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Cloning and characterization of *Thermotoga maritima* β-glucuronidase

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Abstract—The putative β-glucuronidase from *Thermotoga maritima*, comprising 563 amino acid residues conjugated with a Hisx6 tag, was cloned and expressed in *Escherichia coli*. The enzyme has a moderately broad specificity, hydrolysing a range of *p*-nitrophenyl glycoside substrates, but has greatest activity on *p*-nitrophenyl β-D-glucosiduronic acid ($k_{cat} = 68\ s^{-1}$, $k_{cat}/K_M = 4.5 \times 10^5\ M^{-1}\ s^{-1}$). The enzyme also shows a relatively broad pH-dependence with activity from pH 4.5 to 7.5 and a maximum at pH 6.5. As expected the enzyme is stable towards heat denaturation, with a half life of 3 h at 85 °C, in contrast to the mesophilic *E. coli* enzyme, which has a half life of 2.6 h at 50 °C. The identity of the catalytic nucleophile was confirmed as Glu476 within the sequence VT**E**FGAD by trapping the glycosyl–enzyme intermediate using the mechanism-based inactivator, 2-deoxy-2-fluoro-β-D-glucosyluronic acid fluoride and identifying the labeled peptide in peptic digests by HPLC–MS/MS methodologies. Consistent with this, the Glu476Ala mutant was shown to be hydrolytically inactive. The acid/base catalyst was confirmed as Glu383 by generation and kinetic analysis of enzyme mutants modified at that position, Glu383Ala and Glu383Gln. The demonstration of activity rescue by azide is consistent with the proposed role for this residue. This enzyme therefore appears suitable for use in enzymatic oligosaccharide synthesis in either the transglycosylation mode or by use of glycosynthase and thioglycoligase approaches.

Keywords: Thermostable glycosidase; Mechanism; Active site; Labeling; Glucuronidase

1. Introduction

Glucuronic acid plays several important roles in biology. One is as a sugar that is conjugated to unwanted small molecules to increase their solubility and assist excretion. Another is as an important component of several polysaccharides, most notably the glycosaminoglycans. Efficient enzymes for the synthesis and degradation of such species are therefore important—both in the natural biological systems and as tools for the enzymatic assembly and disassembly of such glycoconjugates in the laboratory. To that end, an understanding of the structures and functions of β-glucuronidases (EC 3.2.1.31) is of considerable importance, not only to better comprehend their natural role, but also as a pre-requisite to the engineering of such enzymes to modify their specificity or to convert them into synthetic catalysts. Indeed, *Escherichia coli* β-glucuronidase has already served as a test bed for the development of technologies for directed evolution of protein function. Thus, through combinations of mutagenic PCR, saturation mutagenesis and gene shuffling the specificity of that enzyme has been evolved and broadened to allow hydrolysis of a series of different glycoside substrates.[1,2]

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Two new classes of mutant glycosidases that are capable of oligosaccharide synthesis have been introduced in recent years: the glycosynthases and the thioglycoligases.[3–5] Glycosynthases are mutant retaining glycosidases in which the catalytic nucleophile has been mutated. When used with glycosyl fluoride donor, sugars of opposite configuration to that of the natural substrate such mutants catalyze high-yielding glycosyl transfer. Thioglycoligases are mutants of the acid/base catalyst that catalyze the formation of thioglycoside products from suitable glycoside donors and thiosugar acceptors. As part of a program to ultimately generate a range of glycosynthases and thioglycoligases for enzymatic synthesis, we wished to engineer a glucuronidase. Further, we wished to explore the engineering of a thermostable enzyme for such purposes, since such thermostable enzymes could prove to be attractive catalysts within the biotechnology industry. A suitable candidate organism as the source of such an enzyme is *Thermotoga maritima*, a hyperthermophilic marine eubacterium that thrives at an optimum temperature of ~80 °C. Inspection of the *T. maritima* genome located an open reading frame identified as TM1062 that encodes a putative 563 amino acid β-glucuronidase (NP_22868; GenBank accession number AAD36143). On the basis of Henrissat's glycosidase classification scheme this enzyme has been grouped into CAZY family 2, along with β-galactosidases, β-mannosidases and other β-glucuronidases (Table 1).[6] The three-dimensional structures of two members of family 2 have been determined; *E. coli* LacZ β-galactosidase and the human lysosomal β-glucuronidase.[7,8] Common structural features include a TIM barrel catalytic domain, a jelly-roll barrel and an immunoglobulin constant domain. Enzymes from this family are known to catalyze hydrolysis with net retention of configuration of the anomeric carbon through a two-step, double-displacement mechanism involving a covalent glycosyl enzyme intermediate that is formed and hydrolyzed *via* oxocarbenium ion-like transition states (Chart 1). Two carboxylic acids play key roles in this mechanism, one as the catalytic nucleophile and the other as the general acid/base catalyst. Previous studies on other glycosidases have shown that the covalent glycosyl enzyme intermediate

Table 1. Multiple sequence alignment of β-glucuronidases in CAZY family 2 generated by CLUSTAL W (1.82)

amino acid sequence around the acid-base catalyst

Human (accession no. AAA52561)	438	AVVMWSVANEPASHLESAG	456
African green monkey (AAC34593)	435	AVVMWSVANEPASHLESAG	453
Dog (AAC48809)	437	SVVMWSVANEPTSFLKPAA	455
Cat (AAD01498)	437	AVVMWSVANEPASFLKPAG	455
House mouse (AAA37696)	434	AVVMWSVANEPSSALKPAA	452
Norway rat (AAA41228)	434	AVVMWSVANEPVSSLKPAG	452
Arthrobacter sp. RP10 (AAV91790)	397	SVVMWSIANEPASNEDGAR	415
Penicillium canescens (AAV91785)	439	SVVMWSIANEPASHEDGAR	457
*Scopulariopsis sp.*RP38.3 (AAV91788)	437	SVVSWCVTNEPASAEDGAR	455
Escherichia coli (AAA68923)	400	SVVMWSIANEPDTRPQGAR	418
Clostridium perfringens str.13 (BAB79853)	399	CVVMWSVANEPDSDSEGAK	417
Lactobacillus gasseri (AAK07836)	403	SVIAWSLFNEPESTTQESY	421
Ruminococcus gnavus (AAQ76046)	405	SVIAWSLFNEPETITDYAY	423
Thermotoga maritima MSB8 (AAD36143)	374	SVIMWSVANEPESNHPDAE	392

