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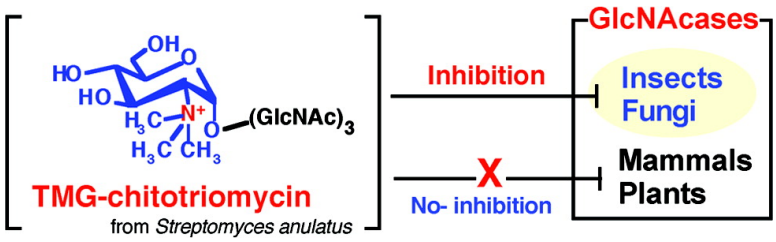

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TMG-chitotriomycin, an Enzyme Inhibitor Specific for Insect and Fungal *β*-*N*-Acetylglucosaminidases, Produced by Actinomycete *Streptomyces anulatus* NBRC 13369

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Abstract: A novel *β*-*N*-acetylglucosaminidase (GlcNAcase) inhibitor named TMG-chitotriomycin (**1**) was isolated from the culture filtrate of *Streptomyces anulatus* NBRC13369. The strain produced **1** only when colloidal chitin was used as the sole carbon source in the production medium. The structure of **1** was determined by spectral and constitutive sugar analyses of the corresponding alditol derivatives to be an equilibrated mixture of α-D-*N*,*N*,*N*-triMeGlcNH$_2$-(1,4)-*β*-D-GlcNAc-(1,4)-*β*-D-GlcNAc-(1,4)-D-GlcNAc and its C-2 epimer of the reducing end residue. TMG-chitotriomycin (**1**) showed potent and selective inhibition of insect and fungal GlcNAcases with no inhibition of mammalian and plant GlcNAcases. In contrast, the known GlcNAcase inhibitor nagstatin potently inhibited all GlcNAcases. It should be emphasized that synthesized D-*N*,*N*,*N*-triMeGlcNH$_2$, which is the component sugar of **1**, showed no inhibition of the insect *Spodoptera litura* GlcNAcase. These results suggest that the (GlcNAc)$_3$ unit positioned at the reducing end of **1** is essential for its enzyme inhibitory activity. The unique inhibitory spectrum of **1** will be useful to study chitinolytic systems and to develop selective fungicides or pesticides.

Introduction

Chitin, a linear polysaccharide composed of *β*-1,4 linked D-*N*-acetylglucosamine (GlcNAc), is widely distributed in nature as a constituent of the insect exoskeleton and fungal cell wall.[1−3] The metabolic turnover of chitin is known to be essential for the normal growth of these organisms. A chitinolytic enzyme system consisting of chitinase and *β*-*N*-acetylglucosaminidase (GlcNAcase) is required for the complete degradation of chitin into corresponding monosaccharides in this pathway.[3−5] Therefore, inhibitors of these enzymes are expected to be potential pesticides or fungicides. However, chitinolytic enzymes are widely distributed in nature, including mammals, plants, insects, and microorganisms, and hence specific inhibitors of chitinolytic enzymes from particular organisms, such as insects and fungi, are desirable.

Chitinase hydrolyzes the polysaccharide chitin into its corresponding oligosaccharides. The resulting oligomers are further decomposed by another chitinolytic enzyme, GlcNAcase, into monosaccharide GlcNAcs. The focus of many researchers on chitinolytic enzyme systems has resulted in the discovery of the chitinase inhibitors allosamidins,[6,7] argifin,[8,9] and argadin.[10] The inhibitory spectra of these inhibitors are very broad, *viz.*, showing inhibition of family 18 chitinases from mammals, insects, plants, and fungi.[11] Analogous to the chitinase inhibitors, the GlcNAcase inhibitors nagstatin[12−15] and NAG-thiazoline[16] have also not been developed as commercial drugs. The limitations are the complexity of their structures, their stability in nature, some physicochemical properties, and their broad inhibitory spectra.

The reason for the wide distribution of the chitinolytic enzymes is still unknown. Nevertheless, their physiological roles would be different in chitin-containing organisms such as insects and fungi, and non-chitin-containing organisms, such as plants

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and mammals. We hypothesized that the chitinolytic enzymes of chitin-containing organisms are different from those of non-chitin-containing organisms. If this conjecture is true, a specific inhibitor of chitinolytic enzymes in insects and/or fungi should be present in nature. Such inhibitors, needless to say, would be ideal for the lead compounds of pesticides or antimicrobial reagents and, of course, molecular probes for biochemical studies. The above considerations prompted us to screen the specific inhibitors of insect and/or fungal chitinases and GlcNAcases.

In a previous paper, we reported the discovery of the novel insect chitinase inhibitor FPS-1 in the culture filtrate of a fungal strain *Sphaeropsis* sp. TNPT116-Cz.[17] FPS-1 showed potent and selective inhibition of insect *Spodoptera litura* chitinase but no inhibition of the chitinase of actinomycete *Streptomyces griseus*. Therefore, our hypothesis was partially satisfied in the case of one chitinolytic enzyme chitinase. The next target was the GlcNAcase positioned downstream of the chitinase in the chitin degradation pathway.

