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Unexpected anomeric acceptor preference observed using dDNP NMR for transglycosylation studies of β -galactosidases

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Supporting Information Placeholder

ABSTRACT: The transglycosylation abilities of β -galactosidases were investigated using hyperpolarized [U-¹³C,U-²H]glucose as acceptor and *o*-nitrophenyl β -galactopyranoside as donor. Several products were readily observable, and at least in the case when O3 acted as acceptor, the enzymes showed a clear selectivity towards the β -anomer of glucose. Additionally, it was possible to determine the relative hydrolysis rates of the formed transglycosylation products, providing information of the selectivity as well. Using this method, the transglycosylation abilities of the enzymes could be studied at a very high temporal resolution as well as with high sensitivity, and due to the relative ease of the setup, this method could be more generally applied to investigate glycosidases.

Introduction

Improving enzymatic production of oligosaccharides is a promising path towards greener and cheaper production of a wide array of carbohydrate products^{1–3}. Glycosidases are a class of enzymes that primarily hydrolyze carbohydrates. After breaking the glycosidic linkage, a water molecule will act as a nucleophile, but not only water can act as acceptor, and new glycosidic linkages can be formed if a carbohydrate is present at the active site in place of water⁴. β -galactosidases (EC 3.2.1.23) of glycoside hydrolase family 2 (GH2) are a promising tool to produce galactooligosaccharides, which can have a wide array of applications including prebiotics. As such, the increased understanding of the transglycosylation abilities of this class of enzymes is an important step towards improving the quality and production of galactooligosaccharides.

Traditionally, NMR spectroscopy is used to study enzyme mechanisms in real time by analyzing reactions at equilibrium or for competition studies^{5,6}. However, due to the limited sensitivity of NMR, sparsely populated or short lived species are difficult to observe in real time experiments, and often products need to be produced in larger scale than what is possible in NMR tubes and purified to be analyzed using signal averaging and longer acquisition times^{7,8}. Using dissolution dynamic nuclear polarization (dDNP), it is possible to increase the signal to noise ratio (SNR) of single scan NMR spectroscopy by up to four orders of magnitude⁹. Utilizing this method, the mechanisms and capabilities of chemical^{10–12} as well as enzymatic^{13–15} reactions can be investigated with high sensitivity and temporal resolution. Recent advances in ultrafast NMR have improved the potential for monitoring reactions^{16,17}, but lacks the sensitivity gained by hyperpolarization. When coupled with dDNP, ultrafast NMR shows great promise for analyzing complex

mixtures, for example for metabolic studies where the temporal information is not required¹⁸.

Previously, the *E. coli lacZ* β -galactosidase was investigated using dDNP NMR with hyperpolarized *o*-nitrophenyl [1-¹³C;1-²H]galactopyranoside as substrate for the enzyme¹⁴. Using galactose as transglycosylation acceptor for the reaction, several previously undescribed transglycosylation products were observed, and they were determined to be better substrates than the expected 1,6-linked product. In particular the β , β -1,1 linked non-reducing galactodisaccharide produced by the enzyme was a much better substrate than the other transglycosylation products formed during the reaction, explaining its transient nature and why it might be very difficult to observe using other methods.

In this study, we expanded the method to be more generally applicable using commercially available [U-¹³C;U-²H]glucose as acceptor for the reaction. This is an established hyperpolarization probe for dDNP NMR studies varying from metal catalyzed reactions¹¹ to living cells^{19,20} as well as in dDNP MRI studies²¹.

[U-¹³C;U-²H]glucose would show the aglycon side of the glycosidic linkage formed in the transglycosylation process, and does not require substrate synthesis using expensive isotopically labelled starting materials. The *E. coli lacZ* β -galactosidase was used as model enzyme, as it is well described in literature and is often used as model enzyme²². The GH2 enzymes have retaining double displacement mechanisms, and their natural substrate is likely lactose, but *o*-nitrophenyl β -D-galactopyranoside (onp-Galp) is often used as model substrate²². Additionally, the commercially available β -galactosidase mixture Lactozyme 2600L, which primarily contains β -galactosidases from *Kluyveromyces lactis* and *Kluyveromyces fragilis*, was also used, as it is known to make a more varied array of transglycosylation products⁷.

