Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 2943

www.rsc.org/obc

PAPER

MS/MS fragmentation-guided search of TMG-chitooligomycins and their structure–activity relationship in specific β-*N*-acetylglucosaminidase inhibition†

Hirokazu Usuki,[a,b] Yukihiro Yamamoto,[a] Yuya Kumagai,[a] Teruhiko Nitoda,[c] Hiroshi Kanzaki[c] and Tadashi Hatanaka*[a]

Received 26th November 2010, Accepted 9th February 2011
DOI: 10.1039/c0ob01090a

The reducing tetrasaccharide TMG-chitotriomycin (**1**) is an inhibitor of β-*N*-acetylglucosaminidase (GlcNAcase), produced by the actinomycete *Streptomyces anulatus* NBRC13369. The inhibitor shows a unique inhibitory spectrum, that is, selectivity toward enzymes from chitin-containing organisms such as insects and fungi. Nevertheless, its structure-selectivity relationship remains to be clarified. In this study, we conducted a structure-guided search of analogues of **1** in order to obtain diverse *N*,*N*,*N*-trimethylglucosaminium (TMG)-containing chitooligosaccharides. In this approach, the specific fragmentation profile of **1** on ESI-MS/MS analysis was used for the selective detection of desired compounds. As a result, two new analogues, named TMG-chitomonomycin (**3**) and TMG-chitobiomycin (**2**), were obtained from a culture filtrate of **1**-producing *Streptomyces*. Their enzyme-inhibiting activity revealed that the potency and selectivity depended on the degree of polymerization of the reducing end GlcNAc units. Furthermore, a computational modeling study inspired the inhibitory mechanism of TMG-related compounds as a mimic of the substrate in the Michaelis complex of the GH20 enzyme. This study is an example of the successful application of a MS/MS experiment for structure-guided isolation of natural products.

Introduction

Discovering a naturally occurring small molecule with unique chemical structure/biological activity is one of the main lines of inquiry in natural product chemistry. Its strategies can be divided into two distinct methods: "activity-guided" and "structure-guided" screening. The former strategy often provides an opportunity to obtain chemicals with novel skeletal structures. In contrast, structure-guided search is specific for obtaining analogues of known compounds and demands the following limitations. The desired compound must possesses the specific structure-reporting groups. Furthermore, it should be routinely and quantitatively detectible with high sensitivity. These conditions were thought to be mainly satisfied by the combination of spectrochemical analysis and liquid chromatography (LC) methods such as LC coupled with ultraviolet photodiode arrays (LC/UV).

Our group has adapted the activity-guided strategy. As a result, we have discovered two new chemicals, TMG-chitotriomycin (**1**)[1] and pochonicine,[2] which we isolated from the metabolites of microorganisms as potent inhibitors of β-*N*-acetylglucosaminidase (GlcNAcase, EC 3.2.1.52.). Here, the term "GlcNAcase" is generally used to refer to several distinct families of enzymes including GH3, GH20, and GH84 (For an overview, see the recent review by Slavova *et al.*[3]). In brief, GH20 and GH84 enzymes have amino acid sequences low in similarity and distinct substrate specificities (For further details, see the CAZY database[4] at http://www.cazy.org/). They possess the same type of reaction mechanism known as a substrate-assisted mechanism. GH20 GlcNAcases catalyze the removal of the non-reducing end D-*N*-acetylglucosamine (GlcNAc) from chitooligosaccharides composed of β-1,4-linked GlcNAcs. The enzymes are required for the complete degradation of the polysaccharide chitin and are responsible for the normal growth of chitin-containing organisms including insects and fungi.[5] GH84 enzymes also remove the non-reducing end GlcNAc. However, their natural substrates are not chitooligosaccharides but *O*-GlcNAc-modified proteins; therefore, they are called *O*-GlcNAcases.[3] The structures of known GlcNAcase inhibitors are shown in Fig. 1. Of these inhibitors, **1** selectively inhibits the GlcNAcases of chitin-containing organisms

[a]*Okayama Prefectural Technology Center for Agriculture, Forestry and Fisheries, Research Institute for Biological Sciences (RIBS), 7549-1 Kibichuo-cho, Kaga-gun, Okayama, 716-1241, Japan. E-mail: hatanaka@bio-ribs.com; Fax: + 81 866 56 9454; Tel: + 81 866 56 9452*
[b]*Research Fellow of the Japan Society for the Promotion of Science (JSPS), Japan*
[c]*Laboratory of Bioresources Chemistry, The Graduate School of Natural Science and Technology, Okayama University, Okayama, 700-8530, Japan*
† Electronic supplementary information (ESI) available: LC conditions, Lineweaver–Burk plots, and NRM spectra of **2** and **3**. See DOI: 10.1039/c0ob01090a

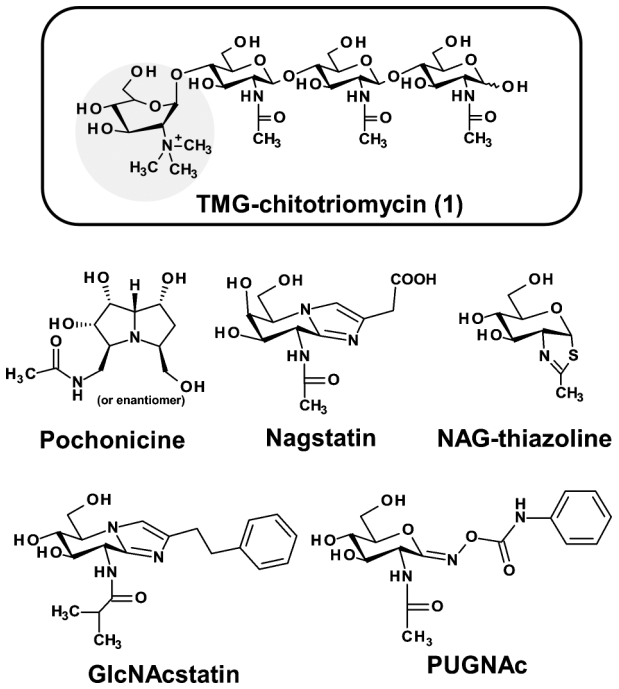

Fig. 1 Structures of TMG-chitotriomycin and known inhibitors of GlcNAcase (GH20) and/or *O*-GlcNAcase (GH84).

including insects and fungi, but not of non-chitin-containing organisms such as plants and mammals. Such species-specific inhibition has never been reported for other known inhibitors. Therefore, clarification of structure-selectivity relationships of **1** will be a challenge. The characteristic structural features of **1** are its molecular size and the presence of an unusual sugar residue, *N*,*N*,*N*-trimethylglucosaminium (TMG), which is linked in a β-1,4 manner. In addition, its conformation is a unique *twist-boat* form, as demonstrated and revised by Yang *et al.*[6] In the case of NAG-thiazoline, its nitrogen atom was proposed to be positioned around the key Asp residue of the enzymes to occupy their -1 subsite.[7,8] Such key nitrogen atoms are also present in the *N*-acetyl groups of pochonicine,[2] nagstatin,[9–11] PUGNAc,[12–14] and GlcNAcstatin.[15,16] The nitrogen atom of the *N*,*N*,*N*-trimethyl group of TMG on **1** may be such a key nitrogen judging from its structure, competitive mode of inhibition, and substrate recognition property of GlcNAcase as *exo*-splitting manner. From the observations and speculations above, we hypothesized that the unique selectivity of **1** might mainly originate from the degrees of polymerization (DP) of the chitooligosaccharide unit at the reducing end. If true, the selectivity for analogues of TMG-chito-"trio"-mycin **1** (TMG-$GlcNAc_3$) with different DPs such as TMG-chito-"mono"-mycin (TMG-GlcNAc) or TMG-chito-"bio"-mycin (TMG-$GlcNAc_2$) should also be distinct from that of **1**. In this paper, we refer such TMG-containing chitooligosaccharides as TMG-chitooligomycins. Here, the fermentation conditions of *Streptomyces anulatus* NBRC13369 for producing **1** inspired the following ideas. As described in a previous paper,[1] **1** was produced only when chitin was the sole carbon source in the production medium. It is reasonable to expect that biosynthesis of **1** will be correlated with the degradation of chitin in the medium because **1** possesses a chitotriose unit, which is also the component unit of chitin. Therefore, TMG-chitooligomycins, our desired chemicals, might also be produced by **1**-producing *Streptomyces*.

