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New analogs of pochonicine, a potent *β-N*-acetylglucosaminidase inhibitor from fungus *Pochonia suchlasporia* var. *suchlasporia* TAMA 87

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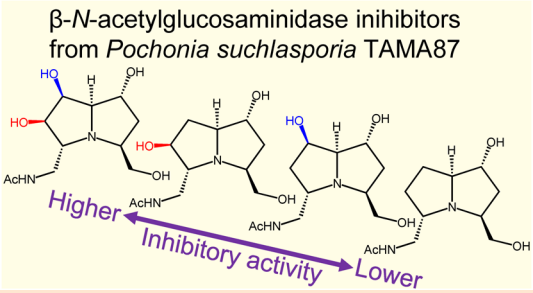
Supplementary material

Three novel analogs of pochonicine (**1**) were isolated from a solid fermentation culture of the fungal strain *Pochonia suchlasporia* var. *suchlasporia* TAMA 87, and their structures were elucidated as 7-deoxypochonicine (**2**), 6-deoxypochonicine (**3**), and 6,7-dideoxypochonicine (**4**). These analogs were found to possess the same stereochemistry as pochonicine. Comparison of *β-N*-acetylglucosaminidase (GlcNAcase) inhibitory activity between these analogs and pochonicine suggested that the C-6 hydroxy group of pochonicine was essential to its potent GlcNAcase inhibitory activity and that the C-7 hydroxy group also contributed to the activity, but to a lesser extent than the C-6 hydroxy group.



Keywords: pochonicine, *β-N*-acetylglucosaminidase inhibitor, *Pochonia suchlasporia*, chitinolytic enzyme, fungi, solid state fermentation.

Introduction

Pochonicine (**1**), the first naturally occurring polyhydroxylated pyrrolizidine alkaloid with an *N*-acetylaminomethyl group on the pyrrolizidine nucleus, was previously isolated from a solid fermentation culture of the fungal strain *Pochonia suchlasporia* var. *suchlasporia* TAMA 87.[1)] The absolute configuration of **1** was determined to be 1*R*, 3*S*, 5*R*, 6*R*, 7*S*, 7a*R* (Fig. 1) *via* synthetic studies.[2,3)] Pochonicine (**1**) is also the first potent *β-N*-acetylglucosaminidase (GlcNAcase) inhibitor having a pyrrolizidine skeleton. It inhibited wide variety of GlcNAcases including those from insects, fungi, mammals, and a plant, and its activity was comparable to that of nagstatin, one of the most potent GlcNAcase inhibitors of natural origin.[1)] GlcNAcase is responsible for numerous physiological functions such as chitin degradation, glycoconjugate processing, signal transduction, fertilization, seed

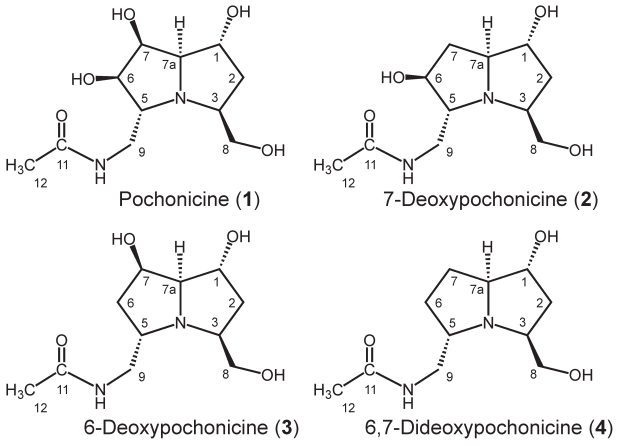


Fig. 1. Chemical structures of pochonicine and its analogs.

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germination, and virus infection.[4] Hence, GlcNAcase inhibitors have potential as research tools and therapeutics.[5]

In the course of purifying pochonicine from the culture extract of *Pochonia suchlasporia* TAMA 87, several analogs of pochonicine were found to be produced along with pochonicine by this strain. However, their amounts were much lower than that of pochonicine. In this work, three novel analogs of pochonicine were isolated and their structures were determined (Fig. 1). Their GlcNAcase inhibitory activities were evaluated and compared with that of pochonicine.

Materials and methods

1. General procedures

Pochonicine (**1**) was purified using the method previously reported.[1] *β-N*-acetylglucosaminidase (GlcNAcase) from *Spodoptera litura* was prepared as described in our previous papers.[6,7] *β-N*-acetylglucosaminidases from the jack bean (*Canavalia ensiformis*) and human placenta were purchased from Sigma-Aldrich (Sigma-Aldrich, Tokyo, Japan). These enzymes were used for enzyme inhibition assay without further purification. Cation-exchange column chromatography was performed on Amberlite CG-50 (H^+ form), which was purchased from the Organo Co. (Organo Co., Tokyo, Japan). HPLC separation was conducted on an Asahipak ES502C (7.5×100 mm, Showa Denko KK, Tokyo, Japan) with a detection wavelength of 210 nm. The NMR spectra were obtained using a Varian Inova AS600 spectrometer (Varian, Palo Alto, CA, USA) in CD_3OD; the spectra were referenced according to the solvent peaks (δ_H 3.35 or δ_C 49.0). High-resolution ESI mass spectra were recorded on a micrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Optical rotation was measured on a P-2200 polarimeter (JASCO, Tokyo, Japan).

