like other members of this family it has been shown to use a two-step catalytic mechanism involving substrate-assisted catalysis that forms a transient oxazoline or oxazolinium ion intermediate (Fig. 1A).^{15,16} Much of the insight into the active site architectures and catalytic mechanisms of GH20 glycosidases and several other glycosidase families has been made

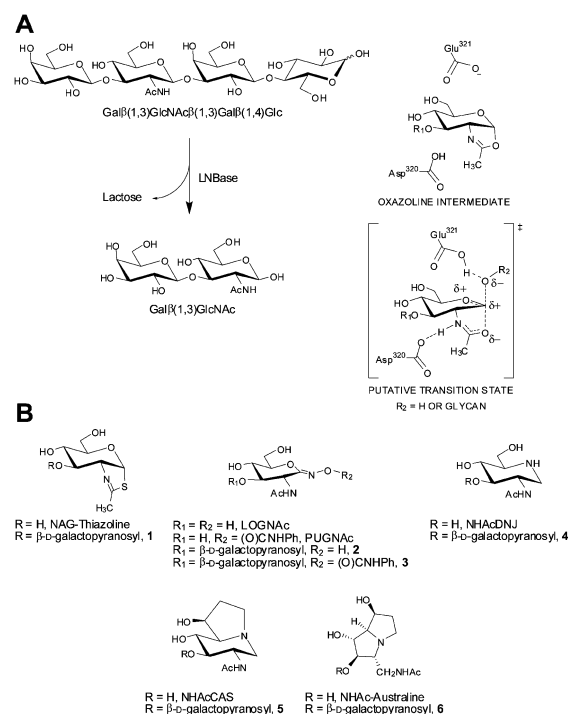


Fig. 1 (A) Reaction catalysed by BbLNBBase with the oxazoline intermediate and putative transition state shown ($R_1 = \beta$ -D-galactopyranose). (B) Inhibitors of other β-*N*-acetylhexosaminidases as well as current and presented inhibitors of LNBBase.

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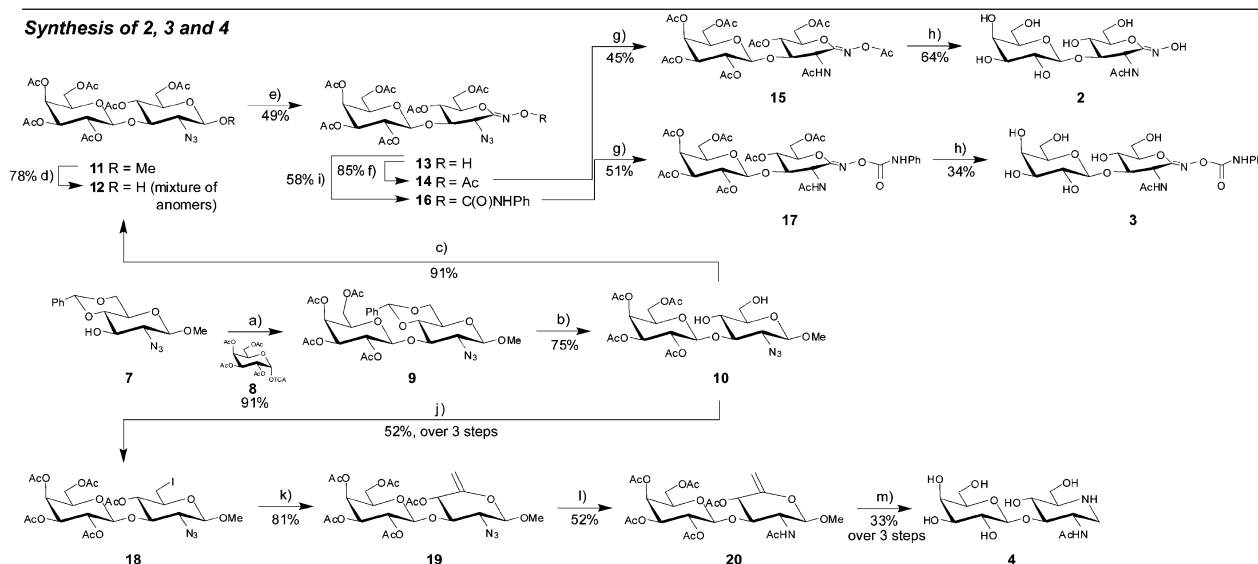
through the synthesis of inhibitors of the respective enzyme being studied. In respect to LNBase, the disaccharide **1** has been prepared,¹⁵ which is based on the known inhibitor NAG-thiazoline,¹⁷ a potent inhibitor of other GH20 *exo*- β -N-acetylhexosaminidases which only cleave monosaccharide GlcNAc (or GalNAc) residues from glycoconjugates. Indeed, the disaccharide **1** was shown to be a potent inhibitor and aided in the confirmation of the catalytic mechanism of LNBase.^{15,16}