We adapted the structure-guided search as our main strategy. One of the major problems is the lack of characteristic UV adsorption profiles of desired compounds. Therefore, the specific MS/MS fragmentation profile of **1** was used to selectively detect the desired compounds by LC/multireaction monitoring (MRM) analysis. As expected, **1**-producing *Streptomyces* proved to be capable of producing putative TMG-chitomonomycin and TMG-chitobiomycin. The compounds were isolated, identified, and evaluated in terms of their GlcNAcase inhibitory activities. Furthermore, their biosynthesis and mechanism of inhibitory action were also investigated.

Results and discussion

ESI-MS/MS analysis of 1

As reported in our previous paper[1] and in an article by another group,[6] **1** possesses a unique physicochemical characteristic, *i.e.*, that the C-2 methine carbon of the reducing end GlcNAc is slowly deuterated in the CD_3OD solution state. Both **1** and deuterated **1** were subjected to ESI-MS/MS analysis (API2000) to clarify the specific fragmentation profiles originated from TMG-containing substructures. As shown in Fig. 2, the observed ions were almost identical except for their remaining precursor ions (*m/z* 831 and 832), indicating that the major fragment ions originated from the non-reducing triose unit containing the TMG residue. The *m/z* values of 168, 186, and 204 were reported as characteristic fragmentations of chitooligosaccharides.[17] Indeed, our analysis of GlcNAc and chitooligosaccharides (DP = 2 to 6) also confirmed such fragmentation profiles (Fig. S2–S7, ESI†). Here, ions of *m/z* 348 were observed in the MS/MS spectra of both intact and deuterated **1**. Furthermore, the ion was not observed in the spectra of $GlcNAc_1$ to $GlcNAc_6$ (Fig. S2–S7†). Therefore, the ion was suggested to be characteristic of TMG-chitooligomycins. The proposed fragmentation profile of **1** is shown in the upper panel of Fig. 2. The removal of the *N*,*N*,*N*-trimethyl group to

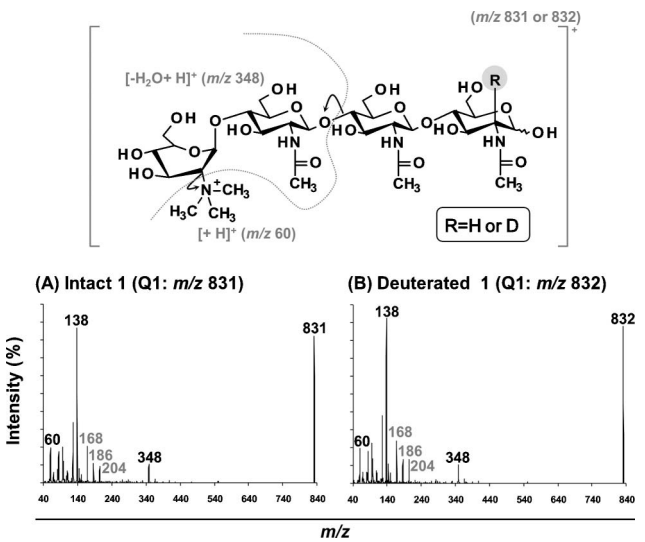

Fig. 2 MS/MS spectra of intact (A) and deuterated **1** (B). Proposed fragmentation profile is shown in the upper panel.

give ions of *m/z* 60 would be characteristic of TMG-relating compounds because such fragmentation was also confirmed for the monosaccharide TMG (Fig. S8†).

Detection of putative TMG-chitotriomycin analogues: TMG-chitomonomycin and TMG-chitobiomycin

As already described, the fragment ion of *m/z* 348 is speculated as characteristic of our desired TMG-chitooligomycins. We tuned the MRM channel for each of the different degrees of polymerization for putative TMG-chitooligomycins (DP = 2 to 4). In brief, authentic **1** was used to optimize the MS/MS instrument to construct the MRM channels: *m/z* 831 as the precursor ion (Q1), *m/z* 348 as the fragment ion (Q3). The corresponding MRM channels for each of the putative TMG-chitooligomycins were constructed by alternatively using their expected *m/z* of the M^+ ion as the Q1 values. MRM channels for monosaccharide TMG, GlcNAc, and chitooligosaccharides (DP = 2–6) were also constructed because the presence of such compounds were speculated as judged from fermentation conditions in which the polysaccharide chitin was contained as the sole carbon source.

S. anulatus NBRC13369 was cultivated in the **1**-producing medium for 8 d as described in the Experimental section. The culture filtrate was analyzed by LC/MRM analysis (Fig. 3). The small inner table indicates the used MRM channels. Fig. 3(B) shows the MRM chromatogram of the authentic mixture composed of TMG, $GlcNAc_1$-$GlcNAc_6$, and deuterated **1**. In our analysis, $GlcNAc_1$ to $GlcNAc_6$ shows two peaks corresponding to its β and α anomers. This phenomenon was in agreement with the study of Suginta *et al.*[18] The monosaccharide TMG, TMG-chitotriomycin (**1**), and putative TMG-chitobiomycin (**2**) were detected by each of the corresponding MRM channels (Fig. 3(A)). Here, **3** is detected by the MRM channel for $GlcNAc_2$; however, its retention time was apparently different from authentic $GlcNAc_2$. It should be noted that the *m/z* of the precursor ions of putative TMG-chitomonomycin and that of $GlcNAc_2$ was the same, *viz.*, *m/z* 425. Therefore, we speculated that **3** might be our desired TMG-chitomonomycin and that its fragmentation profile unfortunately overlapped with that of $GlcNAc_2$. The observations

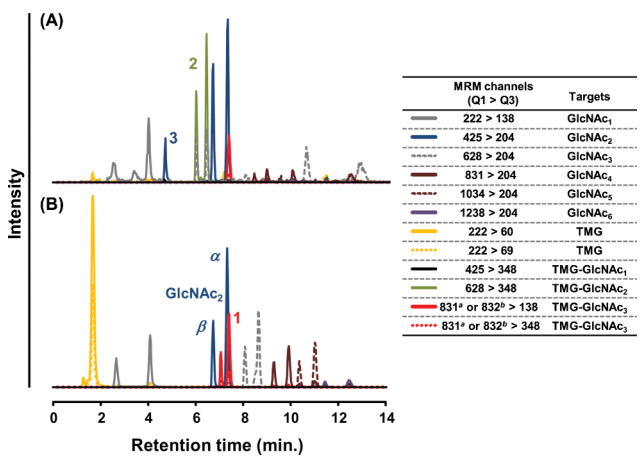

Fig. 3 MRM-guided search of putative TMG-chitooligomycins. MRM chromatograms of the culture filtrate (A) and authentic mixture (B) are shown. Used MRM channels are shown in the inner table. [a] For intact **1** (upper panel). [b] For deuterated **1** (lower panel).

and speculation above prompted us to isolate **2** and **3** using the MRM chromatogram-guide purification procedure. As a result of the fermentation and isolation of **2** and **3** described in the Experimental section, 8.65 mg of **2** and 11.4 mg of **3** were isolated from 4,990 mL of culture filtrate. The purified materials were further analyzed for identification.