amino acid sequence around the catalytic nucleophile

Human (accession no. AAA52561)	530	KKYQK-PIIQSEYGAETI	546
African green monkey (AAC34593)	527	KTYQK-PIIQSEYGAETI	543
Dog (AAC48809)	529	RTYQK-PIIQSEYGAETI	545
Cat (AAD01498)	529	RTYQK-PIIQSEYGADTI	545
House mouse (AAA37696)	526	KTHQK-PIIQSEYGADAI	542
Norway rat (AAA41228)	526	KMYQK-PIIQSEYGADAV	542
Arthrobacter sp. RP10 (AAV91790)	491	EAFGK-PIMMSEYGADTM	507
Penicillium canescens (AAV91785)	534	EKFHR-PIVMTEYGADTL	550
*Scopulariopsis sp.*RP38.3 (AAV91788)	531	DEYDK-PIIMSEYGADTL	547
Escherichia coli (AAA68923)	494	EKLHQ-PIIITEYGVDTL	510
Clostridium perfringens str.13 (BAB79853)	494	ERCPKTPIMFTEYGADTV	511
Lactobacillus gasseri (AAK07836)	499	NLKLNKPFVFTEFGADTL	516
Ruminococcus gnavus (AAQ76046)	501	AKELNVPFVFTEFGTDTM	518
Thermotoga maritima MSB8 (AAD36143)	466	ARHRK-PIFVTEFGADAI	482

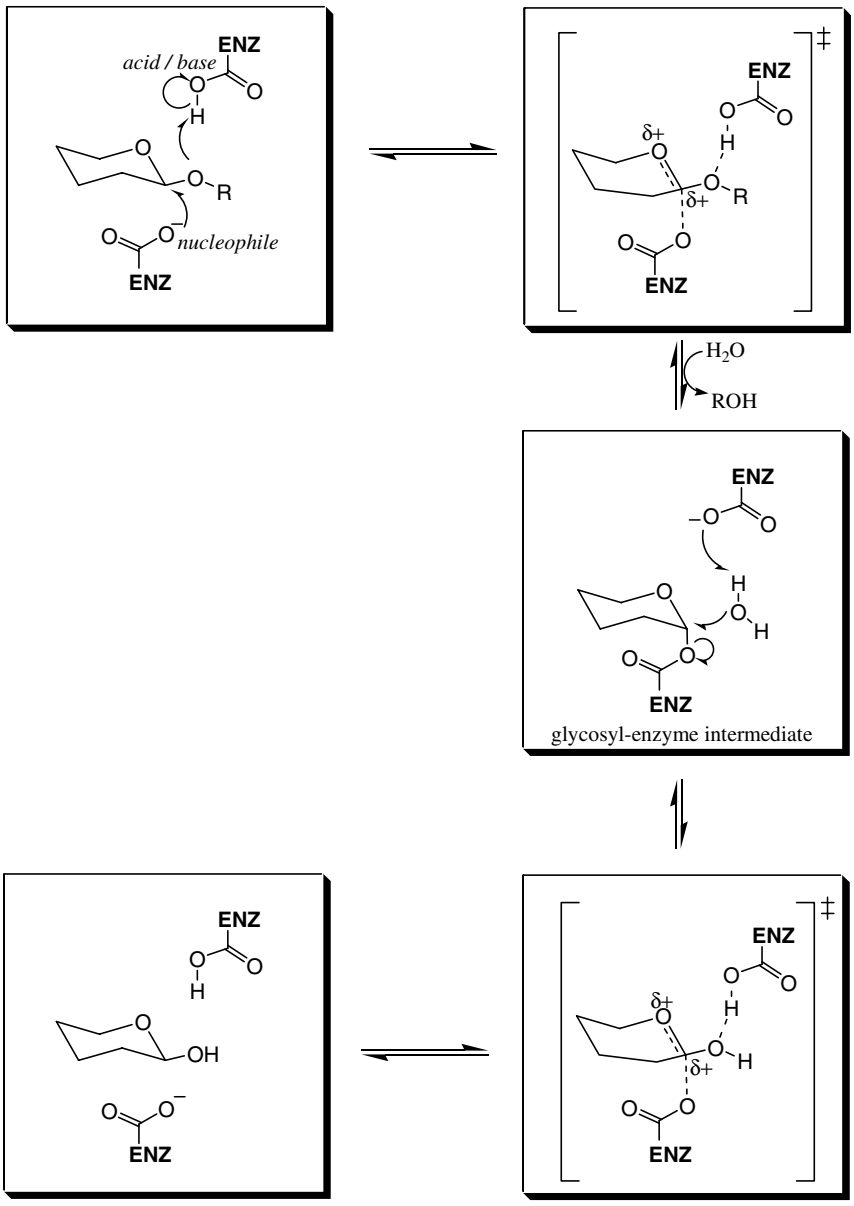

Chart 1. Proposed mechanism for a retaining β-glycosidase.

can be trapped through use of activated 2-deoxy-2-fluoroglycosides.[9,10] The fluorine at C-2 destabilizes the oxocarbenium ion-like transition states thereby slowing the formation and decomposition of the glycosyl–enzyme intermediate, while the activated leaving group ensures that this intermediate is nonetheless kinetically accessible. Such tagging of the active site nucleophile has allowed its identification through peptide mapping protocols.

This paper describes, the cloning, expression, purification, and partial characterization of *T. maritima* β-glucuronidase, including the unequivocal identification of these two key active site residues through labeling and through kinetic analysis of mutants in which these residues have been modified.