Experimental

[U-¹³C,U-²H]glucose (Sigma Aldrich) was prepared for polarization by dissolving it in a 1:1 mixture of glycerol/water (2 M glucose concentration). Subsequently, AH111501 trityl radical and gadoteridol was added, in amounts corresponding to concentrations of 20 mM and 17 mM, respectively. 35–50 μ L of this mixture was polarized for at least 90 min in a HyperSense (Oxford Instruments) operating at 3.35 T and <1.4 K with a 94 GHz microwave source. Dissolution was carried out using 5.0 mL of 180 °C pH 7.4 phosphate buffer (50 mM), mixed with onp-Galp (36 mM final concentration) and 0.5 μ L of the resulting mixture was transferred to the NMR tube containing the enzyme (50 U, Sigma Aldrich), which was already placed in the magnet. NMR acquisition was performed at 9.4 T (DirectDrive console, Agilent, 100.5 MHz for ¹³C) at 310

K, and was started immediately after transfer. 30° flip angles every two seconds were used, and 50000 points obtained. In the first scan, an approximate SNR increase of 28500-32500 was observed, corresponding to approximately 23-26% polarization (figure S1). The data was acquired using VnmrJ 4.2 (Agilent) and was processed using TopSpin 4.0 (Bruker) and Matlab R2018b (MathWorks). The hydrolysis rates of the transglycosylation products were determined by fitting them to the decline in integrals for the corresponding signals, similar to how hydrolysis rates have been determined for the enzyme previously¹⁴, with the polarization loss due to T₁ relaxation and usage by flip angle pulsing being constrained within 10% of the corresponding signals from the free glucose, which was measured after no other signals remained. The experiment without enzyme nor donor was carried out in a similar fashion, albeit without mixing the dissolved hyperpolarized [U-¹³C, U-²H]glucose with donor and with no enzyme being present in the NMR tube.

The thermal NMR experiments were carried out by heating a sample containing 100 mM glucose and 35 mM onp-Galp in 90% of pH 7.4 phosphate buffer (50 mM) and 10% of D₂O to 310 K in the magnet. After the sample was stable, 20 µL of Lactozyme 2600L diluted 20 times in the phosphate buffer (2.6 U) was added, and the sample was quickly reshimmied and acquisition started approximately 2 min after the addition of enzyme. The HSQC data were acquired using standard Bruker pulse sequences, and the HSQC series had a repetition time of 8 min and each spectrum was recorded using 8 scans, 32 F1 increments with an F1 spectral width of 38 ppm and the transmitter offset set to 72 ppm. The data was obtained on a Bruker Avance III (799.90 MHz for ¹H and 201.15 MHz for ¹³C) equipped with a 5 mm TCI ¹H/(¹³C, ¹⁵N) cryoprobe. The data was acquired using TopSpin 3.5 (Bruker) and was processed using TopSpin 4.0 (Bruker).

Results and discussion

Using hyperpolarized [U-¹³C; U-²H]glucose, polarized to approximately 25% after dissolution and transfer, as acceptor and onp-Galp as donor, high formation of 6-substituted glucose (6-Glc, allolactose, labelled **A** in figure 2 and scheme 1) was observed, which is the expected product^{22,23}, and another two signals not originating from the free glucose arose, see figure 1 and 2. Based on chemical shift (table S1), they were assigned as arising from 4-substituted glucose (4-Glc, lactose, labelled **B** in figure 2 and scheme 1) and 3-substituted β-glucose (3-β-Glc, labelled **C** in figure 2 and scheme 1). Curiously, there did not seem to be any direct formation of 3-substituted α-glucose, although very small amounts are observed over time due to mutarotation. Whether this is also the case for the 4-position or 6-position is difficult to determine using NMR, as those positions have very similar chemical shift in their α- and β-configurations. According to literature, the transglycosylation abilities of the enzyme is described as being capable of producing allolactose using glucose as acceptor^{3,22–24}. This was also the major product, as shown in table 1. It should be noted that the 6-position has a shorter T₁ relaxation time constant of approximately 10 s at 9.4 T compared to the remaining positions at around 12 s, due to it having two deuterons contributing to the relaxation²⁰. Consequently, the integrals used in table 1 have been corrected according to the observed T₁ of the corresponding carbon, and are only based on initial spectra where the onp-Galp concentration is still much higher than the formed transglycosylation products. Compared to using galactose as an acceptor¹⁴, there was no apparent signal corresponding to a 1-linked non-reducing glucose, which would be expected to have an anomeric signal around 100 ppm for the β-anomer and around 101 ppm for the α-anomer^{8,14,25}. It is possible that it is very sparsely populated and overlaps with one of the signals arising from a glucofuranoside. To better visualize the

transglycosylation products, the sums of spectra 3-10 is shown in figure 2, and the reaction is schematized in scheme 1.

