Bioorganic & Medicinal Chemistry 17 (2009) 7248–7253

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Pochonicine, a polyhydroxylated pyrrolizidine alkaloid from fungus *Pochonia suchlasporia* var. *suchlasporia* TAMA 87 as a potent β-*N*-acetylglucosaminidase inhibitor

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ARTICLE INFO

Article history:
Received 1 July 2009
Revised 26 August 2009
Accepted 26 August 2009
Available online 31 August 2009

Keywords:
Chitinolytic enzyme system
Metabolic turnover of chitin
Family 20 GlcNAcase
Family 18 chitinase
Enzyme inhibitor

ABSTRACT

A new polyhydroxylated pyrrolizidine alkaloid designated as pochonicine (**1**) was isolated from a solid fermentation culture of the fungal strain *Pochonia suchlasporia* var. *suchlasporia* TAMA 87. The structure of **1** was determined using NMR and MS techniques as (1*R**, 3*S**, 5*S**, 6*S**, 7*R**, 7a *S**)-5-acetamidomethyl-3-hydroxymethyl-1,6,7-trihydroxypyrrolizidine. Pochonicine (**1**) showed potent inhibition against β-*N*-acetylglucosaminidases (GlcNAcases) of various organisms including insects, fungi, mammals, and a plant but no inhibition against β-glucosidase of almond, α-glucosidase of yeast, or chitinase of *Bacillus* sp. The GlcNAcase inhibitory activity of pochonicine (**1**) was comparable to nagstatin, a potent GlcNAcase inhibitor of natural origin.

1. Introduction

Chitin, a linear polysaccharide composed of β-1,4 linked D-*N*-acetylglucosamine (GlcNAc), is widely distributed in natural environments as a constituent of insect exoskeletons and fungal cell walls.[1–3] The metabolic turnover of chitin is known to be necessary for normal growth of these organisms. A chitinolytic enzyme system consisting of chitinase and β-*N*-acetylglucosaminidase (GlcNAcase) is necessary for the complete degradation of chitin into constituting monosaccharide in the degradation pathway.[3–5] Chitinase hydrolyzes the polysaccharide chitin into GlcNAc oligomers, which are further hydrolyzed by another chitinolytic enzyme GlcNAcase into monosaccharide GlcNAc. The inhibitors of this enzyme system are expected to be applicable, potentially, as pesticides or fungicides. Furthermore, recent studies have revealed that non-chitin-containing organisms such as plants and mammals fundamentally produce these enzymes for several physiological reasons including self-defense against pathogens,[6,7] and glycoprotein processing.[8,9] Therefore, specific inhibitors of chitin degrading enzymes would also be helpful tools for biochemical studies.

In an earlier study, we screened microorganisms producing GlcNAcase inhibitor and found four actinomycete strains and four fungal strains.[10] Interestingly, the actinomycete strains produced inhibitors with narrow spectrum specific for insect GlcNAcases, whereas the fungal strains produced those with broader spectrum effective against GlcNAcases of fungal, mammalian, insect, and plant origin. In addition, our continuing studies revealed that one active actinomycete strain—*Streptomyces anulatus* NBRC13369—produced quite a unique inhibitor in biological, structural, and physico-chemical respects. The isolated active compound, which was designated as TMG-chitotriomycin, showed selective and potent inhibition against the GlcNAcases from chitin-containing organisms but no inhibition of enzymes from non-chitin-containing organisms.[11]

Herein, we report the GlcNAcase inhibitor pochonicine (**1**), which is produced by *Pochonia suchlasporia* var. *suchlasporia* TAMA 87, an active fungal strain. Pochonicine (**1**), a new polyhydroxylated pyrrolizidine alkaloid, showed potent inhibition against GlcNAcases from various organisms including insects, fungi, mammals, and a plant, but no inhibition of other glycosidases such as β-glucosidase from almond, α-glucosidase from yeast, or chitinase from bacterium *Bacillus* sp. Its inhibitory activity was comparable to that of nagstatin, an extremely potent GlcNAcase inhibitor.

2. Results

2.1. Identification of pochonicine (1) producing fungus

The fungal strain TAMA 87 was isolated from a soil sample obtained from the premises of Tamagawa University, Tokyo, Japan.