Due to the considerable interest in *exo*- β -N-acetylhexosaminidases from GH20 and other GH families, numerous other potent inhibitors have also been synthesized with the most well known of these being the hydroxylactone-based compounds LOGNAc and PUGNAc,¹⁸ the iminosugars NHAcDNJ,¹⁹ and NHAcCAS and an isomer of the latter compound NHAc-australine²⁰ (Fig. 1B). The inhibitory properties of these compounds towards *exo*- β -N-acetylhexosaminidases is thought to come about through the way each of them mimic, either through shape or charge, the putative transition state of the pyranose ring during catalysis (Fig. 1A).²¹ As LNBase are important enzymes in the degradation of HMOs by bifidobacteria and that thiazoline-based inhibitors suffer from instability in solution,²² the development of a repertoire of suitable tools is necessary to study the roles that this

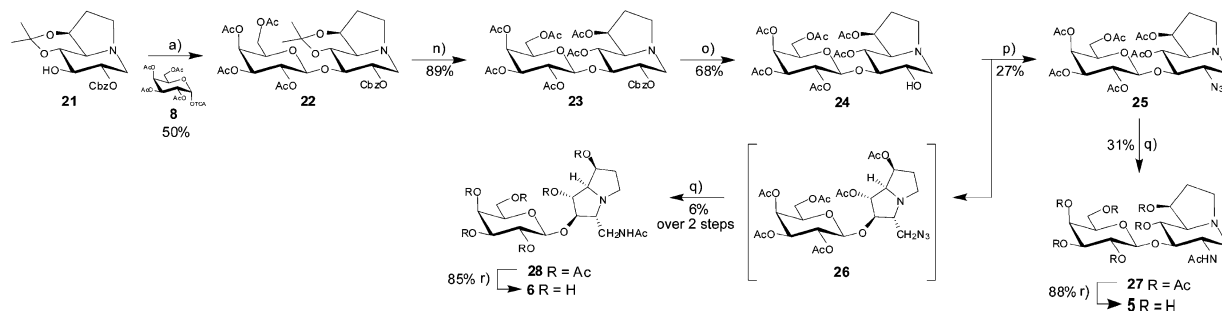
enzyme plays in bacterial growth and establishment within the GI tract. Thus we set about preparing compounds **2–6** which are compounds tailor-made to be potent inhibitors of LNBase.

Compounds **2** and **3** were prepared starting from the alcohol **7**²³ (Scheme 1). TMSOTf-promoted glycosylation with the trichloroacetimidate **8**, gave the disaccharide **9** in good yield. Removal of the benzylidene protecting group gave the diol **10**, which was then converted to the acetate **11**. Of note here is that **10** is a key intermediate to access not only **2** and **3** but also the iminosugar **4**. Treatment of **11** under acetolysis conditions followed by selective removal of the presumed anomeric acetate gave the hemiacetal **12**. This material was then activated with hydroxylamine hydrochloride which yielded the presumed mixture of oximes which was in turn converted to the hydroxylactone **13** in excellent overall yield. For the preparation of **2**, acetylation of **13** gave **14**, and subsequent conversion of the azide to an acetamido group using Pd(OH)₂-mediated hydrogenolysis gave **15**. Global deprotection furnished the desired compound **2** in good overall yield. For **3**, the hydroxylactone **13** was converted to the carbamate **16** and using similar conditions as for the preparation of **15** and **2**, compounds **17** and **3** were prepared respectively.