Identification of 2 and 3

HRESIMS (micrOTOFII-SKA) of **2** and **3** showed M^+ peaks at *m/z* 628.2896 and *m/z* 425.2108, in agreement with the formulas for TMG-chitobiomycin ($C_{25}H_{46}N_3O_{15}$, calcd 628.2923) and TMG-chitomonomycin ($C_{17}H_{33}N_2O_{10}$, calcd 425.2130), respectively. The isolated compounds **2** and **3** were analyzed by MS/MS and NMR experiments including ^1H, ^{13}C, HSQC, HMBC, COSY, 1D-TOCOSY, and NOESY. The MS/MS spectra of **2** and **3** were quite similar except for the remaining precursor ions of each compound (left panels of Fig. 4). The fragment ions of *m/z* 60 as reflecting the removal of *N*,*N*,*N*-trimethyl group, and of *m/z* 348 as characteristic of TMG-chitooligomycins, were also observed. As judged by the *m/z* values of their precursor ions, **2** and **3** were strongly suggested to be TMG-chitobiomycin and TMG-chitomonomycin, respectively. Indeed, detailed analysis of the series of NMR spectra easily deduced their planar structures to be those of **2** and **3** (Section-5 of ESI†). Their ^1H NMR spectra are shown in the right panels of Fig. 4, where key resonances as structure-reporting groups are shaded and expanded. The resonances around δ_H 3.3 (9H, s), which were observed in both **2** and **3**, were those of methyl groups in an *N*,*N*,*N*-trimethyl group of the TMG residue.[1] In the case of **3**, the resonance was significantly affected by the equilibrium of the reducing GlcNAc to give two distinct peaks (δ_H 3.318, δ_H 3.322) because the TMG residue of **3** was directly connected to the reducing GlcNAc. Such a characteristic was observed for A-1 (δ_H 5.41 and δ_H 5.42), B-1 (δ_H 4.71 and δ_H 5.19), and B-8 (δ_H 2.044 and δ_H 2.046). In the case of **2**, the effect was only observed in sugars B and C.

We speculated that the tetrasaccharide **1** (Fig. 1) might be recognized by the chitinase because such enzymes are generally capable of recognizing chitotetraose as a substrate. From this point of view, **1** was treated with *Streptomyces* chitinase and then subjected to LC/MRM analysis. As shown in Fig. 5, tetrasaccharide **1** was almost completely hydrolyzed by the *Streptomyces* chitinase to give the two compounds **A** and **B**, detected by the MRM channels for **3** and **2**, respectively. The co-chromatography experiments of chitinase-treated **1** with **2** or **3** also confirmed the above identity (data not shown). Therefore, the planar structures of **2** and **3** are the same as those of TMG-chitobiomycin and TMG-chitomonomycin, respectively. As a result, 6.6 nmol of **1** gave 5.1 nmol of **2** and 1.5 nmol of **3**, indicating that the enzyme treatment proceeded stoichiometrically. In addition, **2** and **3** were not hydrolyzed by the above chitinase treatments. The results suggest that **1** would be the precursor of **2** and **3**, in which chitinase is involved in their biosynthesis.

In order to determine absolute configurations, **2** and **3** were subjected to butanolysis (1.4 M HCl/2*R*-butanol), *N*-acetylation (Ac_2O/pyridine), and LC/MRM analysis. In this analysis, authentic D-GlcNAc was subjected to the same treatment. The generated 2*R*-butylglycoside was used to construct the MRM channel. It should be noted that our analysis system could

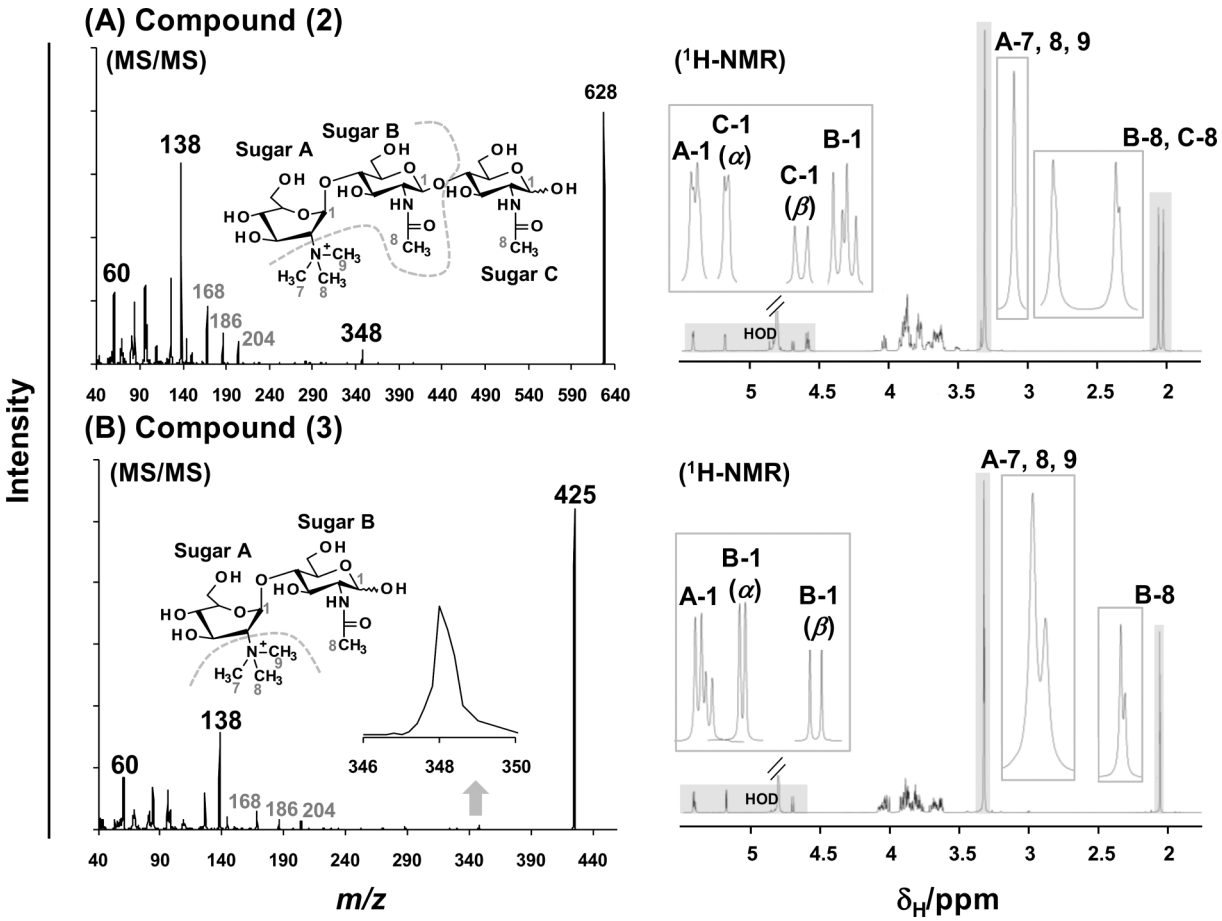

Fig. 4 MS/MS and ^{1}H-NMR spectra of **2** and **3**. Proposed structures and their fragmentation profiles upon MS/MS analysis are shown as small inner images.

sufficiently distinguish the D- and L-forms of glucose as shown in Fig. S1†. As a result, butanolysis of 30.1 nmol of **2** and 43.5 nmol of **3** gave 40.2 and 21.0 nmol, respectively, of D-GlcNAc. The co-chromatography experiment also supports this observation. Therefore, the presence of D-GlcNAc residues in **2** and **3** were demonstrated. The planar structures of **2** and **3** were already clarified as described above. Given that **2** and **3** possess the D-GlcNAc residue, the remaining TMG residues at the non-reducing end of **2** and **3** were logically proposed to be the same as that of **1** (*i.e.*, D-TMG). From these results, compounds **3** and **2** were identified to be the TMG-chitomonomycin and TMG-chitobiomycin, respectively.