Screening microbial producers of such GlcNAcase inhibitors has resulted in the discovery of four actinomycete strains *Streptomyces griseoloalbus* JCM4480, *S. clauifer* JCM5059, *S. anulatus* NBRC13369, and *S. griseus* subsp. *rhodochrous* NBRC13849. The culture filtrates of these four strains showed potent inhibition against GlcNAcases of insects, with little or no inhibition of the enzymes of mammals, fungi, and plants.[18] The data should reveal distinct characteristics of the GlcNAcases of insects and other organisms including mammals, fungi, and plants.

Here we report that one active strain, *S. anulatus* NBRC 13369, produces an inhibitor that is quite unique in biological, structural, and physicochemical aspects. The isolated active compound **1**, named TMG-chitotriomycin, showed selective and potent inhibition of insect and fungal GlcNAcases with no inhibition of the enzymes of mammals and plants.

Experimental Section

General Procedures. The NMR spectra of **1**, 2-deoxy-2-(trimethylammonio)-D-glucopyranose, were obtained with a Varian Inova 600 spectrometer in CD_3OD or D_2O solution. The spectra of **2** and **3** were measured with Bruker dmx 750 and Bruker dmx 500 spectrometers in D_2O solution using TSP as the external standard. Optical rotation was measured on a JASCO P-2200 polarimeter. ESIMS experiments were done on a Micromass Q-Tof or a Bruker Daltonics APEX-Qe. The FABMS experiment was carried out using a JEOL JMS-HX/HX110 or a JMS-SX102A. Asahipak NH2P-50 (0.46 × 25 cm) and ES502C (0.76 × 10 cm) columns were purchased from Showa Denko Co. A Cosmosil 5C18-AR column (0.6 × 25 cm) was purchased from Nacalai Tesque Inc. and a Hypercarb column (0.46 × 10 cm) from Chemco Co. Amberlite CG-50 and Sephadex LH-20 resins were from Organo Co. and GE Healthcare Bio-Sciences AB, respectively. *β-N*-Acetylglucosaminidases from bovine kidney, human placenta, jack bean, and *Aspergillus oryzae* were obtained from Sigma-Aldrich Co. *β-N*-Acetylglucosaminidase from *Penicillium oxalicum* were obtained from Seikagaku Kogyo Co. Chitinases from *Streptomyces griseus* and *Bacillus* sp. were purchased from Sigma-Aldrich and Wako Pure Chemical Co., respectively. These enzymes were used for enzyme inhibition studies without further purification. Chitinase and *β-N*-acetylglucosaminidase from *Spodoptera litura* were prepared as described in our previous papers.[17,18] Nagstatin was a kind gift from the Microbial Chemistry Research Foundation (Japan). All other chemicals were commercially available.

Assay Methods of Enzyme Inhibitory Activity. Inhibition of GlcNAcases was determined by a manner similar to that of our previous work[18] in which the *p*-nitrophenyl-*N*-acetyl-D-glucosaminide (*p*NP-GlcNAc) was used as a substrate, and the following reaction buffers were used as accompanying instructions: (1) human placenta: 100 mM citrate buffer (pH 4.3) containing 100 mM NaCl and 0.01% (w/v) bovine serum albumin, (2) jack bean and *A. oryzae*: 100 mM citrate buffer (pH 5.0) containing 100 mM NaCl and 0.01% (w/v) bovine serum albumin, and (3) *P. oxalicum*: 100 mM citrate buffer (pH 4.5). The IC_{50} values were calculated by plotting the inhibitor concentration vs the rate of hydrolysis. The inhibition constants (*Ki*) and the type of inhibition were determined from Lineweaver−Burk and Dixon plots. In these assays, a blank and several concentrations of inhibitors in duplicate were used. The inhibitions by TMG-chitotriomycin (**1**) of chitinases were determined as reported in our previous paper.[17]

Production of TMG-chitotriomycin (1). A stock culture of *S. anulatus* NBRC 13369 grown on Bennett's medium was inoculated into 10 mL of colloidal chitin-Bennett's medium[18] and incubated on a reciprocal shaker (280 strokes/min) at 28 °C for 2 days. The seed culture was transferred to a 1 L Sakaguchi flask containing 300 mL of the same medium and cultured on a reciprocal shaker (280 strokes/min) at 28 °C for 14 days.

Isolation of TMG-chitotriomycin (1). The culture broth (5.5 L) was centrifuged at 6000 g, and the supernatant was adsorbed onto an active carbon column (6.5 × 33 cm) and washed with 1.1 L of H_2O, followed by elution with 2.5 L of 30% acetone containing 0.01% HCl. The active fractions were combined and evaporated under reduced pressure to remove acetone; then, the remaining aqueous solution was subjected to an Amberlite CG-50 (H^+ form) column (6.5 × 31 cm). After washing with 2 L of H_2O, the active substance was eluted with 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 (1.0 × 50 cm) chromatography with the same solvent. The active fraction was evaporated under reduced pressure, dissolved in small amounts of H_2O, subjected to HPLC using two successive Asahipak ES502C columns (7.6 × 100 mm), and developed with 7.5 mM ammonium carbonate at the flow rate of 0.8 mL/min to yield 46.6 mg of **1**.