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doi:10.1016/j.bmc.2009.08.052

The surfaces of its colonies were white with yellow reverse; the maximum temperature for its growth was less than 30 °C. The erect conidial structures composed of verticillate phialides originated from creeping hyphae or substrates. Subglobose to obovate conidia were born in head, and characteristic chlamydospores (dictyospores) were produced in the substrates. Based on these morphological and physiological characteristics and the phylogenetic analyses of internal transcribed spacer (ITS) sequences, the fungal strain was identified as *P. suchlasporia* var. *suchlasporia* TAMA 87.[12]

2.2. Production, isolation, and structure elucidation of pochonicine (1)

Pochonicine (**1**) was obtained as an optically active ($[\alpha]_D^{17}$ +9.2, MeOH) colorless syrup from the MeOH extract of the culture of *P. suchlasporia* var. *suchlasporia* TAMA 87. The HRFABMS of **1** gave a molecular ion peak at *m/z* 261.1452 $[M+H]^+$, which, together with data obtained from the ^{13}C and ^1H NMR spectra, indicated the molecular formula of **1** to be $C_{11}H_{20}N_2O_5$. In addition, trimethylsilylation of **1** gave a tetra-trimethylsilylated derivative detected as a single peak in GC–MS total ion chromatogram, which showed a characteristic fragment ions at *m/z* 533 $[M-CH_3]^+$, *m/z* 476 $[M-CH_2NHAc]^+$, and *m/z* 445 $[M-CH_2OSiMe_3]^+$, indicating the presence of four hydroxy groups. The two types of fragment ions $[M-CH_3]^+$ and $[M-CH_2OSiMe_3]^+$ were also reported in GC–MS analysis of trimethylsilylated derivatives of hyacinthacines, polyhydroxylated pyrrolizidine alkaloids with no acetamidomethyl group.[13] The HSQC and ^{13}C spectral data of **1** revealed the presence of a single methyl, three methylene, and six methine groups, and a single carbonyl carbon, as presented in Table 1. Its ^1H NMR spectrum showed one singlet signal at δ_H 2.01 (3H, s), which is in agreement with methyl protons of an acetamido group. The ^{13}C NMR spectrum of **1** also supported the presence of this moiety by the resonance of the carbonyl carbon (δ_C 174.5). Furthermore, the HMBC correlation between the carbonyl carbon (δ_C 174.5) and methylene protons of C-9 (δ_H 3.48, δ_H 3.56) indicated that the acetamido group was attached to the methylene carbon of C-9 position. The pyrrolizidine ring structure, including the location of its substituents, was demonstrated easily by ^1H, COSY, HSQC, and HMBC spectra, which also allowed a complete assignment of the ^1H and ^{13}C NMR spectra (Table 1, Fig. 1a, and Supplementary data). Its relative stereochemistry was determined using NOE correlations (Fig. 1b) and $^3J_{H,H}$ coupling constants (Table 1). The NOE interactions between H-1/H-7a, H-7a/H-7, and H-7/H-6, indicated that H-1, H-6, H-7, and H-7a were on the same side of the ring. Moreover, the intensity of the NOE interaction between H-1/H-2b was much stronger than that of between H-1/H-2a (Fig. S6 of Supplementary data) indicating that H-2b and the above four protons were in a *cis* arrangement. Non-vicinal NOE interactions were also observed between H-1/H-7, H-6/H-7a to support above relative stereochemistry. The NOE interaction observed for H-3 with H-2a but not with H-2b indicated that H-3 was on the opposite side of the ring to H-1, H-2b, H-6, H-7 and H-7a. This was supported by the NOE interaction between H-2b/H-8. The NOE interaction between H-5/H-8 and H-5/H-8′without H-5/H-3 indicated the possibility of the *trans* arrangement of H-5 and H-3. But the NOE interaction between H-6/H-9′ and H-8/H-9′ indicated that H-5 had the same orientation with H-3. Furthermore, the coupling constant (8.6 Hz) between H-5/H-6, which was confirmed by the 2D *J*-resolved ^1H NMR experiment (Fig. S7 of Supplementary data), strongly indicated their *trans* orientation. Herein, the case of the absence of the NOE interaction between *cis*-oriented H-5 and H-3 in the pyrrolizidine ring is previously reported for hyacinthacine C_5.[13] Therefore, H-5 and H-3 were assigned to be in a *cis* arrangement. Consequently, isolated (**1**) was determined to be (1*R**, 3*S**, 5*S**, 6*S**, 7*R**, 7a*S**)-5-acetamidomethyl-3-hydroxymethyl-1,6,7-trihydroxypyrrolizidine. The reason for the NOE interaction between H-5/H-8 and H-5/H-8′ are not clear, but it may be due to the distorted conformation of **1** by the effect of the acetamidomethyl group which linked to C-5 of **1**. To date, the distribution of polyhydroxylated pyrrolizidine alkaloids, including australines,[14] alexines,[15] and hyacinthacines,[13,16,17] has been restricted to plants.[18,19] To the best of our knowledge, pochonicine (**1**) is the first example of such a compound isolated from a fungal metabolite.

