Communication

Glycosyl Cations versus Allylic Cations in Spontaneous and Enzymatic Hydrolysis

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Glycosyl Cations versus Allylic Cations in Spontaneous and Enzymatic Hydrolysis

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Supporting Information Placeholder

ABSTRACT: Enzymatic prenyl and glycosyl transfer are seemingly unrelated reactions that yield molecules and protein modifications with disparate biological functions. However, both reactions employ diphosphate-activated donors and each proceed via cationic species: allylic cations and oxocarbenium ions, respectively. In this study, we explore the relationship between these processes by preparing valienyl ethers to serve as glycoside mimics that are capable of allylic rather than oxocarbenium cation stabilization. Rate constants for spontaneous hydrolysis of aryl glycosides and their analogous valienyl ethers were found to be almost identical, as were the corresponding activation enthalpies and entropies. This close similarity extended to the associated secondary kinetic isotope effects (KIEs), indicating very similar transition state stabilities and structures. Screening a library of over 100 β-glucosidases identified a number of enzymes that catalyze hydrolysis of these valienyl ethers with k_{cat} values up to 20 sec^{-1}. Detailed analysis of one such enzyme showed that ether hydrolysis occurs via the analogous mechanisms found for glycosides, and through a very similar transition state. This suggests that the generally lower rates of enzymatic cleavage of the cyclitol ethers reflects evolutionary specialization of these enzymes towards glycosides rather than inherent reactivity differences.

Enzymatic transfer reactions enable organisms to assemble complex molecules by modular or incremental coupling of key, activated donor substrates. Two examples are the prenyl transferases[1] responsible for transfer of isoprene molecules and the glycosyl transferases (GTs) that assemble oligosaccharides and append glycosyl moieties to proteins.[2] While these two enzyme subclasses act on distinct substrates, they accomplish transfer through analogous mechanisms. Not only do both enzymes employ pyrophosphate-containing donor substrates but both reactions also proceed through resonance-stabilized cationic species: oxocarbenium ions during glycosyl transfer (Figure 1a) or allylic cations during isoprene transfer (Figure 1b).[3, 4] Despite the clear similarities between these systems, direct comparison of reactivities and ion stabilities has been complicated by the core structural differences between glycosides and isoprenoids, such as cyclic vs acyclic and substituent effects.

The similarity of these mechanisms was recently highlighted by the discovery that a key step in the biosynthesis of the trehalase inhibitor validamycin A is carried out by an enzyme (VldE), which transfers a 5,6-unsaturated C-7 cyclitol (valienol) moiety from a GDP (Guanosine DiPhosphate) donor onto the amino group of validamine phosphate.[5] Structural analysis of VldE revealed a close similarity to the glycosyltransferase trehalose-6-phosphate synthase (OtsA), which is the enzyme responsible for the assembly of trehalose from UDP-glucose and glucose-6-phosphate. The mechanistic analogy between these two enzymes is clear and it is likely that VldE employs an allylic cation-like transition state in place of the oxocarbenium ion-like transition state found in traditional GT mechanisms (Figure 1a and c).[6] In a broader sense we saw valienol-based substrates as an opportunity to directly compare the stabilities of allylic and oxocarbenium ion-like transition states within an otherwise identical molecular construct.

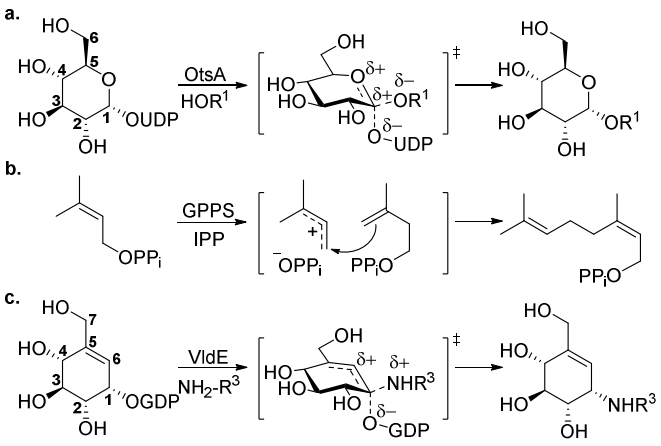

Figure 1. a. Trehalose-6-phosphate synthase (OtsA) transfers a glucosyl moiety from UDP-Glc to glucose-6-phosphate (HOR^1) via oxocarbenium ion-like transition states. **b.** Geranyl pyrophosphate synthase (GPPS) transfers a prenyl moiety from dimethylallyl pyrophosphate to isoprenyl pyrophosphate (IPP) via an allylic cation intermediate. **c.** VldE from *Streptomyces hygroscopicus sp.* catalyzes transfer of valienol from a GDP (guanosine diphosphate) valienol to validamine-7-phosphate (R^3) via a proposed allylic cation-like transition state.