Identification of Reducing End Residue of TMG-chitotriomycin (1). Pyridylamination of **1** was done by the method of Kondo et al.[19] Analytical procedures including the acid hydrolysis of the derivatives and HPLC using a Cosmosil 5C18-AR column (0.6 × 25 cm) or an Asahipak NH2P-50 column (0.46 × 25 cm) were carried out as published.[20]

Preparation of Alditols 2 and 3. Five milligrams of **1** was reduced by 150 μL of 10 mg/mL $NaBH_4$ for 2 h at room temperature. The reduced compounds were applied to column chromatographies with active carbon (6.0 × 35 mm), Asahipak ES502C (7.6 × 100 mm), and Hypercarb (4.6 × 100 mm) columns to give two alditols, **2** (820 μg) and **3** (2.8 mg).

Carbohydrate Component Analysis of 1−3. The carbohydrate compositions of **1**−**3** were analyzed by GLC as trimethylsilyl derivatives after methanolysis (1.5 M methanolic HCl) by the method of Mega and Ikenaga[21] using myo-inositol as an internal standard. For GLC analysis, a Hitachi G-3000 gas chromatograph with a DB-WAX capillary column (30 m × 0.25 mm, J & W Scientific) was used.

Acid Hydrolysis of TMG-chitotriomycin (1) and Determination of Absolute Configuration of Nonreducing Sugar Residue 2-Deoxy-

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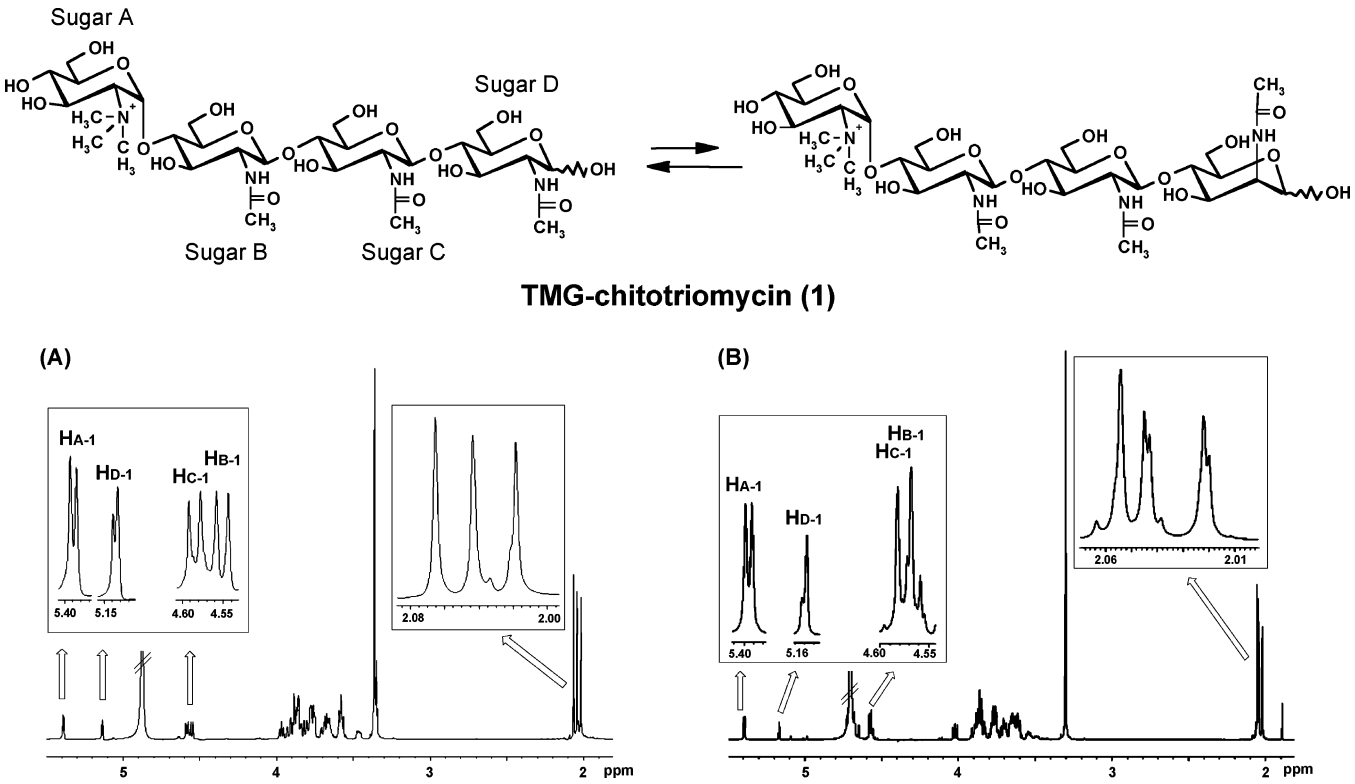

Figure 1. ^{1}H NMR spectra of TMG-chitotriomycin (**1**) in (A) CD_3OD and (B) D_2O.