Synthesis of 2, 3 and 4



Synthesis of 5 and 6



Scheme 1 (a) TMSOTf, 4A sieves, CH₂Cl₂; (b) CH₃COOH : H₂O (4 : 1); (c) Ac₂O, pyr.; (d) i. Ac₂O, H₂SO₄; ii. Aq. MeNH₂, THF; (e) i. NH₂OH·HCl, pyr.; ii. DBU, NCS, CH₂Cl₂; (f) Ac₂O, pyr.; CH₂Cl₂; (g) Pd(OH)₂/C, H₂, Ac₂O, EtOH; (h) NH₃, MeOH; (i) PhNCO, Et₃N, THF; (j) i. TsCl, pyr., CH₂Cl₂; ii. NaI, DMF; iii. Ac₂O, pyr.; (k) DBU, THF; (l) PBu₃, Ac₂O, pyr., THF, H₂O; (m) i. mCPBA, BnOH, CH₂Cl₂; ii. NaOMe, MeOH; iii. NH₄HCOO, Pd(OH)₂/C, H₂, MeOH, H₂O; (n) i. CH₃COOH : H₂O (4 : 1); ii. Ac₂O, pyr.; (o) Pd/C, H₂, MeOH; (p) i. MsCl, pyr.; ii. NaN₃, DMSO; (q) i. Pd/C, H₂, PhMe; ii. Ac₂O, pyr.; (r) NaOMe, MeOH.



The iminosugar **4**, was synthesized from **10** starting with a one-pot activation at O-6, and *in situ* acetylation to give the iodide **18** in excellent yield over three steps. Elimination across C-5/6 was achieved using DBU to give the desired alkene **19**. Treatment of the alkene **19** using Staudinger conditions followed by acetylation gave the amide **20**. Oxidation of **20** with *m*CPBA, followed by deprotection provided the presumed intermediate ulososide which when put under reductive amination conditions in the presence of ammonium formate and hydrogen gratifyingly gave **4**, exclusively as the D-isomer, consistent with previous observations for similar transformations.^{24,25}

The synthesis of the iminosugars **5** and **6** started from the carbamate **21**.²⁶ Again, TMSOTf-promoted glycosylation with the trichloroacetimidate **8**, gave the disaccharide **22** in good yield. Removal of the isopropylidene protecting group and *in situ* acetylation gave **23**. Removal of the Cbz-protecting group yielded the alcohol **24**, which is the critical divergence point to the synthesis of **5** and **6**. Activation of the alcohol on **24** as a presumed mesylate, followed by treatment with sodium azide²⁰ gave the two azides **25** and **26**. For **5**, hydrogenolysis of **25** followed by *in situ* acetylation gave **27**, which after removal of the acetyl protecting groups gave the desired disaccharide **5**. Similar methodologies were used for converting crude **26**, through **28**, to **6**.

With the synthesized set of inhibitors now in hand, we evaluated them against a representative GH20 LNBse found in *Bifidobacterium bifidum* (*Bb*LNBse) and found them all to be potent competitive inhibitors of this enzyme (Table 1). These results demonstrate that these inhibitor scaffolds that have been used previously to inhibit *exo*- β -N-acetyl-hexosaminidases are also useful in inhibiting the disaccharide releasing LNBse-type enzymes. In terms of comparing the potency of the inhibitors **2**–**6** to their monosaccharide derivatives, all the compounds are in good agreement. Typically, PUGNac is a very potent inhibitor of GH20 and GH84 enzymes^{27,28} with LOGNac being less potent.²⁹ The

iminosugars NHAcDNJ and NHAcCAS also have good potency towards enzymes from GH20³⁰ and GH84.^{25,31} NHAc-australine has to date, not been assessed as an inhibitor of *exo*- β -N-acetyl-hexosaminidases.

To gain a more detailed understanding of the molecular basis for the inhibition of *Bb*LNBse co-crystallization trials with **2**–**6** were attempted. We finally determined high resolution (up to 1.60 Å) crystal structures of *Bb*LNBse in complex with compounds **2**, **4**–**6** (Fig. 2) with clear electron densities. Of note is that this is also the first report of an X-ray structure of an *exo*- β -N-acetyl-hexosaminidase in complex with a NHAc-australine-based compound. All of the hydroxyl groups of the inhibitors **2**, **4**–**6** form hydrogen bonds with the surrounding amino acids. Previously it has been established that the amino acid D467 plays a crucial role in recognizing the pyranose ring of LNB and **1** at the –1 subsite, forming bidentate hydrogen bonds with the C4 and C6 hydroxyl groups.¹⁶ In concert with these observations, the bidentate hydrogen bonds from D467 are present in all complex structures, even in **5** and **6**, which have an unusual bicyclic group in the –1 subsite. The hydroxyl group present at the C1 position of the iminosugar in **5** is located at the most appropriate position to form a hydrogen bond whereas the corresponding hydroxyl group in **6** is not ideally positioned. This difference in the geometry of this hydrogen bond is likely a source of the difference in the K_i values obtained for **5** and **6**.