Figure 1. Top spectrum: Hyperpolarized [U-¹³C; U-²H]glucose with no enzyme nor donor. Bottom 10 spectra: First 20 seconds of hyperpolarized [U-¹³C; U-²H]glucose mixed with onp-Galp and *lacZ* β-galactosidase, starting from the bottom. Each spectrum is labelled with the time in seconds after injection that it was acquired. The signal labelled as **A** corresponds to the 6 position in 6-substituted glucose, **B** to the 4 position in 4-substituted glucose and **C** to the 3 position in 3-substituted β-glucose.

Figure 2. Sums of spectra 3-10 (4-18 seconds after transfer to NMR tube) zoomed to the areas containing transglycosylation products. Top: Hyperpolarized [$U\text{-}^{13}\text{C};U\text{-}^2\text{H}$]glucose with no enzyme nor donor. Middle spectrum: Hyperpolarized [$U\text{-}^{13}\text{C};U\text{-}^2\text{H}$]glucose mixed with onp-Galp and Lactozyme 2600L. Bottom spectrum: Hyperpolarized [$U\text{-}^{13}\text{C};U\text{-}^2\text{H}$]glucose mixed with onp-Galp and *lacZ* β -galactosidase. The signal labelled as **A** corresponds to the 6 position in 6-substituted glucose, **B** to the 4 position in 4-substituted glucose and **C** to the 3 position in 3-substituted β -glucose.

Table 1. Relative transglycosylation product formation using glucose as acceptor and onp-Galp as donor.

Enzyme \ substitution	6-Glc	4-Glc	3- β -Glc
<i>LacZ</i> β -galactosidase	95%	1%	4%
Lactozyme 2600L	61%	17%	22%

Scheme 1. Overview of the enzymatic reaction using onp-Galp as substrate and [$U\text{-}^{13}\text{C};U\text{-}^1\text{H}$]glucose (shown in red) as transglycosylation acceptor. In the schematic, the glucose is shown without isotopic labels for simplicity. A is allolactose, B is lactose and C is β -Galp-1,3- β -Glep.

To investigate the apparent lack of 3-substituted α -glucose, the enzyme mixture Lactozyme 2600L, which is a reported to make 1,3-linked transglycosylation products⁷, was also used, see figure 2, 3 and S3. As expected, the signal corresponding to the 3-position in 3-substituted β -glucose increased quite a lot compared to when using the *lacZ* enzyme, as seen in figure 2 and figure 3. Similarly, the peak corresponding to the 4-position in 4-substituted glucose also increased in intensity, while the peak corresponding to the 6-position in 6-substituted glucose decreased by comparison. These results are collected in table 1, where the relative formation of each product corrected for differences in T_1 relaxation is shown. Once

again, the lack of 3-substituted α -glucose was observed, which would suggest that the enzymes prefer using β -glucose over α -glucose as acceptor, at least when the 3-position (O3) acts as the acceptor. A similar observation have previously been made for *lacZ* when using O6 as acceptor at 0 °C, where β -glucose was also found to be preferred²⁶. This would suggest that perhaps it is more common than previously described that glycosidases will distinguish between anomeric configurations of the acceptors during transglycosylation. Anomeric preference has also been observed when using allolactose and lactose as substrate for the enzyme, in which it was observed that the α -anomer of the substrates was hydrolyzed

up to two times more rapidly, as the release of α -glucose was faster than of β -glucose^{26,27}. Perhaps this apparent higher hydrolysis rate of the α -allolactose was because the released α -glucose is less likely to act as acceptor for transglycosylation, thus more α -glucose would be released compared to β -glucose. The biological importance of the anomeric preference is unclear.

Figure 3. Top spectrum: Hyperpolarized [U - ^{13}C ; U - ^2H]glucose with no enzyme nor donor. Bottom 10 spectra: First 20 s of hyperpolarized [U - ^{13}C ; U - ^2H]glucose mixed with onp-Galp and Lactozyme 2600L, starting from the bottom. Each spectrum is labelled with the time in seconds after injection that it was acquired. The signal labelled as **A** corresponds to the 6 position in 6-substituted glucose, **B** to the 4 position in 4-substituted glucose and **C** to the 3 position in 3-substituted β -glucose.