The synthesis of **1** was already achieved by Yang *et al.*[6] Their study revised the originally proposed structure of **1** (α-1,4-linked D-TMG) to a β-1,4 manner. In addition, the study described the interesting characteristic of its non-reducing end TMG as having the *twist-boat* conformation. After their paper was published, we reanalyzed the series of NMR spectra for **1**, and noticed that such a characteristic was also confirmed by our NOESY experiment in which a correlation of H-1/H-2, H-1/H-3, and H-1/H-5 of the non-reducing TMG residue was observed. Those of **2** and **3** were also observed in this study (Section-5 of ESI†), supporting such a *twist-boat* conformation of the TMG residue. Interestingly, the *twist-boat* conformation was not adopted for the monosaccharide TMG because of the absence of the above NOE correlations and the presence of a correlation between H-1/H-2 and H-3/H-5. Rather the $^4C_1$ was a typical one with an α anomeric configuration (Section-6 of ESI†). Logically, these data suggest that the reducing end GlcNAc units of TMG-chitooligomycins distort a $^4C_1$ conformation of TMG into the *twist-boat* form.

Biosynthesis

As already described, **1** was speculated as the precursor of **2** and **3**. Therefore, the time course of the production was monitored by LC/MRM analysis for TMG, **1**, **2**, and **3** (Fig. 6). Compound **1** appeared at 4 d, rapidly reached a maximum after 6 to 7 d, and gradually decreased thereafter. On the other hand, **2** and **3** also appeared at 4 d, rapidly reached a maximum, and remained at a high level for 14 d. These observations support the following biosynthetic steps: chitin, the sole carbon source in the medium, was converted into **1**, followed by the hydrolysis with the chitinase to give **2** and **3**. Here, the notable result in Fig. 6 is the molar ratio of the maximum amounts of **2** and **3** relative to the maximum amount of **1**, which was around 1.3. In addition, the maximum amounts of **2** and **3** during 14 d of cultivation were approximately the same level (around 8 nmol mL^{-1} of culture). This observation might be explained as follows. Several types of TMG-chitooligomycins might be produced as the precursor(s) of **1**. The precursor(s) should be hydrolyzed by chitinases or other

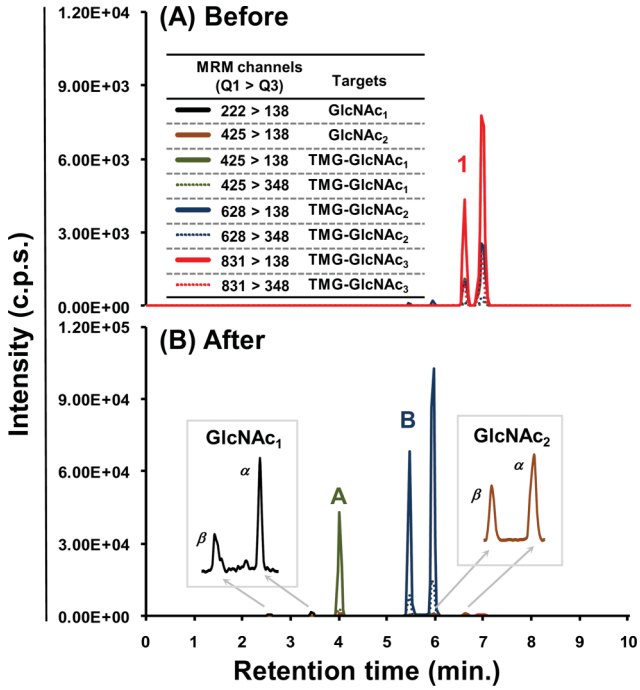

Fig. 5 MRM chromatograms of **1** before (A) and after (B) chitinase treatment. The used MRM channels are shown in the inner table.

chitinolytic enzymes including GlcNAcases to give **1**, **2**, and **3**. In this degradation pathway, **1** would also be hydrolyzed by chitinase to give **2** and **3**. Further works, such as the feeding experiments of **1**, will be needed to clarify this possibility.

Enzyme inhibitory activity and structure-selectivity relationships

Table 1 shows the GlcNAcase inhibitory activities of TMG, **1**, **2**, and **3**. The monosaccharide TMG shows only weak inhibition of Jack bean GlcNAcase with no inhibition of others at 400 μM, indicating that the reducing chitooligosaccharide units were essential for inhibitory potency. SCO2758, the GH3 enzyme from *Streptomyces coelicolor*, was insensitive to **1**, **2**, **3**, and TMG. In contrast, SCO2786, the GH20 GlcNAcase from *S. coelicolor*, was inhibited by **1**, **2**, and **3**. Their mode of inhibition was competitive (Section-4 of ESI†), indicating that the inhibitors bound to the active site of the SCO2786. It is notable that the GlcNAcases of *A. oryzae* and SCO2786 were distinct from their sensitivities to TMG-chitooligomycins. The order of magnitudes for the inhibitory effects on the *A. oryzae* enzyme was **1** (K_i = 0.977 μM), **2** (K_i = 47.4 μM), and **3** (K_i = 360 μM). In addition, **1** (TMG-GlcNAc$_3$) was around 50 times more potent than **2** (TMG-GlcNAc$_2$) toward the *A. oryzae* GlcNAcase. In contrast, SCO2786 was potently inhibited by **1** (IC_{50} = 23.3 μM, K_i was not determined) and **2** (IC_{50} = 51.9 μM, K_i = 3.41 μM) and moderately inhibited by **3** (IC_{50} = 178 μM, K_i = 44.0 μM). Therefore, their DP of reducing chitooligosaccharide units and selectivity are directly correlated. The case of Jack bean GlcNAcase was also informative. The enzyme was weakly inhibited by TMG (43.6% inhibition at 400 μM) and **3** (K_i = 650 μM), but not by **1** and **2**, again indicating a correlation between their selective inhibition and the DP of TMG-chitooligomycins. We also checked whether **1**, **2**, and **3** were hydrolyzed by the used GlcNAcases. LC/MRM analysis of the reaction mixtures of the enzyme inhibition assays above revealed the stable presence of **1**, **2**, **3**, and TMG (data not shown). Therefore, TMG-chitooligomycins were shown to be selective inhibitors, and not substrates, of GlcNAcases with distinct selectivity.