For the hydroxylactone-based compound **2**, similar features are observed with an additional hydrogen bond also being formed between the nitrogen atom of the hydroximo group and E321 (catalytic acid/base). Due to lack of success in obtaining *Bb*LNBse in complex with **3**, a docking analysis was undertaken in an effort to gain insight into the molecular basis for inhibition. Using Autodock Vina,³² a good match was obtained for the binding of **3** with *Bb*LNBse, using the protein structure of *Bb*LNBse–**2** complex as a receptor, with the affinity determined to be -8.7 kcal mol⁻¹ which is in good agreement with the K_i (see figure in ESI†). In addition to similar binding features observed for **2**, **4**–**6** the docking analysis also revealed a potential extra hydrogen bond between the hydroxylactone oxygen of **3** and Y427. Interestingly the docking analysis also revealed that the phenyl ring is positioned in a hydrophobic pocket of *Bb*LNBse surrounded by A424 and V426 which lies ahead of the hydrophobic cage commonly found in GH20 *exo*- β -N-acetyl-hexosaminidases,³³ and this likely also adds to the increased potency of **3** versus **2**.

Table 1 Inhibition constants of inhibitors for the *Bb*LNBse

Compound	K_i (μ M)
1	0.125 ± 0.008^{15}
2	7.7 ± 0.1
3	0.091 ± 0.003
4	0.88 ± 0.012
5	0.52 ± 0.007
6	52 ± 2

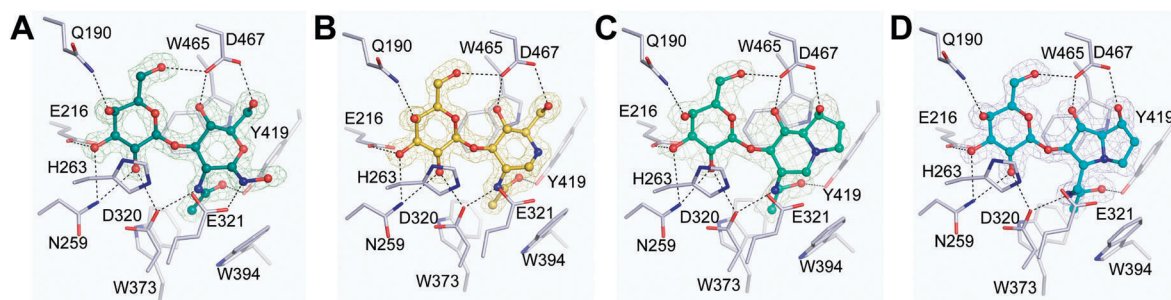


Fig. 2 Active site structures of *Bb*LNBse in complex with **2** (A), **4** (B), **5** (C), and **6** (D). $|F_o| - |F_c|$ omit electron density maps (mesh, 1.5σ) and hydrogen bonds (dashed lines) are shown with a hydrogen bond cut off distance of 3.2 Å used.



One method that can lead to inhibition of glycosidases is to mimic the conformation of the pyranose ring at the intermediate and transition states of catalysis (Fig. 1A).²¹ Thus we analysed the conformations of the pyranose ring containing inhibitors **2**, **4** and **5** in detail using the Cremer–Pople system which is used to determine the conformation of six-membered rings (see table in ESI†).³⁴ The inhibitor **2** is in a typical ⁴E conformation ($244^\circ < \varphi < 247^\circ$ and $59^\circ < \theta < 65^\circ$), similar to what is observed for the binding of corresponding inhibitors with GH20³⁵ and GH84 enzymes.³⁶ This conformation is also close to the putative transition states in the proposed conformational itinerary of the pyranose ring for BbLNBase¹⁶ and other enzymes that utilize a substrate-assisted catalytic mechanism.^{27,33,37} In contrast, the iminosugars **4** and **5** adopt a ^{1,4}B conformation ($234^\circ < \varphi < 246^\circ$ and $75^\circ < \theta < 85^\circ$). Based on these values it seems that both these compounds mimic somewhat the conformation that has been observed for Michaelis-like complex structures of GH20 *exo*- β -N-acetylhexosaminidases.^{38,39} These results give further credence to the proposed reaction pathway of BbLNBase: ^{1,4}B (Michaelis complex)–⁴E (transition state)–⁴C₁ (oxazoline intermediate)–⁴E (transition state)–⁴E (product complex).¹⁶