2. Results and discussion

2.1. Isolation of clones encoding *T. maritima* β-glucuronidase and protein expression in β-glucuronidase-deficient *E. coli* GMS407 strain

To facilitate the characterization of β-glucuronidase from *T. maritima* with the idea of using this enzyme in chemoenzymic synthesis of oligosaccharides and other glycoconjugates, the TM1062 gene that encodes this enzyme was cloned and expressed as follows. Using the published sequence of the ORF TM1062 (NP_22868; GenBank accession number AAD36143) primers flanking the coding region were designed and synthesized to isolate the full-length open reading frame TM1062

Table 2. Substrate specificity of TMGUA-wt at 75 °C

Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}s^{-1}$)
*p*NP β-D-galactosiduronic acid	443 ± 8	3.06 ± 0.15	145
*p*NP β-D-glucosiduronic acid	68 ± 2	0.15 ± 0.01	453
*p*NP β-D-glucoside	9 ± 0.2	145 ± 6	0.06

encoding the putative β-glucuronidase, by PCR. The DNA source for the amplification reaction was commercially available lysed *T. maritima* MS8B. A single PCR product of ~1.8 kb was subcloned and fully sequenced. The ~1.8 kb PCR amplimer was identical in sequence to the ORF identified as TM1062 by the *T. maritima* genome sequencing project encoding a putative 563 amino acid β-glucuronidase. The putative β-glucuronidase gene was subcloned into a pET28a(+) expression vector using *Nde*I and *Xho*I cloning sites, resulting in a recombinant plasmid construct pET-TMGUA-wt. This was then transformed into the β-glucuronidase deficient strain GMS407.

2.2. Purification of *T. maritima* β-glucuronidase from *E. coli* cells

A *T. maritima* β-glucuronidase transformant was induced in a 2 L shake flask culture as described in Experimental and the β-glucuronidase bearing a Hisx6 tag at the N-terminus was purified to apparent homogeneity by heat treatment and immobilized-metal affinity chromatography. The TM1062 gene consisted of a 1689-nucleotide open reading frame encoding a protein of 563 amino acid residues and the theoretical molecular weight calculated from the amino acid composition of the deduced 583 amino acid residues plus linker and Hisx6 tag enzyme is M_r 67846.2 Da. The purified enzyme preparation was resolved by SDS-PAGE and migrated as a band with an apparent molecular mass of ~66 kDa, very close to the predicted size of the polypeptide. Approximately 5 mg of purified protein can be obtained from 1 L of culture medium.

2.3. Characterization of the expressed protein

The polypeptide encoded on the plasmid pET-TMGUA-wt contains a Hisx6 tag conjugated at the N-terminus of β-glucuronidase *via* a thrombin cleavage site. The purified *T. maritima* β-glucuronidase expression product was kinetically characterized using *p*-nitrophenyl β-D-glucosiduronic acid (*p*NP β-GlcUA) without prior removal of the N-terminal 6xHis tag. The expressed enzyme cleaves this synthetic substrate over a pH range of 4.5–7.5, with the highest activity being found at ~pH 6.5. The enzyme activity follows normal Michaelis–Menten kinetics with a K_M of 152 μM for *p*NP β-GlcUA, k_{cat} of 68 s^{-1} and k_{cat}/K_M of 4.5×10^5 M^{-1} s^{-1} measured at pH 6.5 and 75 °C.

The purified enzyme was also tested for substrate specificity in the cleavage of closely related synthetic substrates. As can be seen in Table 2, the enzyme not only hydrolyzes *p*NP β-GlcUA effectively but also catalyzes the hydrolysis of *p*NP β-D-galactosiduronic acid and *p*NP β-glucoside (Table 2). The substrate specificity trend observed for *T. maritima* β-glucuronidase is similar to that of the recombinant β-glucuronidase from *E. coli*.[1]

2.4. Heat inactivation and circular dichroism studies

The thermal stability of the recombinant Hisx6 tagged wild type enzyme was probed by incubation at 85 °C, removing aliquots at time intervals and assaying. Under these conditions, a half life for inactivation of 3 h was measured. By comparison, a parallel analysis of the thermal stability of *E. coli* β-glucuronidase revealed a half life of 2.6 h at 50 °C for that enzyme (Hwang and Withers, unpublished results). Thus, *T. maritima* enzyme can tolerate some 35 °C higher temperatures. The conformational integrity and thermostability of the wild-type and mutant proteins were further probed by circular dichroism spectroscopy. First, spectra from 320 to 190 nm were recorded for each protein at room temperature. Scans obtained were almost identical in all cases, confirming that all the mutants used were folded correctly (data not shown). Next, the thermostability of each enzyme was tested by heating from 40 to 95 °C and observing the change of signal at 217 nm (Fig. 1). The E476A mutant proved to be the most stable of the enzymes with a melting temperature of ~90 °C. The recombinant wild-type and E383A mutant showed similar melting temperatures of ~85 °C, and only the E383Q mutant had a reduced thermostability, becoming unfolded at ~72 °C. Arrows in Figure 1 indicate the approximate melting temperatures of the enzymes. To confirm these results and test at which temperatures enzyme assays could be done with the most unstable mutant, the E383A and E383Q mutants were incubated at 62 °C and the change of the CD signal was observed over time. Not surprisingly, unfolding of the E383Q mutant could be observed within the first 8 min of the experiment while the E383A mutant did not show any denaturation even after 1 h (data not shown) at 62 °C. Subsequent kinetic analyses of the recombinant wild-type, E383A and E476A mutants were carried out at 75 °C and/or 65 °C, while for the E383Q mutant, experiments were performed only at 65 °C and for less than 5 min. This observation of greater stability in mutants

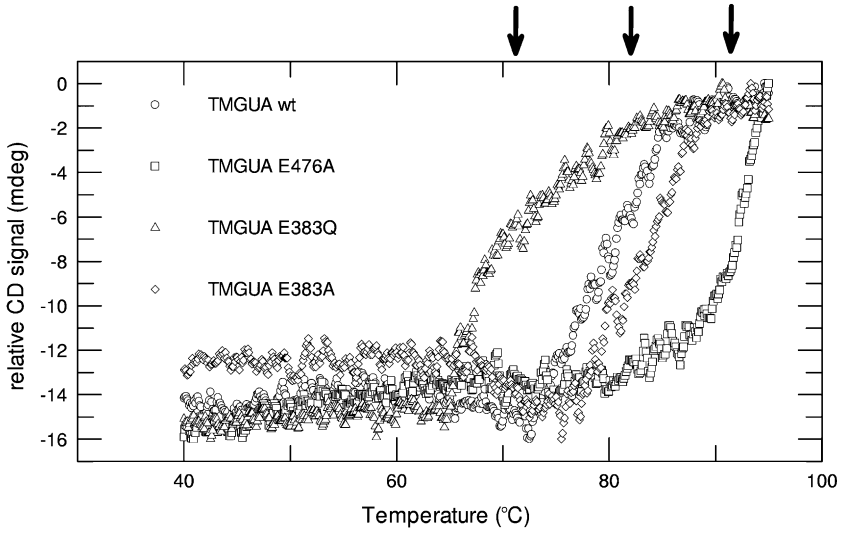

Figure 1. Thermal denaturation of recombinant wild-type and mutants of *T. maritima* β-glucuronidase. Change of circular dichroism signal intensity at 217 nm while proteins were heated at a rate of 1 °C/min (arrows indicate the approximate melting temperatures of the enzymes).