Table 1
^1H and ^{13}C NMR data of 1 in CD_3OD

Position	δ_C	δ_H (*J* in Hz)
1	69.4	4.61 ddd (3.9, 5.9, 5.9)
2a	39.5	2.00 ddd (5.9, 6.2, 12.6)
2b		2.19 ddd (5.9, 6.5, 12.6)
3	63.9	3.70 dddd (3.3, 6.2, 6.3, 6.5)
5	61.5	3.46 ddd (4.4, 4.9, 8.6)
6	77.6	3.91 dd (3.9, 8.6)
7	71.5	4.08 dd (3.9, 3.9)
7a	76.4	3.61 dd (3.9, 3.9)
8	61.7	3.76 dd (6.2, 12.1)
8′		3.90 dd (3.3, 12.1)
9	42.2	3.48 dd (4.9, 13.8)
9′		3.56 dd (4.4, 13.8)
11	174.5	
12	22.5	2.01 s

2.3. Enzyme inhibitory activity of pochonicine (1)

Pochonicine (**1**) showed potent inhibition of the GlcNAcases from various organisms including an insect, fungi, mammals, and a plant (Fig. 2, Table 2). Interestingly, **1** showed distinct three types of modes of inhibition such as competitive toward *Spodoptera litura* (insect) and Jack bean (plant), as non-competitive toward *Aspergillus oryzae* (fungus) and bovine kidney (mammal), and as mixed manners toward *Penicillium oxalicum* (fungus) and human placenta (mammal) as shown in Figure 2. In addition, **1** did not inhibit chitinase from *Bacillus* sp. (no inhibition at 209 μM of **1**), β-glucosidase from almond (no inhibition at 2.38 μM of **1**), or α-glucosidase from yeast (no inhibition at 2.38 μM of **1**).

3. Discussion

To our knowledge, this is the first report of the inhibition of GlcNAcase by pyrrolizidine derivatives. It must be emphasized that GlcNAcase inhibitory activity of **1** was comparable to nagstatin, an extremely potent GlcNAcase inhibitor of natural origin, as presented in Table 2. Polyhydroxylated pyrrolizidines such as hyacinthacines are reportedly distributed throughout the plant kingdom as inhibitors of several glycosidases.[13,16,17] The characteristic distinction of the chemical structures between **1** and known polyhydroxylated pyrrolizidines is the presence of an acetamidomethyl group at the C-5 position. Such a substitution on a pyrrolizidine ring had been hitherto unknown in nature. The known GlcNAcase inhibitor including nagstatin and PUGNAc contains the acetamido group.[20–23] Tatsuta et al. synthesized nagstatin analogs to elucidate the essential effect of the acetamido group for the GlcNAcase inhibition.[21] Therefore, the acetamidomethyl group of **1** is also important for affinity between the enzymes and **1**. In addition, the known GlcNAcase inhibitors possess the nitrogen atom to interact to the catalytic amino acid residue.[20–24] For **1**, 3° nitrogen atom in a pyrrolizidine ring might be positively charged during the interaction to the targeted enzymes to act as such a key nitrogen atom of known inhibitors.