In conclusion the study of LNBase is critical to understanding how Bifidobacteria degrade HMOs and thus occupy a niche in the GI tract. The inhibitors prepared here are all potent inhibitors of BbLNBase and, through structural analysis, reasons for their potency are presented. Further detailed structural analysis of BbLNBase in complex with inhibitors synthesized by rational design will facilitate the development of more potent and stable inhibitors of LNBase. Additionally, these compounds will also prove useful for studying the roles that this enzyme plays in the bifidobacteria life cycle, HMO degradation and other biological pathways.⁴⁰

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Notes and references

‡ A LNBase from *B. longum* is currently non-classified.

- 1 I. Cho and M. J. Blaser, *Nat. Rev. Genet.*, 2012, **13**, 260–270.
- 2 T. Katayama, K. Fujita and K. Yamamoto, *J. Biosci. Bioeng.*, 2005, **99**, 457–465.
- 3 F. Turrone, F. Bottacini, E. Foroni, I. Mulder, J. H. Kim, A. Zomer, B. Sánchez, A. Bidossi, A. Ferrarini, V. Giubellini and M. Delledonne, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 19514–19519.
- 4 N. Iwabuchi, N. Takahashi, J. Z. Xiao, K. Miyaji and K. Iwatsuki, *Microbiol. Immunol.*, 2007, **51**, 649–660.
- 5 B. Sánchez, L. Ruiz, M. Gueimonde, P. Ruas-Madiedo and A. Margolles, *Pharmacol. Res.*, 2013, **69**, 127–136.
- 6 K. Pokusaeva, G. F. Fitzgerald and D. van Sinderen, *Genes Nutr.*, 2011, **6**, 285–306.
- 7 A. Marcobal and J. Sonnenburg, *Clin. Microbiol. Infect.*, 2012, **18**, 12–15.
- 8 C. Kunz, S. Rudloff, W. Baier, N. Klein and S. Strobel, *Annu. Rev. Nutr.*, 2000, **20**, 699–722.
- 9 T. Urashima, T. Saito, T. Nakamura and M. Messer, *Glycoconjugate J.*, 2001, **18**, 357–371.
- 10 R. G. LoCascio, M. R. Ninonuevo, S. L. Freeman, D. A. Sela, R. Grimm, C. B. Lebrilla, D. A. Mills and J. B. German, *J. Agric. Food Chem.*, 2007, **55**, 8914–8919.
- 11 M. Kitaoka, J. Tian and M. Nishimoto, *Appl. Environ. Microbiol.*, 2005, **71**, 3158–3162.
- 12 S. Asakuma, E. Hatakeyama, T. Urashima, E. Yoshida, T. Katayama, K. Yamamoto, H. Kumagai, H. Ashida, J. Hirose and M. Kitaoka, *J. Biol. Chem.*, 2011, **286**, 34583–34592.
- 13 J. Wada, T. Ando, M. Kiyohara, H. Ashida, M. Kitaoka, M. Yamaguchi, H. Kumagai, T. Katayama and K. Yamamoto, *Appl. Environ. Microbiol.*, 2008, **74**, 3996–4004.
- 14 S. Asakuma, T. Urashima, M. Akahori, H. Obayashi, T. Nakamura, K. Kimura, Y. Watanabe, I. Arai and Y. Sana, *Eur. J. Clin. Nutr.*, 2008, **62**, 488–494.
- 15 M. Hattie, A. W. Debowski and K. A. Stubbs, *ChemBioChem*, 2012, **13**, 1128–1131.
- 16 T. Ito, T. Katayama, M. Hattie, H. Sakurama, J. Wada, R. Suzuki, H. Ashida, T. Wakagi, K. Yamamoto, K. A. Stubbs and S. Fushinobu, *J. Biol. Chem.*, 2013, **288**, 11795–11806.