2-(trimethylammonio)glucopyranose (4). Thirteen milligrams of **1** was hydrolyzed with 2M TFA (2.4 mL) at 121 °C for 6 h in a sealed tube. The solution was lyophilized, and the material was dissolved in small amounts of H_2O and then subjected to the column chromatography with Asahipak ES502C and developed with MeOH/25 mM HCO_3NH_4 (1:10, v/v) at the flow rate of 0.8 mL/min to give 1.24 mg of **4**, the non-reducing sugar residue 2-deoxy-2-(trimethylammonio)glucopyranose. Specific rotation of the isolated **4** was compared to the authentic compound, which was synthesized according to the procedure by Falkowski et al.[22]

Results

Fermentation and Isolation of 1. *S. anulatus* strain NBRC13369 was cultured in a modified Bennett's medium containing colloidal chitin as the sole carbon source. The culture filtrate showed potent inhibition against the insect *S. litura* GlcNAcase with no inhibition of bovine kidney GlcNAcase (data not shown), indicating the presence of a unique and selective GlcNAcase inhibitor. Interestingly, the productivity of the active compound was dependent on the initial amounts of colloidal chitin in the production media (Figure S1), and the absence of colloidal chitin in the medium prevented production of the active compound (data not shown). These results suggest the essential effect of the chitin in the production of the active compound.

Activity-guided purification, described in the Experimental Section, resulted in the isolation of **1**. Purified **1** was subjected to the further experiments.

Elucidation of Structure of 1. HRESIMS (Bruker Daltonics APEX-Qe) of **1** showed the M^+ peak at *m*/*z* 831.37183, in agreement with the formula $C_{33}H_{59}O_{20}N_4^+$ (calcd 831.37172). Forty milligrams of **1** was subjected to NMR experiments (^{1}H, ^{13}C, HSQC, HMBC, TOCSY, and ROESY) in CD_3OD at 30 °C for 2 days. The ^{1}H NMR spectrum of **1** (Figure 1A) was typical for reducing oligosaccharides.

Four anomeric protons (δ 5.38, sugar A; δ 5.13, sugar D; δ 4.55, sugar B; δ 4.58, sugar C) suggested the presence of four corresponding sugar units. The resonance at δ 5.13 (sugar D) was weaker than those of other anomeric protons, indicating that the sugar D residue was positioned at the reducing end. The resonances at δ 2.06 (3H, s), δ 2.04 (3H, s), and δ 2.01 (3H, s) corresponding to the methyl protons of *N*-acetyl groups suggested three *N*-acetylhexose residues. It was noteworthy that the multiplicity of the resonance at δ 5.13, corresponding to the anomeric proton of the reducing end residue, was an unusual doublet. In addition, ESIMS (Micromass Q-Tof) of **1** after the above NMR experiments showed an M^+ peak at *m*/*z* 832 as the strongest intensity, with *m*/*z* 831 as the much less intense peak (Figure 2B). Therefore, partial deuterium exchange of **1** at the C-2 position of the reducing sugar residue was suggested.

^{1}H NMR analyses of **1** in D_2O provided more clear evidence for the deuterium exchange as shown in Figure 1B. The anomeric proton of sugar D (δ_H 5.16) clearly showed an unusual doublet and five singlet resonances between δ_H 2.02 and 2.05, which were identical to the methyl protons of *N*-acetyl groups. These observations also supported the deuterium exchange of **1** at the C-2 position of the reducing sugar residue. In addition, the resonance at δ_H 3.30 (9H, s) was characteristic of the methyl group of a quaternary ammonium,[23,24] suggesting the presence of an $N(CH_3)_3$ group. Further analyses of the spectra were impossible because the signals derived from the equilibrium of the reducing end residue sugar D were so overlapped.

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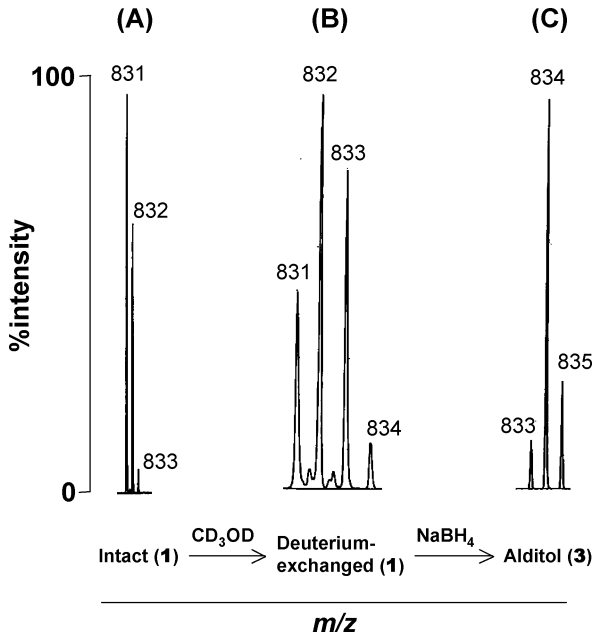

Figure 2. ESIMS spectra of **1** and **3**. (A) **1** before NMR experiments. (B) **1** after NMR experiments in CD_3OD at 30 °C for 2 days. (C) **3** prepared from **1** after NMR experiments in CD_3OD at 30 °C for 2 days.