Neither the *lacZ* β -galactosidase nor the Lactozyme 2600L enzyme mixture produced any detectable amounts of 1- or 2-substituted glucose. By fitting the hydrolysis rates and polarization relaxation to the integrals of the signals corresponding to each of the three transglycosylation products, shown in figure 4, it was found that allolactose was the preferred substrate for Lactozyme 2600L, having a hydrolysis rate approximately 40% and 60% faster than those of 3- and 4-substituted glucose, respectively. For *lacZ* β -galactosidase it was more difficult to determine, but it would seem that the 1,6- and 1,4-linked products had similar hydrolysis rates, which was slightly faster than that of the 1,3-linked product. According to literature, for *lacZ* β -galactosidase allolactose should be as good a substrate as lactose²⁷, which agrees well with the observed rates. For both enzymes, the total amount of transglycosylation product formed using hyperpolarized glucose as acceptor corresponded to approximately 5% of the added glucose.

Figure 4. Integrals of the three transglycosylation products by Lactozyme 2600L with fits for the decline of the integrals.

Additionally, the lack of formation of the 3-substituted α -glucose was further investigated using traditional NMR spectroscopy, but due to spectral overlap it was not possible to distinguish the ^1H NMR signals arising from transglycosylation products from the starting materials. To overcome this difficulty, heteronuclear single quantum coherence (HSQC) spectroscopy was utilized, and by using a narrow spectral width in the ^{13}C dimension accompanied with few increments and a low number of scans, it was possible to obtain a spectrum of the reaction mixture before the equilibrium of mutarotation had been reached using Lactozyme 2600L with natural abundance glucose as acceptor and onp-Galp as donor. At the time of the first HSQC (average of 2–10 min after addition of enzyme), the ratio between 3-substituted β - and α -glucose was approximately 8:2, whereas at equilibrium it is 58:42, shown in figure S2. These results supported the observation made using dDNP NMR, that O3 of β -glucose is a superior substrate to O3 of α -glucose. However, due to the low temporal resolution of 8 min compared to the 2 s in the dDNP NMR experiment, the formation rates are harder to determine as mutarotation has a much larger impact on this time scale.

Conclusion

Using dDNP NMR to investigate the transglycosylation abilities of these β -galactosidases clearly showed the possible transglycosylation products when using glucose as acceptor as well as relative ratios between them. Furthermore, when using O3 of glucose as acceptor for transglycosylation, the enzymes preferred the β - over α -glucose as at least 25 times more β -glucose acted as acceptor, despite there being a 6:4 ratio between the free glucopyranoses. Additionally, despite the *lacZ* β -galactosidase being described as capable of forming allolactose^{22–24}, it was found to also being capable of forming lactose and β -Galp-1,3- β -Glc. And lastly, it was possible to determine the relative formation ratios between the three products, as well as the hydrolysis rates for the Lactozyme 2600L enzyme mixture, where it was found that allolactose was the preferred substrate, as well as for the *lacZ* β -galactosidase, where the allolactose and lactose had similar hydrolysis rates. As all the materials used here were commercially available, any glycosidase where the transglycosylation onto glucose is of interest could be

investigated using this method. Finally, considering the difficulty of observing the β -anomeric preference when using O3 as donor with traditional ^1H NMR, even when optimizing for it, this also further highlights the potential of dDNP NMR for reaction monitoring.

ASSOCIATED CONTENT

Supporting Information

Contains a figure with two overlaid HSQC spectra, one where the reaction has been quenched at approximately 70% conversion, and one obtained during the reaction as well as a figure showing the polarization of the first scan. Also contains a figure with label assignments of all peaks and a table with ^{13}C chemical shift assignments of the glucose in the different species discussed. The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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REFERENCES