The absolute configuration of **1** has not yet been determined. Nevertheless, an attractive idea related to the potent inhibitory

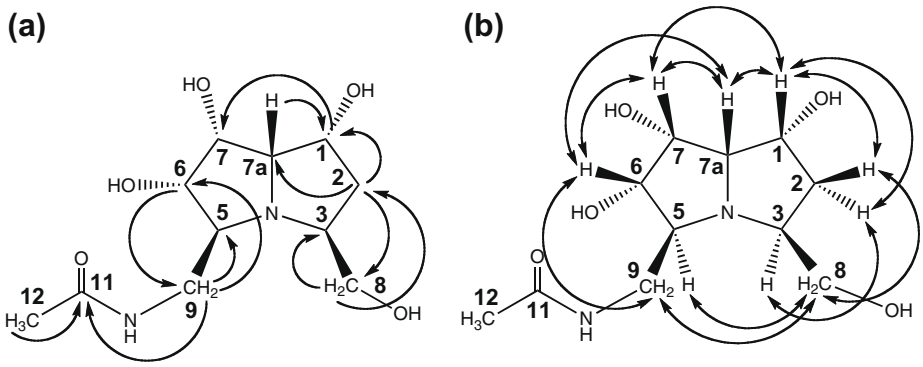


Figure 1. Selected HMBC (a) and NOE (b) correlations of pochonicine (**1**).

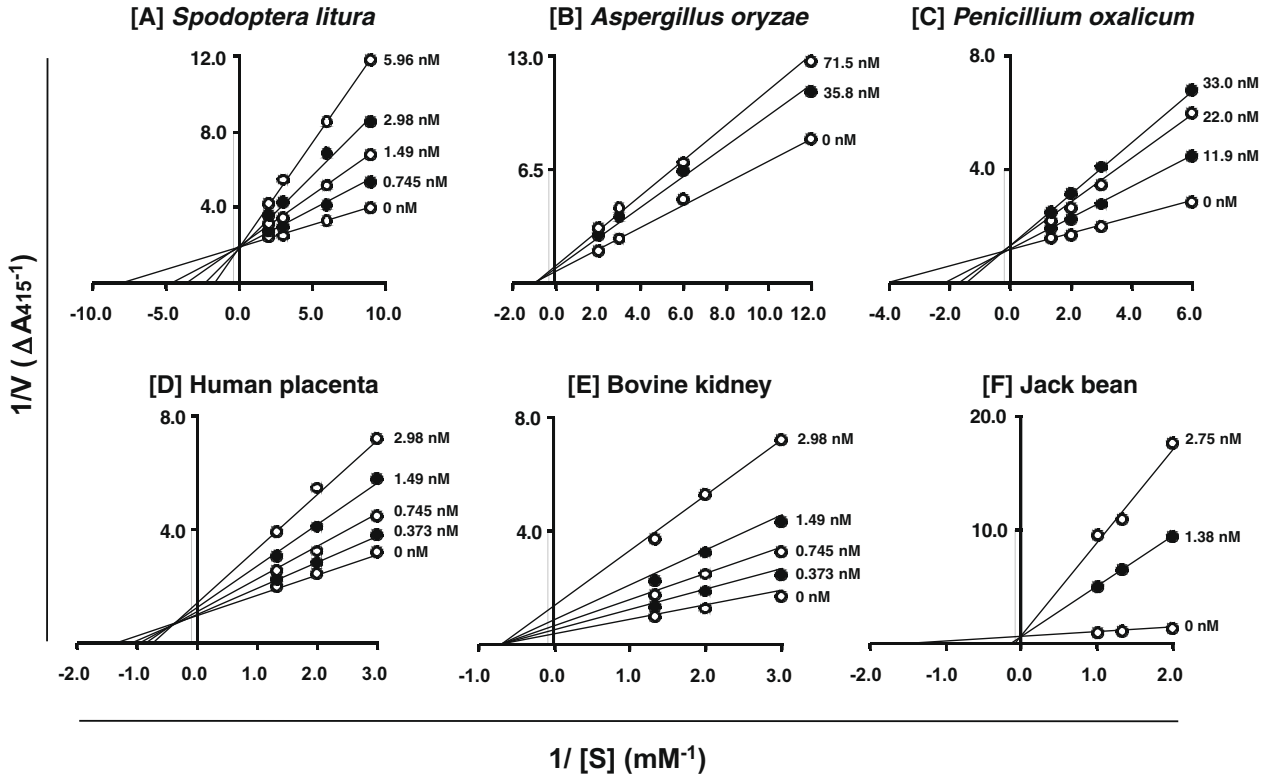


Figure 2. Lineweaver–Burk plots of pochonicine (**1**) against several GlcNAcases. Represented concentrations of pochonicine were assayed toward the GlcNAcases from *Spodoptera litura* (A), *Aspergillus oryzae* (B), *Penicillium oxalicum* (C), human placenta (D), bovine kidney (E), and jack bean (F).