$NaBH_4$ reduction of the deuterium-exchanged **1** (5 mg) gave us two alditol derivatives, **2** (820 μg) and **3** (2.8 mg). NMR analyses of **3**, measured in D_2O, revealed three anomeric protons at δ_H 5.42 (sugar A′), δ_H 4.62 (sugar C′), and δ_H 4.60 (sugar B′), as shown in Figure S2. A glucopyranose form of sugar A′ was easily deduced by a series of NMR spectra (^1H, ^{13}C, HSQC, TOCSY, and NOESY). The resonances at δ_H 3.33 (9H, s) and δ_c 56.59, which are characteristic of the methyl group of a quaternary ammonium,[23,24] revealed the presence of a $N(CH_3)_3$ group. The connection of the $N(CH_3)_3$ to the C-2 position of sugar A′ was confirmed by the HMBC correlation between methyl protons (δ_H 3.33, 9H, s) and the C-2 carbon (δ_c 81.12). From these spectral data, sugar A′ was deduced to be a 2-deoxy-2-(trimethylammonio)glucopyranose (*N*,*N*,*N*-triMeGlcNH$_2$).

To obtain further information and clarify the absolute configuration of the residue, 13.0 mg of deuterium-exchanged **1** was hydrolyzed with TFA to isolate 1.24 mg of **4**, which was compared to the synthesized *N*,*N*,*N*-triMe-D-GlcNH$_2$. Physicochemical properties of **4** were a good agreement with those of authentic one as described follow and listed in Table S1. The ^1H NMR spectrum of **4** measured in D_2O was identical to that of the synthesized *N*,*N*,*N*-triMe-D-GlcNH$_2$ (Figure 3).

Interestingly, the multiplicity of anomeric protons (δ_H 5.69) of *N*,*N*,*N*-triMe-D-GlcNH$_2$ was an unusual doublet. Furthermore, the resonance of a methine proton at the C-2 position (δ_H 3.52) was weaker than that of the other methine proton, indicating a partial deuterium exchange at the position.

FABMS (JEOL JMS-SX102A) of **4** and the synthesized *N*,*N*,*N*-triMe-D-GlcNH$_2$ after the above NMR experiments showed an M^+ peak at *m*/*z* 222 as the strongest intensity, with *m*/*z* 223 as the less intense peak (Figure S3). Their high-resolution spectra were in agreement with the molecular formulas $C_9H_{20}O_5N$ and $C_9H_{19}DO_5N$, respectively (Table S1). Furthermore, the specific rotation of isolated **4** was in a good agreement with that of the synthesized compound [**4**: $[\alpha]_D^{32}$ +80.1(*c* 0.11, MeOH), synthesized *N*,*N*,*N*-triMe-D-GlcNH$_2$:

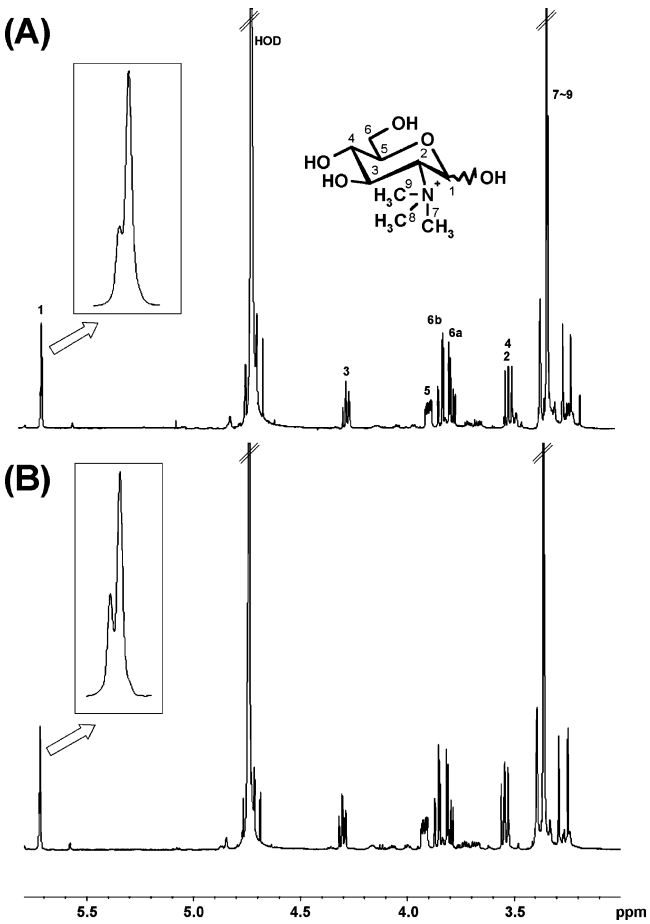

Figure 3. ^1H NMR spectra of **4** and synthesized *N*,*N*,*N*-triMe-D-GlcNH$_2$. (A) Compound **4** prepared from the isolated **1**. (B) Synthesized *N*,*N*,*N*-triMe-D-GlcNH$_2$.