Given these analogies, and since glycoside hydrolases (GHs) likewise effect hydrolysis via oxocarbenium-ion-like transition states, we investigated whether GHs could carry out hydrolysis of unsaturated cyclitol ethers, and if so, how such reactions compare with those of glycosides. This report describes both the non-enzymatic (spontaneous) solvolysis and enzymatic hydrolysis of valienyl ether substrates, alongside an analysis of their mechanisms.

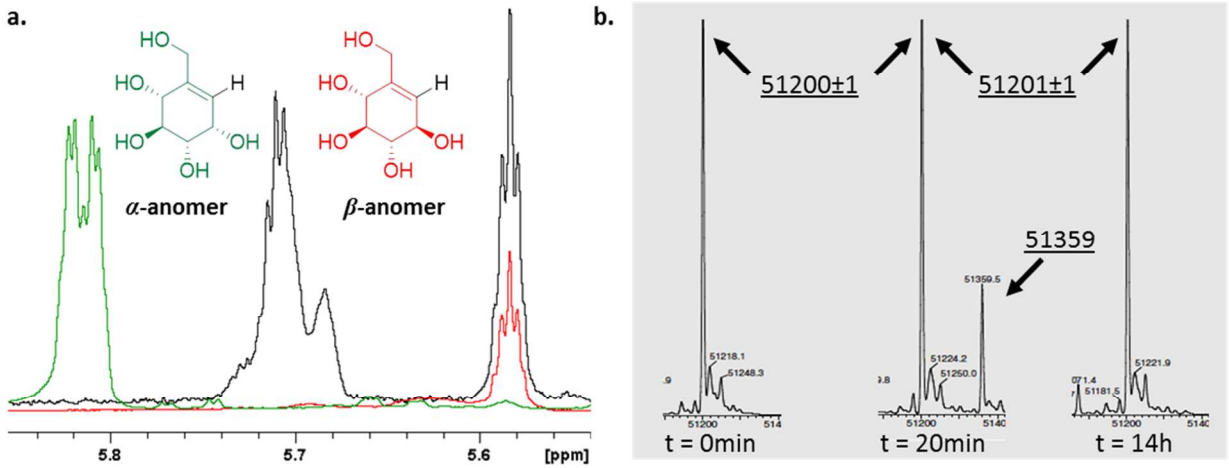

Figure 2: **a.** Product stereochemistry determined by ^{1}H-NMR, following reaction of Abg with TFMU-Valienol. Green trace is the α-anomer, red is the β-anomer and in black the reaction mixture. The region shown corresponds to the chemical shift of the alkenyl proton. Also present is a peak corresponding to unreacted TFMU-Val. **b.** Intact mass spectrometry of wild type Abg (left) and Abg incubated with DNP-Valienol for 20 min (centre) or 14 h (right). Observed MW increase of 158 corresponds to covalent Valienyl-Enzyme intermediate.

Synthesis of valienol from δ-D-gluconolactone was achieved following published protocols.[7] Installation of chromogenic leaving groups at the C-1 position (see Figure 1c for numbering convention) was completed using Mitsunobu chemistry[8] to couple trifluoromethylumbelliferyl alcohol (to give TFMU-Valienol, TFMU-Val) or by nucleophilic aromatic substitution with fluoro-2,4-dinitrobenzene to produce the dinitrophenyl ether (DNP-Valienol, DNP-Val). We focused on substrates that mimic β-glycosides as a large body of reliable data is available on both their non-enzymatic and enzymatic hydrolysis.[9, 10]

We first established the rate constants for non-enzymatic hydrolysis to determine relative intrinsic reactivities by comparison with data on the spontaneous hydrolysis of 2,4-dinitrophenyl (DNP) β-glucosides.[9, 11-13] Use of a 2,4-dinitrophenyl leaving group simplified interpretation of kinetic data on glucoside hydrolysis, for which rates were shown to be pH-independent between 2 and 8.5.[13] Thus, within this range, rate constants reflect the simple heterolysis of the C-O bond, without any involvement of acid-/base-catalyzed processes. We measured rate constants for non-enzymatic hydrolysis of DNP-Val at 70 °C at a series of pH values and showed them to be independent of pH between pH 4 and 7.5 (Fig S2). All further studies of uncatalyzed hydrolysis were performed at pH 6.5 under the same conditions used for DNP-Glc hydrolysis.[13]