Table 2
Inhibition of **1** and nagstatin against GlcNAcases

Origin of enzymes	K_m[a] (mM)	**1**			Nagstatin[e]		
		IC_{50} (nM)	K_i (nM)	Mode of inhibition	IC_{50} (nM)	K_i (nM)	Mode of inhibition
Spodoptera litura	0.121	5.96	1.77	Comp.[b]	5.85	1.10	Comp.
Aspergillus oryzae	0.955	20.3	141	Non-comp.[c]	10.6	9.69	Comp.
Penicillium oxalicum	0.246	42.1	13.2	Mixed[d]	60.6	8.11	Comp.
Human placenta	0.858	2.39	1.25	Mixed	1.83	0.925	Comp.
Bovine kidney	0.712	1.06	1.04	Non-comp.	1.71	1.64	Comp.
Jack bean	0.615	0.288	0.162	Comp.	1.87	1.41	Comp.

[a] For *p*NP-GlcNAc.
[b] Competitive manner.
[c] Non-competitive manner.
[d] Mixed competitive and non-competitive manner.
[e] These data were referred from the authors' previous paper.[11a]

activity of **1** might be the following one, which was inspired by a report of Takaoka et al.[25] The paper reported the structure–activity relations of two epimers of a five-membered acetamide aza sugar derivative, compounds **2** and **3** as inhibitors of GlcNAcases from bovine kidney and jack bean. Figure 3b and c shows that their only structural difference was the configuration at their C-2 position. Herein, both compounds showed potent inhibitory activity against the distinct GlcNAcases described above. In particular, **2** reportedly shows more potent inhibitory activity toward both enzymes than **3** as mimicking the transition-state nature of enzyme reaction, indicating the critical role of configuration of C-2 acetamidomethyl group for the enzyme inhibition. It is noteworthy that one enantiomer of **1**, viz. (1*S*,3*R*,5*R*,6*R*,7*S*,7a *R*)-5-acetamidomethyl-3-hydroxymethyl-1,6,7-trihydroxypyrrolizidine can reasonably adopt conformational structure corresponding to above two reported inhibitors, especially to **2**, as portrayed in Fig. 3a. The apparent structural differences between **1** and a set of reported inhibitors **2** and **3** were the heterobicyclic template for the former or heteromonocyclic one for the latter. Therefore, a potent inhibitory activity of **1** might be due to the structural rigidity of the bicyclic pyrrolizidine ring. It is particularly noteworthy that the hydroxyl groups at C-1 and C-3 of the enantiomer of **1** are conformationally and configurationally fixed by the nature of the bicyclic template. Therefore, clarification of the role of such characteristics is expected to yield new insight into the development of potent GlcNAcase inhibitors. Furthermore, the configuration of hydroxyl group at C-7 of **1** would not be fitted to those at C-4 of reported compounds **2** and **3**. Therefore, preparation of such pochonicine (**1**) analogues, and of course, a set of its enantiomers, to clarify the enzyme inhibitory activity will be a challenging undertaking for the development of potent GlcNAcase inhibitors with a polyhydroxylated pyrrolizidine skeleton.