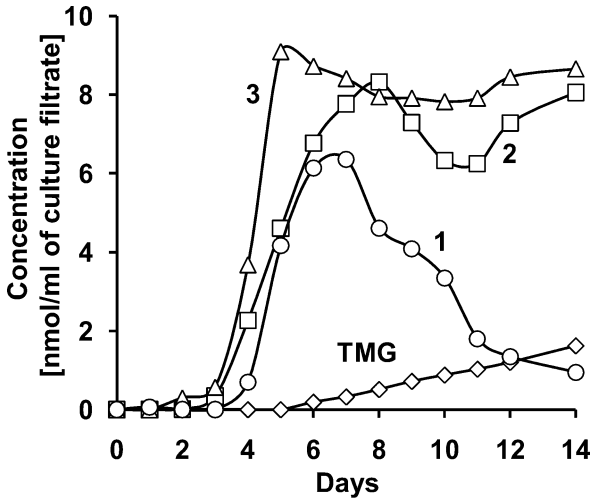

Fig. 6 Time course of production for TMG, **1**, **2**, and **3**.

Preparing the larger size of new TMG-chitooligomycins, such as TMG-GlcNAc$_4$, is also attractive for the clarification of the structure-selectivity relationship of TMG-chitooligomycins. It should be noted that during this manuscript's reviewing process, Yang *et al.* reported the synthesis of new TMG-chitooigosaccharides including TMG-GlcNAc$_{1-4}$ and their inhibitory activities toward several GH 20 GlcNAcases.[19] The two papers differ somewhat in the content and are essentially

Table 1 Inhibitory activity of **1**, **2**, and **3** toward GlcNAcases

Enzymes			TMG	**3** (TMG-GlcNAc)		**2** (TMG-GlcNAc$_2$)		**1** (TMG-GlcNAc$_3$)	
Origin	Family	K_m[b] (μM)	%inhibition at 400 μM	IC_{50} (μM)	K_i[b] (μM)	IC_{50} (μM)	K_i[c] (μM)	IC_{50} (μM)	K_i[c] (μM)
Aspergillus oryzae	Unknown	368	6.62	604	360	73.0	47.4	4.42	0.977[g]
Jack bean	Unknown	347	43.6	677	650	>1123 (38.9%)[e]	n.t.[d]	>24.0 (38.7%)[f]	n.t.[d]
Streptomyces coelicolor (SCO2786)[a]	20	79.3	8.64	178	44.0	51.9	3.41	23.3	n.t.[d]
S. coelicolor (SCO2758)[a]	3	70.0	9.98	>400	n.t.[d]	>400	n.t.[d]	>24.0	n.t.[d]

[a] Gene IDs of *Streptomyces coelicolor* genome project. [b] For *p*NP-GlcNAc. [c] Mode of inhibition was competitive in all cases (Fig. S10, S11†). [d] Not tested. [e] Inhibition at 1123 μM. [f] Inhibition at 24.0 μM. [g] Cited from a previous paper.[1]

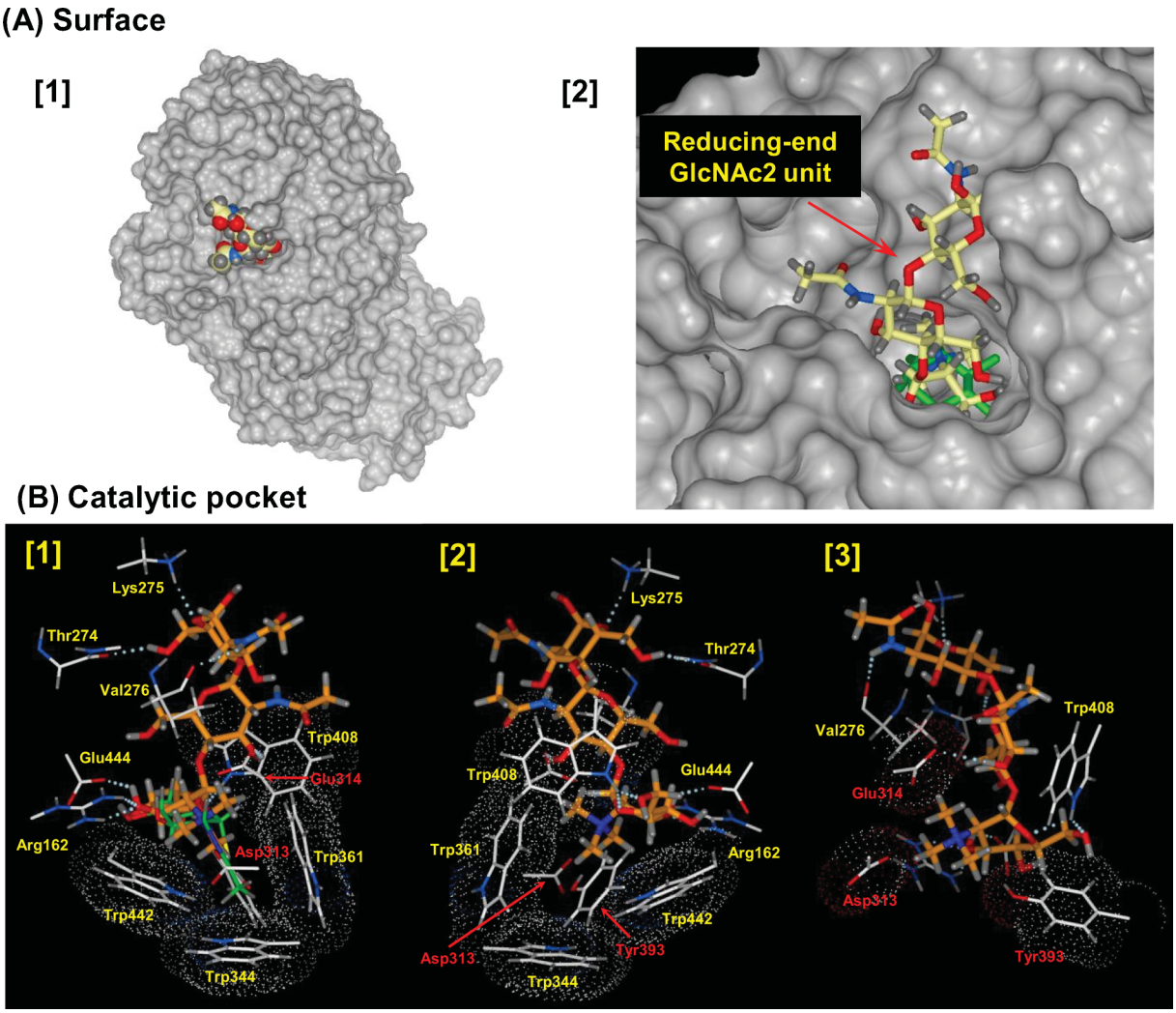

Fig. 7 Computational molecular model of **2** bound in the active site of *Sp*Hex. (A-1) **2** is shown as a calotte model (A-2). **2** is shown as stick model. The green sticks indicate the NAG-thiazoline bound in the -1 subsite of *Sp*Hex. (B) **2** is shown as stick model with orange. NAG-thiazoline bound in the -1 subsite is shown with green sticks. Predicted hydrogen bonds between **2** and *Sp*HEX are shown in dotted lines.

co-incident in the experimental results of their enzyme inhibitory activities. Therefore, a complementary understanding of this study and their one[19] will be informative for the development of more selective and potent TMG-chitooligomycins.