Heterolysis of the DNP-Valienol ether bond was followed by monitoring dinitrophenol release at a range of temperatures, over at least 3 half-lives. Rate constants were determined by fitting these progress curves to a first order equation. The resultant Eyring plots (Figure S4) were of good quality (r^2 = 0.9995) and yielded enthalpies and entropies of activation as well as allowing interpolation of rate constants at any temperature (Table 1).[9, 14] Rate constants for solvolysis of the analogous DNP-glucoside were also re-determined, under the same conditions, and are in agreement with previously published data (Table 1).[9, 11, 15, 16]

Remarkably, the rate constants for solvolysis of the two compounds are extremely similar, as are the activation enthalpies and entropies. In previous work Cocker and Sinnott showed that tertiary carbocations and oxocarbenium ions had broadly similar stabilities.[12] This result was reinforced by nearly identical lifetimes observed for glycosyl oxocarbenium cations[17, 18] and t-butyl cations.[19] To our knowledge allyl cations or hydroxyl-substituted systems have not been explored.

Table 1. Rate Constants and Activation Parameters for Heterolysis of DNP Valienol and DNP Glucoside

Substrate:	DNP-Val	DNP-Glc
*Lit. k (37°C) /s^{-1}	-	5.58 x 10^{-6}
Exp. k (37°C) /s^{-1}	4.91x10^{-6}	7.41x10^{-6}
$\Delta G^{\ddagger}$ /kJ·mol^{-1}	108 (1.5)	106 (1.3)
ΔH(37°C)/kJ·mol^{-1}	112 (1.5)	111 (1.3)
ΔS /J·mol^{-1}·K^{-1}	13.1 (0.3)	14.4 (0.3)

*Literature values from Namchuk and Withers.[9] Error in parentheses propagated from linear regression of Eyring plots (Figure S4).

The non-enzymatic solvolysis of aryl glycosides is known to proceed via an oxocarbenium ion-like transition state, as supported by the alpha-deuterium KIE of 1.09 ± .02 on spontaneous hydrolysis of DNP-Glc.[11] Likewise, Bennet and Sinnott [20] reported the same value (k_H/k_D = 1.089 ± 0.006) for acid-catalyzed cleavage of methyl β-D-glucopyranoside concluding that the reaction proceeds via a S_N1-like (D_N*A_N) process with a short-lived oxcarbenium ion intermediate. To enable a direct comparison of transition state structures we synthesized the analogous 1-^{2}H-DNP-Val by stereospecific reduction of the protected valienone using sodium borodeuteride under Luche reduction conditions, followed by coupling with fluorodinitrobenzene and deprotection as described above. Integration of the corresponding ^{1}H-NMR confirmed >96 % deuteration. The KIE measured in this case was (k_H/k_D = 1.11 ± 0.01), fully consistent with the anticipated allylic cation-like transition state with sp^{2}-hybridization at C1.

The stereochemical outcome of the solvolysis of DNP-Val was evaluated by ^{1}H-NMR analysis of the product mixture, revealing a 3:1 ratio of the α- and β- 'anomers' (using carbohydrate terminology). Also present, as approximately 50% of the total, were the products of water attack at the tertiary C5 position of the allylic cation (Figure S5). Such an outcome is not, of course, possible for pyranosyl systems, but should not confound the kinetic analysis since the rate-limiting step, heterolysis, precedes the product-determining step, as was confirmed by the KIE. These are indeed kinetically controlled products since incubation of pure β-valienol

product at 70 °C for 3 hours under the same conditions revealed no interconversion to products of different stereochemistry at C1 nor positional isomerization (Figure S6). Alongside the KIE measurements these results are consistent with a reaction mechanism that is largely dissociative (S_N1-like). Assuming similar ground state energies these conclusions suggest that the thermodynamic stabilities of the transition states preceding the allylic and oxocarbenium ions are also nearly identical.