of retaining glycosidases in which catalytic carboxylic acids have been replaced by alanine is precedented and logical. Studies of pH-dependence of catalysis of retaining glycosidases revealed that the pK_a of the acid/base residue is raised 2–3 units above its 'normal' pK_a in the free enzyme, largely due to electrostatic interactions with the deprotonated active site nucleophile. Upon formation of the glycosyl enzyme intermediate (thus removal of the charge on the nucleophile) the pK_a of the acid/base typically drops 2.5 units as was shown directly by ^{13}C NMR for a retaining xylanase.[11] This electrostatically perturbed pK_a in the free enzyme is a factor that destabilizes the free enzyme, therefore mutation of either of these two residues to a neutral residue, thereby removing this electrostatic interaction, generally results in stabilization unless some other structural element is adversely affected.[12]

2.5. Inactivation of *T. maritima* β-glucuronidase

In β-retaining glycosidases, including family 2 glycosylhydrolases, two carboxylic acid functional groups have been implicated in catalysis: one acting as a catalytic nucleophile and the other as an acid/base catalyst. In the present study, incubation of purified recombinant *T. maritima* β-glucuronidase with a known specific inactivator of β-glucuronidases, 2-deoxy-2-fluoro-β-D-glucosyluronic acid fluoride (2FGlcUAF), inactivated the enzyme in a concentration- and time-dependent manner according to pseudo first-order kinetics (Fig. 2A). Unfortunately, saturation behavior could not be observed since very rapid inactivation of the enzyme occurs at higher concentrations of inactivator, thereby preventing accurate sampling. Hence, we were unable to determine the inactivation rate constant, k_i, and the reversible dissociation constant, K_i. However, an accurate second-order rate constant of $k_i/K_i = 0.16 \pm 0.01$ min^{-1} mM^{-1}, was extracted from the slope of the plot of apparent

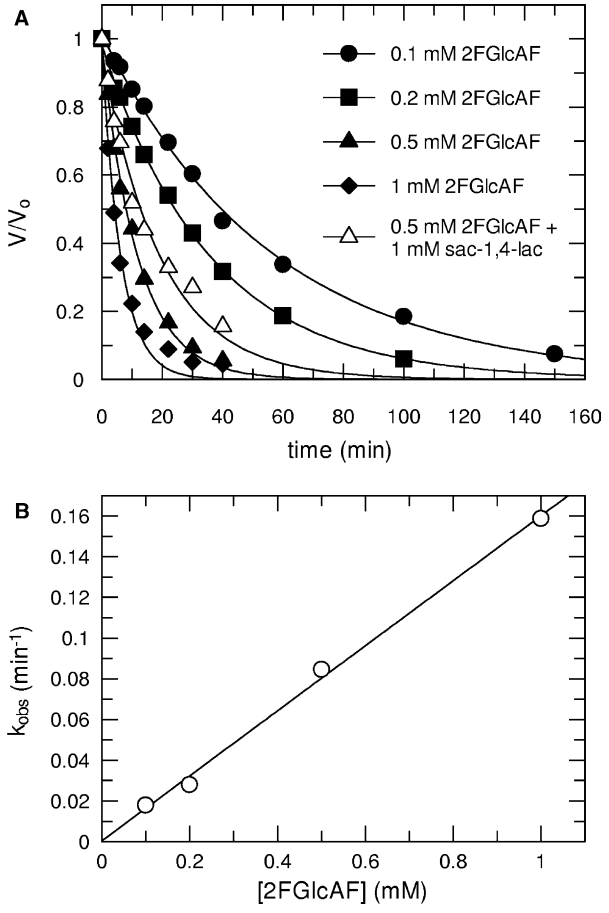

Figure 2. Inactivation of recombinant *T. maritima* β-glucuronidase by 2FGlcUAF. (A) Plot of residual activity (V/V_o) versus time in the presence of various concentrations of 2FGlcUAF. (B) Replot of the apparent first-order rate constants from A.

first-order rate constants versus inactivator concentration (Fig. 2B). Incubation of the purified enzyme with 2FGlcUAF (500 μM) in the presence of a competitive inhibitor, D-saccharic acid 1,4-lactone (K_i = 24 μM) protected the enzyme against inactivation by 2FGlcUAF as shown in Figure 2A inferring that 2FGlcUAF is a specific, active site-directed inactivator of *T. maritima* β-glucuronidase. Confirmation that inactivation is caused by reaction with a single 2FGlcUAF molecule is provided by the ESIMS analysis of the inactivated protein: the molecular mass of wild-type β-glucuronidase increased by 180 (±5) Da after incubation with 2FGlcUAF (data not shown).

2.6. Identification of the labeled active-site peptides

The sequence motifs of W(S/C)XXN**E**PX(S/T) and PXXX(S/T)**E**(Y/F)G are found to be conserved in β-glucuronidases (Table 1) and these motifs have been shown to correspond to regions of the active site of the glycoside hydrolases of family 2 that surround the acid/base and nucleophile residues, respectively.[13,14] To confirm the roles of the glutamate residues in these motifs within thermophilic members of family 2, peptic digestion of recombinant wild-type *T. maritima* β-glucuronidase and of the modified enzyme (2-deoxy-2-fluoro-α-D-glucosyluronic acid-enzyme) were performed. The peptic digests yielded mixtures of peptides, which were separated by reverse-phase HPLC using the ESI mass spectrometer as detector. The labeled peptides were located within the total ion chromatogram by comparison of the peptides present within digests of labeled and unlabeled enzyme, using the expected mass difference of 178 Da between the labeled and unlabeled peptides as confirmation of the locations of the peptides of interest. Only a single pair of peaks fulfilled this requirement of difference in mass by the appended label: a peak at *m*/*z* 738 in the native digest and a peak at *m*/*z* 916 in the labeled digest, which was not detected in the native digest. To gain information on the peptide sequence, fragmentation of the *m*/*z* 916 peptide was carried out in the product ion scan mode. The parent ion of *m*/*z* 916 was subjected to collision-induced dissociation, yielding the spectrum shown in Figure 3. The presence of B-ions with masses of 201, 330, 477, 534, 605 and 720, and of Y″-ions with masses of 738, 639, 538, 409, 262 and 205 strongly supports the sequence of this peptide as VTEFGAD, corresponding to residues 474–480 of *T. maritima* β-glucuronidase. Identification of the point of attachment of the sugar moiety was achieved through inspection of the fragment ions of the 2FGlcUA-labeled peptide. A series of B-ions corresponding to peptides bearing the 2FGlcUA label [VTE (*m*/*z* 380), VTEY (*m*/*z* 509), VTEYG (*m*/*z* 656) and VTEYGA (*m*/*z* 713)] were observed (Fig. 3). Since the B-ions bearing the label include only a single glutamic