Docking simulations

The SCO2786 (GH 20 family), which is potently inhibited by **2** (K_i = 3.41 μM), shared 94% identity in the primary sequence with the GlcNAcase (also known as hexosaminidase) from actinomycete *Streptomyces plicatus* (*Sp*Hex), for which the reaction mechanism has been well characterized.[7,8,20–24] We conducted computational analysis of **2** bound in the active site of *Sp*Hex (PDB entry 1HP5) in order to clarify the mechanisms of its inhibitory action. In this analysis, the conformation of **2** as a ligand was optimized using the MMFF algorithm to shape the non-reducing end TMG in its typical *twist-boat* form (Fig. S13†). As shown in Fig. 7(A-1), **2** occupied the catalytic pocket of *Sp*Hex. The non-reducing TMG was inserted into the enzyme as shown in Fig. 7(A-2). The observations reasonably explain the mode of inhibition as competitive. Details around the catalytic pocket are shown in Fig. 7(B). The green sticks indicate the NAG-thiazoline bound in the -1 subsite of *Sp*Hex. In the figure, nitrogen, oxygen, sulfur, and hydrogen atoms are colored in blue, red, yellow, and gray, respectively. Compound **2** is shown with orange sticks. The key amino acid residues of *Sp*Hex are also shown with white sticks in those tryptophan residues constructing the hydrophobic pocket shown as dot models in B-1 and B-2. The dot models of acidic Asp313, Glu314, and Tyr393 are also shown in B-3. According to this computational model, the non-reducing end TMG was positioned around the -1 subsite at which the NAG thiazoline was also positioned (B-1). The TMG residue should be tightly locked by the predicted hydrogen bonding between Arg162, Glu444, and Trp408 (B-1 and B-2). Indeed, the roles of the three residues were well characterized as those of forming similar hydrogen bonds with the hydroxyl groups of the non-reducing end GlcNAc to lock it at the -1 subsite.[7,8] In addition, the positively charged *N*,*N*,*N*-trimethyl group of TMG was oriented toward the inside of the

hydrophobic pocket constructed by Trp442, Trp344, and Trp361 (B-2). The most important residues, Asp313 and Tyr393, were positioned around this region (B-1 and B-2). The key Asp residues are believed to be primarily responsible for stabilizing positively charged oxazolinium intermediates in the hydrolysis reaction of GH20 enzymes.[7,8] Another key residue, Glu314, being proposed as a general acid/base possibly interacted with the hydroxyl group at the C-3 atom of the middle-positioned GlcNAc (B-1 and B-3) rather than with the glycosidic oxygen. Furthermore, the reducing end GlcNAc could interact with Thr274, Lys275, and Val276 (B-1 and B-3). Here, the reaction mechanism of *Sp*Hex was characterized as follows. The substrate (typically $GlcNAc_2$) bound to the active site, and this was followed by the distortion of $^4C_1$ conformation of non-reducing end GlcNAc (bound in -1 subsite of enzyme) into the sofa/boat form in the Michaelis complex.[7,8] In this process, the C-1 atom of the non-reducing end GlcNAc at the -1 subsite was driven from the position below the plane between C-2, C-3, C-5, and O-5 of the sugar residue to the position above it. For this process, the Glu314 residue of the *Sp*Hex was assumed to be the proton donor that activates the glycosidic oxygen atom of the substrate. The acidic Asp313 was as the counter ion to stabilize the cationic oxisazoline intermediate. Such characteristics were also proposed for other related enzymes including GH18,[25] GH20,[7,8,26,27] and GH84.[14,28–30] In this aspect, remark finding is the *twist-boat* conformations of non-reducing TMG on TMG-chitooligomycins, in which the orientation of the C-1 atom was comparable to that of the Michaelis complex above (Fig. 7, S13†). Furthermore, the key acidic residues of the enzyme including Asp313, Tyr393 (stabilizing oxazolinium intermediate), and Glu314 (general acid/base) could be positioned around the cationic *N*,*N*,*N*-trimethyl group residue of **2** (B-3). Therefore, the following model would reasonably explain the inhibitory mechanism of TMG-chitooligomycins. The non-reducing end TMG of TMG-chitooligomycins would interact with the -1 subsite of the family 20 GlcNAcase as a mimic of the substrate in the Michaelis complex of the enzyme reaction, and as an ionic counter toward key acidic residues such as Asp313 and Tyr 393 of *Sp*Hex (B-3). For this interaction, the *twist-boat* conformation would be essential because the free TMG ($^4C_1$ conformation) showed no inhibition toward several GlcNAcases (Table 1). Furthermore, the DP of the reducing chitooligosaccharide units on TMG-chitooligomycins would be responsible for the binding affinity between the inhibitor and the plus subsite of the enzymes (Table 1, Fig. 7). From this point of view, the importance of the reducing chitooligosaccharide units on the inhibitors could be to help place the cationic TMG residue at the appropriate anionic position—the -1 subsite of the enzymes. Such structure-selectivity relationships of GlcNAcase inhibitors were recently demonstrated in a report on iminocyclitol derivatives.[31] In this report, the authors clearly demonstrated that GlcNAc-type iminocyclitols inhibited the both GH20 GlcNAcase and GH84 *O*-GlcNAcase. Their selectivity and inhibitory potency were well controlled by the modification of its ring nitrogen atom. Briefly, the long length of modification, which was speculated by computational modeling to occupy a hydrophobic cleft on the protein surface near the catalytic pocket, had a significant effect on selective inhibition toward GH20 human Hex B. It will likewise be challenging to prepare TMG residue-containing glycosides with diverse aglycon moieties and to evaluate their inhibitory activities toward family 20 enzymes as well as related enzymes such as GH18 and GH84. For the comparison of this analysis, we also constructed another TMG-chitobiomycin, which was composed of $^4C_1$ form of TMG residue at the non-reducing end, and simulated with the same manner (Section 8 of ESI†). Interestingly, such a $^4C_1$ type of TMG-chitobiomycin was predicted to be positioned at the surface of the protein, not the inside of the catalytic pocket (Fig. S14†). This result might also support the above mentioned inhibitory mechanism of TMG-chitooligomycins, that is, the *twist-boat* form of TMG residue would be essential to occupy the -1 subsite of the enzyme. It should be noted that during this manuscript's reviewing process, the crystal structure of insect GlcNAcase OfHex1 complexed with TMG-chitotriomycin was reported (PDB code: 3NSN).[32] As a result, the TMG residue of TMG-chitotriomycin was proved to be positioned around the -1 subsite of the enzyme. Their data also support the above inhibitory mechanism of TMG-chitooligomycins.

Conclusions

Our desired compounds, TMG-chitomonomycin and TMG-chitobiomycin, were successfully obtained using the LC/MRM guided approach. One issue emphasized in this study is the applicability of MRM techniques in the field of natural product chemistry. In particular, we expected that our desired TMG-chitooligomycins would show non-characteristic UV adsorption profiles, and therefore, that their selective detection by well established LC/UV techniques would be difficult. From this perspective, this study is a unique example of the successful application of MRM techniques in the structure-guided isolation of naturally occurring small molecules. Especially, such a MRM-guided search will be a powerful technique for gaining the novel analogues of known and well characterized biologically active compounds. In addition, further developments and applications of this strategy may lead to opportunities for obtaining attractive biologically active chemicals.

TMG-chitooligomycins were shown to be specific inhibitors of GlcNAcases with distinct selectivities (Table 1). The DPs of their reducing chitooligosaccharide units were responsible for their selectivity and inhibitory potency (Table 1). The inhibitory mechanism was predicted with the assistance of computational modeling as follows. The structures of TMG-chitooligomycins could be divided into two moieties such as non-reducing end TMG and reducing end chitooligosaccharide unit. The non-reducing TMG serves two roles; one as a mimic of the substrate in the Michaelis complex of the enzyme reaction, and another as the ionic counter toward key acidic residues around the catalytic pocket such as Asp313, Glu314, and Tyr393 of *Sp*Hex. In this respect, the unique *twist-boat* conformation of non-reducing TMG would essentially need to occupy the -1 subsite of the enzyme (Fig. 7-B). Such conformational distortion was the origin of the reducing chitooligosaccharide unit of the TMG-chitooligomycin. Therefore, the reducing chitooligosaccharide units of TMG-chitooligomycins would also posses two roles; one to distort the $^4C_1$ TMG into the *twist-boat* conformation, and the other to give the binding affinity between the inhibitor and enzyme. Further work will be needed to clarify these considerations, which will be our next challenge.