- Moreno, F. J.; Corzo, N.; Montilla, A.; Villamiel, M.; Olano, A. Current State and Latest Advances in the Concept, Production and Functionality of Prebiotic Oligosaccharides. *Curr. Opin. Food Sci.* **2017**, *13*, 50–55. <https://doi.org/10.1016/j.cofs.2017.02.009>.
- Mano, M. C. R.; Neri-Numa, I. A.; da Silva, J. B.; Paulino, B. N.; Pessoa, M. G.; Pastore, G. M. Oligosaccharide Biotechnology: An Approach of Prebiotic Revolution on the Industry. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 17–37. <https://doi.org/10.1007/s00253-017-8564-2>.
- Lu, L.; Guo, L.; Wang, K.; Liu, Y.; Xiao, M. β -Galactosidases: A Great Tool for Synthesizing Galactose-Containing Carbohydrates. *Biotechnol. Adv.* **2020**, *39* (March–April), 107465. <https://doi.org/10.1016/j.biotechadv.2019.107465>.
- Raich, L.; Borodkin, V.; Fang, W.; Castro-López, J.; van Aalten, D. M. F.; Hurtado-Guerrero, R.; Rovira, C. A Trapped Covalent Intermediate of a Glycoside Hydrolase on the Pathway to Transglycosylation. Insights from Experiments and QM/MM Simulations. *J. Am. Chem. Soc.* **2016**, *138* (19), 3325–3332. <https://doi.org/10.1021/jacs.5b10092>.
- Robillard, G.; Shaw, E.; Shulman, R. G. ^{13}C High-Resolution Nuclear Magnetic Resonance Studies of Enzyme-Substrate Reactions at Equilibrium. Substrate Strain Studies of Chymotrypsin-N-Acetyltyrosine Semicarbazide Complexes. *Proc Natl Acad Sci USA* **1974**, *71* (7), 2623–2626.
- Withers, S. G.; Dombroski, D.; Berven, L. A.; Kilburn, D. G.; Miller, R. C.; J. Warren, R. A.; Gilkes, N. R. Direct ^1H N.M.R. Determination of the Stereochemical Course of Hydrolyses Catalysed by Glucanase Components of the Cellulase Complex. *Biochem. Biophys. Res. Commun.* **1986**, *139* (2), 487–494. [https://doi.org/10.1016/S0006-291X\(86\)80017-1](https://doi.org/10.1016/S0006-291X(86)80017-1).
- Martínez-Villaluenga, C.; Cardelle-Cobas, A.; Corzo, N.; Olano, A.; Villamiel, M. Optimization of Conditions for Galactooligosaccharide Synthesis during Lactose Hydrolysis by β -Galactosidase from *Kluyveromyces Lactis* (Lactozym 3000 L HP G). *Food Chem.* **2008**, *107* (1), 258–264. <https://doi.org/10.1016/j.foodchem.2007.08.011>.
- Visnapuu, T.; Teze, D.; Kjeldsen, C.; Lie, A.; Duus, J. Ø.; André-Miral, C.; Pedersen, L. H.; Stougaard, P.; Svensson, B. Identification and Characterization of a β -N-Acetylhexosaminidase with a Biosynthetic Activity from the Marine Bacterium *Paraglacicola Hydrolytica* S66T. *Int. J. Mol. Sci.* **2020**, *21* (2), 417. <https://doi.org/10.3390/ijms21020417>.
- Ardenkjær-Larsen, J. H.; Fridlund, B.; Gram, A.; Hansson, G.; Hansson, L.; Lerche, M. H.; Servin, R.; Thaning, M.; Golman, K. Increase in Signal-to-Noise Ratio of > 10,000 Times in Liquid-State NMR. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100* (18), 10158–10163. <https://doi.org/10.1073/pnas.1733835100>.
- Lee, Y.; Heo, G. S.; Zeng, H.; Wooley, K. L.; Hilty, C. Detection of Living Anionic Species in Polymerization Reactions Using Hyperpolarized NMR. *J. Am. Chem. Soc.* **2013**, *135* (12), 4636–4639. <https://doi.org/10.1021/ja4001008>.
- Meier, S.; Karlsson, M.; Jensen, P. R. Detecting Elusive Intermediates in Carbohydrate Conversion: A Dynamic Ensemble of Acyclic Glucose–Catalyst Complexes. *ACS Sustain. Chem. Eng.* **2017**, *5* (6), 5571–5577. <https://doi.org/10.1021/acssuschemeng.7b00985>.
- Boeg, P. A.; Duus, J. Ø.; Ardenkjær-Larsen, J. H.; Karlsson, M.; Mossin, S. Real-Time Detection of Intermediates in Rhodium-Catalyzed Hydrogenation of Alkynes and Alkenes by Dissolution DNP. *J. Phys. Chem. C* **2019**, *123* (15), 9949–9956. <https://doi.org/10.1021/acs.jpcc.9b01376>.