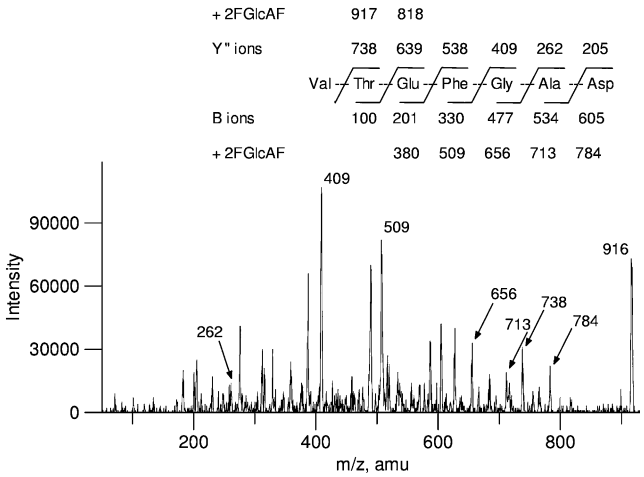

Figure 3. Electrospray tandem mass-spectrometry product ion spectrum of labeled peptide. (The sizes of B and Y″ series fragments are shown.)

acid, we concluded that the label must be attached to E476. Further corroboration of E476 as the catalytic nucleophile in the active site was obtained from analysis of the E476A mutant modified at this position. The E476A mutant protein is totally devoid of any measurable hydrolytic activity towards the synthetic substrate *p*NP β-GlcUA, even when milligram quantities of the E476A mutant enzyme were used in enzyme assays.

2.7. Analysis of acid–base mutants

Sequence alignment analysis of the open reading frame of TM1062 with other members of the family 2 β-glucuronidase grouping (Table 1) suggests that E383 is the likely candidate for the acid/base catalyst in *T. maritima* β-glucuronidase. To identify and quantify the exact catalytic role of E383, two mutants E383A and E383Q were constructed and expressed in the β-glucuronidase-deficient *E. coli* GMS407 strain. Due to the possibility of instability of E383Q mutant enzyme, noted earlier, the heat treatment step normally performed on the crude extract during purification of this enzyme was omitted. The purified E383Q was stored at 4 °C in phosphate buffer containing 500 mM $(NH_4)_2SO_4$. Using *p*NP β-GlcUA as a substrate, E383A exhibits a lower K_M (6.5 μM) than that of the wild-type enzyme, and a k_{cat}/K_M which is ~7-fold lower than that of the wild-type (Table 3). This 7-fold reduction in k_{cat}/K_M for the E383A relative to wild type enzyme is smaller than that seen for many other retaining glycosidases, including mutant human β-glucuronidase, but still in a range that supports its role as acid/base catalyst.[15–18] The very low K_M value observed for E383Q is a consequence of accumulation of the glycosyl–enzyme intermediate. The greater effect on K_M for E383Q than for E383A suggests that the relative rates of formation and hydrolysis of the intermediate are affected more severely for the E383Q

Table 3. Kinetic parameters of *T. maritima* β-glucuronidase and its E383A and E383Q mutants in the absence and presence of 100 mM azide

Enzyme	Temperature (°C)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}\ s^{-1}$)
β-Glucuronidase	75	68 ± 2	0.15 ± 0.01	453
β-Glucuronidase	65	29 ± 1	0.083 ± 0.009	352
E383A	65	0.33 ± 0.007	0.0065 ± 0.0004	51
E383Q	65	0.98 ± 0.01	0.0046 ± 0.0002	213
E383A (100 mM azide)	65	0.46 ± 0.01	0.012 ± 0.0006	40
E383Q (100 mM azide)	65	11.0 ± 0.1	0.069 ± 0.003	160

mutant, with the deglycosylation step being affected more than the glycosylation step. This is consistent with recent observations on other retaining β-glycosidases that departure of the *p*-nitrophenol leaving group is assisted by Gln at the acid/base position, whereas no such assistance is provided by Ala.[19]

Activity rescue of catalytic mutants with added anionic nucleophiles such as azide anion, is one of the most reliable methods for the identification of the acid/base residues of retaining glycosidases.[17,18,20] When assayed with substrates bearing good leaving groups (*p*-nitrophenol or better), mutants modified at the acid/base position undergo rapid formation of the glycosyl–enzyme intermediate (glycosylation), but hydrolysis of this intermediate (deglycosylation) is typically very slow due to the absence of general base catalysis. However, inclusion of anionic nucleophiles such as azide, that do not need general base catalysis, results in rapid turnover of the intermediate, thus rescue of steady state activity, as a result of efficient nucleophilic attack by azide with formation of a glycosyl azide product.

In the present study, the activities of wild-type *T. maritima* β-glucuronidase and the E383A and E383Q mutant proteins were tested in the presence of different concentrations of sodium azide (Fig. 4). Increasing concentrations of azide resulted in increased reaction rates in each case. In the case of E383Q, the rates increased over 10-fold, apparently reaching saturation at approximately 100 mM azide. This apparent saturation behavior is most likely not a reflection of true saturation of an azide binding site, but more likely reflects a change in rate-limiting step such that at higher concentrations of azide the glycosylation step now becomes rate limiting. Much smaller rate increases of approximately 40% were seen with the E383A mutant (Fig. 4, small inset), probably due to a lower rate constant for formation of the glycosyl–enzyme intermediate with the alanine mutant. Thus, the glycosylation step becomes rate-limiting more easily in this case. The very modest increase seen with the wild-type enzyme most likely reflects simple salt effects, as supported by the similar effects of sodium chloride. TLC analysis of reaction products confirmed the formation of β-D-glucosyluronic acid azide. Identical R_f values of 0.15 were seen for the sugar reaction product and for authentic β-D-glucosyluronic acid azide prepared *via* chemical synthesis.