Having explored the non-enzymatic hydrolysis, we turned our attention towards enzyme-catalyzed hydrolysis of these cyclitol ethers. Substrates were tested initially with the β-glucosidase from *Agrobacterium sp.* (Abg), a very well characterized member of CAZY family GH1 (http://www.cazy.org/).[15, 16, 21, 22]

Kinetic studies with fluorogenic TFMU-Val, revealed that Abg is indeed able to cleave the pseudo-glycosidic bond, yielding the kinetic parameters shown in Table 2 alongside those for the analogous TFMU-glucoside substrate. As can be seen, the k_{cat} value is approximately 10^3-fold lower than that for the corresponding glucoside Given their similar K_m values this difference extends to the specificity constants (k_{cat}/K_m) as well. Since the inherent reactivities of the two substrates are the same, this substantial variation presumably reflects differences in recognition of the two substrates and their transition states as a consequence of the evolutionary optimization of Abg towards glucoside hydrolysis.

Table 2. Kinetic Parameters for Hydrolysis of TFMU-Valienol by Abg at 25°C

Substrate:	k_{cat} /min^{-1}	K_m /μM	k_{cat}/K_m /min^{-1}·μM^{-1}
TFMU-Val	1.44 ± 0.13	356 ± 80	0.004 ± 0.001
TFMU-Glc	2682 ± 170	204 ± 30	13.1 ± 2.1

Given this considerable difference in rates it was necessary to establish whether the enzyme employs the same mechanisms for the two substrates. We thus first established that catalysis was, in fact, occurring at the normal active site by showing that preincubation of Abg with the active site-directed mechanism-based inactivator cyclophellitol abolishes hydrolase activity (Figure S9).[23, 24] Secondly, we confirmed the 'retaining' stereochemical outcome of Abg-catalyzed valienyl ether cleavage by NMR analysis against prepared standards (Figure 2a). Only the equatorial β product is observed, confirming that the enzyme completes valienyl ether cleavage by a retaining mechanism (Figure 3), in contrast to the product distribution observed for heterolysis.

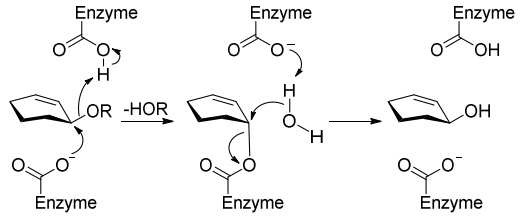

Figure 3. Proposed general mechanism of enzyme-catalyzed hydrolysis of β-valienyl ethers. Substituent hydroxyls as well as transition states or intermediates are omitted for clarity.

We next confirmed that the same active site residues were involved in hydrolysis of valienol substrates by demonstrating that a mutant missing the catalytic nucleophile (mutation: E358G) had no valienyl ether cleavage activity, but that cleavage could be rescued by addition of an exogenous nucleophile (1 M azide), (Figure S11, Table S4), exactly as had been shown previously for glucoside cleavage.[25, 26] Direct detection of the intermediate and its turnover were achieved by mass spectrometry (Figure 2b) showing both free Abg and its covalent valienyl–enzyme intermediate. Further incubation for 14 hours resulted in complete turnover as seen by the return of free enzyme.

The successful observation of the valienyl-enzyme intermediate strongly suggests that the subsequent 'deglycosylation' step of this process, which releases valienol, is rate limiting, as also do the comparably low K_m values for the two substrates, likely due to accumulation of the covalent intermediate. Confirmation of this was sought through nucleophilic competition experiments parallel to those performed on DNP-Glc hydrolysis in which addition of DTT increased the steady state rate.[15] Accordingly, addition of DTT also increased the turnover of TFMU-Val (Fig. S13): control experiments confirmed that this does not arise from background solvolysis. The reagents therefore function as transient inactivators. Finally, we confirmed that the enzymatic transition state also has substantial allylic cation character by measuring a secondary KIE, under Vmax conditions, for 1-^{2}H-DNP-Valienol hydrolysis of $k_H/k_D = 1.15 \pm 0.03$. This is again very similar to that of $k_H/k_D = 1.10 \pm 0.02$ measured for Abg-catalyzed DNP-Glc hydrolysis.[15] Taken together these results suggest the overall reaction is double displacement with the most rate-limiting transition state having considerable allylic-cation-like character. It must be noted that the hydrolysis of the enzyme-substrate complex may be S_N2-like (A_ND_N) (with significant dissociative character) or S_N1-like (D_N*A_N or $D_N + A_N$); both are consistent with the KIEs.