- Jensen, P. R.; Meier, S.; Ardenkjær-Larsen, J. H.; Duus, J. Ø.; Karlsson, M.; Lerche, M. H. Detection of Low-Populated Reaction Intermediates with Hyperpolarized NMR. *Chem. Commun. (Camb.)* **2009**, No. 34, 5168–5170. <https://doi.org/10.1039/b910626j>.
- Kjeldsen, C.; Ardenkjær-Larsen, J. H.; Duus, J. Ø. Discovery of Intermediates of LacZ β -Galactosidase Catalyzed Hydrolysis Using DDNP NMR. *J. Am. Chem. Soc.* **2018**, *140* (8), 3030–3034. <https://doi.org/10.1021/jacs.7b13358>.
- Sapir, G.; Harris, T.; Uppala, S.; Nardi-Schreiber, A.; Sosna, J.; Gomori, J. M.; Katz-Brull, R. [$^{13}\text{C}_6\text{D}_8$]2-Deoxyglucose Phosphorylation by Hexokinase Shows Selectivity for the β -Anomer. *Sci. Rep.* **2019**, *9* (1), 1–14. <https://doi.org/10.1038/s41598-019-56063-0>.
- Queiroz, L. H. K.; Giraudeau, P.; Dos Santos, F. A. B.; De Oliveira, K. T.; Ferreira, A. G. Real-Time Mechanistic Monitoring of an Acetal Hydrolysis Using Ultrafast 2D NMR. *Magn. Reson. Chem.* **2012**, *50* (7), 496–501. <https://doi.org/10.1002/mrc.3827>.
- Giraudeau, P.; Frydman, L. Single-Scan 2D NMR: An Emerging Tool in Analytical Spectroscopy. *Annu Rev Anal Chem (Palo Alto Calif)* **2014**, *7*, 129–161. <https://doi.org/10.1146/annurev-anchem-071213-020208>. Single-scan.
- Frydman, L.; Blazina, D. Ultrafast Two-Dimensional Nuclear Magnetic Resonance Spectroscopy of Hyperpolarized Solutions. *Nat. Phys.* **2007**, *3* (6), 415–419. <https://doi.org/10.1038/nphys597>.
- Meier, S.; Karlsson, M.; Jensen, P. R.; Lerche, M. H.; Duus, J. Ø. Metabolic Pathway Visualization in Living Yeast by DNP-NMR. *Mol. Biosyst.* **2011**, *7* (10), 2834–2836. <https://doi.org/10.1039/c1mb05202k>.
- Meier, S.; Jensen, P. R.; Duus, J. Ø. Real-Time Detection of Central Carbon Metabolism in Living *Escherichia Coli* and Its Response to Perturbations. *FEBS Lett.* **2011**, *585* (19), 3133–3138. <https://doi.org/10.1016/j.febslet.2011.08.049>.
- Rodrigues, T. B.; Serrao, E. M.; Kennedy, B. W. C.; Hu, D.-E.; Kettunen, M. I.; Brindle, K. M. Magnetic Resonance Imaging of Tumor Glycolysis Using Hyperpolarized ^{13}C -Labeled Glucose. *Nat. Med.* **2014**, *20* (1), 93–97. <https://doi.org/10.1038/nm.3416>.
- Juergens, D. H.; Matthews, B. W.; Huber, R. E. LacZ β -Galactosidase: Structure and Function of an Enzyme of Historical and Molecular Biological Importance. *Protein Sci.* **2012**, *21* (12), 1792–1807. <https://doi.org/10.1002/pro.2165>.
- Brás, N. F.; Fernandes, P. A.; Ramos, M. J. QM/MM Studies on the β -Galactosidase Catalytic Mechanism: Hydrolysis and Transglycosylation Reactions. *J. Chem. Theory Comput.* **2010**, *6* (2), 421–433. <https://doi.org/10.1021/ct900530f>.
- Juergens, D. H.; Heighman, T. D.; Vasella, A.; McCarter, J. D.;

- Mackenzie, L.; Withers, S. G.; Matthews, B. W. A Structural View of the Action of Escherichia Coli (LacZ) β -Galactosidase. *Biochemistry* **2001**, *40* (49), 14781–14794. <https://doi.org/10.1021/bi011727i>.
- (25) Koto, S.; Inada, S.; Zen, S. Upfield Shift of CMR of Anomeric Carbons of 1,1'-Linked Glycopyranosyl Glycopyranosides. *Chem. Lett.* **1980**, No. 4, 403–406.
- (26) Huber, R. E.; Kurz, G.; Wallenfels, K. A Quantitation of the Factors Which Affect the Hydrolase and Transgalactosylase Activities of β -Galactosidase (E. Coli) on Lactose. *Biochemistry* **1976**, *15* (9), 1994–2001. <https://doi.org/10.1021/bi00654a029>.
- (27) Huber, R. E.; Wallenfels, K.; Kurz, G. The Action of β -Galactosidase (Escherichia Coli) on Allolactose. *Can. J. Biochem.* **1975**, *53* (9), 1035–1038. <https://doi.org/10.1139/o75-142>.