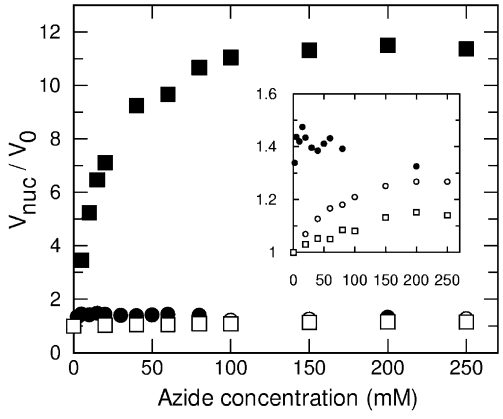

Figure 4. Effect of added azide nucleophile on *T. maritima* β-glucuronidase activity. Enzyme activity was measured at increasing concentrations of azide (0–250 mM). (■) TMGUA E383Q; (●) TMGUA E383A; (○) TMGUA-wt; (□) TMGUA-wt (NaCl). Inset: expansion of vertical axis.

The kinetic parameters for the E383Q and E383A mutants were measured in the presence of 100 mM azide and are also presented in Table 3. As can be seen in both cases, k_{cat} indeed increases (1.4-fold and 11-fold for the Ala and Gln mutants, respectively) as seen in Figure 4. Importantly, K_M values increase by approximately corresponding amounts, with the net effect that k_{cat}/K_M values remain essentially unchanged. These results are completely consistent with the proposed effects of azide, exclusively accelerating the deglycosylation step. The low K_M value for E383Q is due to accumulation of the glycosyl enzyme intermediate, as noted earlier. Since azide increases the deglycosylation rate, there should be less accumulation of intermediate as azide concentrations increase, and K_M values should indeed rise in parallel. Likewise, since azide has no effect on the glycosylation step, k_{cat}/K_M values, which reflect the first irreversible step—glycosylation—should indeed remain unaffected, as is seen.

3. Conclusions

To facilitate the characterization of the β-glucuronidase from *T. maritima* and the utilization of this enzyme in chemoenzymatic synthesis of oligosaccharides, we have

cloned the TM1092 gene that encodes this enzyme. Using the expression plasmid pET28a, the recombinant enzyme was overproduced in *E. coli* and we were able to obtain ~5 mg of the pure and fully active recombinant enzyme from a 1 L culture of *E. coli* carrying pET-TMGUA-wt.

From the conserved sequence motifs W(S/C)-XXN**E**PX(S/T) and PXXX(S/T)**E**(Y/F)G in family 2 β-glucuronidases, amino acid substitution by site-directed mutagenesis revealed that E383 and E476 residues in these motifs were essential for *T. maritima* β-glucuronidase activity. The catalytic nucleophile of *T. maritima* β-glucuronidase was also identified by labeling of the wild-type recombinant enzyme with a mechanism-based inactivator, 2FGlcUAF. Peptic digestion of the 2-fluoro-α-D-glucopyranosyluronic acid–enzyme intermediate and subsequent analysis by HPLC and tandem mass spectrometry allows the identification of E476 as the catalytic nucleophile contained in the sequence VT**E**FGAD, corresponding to residues 474–480 of *T. maritima* β-glucuronidase.

CD spectroscopy studies indicate that E476A and E383A mutant proteins exhibit conformational integrity and thermostability comparable to or better than that of the recombinant wild-type enzyme while the E383Q mutant protein has somewhat lower thermostability. Consequently, mutants of this enzyme may be useful as catalysts for the syntheses of O- and S-linked glycosides as long as reaction temperatures are suitably modulated.

4. Experimental

4.1. Materials

Biochemical reagents for molecular biological procedures were obtained from New England Biolabs (Mississauga, ON, Canada), Gibco-BRL (Burlington, ON, Canada) and Boehringer Mannheim (Laval, QQ, Canada). *p*-Nitrophenyl β-D-glycosides, D-saccharic acid 1,4-lactone, and 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlcUA) were purchased from Sigma (St. Louis, USA). Isopropyl β-D-thiogalactoside (IPTG) was obtained from Rose Scientific (Edmonton, Canada). The synthesis of 2-deoxy-2-fluoro β-D-glucosyluronic acid fluoride (2FGlcUAF) has been described elsewhere.[21] β-D-Glucosyluronic acid azide was synthesized as described.[22]

4.2. Bacterial strains, plasmids, and media

Thermotoga maritima strain MSB8 was obtained from ATCC (American Type Cell Culture, USA). *E. coli* β-glucuronidase-deficient strain, GMS407, was obtained from *E. coli* Genetic Stock Centre, USA (CGSC #5497). Lysogenization of λDE3 prophage into GMS407 was accomplished using λDE3 Lysogenization Kit (Novagen, Madison, WI) following a protocol recommended by the supplier. Other *E. coli* strains used were TOP10 and DH5α. The plasmids used were pCR-Blunt (Zero Blunt PCR Cloning Kit, Invitrogen) and pET28a(+) (Novagen, Madison, WI).

4.3. DNA manipulations

Oligodeoxynucleotide primers were synthesized by the Nucleic Acid and Protein Service (NAPS) Unit at the University of British Columbia. Small scale plasmid preparation was conducted using Qiaprep Spin Miniprep kits (Qiagen, CA). Restriction endonucleases were utilized according to recommended protocols from the suppliers. Bacterial transformations and agarose gel electrophoresis were done according to standard procedures. DNA fragments were extracted from agarose gels with Qiaex II or QIAquick DNA purification kit (Qiagen, CA). DNA was sequenced by the NAPS Unit employing an Applied Biosystems model 377 sequencer and the AmpliTaq dye termination cycle sequencing protocol.

4.3.1. PCR and cloning of the gene encoding a thermostable β-glucuronidase. The gene encoding the putative β-glucuronidase was amplified by polymerase chain reaction (PCR) from lysed *T. maritima* strain MSB8 using *Pwo* DNA polymerase and primers TMgusf (5′ GATATTTCGGATCCAACAAGTGATTACGG 3′) and TMgusr (5′ CAAGCGATCCGCTGAGCAAA-GTTCAAGAGG 3′). Unless otherwise stated, the conditions for all PCR reactions used in this study consisted of 30 cycles of 30 s, 94 °C; 30 s, 53 °C; 60 s, 72 °C. A final extension of 5 min at 72 °C was conducted at the end of the 30 cycles to complete the PCR. The desired ~1.8 kb DNA fragment was excised from a 1% agarose gel and recovered using a QIAquick gel purification kit and cloned into pCR-Blunt to give pCR-TMGUA-wt and subsequently sequenced.