Experimental

Generals

NMR spectra were obtained using a Varian NMR-System 600 (600 MHz) spectrometer in D_2O solution using acetone as the external standard (δ_H 2.220 or δ_C 30.894). HRESIMS experiments were performed on a micrOTOFII-SKA (Bruker Daltonics). An ACQUITY™ ultra performance liquid chromatography system (Waters, Milford MA) and an API 2000 LC/MS/MS system (AB SCIEX) were used for LC/MRM analysis. Hypercarb (5 μm, 2.1 × 100 mm, Thermo Scientific) was used as the column for oligosaccharide separation. Amberlite CG-50 and Sephadex LH-20 resins were obtained from Organo. *β*-*N*-acetylglucosaminidases from jack bean and *Aspergillus oryzae*, as well as the chitinase from *Streptomyces griseus*, were obtained from Sigma–Aldrich. All other chemicals were commercially available.

Cloning, overexpression, and purification of GH3 and GH20 GlcNAcases from *Streptomyces coelicolor* A3(2)

Genomic DNA was prepared from *S. coelicolor* A3 (2)[33] using the methods described by Hopwood *et al.*[34] The gene encoding SCO2758 (http://streptomyces.org.uk/) was amplified using PCR with a set of sense primers incorporating the *Nde*I site upstream of a start codon (5-ATATATATCATATGCACCA-CAGCAGCACGGC-3), the anti-sense primer incorporating the *Hin*dIII site downstream of a stop codon (5-ATATAAGCT-TCTACGACCGGTAGGTCAGCC-3), and the genomic DNA of *S. coelicolor*. Similarly, the gene encoding SCO2786 was amplified using the sense primer (5′-ATATAT-ATCATATGAGACCTCATCGACGGCACC-3′) and antisense primer (5′-ATATAAGCTTCAGGTCCAGGGCACCTGC-3′). In addition, a set of Prime Star GXL and Prime Star GXL buffers (Takara) was used for the PCR reaction. The PCR products were digested using *Nde*I and *Hin*dIII to obtain the gene fragments encoding SCO2758 and SCO2786. The fragments were used for the construction of final expression plasmids as follows. The *Streptomyces* hyperexpression system[35] was adopted for the overexpression of the enzymes. In brief, the above fragment was introduced into the *Nde*I/*Hin*dIII gap of pTONA-5a to generate the expression plasmid, after which it was transferred from *Escherichia coli* to *S. lividans*1326. The enzyme was secreted at high levels in the culture broth. The culture filtrates were dialyzed followed by analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under a reducing condition with Coomassie Blue staining. As shown in Fig. S9†, the two enzymes were obtained as almost pure, and therefore, the fractions were used as purified enzymes. Enzymatic characteristics of SCO2758 and SCO2786 were summarized in Table S1†.

Enzyme inhibition assays

Inhibition of GlcNAcases was determined using the method described in our previous work[36] with the following modifications. The reaction volume was 100 μL, and the enzyme reaction was quenched by adding 100 μL of 1.0 M NaOH. This was followed by the quantification of released *p*NP at 405 nm. The reaction buffer for the GlcNAcases of jack bean, *A. oryzae*, and SCO2786 was a 50 mM citrate-phosphate-borate buffer containing 0.01% BSA (w/v) at pH 5.0. The same buffer at pH 6.4 was used for SCO2758. The IC_{50} values of the inhibitors were calculated by plotting the inhibitor concentration *versus* the rate of hydrolysis. The inhibition constants (K_i) and the type of inhibition were determined from Lineweaver–Burk and Dixon plots. In these assays, a blank and several concentrations of inhibitors were used in duplicate.

Fermentation and isolation of 2 and 3

A stock culture of *S. anulatus* NBRC 13369 was inoculated into 150 mL of Bennett's medium[36] and incubated on a reciprocal shaker (123 strokes/min) at 28 °C for 2 d. A seed culture of 5 mL was transferred to a 500 mL Sakaguchi flask containing 150 mL of the colloidal chitin-Bennett's medium[36] and cultured on a reciprocal shaker (123 strokes/min) at 28 °C for 8 d. The isolation of **2** and **3** began with 4,990 mL of the culture filtrate. At each step of purification, a portion of the fraction was analyzed by LC/MRM analysis using a Hypercarb column to monitor the presence of **2** and **3**. The isolation procedure was as follows. The culture broth was centrifuged at 12,000 *g*, and the supernatant was adsorbed onto an active carbon column (6.5 × 26.5 cm), washed with 1.7 L each of H_2O and 0.01% HCl, and eluted with 1.2 L of 30% acetone containing 0.01% HCl. The active fractions were combined and evaporated under reduced pressure to remove acetone; the remaining aqueous solution was then subjected to an Amberlite CG-50 (H^+ form) column (6.5 × 16 cm). After washing with 4 L of H_2O, the active substances were eluted with 4 L of 0.1 M NaCl. The active fraction was desalted by active carbon column chromatography in a manner similar to that described above and lyophilized. The powder obtained was dissolved in small amounts of MeOH and subjected to Sephadex LH-20 column (1.6 × 70 cm) chromatography with the same solvent. The active fraction was evaporated under reduced pressure, dissolved in small amounts of H_2O, and subjected to HPLC using at least three successive Hypercarb columns (4.6 × 150 mm) with gradient elution protocols of H_2O to 0.01% HCOOH for **3** and 0.01% HCOOH to 30% MeOH containing 0.01% HCOOH for **2**. As a result, 8.65 mg of **2** and 11.4 mg of **3** were obtained with these procedures.

Identification of D-GlcNAc residue in 2 and 3

Thirty point one nmol of **2** and 43.5 nmol of **3** were subjected to partial butanolysis treatment (1.4 M HCl/2*R*-butanol) at 80 °C for 6 h using D-glucose as an internal standard. The solutions were dried under reduced pressure, *N*-acetylated (Ac_2O/pyridin), and subjected to LC/MRM analysis to quantify the corresponding butyl glycoside. For this analysis, D-GlcNAc was used as an authentic standard to construct the corresponding multiple-reaction monitoring (MRM) channel. The LC conditions are described in Section-1 of the Electronic Supplementary Information (ESI)†.

Chitinase treatment of TMG-chitotriomycin (1)

For this experiment, the previously reported compound **1**[1] and its analogues **2** and **3** were used. The sample was treated with 15.5 μg of *Streptomyces* chitinase (Sigma–Aldrich) in 200 mM sodium phosphate buffer (pH 6.0) containing 2.0 mM $CaCl_2$ at 25 °C in a final volume of 300 μL. After 4.5 h, 150 μL of the reaction mixture was added to the same volume of 5% HCOOH for quenching. This was followed by LC/MRM analysis to monitor GlcNAc,

$GlcNAc_2$, **3**, **2**, and **1**. The LC conditions are described in Section-1 of the ESI†.

Docking simulations between 2 and *Sp*Hex

A MMFF algorithm was used to construct the ligand **2**, in which the conformation of the non-reducing TMG was of a typical *twist-boat* form. Spartan'08 (Wavefunction, Inc. Irvine, CA) was used for this procedure. The constructed ligand (Fig. S12†) was used for docking simulations with *Sp*Hex (PDB entry 1HP5) using ICM-Pro 3.3-04a (Molsoft LLC, CA). Default settings were used, with the exception that the parameter "Flexible ring sampling level" was set to "2," meaning that the ring of the ligand was flexible throughout the simulations.