$[\alpha]_D^{32}$ +75.7(*c* 0.14, MeOH)]. From these observations, **4** was identified to be an *N*,*N*,*N*-triMe-D-GlcNH$_2$.

Sugars B′ and C′ were identified as GlcNAc by a series of NMR data. The connection between these sugar units was confirmed by the HMBC correlation between the anomeric carbon of sugar B′ (δ_C 104.18) and the H-4 of sugar C′ (δ_H 3.67), and the stereochemistry was defined as β by its coupling constant (J = 8.5). The HMBC correlations between the anomeric position (δ_H 5.42, δ_C 98.56) of sugar A′ and the C-4 position (δ_H 3.92, δ_C 79.69) of sugar B′ indicated the connection of sugar A′ to sugar B′. The stereochemistry at the glycosidic position was defined as α by its coupling constant (J = 3.7).

Sugar D′ was easily deduced to be D-*N*-acetylglucosaminitol (GlcNAc-ol) by component analysis and the NMR spectra. The HMBC correlation between H-4 (δ_H 3.80) of sugar D′ and C-1 (δ_C 103.87) of sugar C′ confirmed the connection to sugar C′ by β-1,4 linkage.

The ESIMS (Micromass Q-Tof) of **3** showed that the M^+ peak at *m*/*z* 834 had the strongest intensity, with a much weaker peak at *m*/*z* 833 (Figure 2C). The HRFABMS (JMS-HX/HX110) of **3** showed the M^+ peak at *m*/*z* 834.3962, in agreement with the molecular formula $C_{33}H_{60}D_1O_{20}N_4^+$ (calcd 834.3942). It is noteworthy that the intensity of the methine proton at H-2 (δ_H 4.26) of sugar D′ was much weaker than those of other protons (Figure S2). The observation could be explained as the deuterium exchange at H-2 of the reducing end GlcNAc of **1**. This consideration was also supported by the MS data as

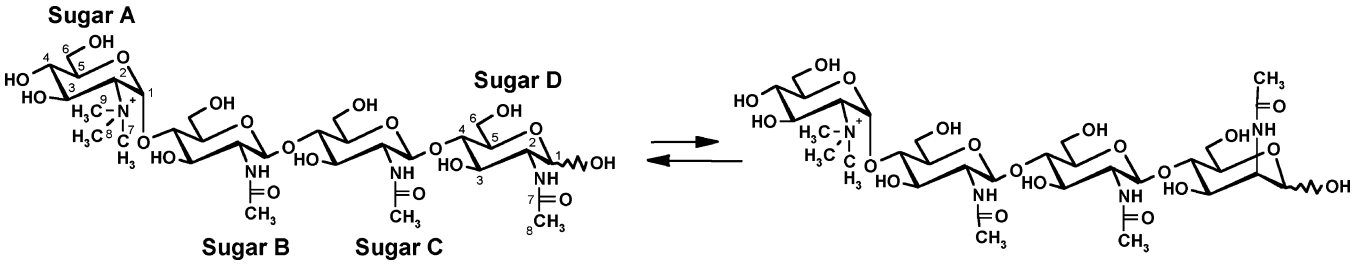

Figure 4. Structure of TMG-chitotriomycin (**1**).

Table 1. Inhibitory Activity of TMG-chitotriomycin (**1**) and Nagstatin against GlcNAcases

enzymes			**1**[c]		nagstatin	
origins	family[a]	K_m [b] (mM)	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)
Spodoptera litura	unknown	0.367	526	290	5.85	1.10
Aspergillus oryzae	unknown	0.512	1460	977	10.6	9.69
Penicillium oxalicum	unknown	0.306	439	55.7	60.6	8.11
human placenta	20	0.733	>70100[d]	n.e.[e]	1.83	0.925
bovine kidney	20	0.513	>70100[d]	n.e.[e]	1.71	1.64
jack bean	20	0.718	>70100[d]	n.e.[e]	1.87	1.41

[a] The GlcNAcases from bovine kidney, human placenta, and jack bean were classified into family 20 as judged by the published data.[25−29] The glycoside hydrolase families of the other GlcNAcases have not yet been determined. [b] For *p*NP-GlcNAc. [c] As a hydrogen carbonate salt. [d] <30% inhibition at 70.1 μM of **1**. [e] Could not be evaluated.

described above. From all the above-mentioned data, the structure of **3** was determined to be (2-deoxy-2-(trimethylammonio)-α-D-glucopyranosyl)-(1,4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1,4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1,4)-2-acetamido-2-deoxy-D-glucitol.