4.3.2. Construction of the plasmid for protein expression. To amplify and to subclone the DNA fragment encoding β-glucuronidase into an expression plasmid, TMAugf sense primer (5′ GGAGGATAAA<u>CATATG</u>GTAAGACCGCAACG 3′), TMAugr anti-sense primer (5′ GCCGAACATCAGTC<u>CTCGAG</u>CTCAA-ACCTCACTCC 3′) carrying *Nde*I and *Xho*I restriction sites (underlined) and pCR-TMGUA-wt as template were used in a PCR reaction to amplify a ~1.7 kb fragment that was subsequently subcloned into pET28a(+) to give pET-TMGUA-wt. The expression plasmid construct was verified by DNA sequencing.

4.3.3. Site-directed mutagenesis of amino acid residues in the putative active site. To generate the desired site-directed mutants, a two-step, four primer PCR method was employed. To create pET-TMGUA-E476A, pET-TMGUA-wt as template and the following primer combinations were used in the first amplification step: TME476Af (5′ GCCCATCTTTGTCACA**GC**ATTCG-GTGCGGACG 3′) with TMAugr and TME476Ar (5′ CGTCCGCACCGAAT**GC**TGTGACAAAGATGGGC 3′) with TMKpnI (5′ CACGTTGGTATCACA-AGGTACCACTACAATC 3′), the nucleotide substitutions are in boldface, and the mutated codons and the *Kpn*I site are underlined. The amplified fragments with a length of 303 and 417 bp, respectively were used as templates in a second reaction with TMKpnI and TMAugr as primers. The 689-bp PCR product was then subcloned into the *Kpn*I and *Xho*I sites of the expression plasmid pET-TMGUA-wt cut with the same restriction enzymes generating the mutated expression plasmid pET-TMGUA-E476A. Construction of other mutants was similar, using primers TME383Af (5′ GTGTGG-CGAAC**GCC**CCAGAGTCCAACC 3′), TME383Ar (5′ GGTTGGACTCTGG**GGC**GTTCGCCACAC 3′), TMAugr and TMKpnI to create pET-TMGUA-E383A for PCR and subcloning *via* the restriction enzymes *Kpn*I and *Xho*I to give pET-TMGUA-E383A. Primers T7, T7term, TM1062-E383Q Rv (5′ GGACTCTGG**TTG**GTTCGCCAC 3′) and TM1062-E383Q Fd (5′ GTGGCGAAC**CAA**CCAGAGTCC 3′) were used to amplify TMGUA-E383Q and restriction enzymes *NdeI* and *Xho*I to subclone the resulting fragment into pET28a and give the plasmid pET-TMGUA-E383Q. All constructs were verified by DNA sequencing and found to be free of any errors that could have been introduced by PCR amplification.

4.4. Production and purification of *T. maritima* β-glucuronidase and mutants

To express *T. maritima* β-glucuronidase-encoding gene, lysogenized *E. coli* GMS407 cells harboring the appropriate expression plasmid were grown at 37 °C in eight 2 L Erlenmeyer flasks each containing 500 mL LB medium supplemented with 50 μg/mL kanamycin until the $OD_{600\,nm}$ reached 0.6. IPTG was added to 0.4 mM, and incubation was continued for 5 h. The cells were harvested by centrifugation at 5000*g* for 15 min at 4 °C, and stored at −20 °C until used.

Unless otherwise stated, all purification steps were carried out at ~4 °C. Cell pellet (4 L culture) was resuspended in 30 mL lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10 mM imidazole). The resuspended cells were ruptured by three passages through a French pressure cell (16,000 psi), and cell debris was removed by centrifugation at 30,000*g* for 30 min. The supernatant was transferred to clean centrifuge tubes and immersed in a 75–80 °C water bath for 10 min followed by centrifugation for 10 min at 30,000*g*. β-Glucuronidase was purified from the clarified heat-treated supernatant by immobilized metal affinity chromatography using a column containing approximately 25 mL packed vol of His-Bind resin (Novagen, Madison, WI) or Hi-Prep chelating columns (Amersham) attached to an FPLC system (Amersham-Biosciences). Column preparation, equilibration, and sample loading followed standard procedures recommended by the resin manufacturer. Throughout the manipulation the flow rate was maintained at 1 mL/min. The bound protein was finally eluted with a sharp 15–100% linear gradient of elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 250 mM imidazole). Fractions were analyzed for the presence of enzyme by assay at 75 °C with *p*-nitrophenyl β-D-glucosyluronic acid (*p*NP β-GlcUA) substrate and/or SDS-PAGE analysis (10% gels) for purity. In some preparations, impurities could still be seen by SDS-PAGE analysis. These were readily removed by hydrophobic interaction chromatography on a Phenyl Sepharose column. Elution with a 0.5–0 M descending gradient of ammonium sulfate in 25 mM phosphate buffer pH 7 yielded pure enzyme. Enzyme-containing fractions were pooled and dialyzed against 50 mM NaH_2PO_4, pH 7.0 until the imidazole or ammonium sulfate concentration was diluted 1000-fold. The dialyzed sample was concentrated in an ultrafiltration stirred cell (Amicon; NMW 5 kDa cutoff membrane) or Centricon (NMW 10 kDa cutoff). For the purification of the E383Q mutant enzyme the heat treatment step was omitted. In addition, the fractions containing the mutant enzyme from the immobilized-metal affinity chelation chromatography were pooled and concentrated without prior dialysis using an ultrafiltration stirred cell (10 kDa cut-off, Amicon). The sample was buffer exchanged by size exclusion using a PD-10 Sephadex G-25M column (Amersham-Biosciences) that was pre-equilibrated in storage buffer (50 mM NaH_2PO_4, 500 mM $(NH_4)_2SO_4$, pH 6.5). The inclusion of ammonium sulfate in the storage buffer prevented the formation of precipitate. All enzymes were stored at 4 °C until used.