- 17 S. Knapp, D. Vocadlo, Z. N. Gao, B. Kirk, J. P. Lou and S. G. Withers, *J. Am. Chem. Soc.*, 1996, **118**, 6804–6805.
- 18 D. Beer, J. L. Maloisel, D. M. Rast and A. Vasella, *Helv. Chim. Acta*, 1990, **73**, 1918–1922.
- 19 E. Kappes and G. Legler, *J. Carbohydr. Chem.*, 1989, **8**, 371–388.
- 20 R. H. Furneaux, G. J. Gainsford, J. M. Mason and P. C. Tyler, *Tetrahedron*, 1994, **50**, 2131–2160.
- 21 D. J. Vocadlo and G. J. Davies, *Curr. Opin. Chem. Biol.*, 2008, **12**, 539–555.
- 22 S. A. Yuzwa, M. S. Macauley, J. E. Heinonen, X. Shan, R. J. Dennis, Y. He, G. E. Whitworth, K. A. Stubbs, E. J. McEachern, G. J. Davies and D. J. Vocadlo, *Nat. Chem. Biol.*, 2008, **4**, 483–490.
- 23 B. K. S. Yeung, D. C. Hill, M. Janicka and P. A. Petillo, *Org. Lett.*, 2000, **2**, 1279–1282.
- 24 A. J. Steiner, G. Schitter, A. E. Stutz, T. M. Wronnigg, C. A. Tarling, S. G. Withers, D. J. Mahuran and M. J. Tropak, *Tetrahedron: Asymmetry*, 2009, **20**, 832–835.
- 25 K. A. Stubbs, J. P. Bacic, G. E. Perley-Robertson, G. E. Whitworth, T. M. Gloster, D. J. Vocadlo and B. L. Mark, *ChemBioChem*, 2013, **14**, 1973–1981.
- 26 P. S. Liu, M. S. Kang and P. S. Sunkara, *Tetrahedron Lett.*, 1991, **32**, 719–720.
- 27 M. S. Macauley, G. E. Whitworth, A. W. Debowski, D. Chin and D. J. Vocadlo, *J. Biol. Chem.*, 2005, **280**, 25313–25322.
- 28 K. A. Stubbs, M. S. Macauley and D. J. Vocadlo, *Angew. Chem., Int. Ed.*, 2009, **48**, 1300–1303.
- 29 K. A. Stubbs, M. Balcewich, B. L. Mark and D. J. Vocadlo, *J. Biol. Chem.*, 2007, **282**, 21382–21391.
- 30 M. B. Tropak, S. P. Reid, M. Guiral, S. G. Withers and D. Mahuran, *J. Biol. Chem.*, 2004, **279**, 13478–13487.
- 31 M. S. Macauley, Y. He, T. M. Gloster, K. A. Stubbs, G. J. Davies and D. J. Vocadlo, *Chem. Biol.*, 2010, **17**, 937–948.
- 32 O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455–461.
- 33 B. L. Mark, D. J. Vocadlo, S. Knapp, B. L. Triggs-Raine, S. G. Withers and M. N. James, *J. Biol. Chem.*, 2001, **276**, 10330–10337.
- 34 D. Cremer and J. A. Pople, *J. Am. Chem. Soc.*, 1975, **97**, 1354–1358.
- 35 T. Liu, H. Zhang, H. Liu, L. Chen, X. Shen and Q. Yang, *Biochem. J.*, 2011, **438**, 467–474.
- 36 F. V. Rao, H. C. Dorfmueller, F. Villa, M. Allwood, I. M. Eggleston and D. M. van Aalten, *EMBO J.*, 2006, **25**, 1569–1578.
- 37 Y. He, M. S. Macauley, K. A. Stubbs, D. J. Vocadlo and G. J. Davies, *J. Am. Chem. Soc.*, 2010, **132**, 1807–1809.
- 38 I. Tews, A. Perrakis, A. Oppenheim, Z. Dauter, K. S. Wilson and C. E. Vorgias, *Nat. Struct. Biol.*, 1996, **3**, 638–648.
- 39 G. Prag, Y. Papanikolaou, G. Tavlas, C. E. Vorgias, K. Petratos and A. B. Oppenheim, *J. Mol. Biol.*, 2000, **300**, 611–617.
- 40 A. Gotoh, T. Katoh, Y. Sugiyama, S. Kurihara, Y. Honda, H. Sakurama, T. Kambe, H. Ashida, M. Kitaoka, K. Yamamoto and T. Katayama, *Carbohydr. Res.*, 2015, **408**, 18–24.