Time course experiment

The fermentation conditions were the same as those for the production of **2** and **3**. This experiment was conducted in triplicate. During each of the 14 days of fermentation, around 1.0 mL of culture broth was recovered and immediately stored at – 20 °C. The culture broths were centrifuged, diluted with 1% HCOOH, and subjected to LC/MRM analysis using a Hypercarb column. The MRM channels were constructed with synthesized TMG, intact **1**, isolated **2**, and isolated **3** to detect each compound. The LC conditions are described in Section-1 of the ESI†.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology to TN (21580128) and a Grant-in-Aid for JSPS Fellows to HU (22·8342). The authors are grateful to the SC-NMR Laboratory of Okayama University and the MS Laboratory of the Faculty of Agriculture, Okayama University, respectively, for assistance in NMR and MS experiments. Dr Hajime Komura and Dr Takashi Iwashita (SUNBOR, Suntory) for their helpful discussions and suggestions are also acknowledged.

References

1 H. Usuki, T. Nitoda, M. Ichikawa, N. Yamaji, T. Iwashita, H. Komura and H. Kanzaki, *J. Am. Chem. Soc.*, 2008, **130**, 4146–4152.
2 H. Usuki, M. Toyo-oka, H. Kanzaki, T. Okuda and T. Nitoda, *Bioorg. Med. Chem.*, 2009, **17**, 7248–7253.
3 K. Slamova, P. Bojarova, L. Petraskova and V. Kren, *Biotechnol. Adv.*, 2010, **28**, 682–693.
4 B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard and B. Henrissat, *Nucleic Acids Res.*, 2009, **37**, D233–238.
5 H. Merzendorfer and L. Zimoch, *J. Exp. Biol.*, 2003, **206**, 4393–4412.
6 Y. Yang, Y. Li and B. Yu, *J. Am. Chem. Soc.*, 2009, **131**, 12076–12077.
7 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.
8 S. J. Williams, B. L. Mark, D. J. Vocadlo, M. N. James and S. G. Withers, *J. Biol. Chem.*, 2002, **277**, 40055–40065.
9 T. Aoyagi, H. Suda, K. Uotani, F. Kojima, T. Aoyama, K. Horiguchi, M. Hamada and T. Takeuchi, *J. Antibiot. (Tokyo)*, 1992, **45**, 1404–1408.
10 T. Aoyama, H. Naganawa, H. Suda, K. Uotani, T. Aoyagi and T. Takeuchi, *J. Antibiot. (Tokyo)*, 1992, **45**, 1557–1558.
11 K. Tatsuta, Y. Ikeda and S. Miura, *J. Antibiot. (Tokyo)*, 1996, **49**, 836–838.
12 M. Perreira, E. J. Kim, C. J. Thomas and J. A. Hanover, *Bioorg. Med. Chem.*, 2006, **14**, 837–846.
13 B. Shanmugasundaram, A. W. Debowski, R. J. Dennis, G. J. Davies, D. J. Vocadlo and A. Vasella, *Chem. Commun.*, 2006, **42**, 4372–4374.
14 G. E. Whitworth, M. S. Macauley, K. A. Stubbs, R. J. Dennis, E. J. Taylor, G. J. Davies, I. R. Greig and D. J. Vocadlo, *J. Am. Chem. Soc.*, 2007, **129**, 635–644.
15 H. C. Dorfmueller, V. S. Borodkin, M. Schimpl, S. M. Shepherd, N. A. Shpiro and D. M. van Aalten, *J. Am. Chem. Soc.*, 2006, **128**, 16484–16485.
16 H. C. Dorfmueller, V. S. Borodkin, M. Schimpl and D. M. van Aalten, *Biochem. J.*, 2009, **420**, 221–227.
17 B. A. Budnik, K. F. Haselmann, Y. N. Elkin, V. I. Gorbach and R. A. Zubarev, *Anal. Chem.*, 2003, **75**, 5994–6001.
18 W. Suginta, A. Vongsuwan, C. Songsiriritthigul, J. Svasti and H. Prinz, *FEBS J.*, 2005, **272**, 3376–3386.
19 Y. Yang, T. Liu, Q. Wu, Q. Yang and B. Yu, *ChemBioChem*, 2011, **12**, 457, DOI: 10.1002/cbic.201000561.
20 B. L. Mark, G. A. Wasney, T. J. Salo, A. R. Khan, Z. Cao, P. W. Robbins, M. N. James and B. L. Triggs-Raine, *J. Biol. Chem.*, 1998, **273**, 19618–19624.
21 D. J. Vocadlo and S. G. Withers, *Biochemistry*, 2005, **44**, 12809–12818.
22 B. Amorelli, C. Yang, B. Rempel, S. G. Withers and S. Knapp, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 2944–2947.
23 I. R. Greig, F. Zahariev and S. G. Withers, *J. Am. Chem. Soc.*, 2008, **130**, 17620–17628.
24 B. L. Mark, D. J. Vocadlo, S. Knapp, S. G. Withers and M. N. James, *J. Biol. Chem.*, 2001, **276**, 42131–42137.
25 I. Tews, A. Perrakis, A. Oppenheim, Z. Dauter, K. S. Wilson and C. E. Vorgias, *Nat. Struct. Biol.*, 1996, **3**, 638–648.
26 B. L. Mark, D. J. Mahuran, M. M. Cherney, D. Zhao, S. Knapp and M. N. James, *J. Mol. Biol.*, 2003, **327**, 1093–1109.
27 M. J. Lemieux, B. L. Mark, M. M. Cherney, S. G. Withers, D. J. Mahuran and M. N. James, *J. Mol. Biol.*, 2006, **359**, 913–929.
28 M. S. Macauley, G. E. Whitworth, A. W. Debowski, D. Chin and D. J. Vocadlo, *J. Biol. Chem.*, 2005, **280**, 25313–25322.
29 F. Marcelo, Y. He, S. A. Yuzwa, L. Nieto, J. Jimenez-Barbero, M. Sollogoub, D. J. Vocadlo, G. D. Davies and Y. Bleriot, *J. Am. Chem. Soc.*, 2009, **131**, 5390–5392.
30 F. V. Rao, H. C. Dorfmueller, F. Villa, M. Allwood, I. M. Eggleston and D. M. van Aalten, *EMBO J.*, 2006, **25**, 1569–1578.
31 C. W. Ho, S. D. Popat, T. W. Liu, K. C. Tsai, M. J. Ho, W. H. Chen, A. S. Yang and C. H. Lin, *ACS Chem. Biol.*, 2010, **5**, 489–497.
32 T. Liu, H. Zhang, F. Liu, Q. Wu, X. Shen and Q. Yang, *J. Biol. Chem.*, 2010, **286**, 4049–4058.
33 S. D. Bentley, K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill and D. A. Hopwood, *Nature*, 2002, **417**, 141–147.
34 D. A. Hopwood, M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser , D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf, *Laboratory Manual*, The John Ines Foundation, Norwich, 1985, 70–84.
35 T. Hatanaka, H. Onaka, J. Arima, M. Uraji, Y. Uesugi, H. Usuki, Y. Nishimoto and M. Iwabuchi, *Protein Expression Purif.*, 2008, **62**, 244–248.
36 H. Usuki, T. Nitoda, T. Okuda and H. Kanzaki, *J. Pestic. Sci.*, 2006, **31**, 41–46.