4.5. Circular dichroism studies

CD scans were recorded on a JASCO spectrophotometer in a 0.1 cm cuvette. The purified protein was diluted with filter-sterilized (10 mM) HEPES buffer (pH 7) to approximately 200 ng/μL. Samples were analyzed in scanning mode in a wavelength range from 230 to 190 nm. Thermostability of the enzymes was tested by heating at a rate of 1 °C/min or by incubation at a fixed temperature of 62 °C for 1 h and recording the signal at 217 nm.

4.6. Enzyme assay and kinetics

Discontinuous assays were used at 75 °C. Enzymes were tested for the release of *p*-nitrophenolate from the hydrolysis of *p*NP β-GlcUA, *p*NP β-D-galactosiduronic acid and *p*NP β-glucoside. The standard assays contain 1 mM substrate and 50 mM sodium phosphate buffer, pH 6.5, or if possible, substrate concentrations from 0.2 to 5 times K_M for determination of kinetic parameters. The soln of the substrate was pre-incubated for five minutes at 75 °C, and the reaction was initiated by the addition of the enzyme (0.6 μg) to a total reaction vol of 2.0 mL. At 30 s intervals, a 50 μL aliquot of the reaction mixture was withdrawn, and the reaction was stopped in 950 μL of 100 mM sodium carbonate. The absorbance of *p*-nitrophenolate in each of the stopped reaction mixtures was measured at 405 nm using a 1 cm cuvette. The amount of *p*-nitrophenolate was calculated using a molar absorptivity value of 18,000 M^{-1} cm^{-1}.[23]

At 65 °C, the reaction progress was monitored by continuous spectrophotometric assay using a CARY-4000 spectrophotometer (Varian Inc.). Unless otherwise noted the assays were performed in 50 mM sodium phosphate buffer (pH 6.5 at room temperature) and 0.1% v/w BSA at 65 °C. In brief, the pre-warmed capped quartz cuvette (1 cm pathlength) was loaded with 700 μL of reaction buffer containing the substrate (if possible at least seven points from 0.2 to 5 times K_M) and incubated at 65 °C for about 7 min. Spontaneous hydrolysis of substrates was monitored prior to the addition of enzymes. Diluted enzyme soln (50 μL) was added to the cuvette and the change in absorption was monitored at 405 nm. GraFit 4.0 (Erithacus Software) was used to generate the apparent kinetic parameters k_{cat} and K_M.[24] Azide rescue studies were performed either at a fixed concentration of substrate (1 mM) and increasing concentrations of azide (20–250 mM) or with increasing *p*NP β-GlcUA concentrations (0.02–2 mM) at a fixed concentration of 100 mM azide (pH 6.5 at room temperature). An absorption coefficient of 9820 M^{-1} cm^{-1} was determined for *p*-nitrophenolate in 50 mM sodium phosphate buffer at 65 °C. To determine thermostability at elevated temperature TMGUA wild type enzyme was incubated at 80 and 85 °C, respectively, in 50 mM sodium phosphate buffer pH 6.5. Aliquots were withdrawn after certain time points and assayed under standard conditions using the stopped assay at 75 °C.

4.7. TLC analysis of reaction products

Thin layer chromatography was done using Silica Gel 60 F254 (E. Merck) and a mixture of 7:2:1 EtOAc–MeOH–water. Separated compounds were visualized by UV and by charing in 10% H_2SO_4 in MeOH and subsequent heating.

4.8. Inactivation of *T. maritima* β-glucuronidase

The inactivation of *T. maritima* β-glucuronidase by 2FGlcUAF was performed by incubation of the purified recombinant enzyme (1.4 μg) with various concentrations (0.1–1.0 mM) of 2FGlcUAF at 37 °C in a total vol of 100 μL, containing 50 mM phosphate buffer, pH 6.5. The residual enzyme activity was monitored at various time intervals by addition of a 5 μL aliquot of the inactivation mixture to a soln of *p*NP β-GlcUA (2 mM, 495 μL) in 50 mM phosphate buffer, pH 6.5 and measurement of the rates of *p*-nitrophenolate release over a period of 3 min at 75 °C.

4.9. Labeling, proteolysis and electrospray mass spectrometric analysis

The labeling of *T. maritima* β-glucuronidase, the proteolytic digestion and subsequent ESIMS analysis of the labeled enzyme were carried out essentially according to the procedures described by Wong et al.[21] Purified *T. maritima* β-glucuronidase (85 μg) was incubated with 2FGlcUAF (5 mM) in a total vol of 150 μL in 50 mM sodium phosphate buffer, pH 6.5 for 1 h at 37 °C. Almost complete inactivation (>95%) was confirmed by enzyme assay. The inactivated enzyme sample was immediately digested with pepsin (30 μL, 15 mg/mL in 2 M phosphate buffer pH 2) for 1 h at 37 °C, frozen and analyzed immediately by LC/MS upon thawing. A control consisting of native recombinant *T. maritima* β-glucuronidase enzyme sample (85 μg) was also subjected to peptic digestion, frozen, and analyzed as above.

Mass spectra were recorded on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an Ionspray ion source. Peptides were separated by reverse phase HPLC on an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA) directly interfaced with the mass spectrometer. In each of the MS experiments, the proteolytic digest was loaded onto a C18 column (Reliasil, 1 × 150 mm), then eluted with a gradient of 0–60% solvent B over 60 min, followed by 100% B over 2 min at a flow rate of 50 μL/min (solvent A: 0.05% trifluoroacetic acid, 2% MeCN in water; solvent B: 0.045% trifluoroacetic acid, 80% MeCN in water). Spectra were obtained in either the single-quadrupole scan mode (LC/MS) or the tandem MS product ion scan mode (LC/MS/MS). In the single quadrupole mode (LC/MS), the quadrupole mass analyzer was scanned over a m/z range of 300–2200 Da with a step size of 0.5 Da and a dwell time of 1 ms/step. The ion source voltage (ISV) was set at 5 kV, and the orifice energy (OR) was 50 V. In the tandem MS product ion scan mode, the spectrum

was obtained by selectively introducing the precursor ion (m/z 916) from the first quadrupole (Q1) into the collision cell (Q2) and observing the product ions in the third quadrupole (Q3). Thus, Q1 was locked on m/z 916; Q3 scan range: 50–930; step size: 0.3; dwell time: 2 ms; ISV: 5 kV; OR: 50; Q0 = −10 V; IQ2 = −49 V.

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