The HRESIMS (Bruker Daltonics APEX-Qe) of **2** showed the M^+ peak at *m*/*z* 834.39389, in agreement with the molecular formula $C_{33}H_{60}D_1O_{20}N_4^+$ (calcd 834.39364). Component analysis of **2** showed the presence of GlcNAc residues and D-*N*-acetylmannosaminitol (ManNAc-ol). The ^{1}H NMR spectrum of the compound was quite a similar to that of **3** (Figure S2). The clear differences between the spectra of **2** and **3** were the resonances of H-1a, H-1b, H-2, and H-5 of sugar D′ (GlcNAc-ol and ManNAc-ol), as shown in Table S2. The intensity of the methine proton at H-2 (δ_H 4.18) of sugar D′ was much weaker than those of other protons by analogy with the case of **3**. From NMR spectra (Figure S2, Table S2), the structure of **2** can reasonably be expected to be the epimer of **3** at the C-2 position of sugar D′.

From the elucidated structures of the corresponding alditols, **1** was revealed to be the equilibrated mixture of (2-deoxy-2-(trimethylammonio)-α-D-glucopyranosyl)-(1,4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1,4)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1,4)-2-acetamido-2-deoxy-β-D-glucopyranose and its C-2 epimer of the reducing end residue (Figure 4). On the basis of the structural information, **1** was named TMG (trimethylglucosaminium)chitotriomycin.

Methanolysis of intact **1** with methanolic HCl (92 °C, 2 h) followed by GC of the trimethylsilyl derivatives of the monosaccharides confirmed the presence of D-GlcNAc residues. In addition, HPLC of the acid hydrolysate of pyridylaminated (PA-) **1** showed the presence of PA-GlcNAc as the major hydrolysate with minor PA-sugars, suggesting that the equilibrium of the reducing end sugar would be strongly shifted toward a GlcNAc form.

Enzyme Inhibitory Activity. The effects of intact **1** and a known GlcNAcase inhibitor nagstatin were tested on GlcNAcases from a wide variety of organisms. As shown in Table 1 and Figure S10, **1** showed potent inhibition of the GlcNAcases of an insect (*S. litura*) and fungi (*A. oryzae*, *P. oxalicum*) in a competitive manner but no inhibition against the enzymes from mammals (human placenta, bovine kidney) and a plant (jack bean). In contrast, the known GlcNAcase inhibitor nagstatin[12−15] showed inhibition of all the GlcNAcases in a competitive manner with K_i values in nM ranges. In addition, **1** showed no inhibition against the family 18 chitinases from *S. litura* (insect), *S. griseus* (actinomycete), and *Bacillus* sp. (bacterium). Therefore, we concluded that TMG-chitotriomycin is a GlcNAcase inhibitor specific for GlcNAcases of insect and fungal origin. It should be emphasized that such a selective inhibitor is hitherto unknown.

The synthesized *N*,*N*,*N*-triMe-D-GlcNH$_2$ was also tested for the GlcNAcase of insect *S. litura*. The compound showed no inhibition against the enzyme (<30% inhibition at 2.6 mM in the standard assay condition).

Discussion

This paper clearly shows the unique inhibitory spectrum of the novel GlcNAcase inhibitor TMG-chitotriomycin. It showed specific inhibition against insect and fungal GlcNAcases, whereas the known inhibitor nagstatin[12−15] showed a broad inhibitory spectrum against GlcNAcases from a wide variety of organisms such as insects, mammals, and plants (Table 1). Insects and fungi, the producers of the TMG-chitotriomycin-sensitive GlcNAcases, are considered to produce chitinolytic enzymes for the metabolic turnover of chitin in ecdysis or normal growth of mycelium, respectively.[3,11] In contrast,

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mammals and plants, the producers of the TMG-chitotriomycin-insensitive GlcNAcases, were reported to produce the enzymes for several physiological roles such as self-defense against pathogens[30,31] and involvement in some human diseases.[32] The unique selectivity of TMG-chitotriomycin would be helpful for the biochemical study of the chitinolytic enzyme system.

The chitinolytic enzyme system consisting of chitinase and GlcNAcase is necessary for the complete degradation of chitin into GlcNAcs. Each enzyme is classified into two distinct groups, family 18 or 19 chitinases and family 3 or 20 GlcNAcases, based on their amino acid sequences[33,34] (http://afmb.cnrs-mrs.fr/CAZY/). These enzymes are widely distributed in natural organisms including mammals, plants, insects, and microorganisms, and the majority of chitinases were classified into family 18. In contrast, distribution of family 19 chitinases is reported to be limited to organisms such as plants[30,35] and actinomycetes.[36,37] By analogy with chitinases, the majority of the GlcNAcases were classified into family 20. Interestingly, family 18 chitinases and family 20 GlcNAcases possess the same catalytic mechanism called substrate-assisted catalysis.[16,38−40] The glycoside hydrolase families of the TMG-chitotriomycin-sensitive GlcNAcases used in the study are still unknown, and therefore it will be interesting to attempt the classification of the GlcNAcases by whether their sensitivities to TMG-chitotriomycin are in agreement with the classification of glycoside hydrolase families or not. In addition, our group has demonstrated that the chitinase inhibitor FPS-1 inhibited the chitinase from the chitin-containing organism *S. litura* but not the enzyme from the non-chitin-containing organism, *S. griseus*.[17] Both enzymes were potently inhibited by the family 18 chitinase-selective inhibitor allosamidin.[17] Of particular interest is the finding that the inhibitory spectrum of the chitinase inhibitor FPS-1 is in good agreement with that of the GlcNAcase inhibitor TMG-chitotriomycin. The selective inhibitions of FPS-1 and TMG-chitotriomycin against the chitinolytic enzymes of chitin-containing organisms strongly support the hypothesis described in the Introduction that the chitinolytic enzymes of chitin-containing organisms are different from those of non-chitin-containing organisms.