In order to assess whether these enzymatic studies were generally representative we screened a library of over 100 expressed GH1 glycosidases, made available from the Joint Genome Institute, for their ability to hydrolyze TFMU-Val.[27] Seven of these enzymes exhibited activities that are >2 standard deviations above the average activity across the library. Since at least 23 members of the library cleave only 6-phospho-sugars and are thus unlikely to work in our assay this represents an initial hit rate of close to 5%. These hits were purified, concentrations determined by active site titration, and kinetic parameters determined (Table 3).

Table 3. Kinetic Parameters for Cleavage of TFMU-Val by GH1 enzymes.

ID*	k_{cat} /min^{-1}	K_m/μM	k_{cat} (Glc)/ k_{cat}(Val)
A.flav	1.81	112	481
Bac.	0.26	27	6007
C.sacc	0.95	82	141
Exi.	0.63	271	1810
P.chrys	0.58	41	1004
Ssβ	2.67	117	24
V.vul	0.89	710	1316
Abg	1.44	355	1863

* See Table S8 for description of species, parameters and errors.

Turnover numbers (Table 3) fall roughly within one order of magnitude but more interesting are the relative rates between the two substrates. Unsurprisingly all enzymes were better able to process the TFMU-glycoside substrate over the valienol derivative. Nevertheless, several enzymes appear to be less highly specific for the glucoside, exhibiting a greater tolerance for valienyl ether cleavage. We selected the thermostable β-glucosidase from *Sulfolobus solfataricus* (Ssβ-Glc) for further study since it has undergone substantial characterization.[28,29] We were interested in seeing what turnover numbers could be achieved at higher temperatures. The kinetic parameters we determined are found in Table 4. As anticipated the k_{cat} value increases 20-fold from 25 ^{o}C to 70 ^{o}C, arriving at a very respectable value of almost 20 sec^{-1}.

Table 4. Kinetic Parameters for Hydrolysis of DNP-Val and DNP-Glc by Ssβ-Glc

Substrate:	k_{cat} /s^{-1}	K_m /μM	k_{cat}/K_m /s^{-1}·μM^{-1}
DNP-Val (25^{o})	0.92 ± 0.04	938 ±95	0.001
DNP-Val (70^{o}C)	18.9 ± 1.2	1500 ±176	0.03
DNP-Glc (25^{o}C)	44.7 ± 0.4	373 ±8	0.05
DNP-Glc (70^{o}C)	645 ± 36	129 ±19	5

In conclusion, relative to ground states, the transition states leading to the allylic and oxocarbenium ions are nearly identical, when isolated within similar molecules. Allylic isoprenyl cations are invoked as formal intermediates for prenyl transfer whereas oxocarbenium ions feature as transition states in glycosyl transfer. This difference is not due to allylic cations being inherently more stable than their oxocarbenium cousins. Rather, we suggest the differences lie in the electronegative hydroxyl substituents on glycosides, which inductively destabilize the positive charge, in conjunction with constraints on planar geometry imposed by the ring. We have also shown the first examples, to our knowledge, of valienyl ether hydrolysis by glycoside hydrolases. Indeed, several such enzymes were identified from screening efforts, demonstrating that this capability is not limited to a single enzyme. Further, enzymes with sufficient activity to be of practical utility in effecting valienol transfer were identified. Detailed characterization of one model enzyme, the β-glucosidase from *Agrobacterium sp.* implies that the mechanism of hydrolysis is the same as that shown for glycoside cleavage, cementing notions of the mechanistic parallels between prenylation and glycosylation reactions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Syntheses and characterization of novel compounds, fluorescence and UV/vis data for kinetics and screening. (PDF).

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Notes

The authors declare no competing financial interests.

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REFERENCES:

1. Liang, P. H.; Ko, T. P.; Wang, A. H. J., *Eur. J. Biochem.* **2002**, *269*, 3339.
2. Lairson, L. L.; Henrissat, B.; Davies, G. J.; Withers, S. G., *Annu. Rev. Biochem.* **2008**, *77*, 521.
3. Poulter, C. D.; Satterwhite, D. M., *Biochemistry* **1977**, *16*, 5470.
4. Poulter, C. D.; Argyle, J. C.; Mash, E. A., *J. Biol. Chem.* **1978**, *253*, 7227.
5. Asamizu, S.; Yang, J.; Almabruk, K. H.; Mahmud, T., *J. Am. Chem. Soc.* **2011**, *133*, 12124.
6. Lee, S. S.; Hong, S. Y.; Errey, J. C.; Izumi, A.; Davies, G. J.; Davis, B. G., *Nat. Chem. Biol.* **2011**, *7*, 631.
7. Shing, T. K. M.; Chen, Y.; Ng, W. L., *Synlett* **2011**, *9*, 1318.
8. Mitsunobu, O.; Yamada, M., *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2380.
9. Namchuk, M. N.; McCarter, J. D.; Becalski, A.; Andrews, T.; Withers, S. G., *J. Am. Chem. Soc.* **2000**, *122*, 1270.
10. Zechel, D. L.; Withers, S. G., *Acc. Chem. Res.* **2000**, *33*, 11.
11. Namchuk, M. N. Substituted aryl glycosides as probes of the mechanism of spontaneous and enzyme-catalyzed glycoside hydrolysis. Ph.D. Thesis, The University of British Columbia (Canada), 1993.
12. Cocker, D.; Sinnott, M. L., *J. Chem. Soc., Chem. Commun.* **1972**, *7*, 414.
13. Cocker, D.; Sinnott, M. L., *J. Chem. Soc., Perkin Trans. 2* **1975**, *13*, 1391.
14. Eyring, H., *J. Chem. Phys.* **1935**, *3*, 107.
15. Kempton, J. B.; Withers, S. G., *Biochemistry* **1992**, *31*, 9961.
16. Namchuk, M. N.; Withers, S. G., *Biochemistry* **1995**, *34*, 16194.
17. Amyes, T. L.; Jencks, W. P., *J. Am. Chem. Soc.* **1989**, *111* 7888.
18. Huang, X. C.; Surry, C.; Hiebert, T.; Bennet, A. J *J. Am. Chem. Soc.* **1995**, *117*, 10614.
19. Toteva, M. M.; Richard, J. P., *J. Am. Chem. Soc.* **1996,** *118*, 11434.
20. Bennet, A. J.; Sinnott, M. L., *J. Am. Chem. Soc.* **1986**, *108*, 7287.
21. Wakarchuk, W. W.; Greenberg, N. M.; Kilburn, D. G.; Miller, R. C.; Warren, R. A. J., *J. Bacteriol.* **1988**, *170*, 301.
22. Withers, S. G.; Street, I. P., *J. Am. Chem. Soc.* **1988**, *110*, 8551.
23. Caner, S.; Zhang, X. H.; Jiang, J. B.; Chen, H. M.; Nguyen, N. T.; Overkleeft, H.; Brayer, G. D.; Withers, S. G., *FEBS Lett.* **2016**, *590*, 1143.
24. Gloster, T. M.; Madsen, R.; Davies, G. J., *Org. Biomol. Chem.* **2007**, *5*, 444.
25. Kim, Y. W.; Lee, S. S.; Warren, R. A. J.; Withers, S. G., *J. Biol. Chem.* **2004**, *279*, 42787.
26. Moracci, M.; Trincone, A.; Perugino, G.; Ciaramella, M.; Rossi, M., *Biochemistry* **1998**, *37*, 17262.
27. Heins, R. A.; Cheng, X. L.; Nath, S.; Deng, K.; Bowen, B. P.; Chivian, D. C.; Datta, S.; Friedland, G. D.; D'Haeseleer, P.; Wu, D. Y.; Tran-Gyamfi, M.; Scullin, C. S.; Singh, S.; Shi, W. B.; Hamilton, M. G.; Bendall, M. L.; Sczyrba, A.; Thompson, J.; Feldman, T.; Guenther, J. M.; Gladden, J. M.; Cheng, J. F.; Adams, P. D.; Rubin, E. M.; Simmons, B. A.; Sale, K. L.; Northen, T. R.; Deutsch, S., *ACS Chem. Biol.* **2014**, *9*, 2082.
28. Pisani, F. M.; Rella, R.; Raia, C. A.; Rozzo, C.; Nucci, R.; Gambacorta, A.; Derosa, M.; Rossi, M., *Eur. J. Biochem.* **1990**, *187*, 321.
29. Gloster, T. M.; Roberts, S.; Ducros, V. M. A.; Perugino, G.; Rossi, M.; Hoos, R.; Moracci, M.; Vasella, A.; Davies, G. J., *Biochemistry* **2004**, *43*, 6101.

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