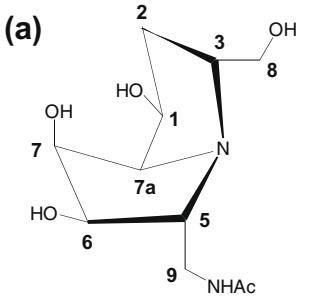
4. Experimental

4.1. General procedures

The NMR spectra were obtained using a Varian Inova AS600 spectrometer in CD_3OD; the spectra were referenced according to the solvent peaks (δ_H 3.35 or δ_C 49.0). The FABMS experiment was carried out on a JEOL JMS-SX102A. Cation-exchange column chromatography was performed on Amberlite CG-50 (H^+ form), which was purchased from Organo Co. HPLC separation was conducted on an Asahipak ES502C (7.5 × 100 mm, Showa Denko K. K.) with a detection wavelength of 210 nm. The β-*N*-acetylglucosaminidases from bovine kidney, human placenta, jack bean, and *A. oryzae* used for this study were obtained from Sigma–Aldrich Corp. The β-*N*-acetylglucosaminidase from *P. oxalicum* was obtained from Seikagaku Corporation. The chitinase from *Bacillus* sp. was purchased from Wako Pure Chemical Industries Ltd. The β-glucosidase from almond and α-glucosidase from bakers' yeast were purchased from Sigma–Aldrich Corp. These enzymes were used for enzyme inhibition studies without further purification. The β-*N*-acetylglucosaminidase from *S. litura* was prepared as described in a previous paper.[10] Nagstatin was a kind gift from the Microbial Chemistry Research Foundation (Japan). All other chemicals were commercially available.

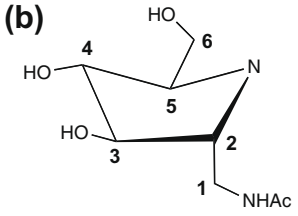
4.2. Organism collection and identification

The fungal strain *P. suchlasporia* var. *suchlasporia* TAMA 87 was isolated on 12 June, 1999 from a soil sample on Miura medium (LCA) with antibiotics using a serial dilution method. The soil was collected under *Acer* and *Pinus* trees on the premises of Tamagawa University, Machida, Tokyo, Japan (10 May, 1999). This fungus TAMA 87 was identified based on its morphological, physiological, and molecular phylogenetic characteristics. For morphological observation, the strain was grown on oatmeal agar (OA), malt extract agar (MA) and Miura medium (LCA) for 1–2 weeks at 25 °C in the dark. Then DNA was extracted from mycelia grown on MA using a kit (QIAamp DNA Mini; Qiagen Inc., Tokyo). Primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTC GTAACAAGG) were used for amplifying internal transcribed spacer (ITS) 1, 2, and 5.8s rDNA regions.[26] The DNA samples were purified using a PCR purification kit (High Pure; Roche Diagnostics Corp., Tokyo) followed by amplification and sequencing using a cycle sequencing kit and an analyzer (Big Dye Terminator v3.1,

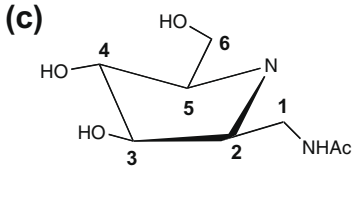


One of enantiomers of pochonicine **(1)**

	*K*i (nM)		
Origin of enzymes	**1**	**2**	**3**
Bovine kidney	1.04	9,800	68,600
Jack bean	0.162	1,900	3,600



Compound (**2**)



Compound (**3**)

Figure 3. Comparison of conformational structures between that of enantiomers of pochonicine (**1**) and a set of reported inhibitors, five-membered aza *N*-acetylglucosamine **2** and **3**, and their GlcNAcase inhibitory activities. The K_i values of **1** in the inner small table are referred from Table 2 of this paper. Those of **2** and **3** are referred from an earlier published paper.[25]

Genetic Analyzer ABI310; Applied Biosystems Japan). The obtained ITS data were compared to those in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-e.html) using a BLAST search. The nucleotide sequences were aligned manually using ClustalW; phylogenetic analyses were completed using PAUP (ver. 4.0b8) to construct the neighbor-joining and maximum parsimony trees. The stability of clades was evaluated using bootstrap tests with 1000 replications. *P. suchlasporia* var. *suchlasporia* TAMA 87 was deposited at the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan as FERM AP-21204.