The known GlcNAcase inhibitors, including NAG-thiazoline,[16] PUGNAc,[28] and nagstatin,[12−15] possess nitrogen atoms. In the case of the best characterized inhibitor NAG-thiazoline, the nitrogen atom of the thiazoline ring was positively charged to interact with the catalytic aspartate residue at −1 subsite of family 20 enzymes.[41] The mechanism of the selective inhibition of TMG-chitotriomycin remains to be clarified, but the charged nitrogen atom of the *N*,*N*,*N*-triMe-D-GlcNH$_2$ residue is just such a cationic atom, and therefore should interact with the catalytic −1 subsite of the enzymes. An interesting observation was that the unusual sugar *N*,*N*,*N*-triMe-D-GlcNH$_2$ was inactive against insect *S. litura* GlcNAcase, suggesting the essential effect of the (GlcNAc)$_3$ unit in the binding of TMG-chitotriomycin to the enzymes. Work on the synthesis of analogues composed of different degrees of the reducing end GlcNAc unit attached to an *N*,*N*,*N*-triMe-D-GlcNH$_2$ residue and evaluation of their enzyme inhibitory activities and selectivities is now in progress.

The *N*,*N*,*N*-triMe-D-GlcNH$_2$ residue positioned at the non-reducing end is a characteristic of TMG-chitotriomycin. The unusual sugar residue is hitherto unknown in nature. The structurally analogous compounds *N*-methyl-L-glucosamine and *N*,*N*-dimethyl-L-glucosamine have already been reported as the component sugar of the major antibiotics streptomycin or bluensomycin, which are produced by an actinomycete *Streptomyces* sp.[42] It should be emphasized, however, that their configurations are L, not D, forms, suggesting that the biosynthesis of the *N*,*N*,*N*-triMe-D-GlcNH$_2$ residue of TMG-chitotriomycin would be independent of those of known compounds such as streptomycin and bluensomycin.

A unique aspect of the physicochemical properties of TMG-chitotriomycin was the epimerization at the C-2 position of the reducing end GlcNAc. In general, the isomerization of GlcNAc to ManNAc is known to occur in alkaline pH conditions. An aldehyde form of the reducing sugar residue and its α-deprotonation are required for the epimerization. Blayer et al. reported that the epimerization of the monosaccharide GlcNAc was not detectable after 8 h below pH 9 at 25 °C.[43] However, the reducing end GlcNAc of TMG-chitotriomycin was slowly isomerized to ManNAc under the CD_3OD solution state. The reason for this interesting characteristic remains to be clarified, but the charged nitrogen atom of an *N*,*N*,*N*-triMe-D-GlcNH$_2$ residue at the nonreducing end is possibly involved in the epimerization because, of course, the isomerization has not been reported in oligomers of β-1,4-linked GlcNAc.

Interestingly, *N*,*N*,*N*-triMe-D-GlcNH$_2$ was slowly isomerized to *N*,*N*,*N*-triMe-D-ManNH$_2$ in the D_2O solution (Figure 3, Figure S3). The incorporation of the unusual sugar residue into the non-reducing end of the β-1,4 linked (GlcNAc)$_3$ unit resulted in the isomerization of the reducing end D-GlcNAc to D-ManNAc with no effect on the nonreducing end *N*,*N*,*N*-triMe-D-GlcNH$_2$ (Figures 1, 2). This observation strongly supports our idea that the cationic nitrogen atom of *N*,*N*,*N*-triMe-D-GlcNH$_2$ residue contributes directly to the epimerization of TMG-chitotriomycin.

In conclusion, we discovered TMG-chitotriomycin as a novel GlcNAcase inhibitor from the culture filtrate of actinomycete *Streptomyces anulatus* NBRC13369 strain. The inhibitor will give new insights for the development of selective antimicrobial and insecticidal reagents and studies of the molecular mechanisms of chitin-degrading systems in a wide variety of organisms.

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Supporting Information Available: The characterization data (NMR, MS, specific rotation) of *N*,*N*,*N*-triMe-D-GlcNH_2, additional NMR spectrum data of **2**−**4**, and the Lineweaver−Burk and Dixon plots of **1** and nagstatin. This material is available free of charge via the Internet at http://pubs.acs.org.

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