4.3. Fermentation and isolation

The stock culture of fungal strain *P. suchlasporia* var. *suchlasporia* TAMA87 grown on yeast extract agar slant was scraped and suspended in 4 ml of sterilized water; 1 ml of the suspension was transferred to a 250 ml plastic flask containing rolled barley solid medium consisting of 10 g of rolled barley (Kyowa Seibaku Co.), 10 ml of water, 20 mg of yeast extract (Aidemar Co.), 10 mg of sodium tartarate, and 10 mg of KH_2PO_4. Then, they were statically cultivated at 22 °C for 19 days. After fermentation, 25 ml of methanol was added to each flask and mixed well and the mixture was kept at room temperature overnight. The supernatants were combined to yield 288 ml of MeOH extract, which was used for isolation of the active compound. Purification of the active compound was done with the guidance of the inhibitory activity against the GlcNAcase of *S. litura* (insect) as follows. In brief, a portion of each fraction was assayed for inhibitory activity against the GlcNAcase of *S. litura* and each active fraction was dried under reduced pressure to measure the weight followed by determination of its ID_{50} value. The MeOH extract was evaporated under reduced pressure to remove the methanol. The resulting aqueous suspension was washed with ethyl acetate to obtain the water-soluble fraction. The fraction was applied to the active carbon column and washed with H_2O followed by elution with 50% methanol. The active fractions were combined and evaporated to remove the methanol. The remaining solution was chromatographed on Amberlite CG-50 (H^+ form). The column was washed with water, then subsequently with 1 mM NaCl, and eluted with 5 mM NaCl. The active fractions were combined and desalted with active carbon column chromatography in a manner similar to that described above, and lyophilized. The material (4.32 mg) was dissolved in small amounts of H_2O, subjected to HPLC using an Asahipak ES502C column (7.5 × 100 mm), and developed with 50 mM ammonium acetate at the flow rate of 0.6 ml/min to yield 1.99 mg of **1**.

Compound **1**. Obtained as colorless syrup; $[\alpha]_D^{17}$ +9.2 (*c* 0.89, MeOH); ^1H and ^{13}C NMR, see Table 1 and Supplementary data; HRFABMS *m/z* 261.1452 $[M+H]^+$ ($C_{11}H_{20}N_2O_5$ requires 261.1450).

4.4. GC–MS analysis of (1)

Samples were dried and silylate at room temperature for 120 min using 100 μl of silblender-HTP (Nacalai Tesque) per 100 μg of material, and then analyzed with a GC–MS system (JEOL Automass 20). GC–MS analysis was carried out under the following conditions: GC, Hewlett–Packard HP5890 Series II; column, DB-1 (30 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific); carrier gas, He; injection temperature, 230 °C; column temperature program, 150 °C (2 min), 150–250 °C at 10 °C/min, 280 °C (20 min); MS, electron ionization positive mode; ionization voltage, 70 eV; ion source temperature, 210 °C.

4.5. Assay methods of enzyme inhibitory activity

Inhibition of GlcNAcases was determined using the method described in our previous work[10] in which the *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-GlcNAc) was used as substrate. Inhibitions of β-glucosidase from almond, α-glucosidase from yeast, and chitinase from *Bacillus* sp. were tested similarly to the above GlcNAcase inhibition assay in which *p*-nitrophenyl-β-D-glucoside (*p*NP-β-Glc) for β-glucosidase and *p*-nitrophenyl-α-D-glucoside (*p*NP-α-Glc) for α-glucosidase, *p*-nitrophenyl *N,N′*-diacetyl-β-D-chitobioside (*p*NP-$GlcNAc_2$) for the chitinase were used as substrates. The following conditions were used for each enzyme inhibition assay: (1) β-glucosidase from almond: 0.5 mM *p*NP-β-Glc, 50 mM citrate/phosphate/borate buffer (pH 5.0), 37 °C, 60 min. (2) α-Glucosidase from yeast: 0.5 mM *p*NP-α-Glc, 50 mM citrate/phosphate/borate buffer (pH 6.8), 37 °C, 60 min. (3) Chitinase from *Bacillus* sp.: 0.5 mM *p*NP-$GlcNAc_2$, 50 mM citrate/phosphate/borate buffer (pH 7.0), 37 °C, 60 min. The IC_{50} values 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.

Acknowledgments

We are grateful to the Microbial Chemistry Research Foundation (Japan) for the kind gift of nagstatin. We are also 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. Technical participations of Ms. Asami Yamada (Okayama University) for GC–MS experiments were also acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.052.

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