2. Fungal strain

The fungal strain *P. suchlasporia* var. *suchlasporia* TAMA 87 was isolated from a soil sample in Machida, Tokyo.[1] The *P. suchlasporia* TAMA 87 culture was grown on a yeast extract agar slant. The fungal mycelial discs for inoculation were prepared as described in our previous paper.[8]

3. Fermentation

One fungal mycelial disc was inoculated into each of 43 Erlenmeyer flasks (200 mL), each containing an autoclaved rolled barley-based solid medium consisting of 9 g of rolled barley (Kyowa Seibaku Co., Kanagawa, Japan), 1 g of peeled oats (DoggyMan H. A. Co. Ltd., Osaka, Japan), 10 mL of water, 20 mg of yeast extract (Nacalai Tesque Inc., Kyoto, Japan), 10 mg of sodium L-tartrate dihydrate, and 10 mg of KH_2PO_4. The fermentation was performed for 22 days under static conditions at 22°C. This fermentation procedure was repeated twice on separate days (a total of 86 Erlenmeyer flasks were used).

*4. Isolation of compounds **2–4***

The fungal culture was extracted by adding 50 mL of MeOH to each flask, shaking the flasks well, and keeping them overnight at room temperature. The mixture was filtered, combined and concentrated *in vacuo* to remove the methanol. The obtained aqueous solution (640 mL) was washed with ethyl acetate to obtain the water-soluble fraction (885 mL). The fraction was chromatographed on Amberlite CG-50 (H^+ form). The column was washed with water, then eluted with aqueous NaCl solution (stepwise gradient, 1, 5, 10, 50, and 100 mM NaCl). The active fractions were obtained in 10 and 50 mM NaCl fractions, which were combined and applied to the active carbon column and washed with H_2O followed by elution with increasing concentra-

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR data of compounds **1–4** in CD_3OD (δ in ppm, J in Hz)

Position	Compound **1** (pochonicne)[a]		Compound **2**		Compound **3**		Compound **4**	
	δ_C	δ_H (mult., J)	δ_C	δ_H (mult., J)	δ_C	δ_H (mult., J)	δ_C	δ_H (mult., J)
1	69.4	4.61 (ddd, 3.9, 5.9, 5.9)	77.5	4.21 (ddd, 3.0, 3.0, 6.0)	69.2	4.49 (ddd, 4.3, 6.2, 6.4)	77.2	3.99 (ddd, 2.8, 2.9, 5.9)
2	39.5	2.00 (ddd, 5.9, 6.2, 12.6)	37.8	2.02 (ddd, 6.0, 10.1, 13.0)	41.0	2.11 (ddd, 6.2, 6.2, 12.6)	38.1	1.82 (ddd, 6.1, 10.3, 13.2)
2′		2.19 (ddd, 5.9, 6.5, 12.6)		1.76 (ddd, 3.0, 5.5, 13.0)		2.04 (dd, 6.4, 12.6)		1.76 (ddd, 2.8, 5.6, 13.2)
3	63.9	3.70 (dddd, 3.3, 6.2, 6.3, 6.5)	64.3	3.63 (dddd, 5.1, 5.5, 6.7, 10.1)	62.3	3.52 (m)	64.1	3.59 (dddd, 4.9, 5.6, 7.1, 10.3)
5	61.5	3.46 (ddd, 4.4, 4.9, 8.6)	65.8	3.21 (ddd, 4.6, 4.8, 6.6)	56.3	3.54 (m)	58.0	3.31 (dddd, 5.3, 6.5, 6.5, 7.4)
6	77.6	3.91 (dd, 3.9, 8.6)	76.3	4.03 (ddd, 4.6, 4.8, 5.6)	42.7	2.06 (ddd, 2.3, 5.9, 13.3)	31.9	1.96 (m)
6′						1.74 (ddd, 4.1, 9.6, 13.3)		1.56 (m)
7	71.5	4.08 (dd, 3.9, 3.9)	38.4	2.27 (ddd, 5.6, 7.8, 13.3)	70.9	4.23 (ddd, 2.1, 4.1, 4.2)	30.4	2.08 (m)
7′				1.77 (ddd, 4.8, 4.8, 13.3)				1.61 (m)
7a	76.4	3.61 (dd, 3.9, 3.9)	74.9	3.41 (ddd, 3.0, 4.8, 7.8)	79.8	3.49 (dd, 4.2, 4.3)	76.7	3.42 (ddd, 2.9, 5.9, 6.7)
8	61.7	3.76 (dd, 6.2, 12.1)	62.4	3.84 (dd, 5.1, 10.7)	62.4	3.89 (dd, 3.8, 11.9)	62.3	3.88 (dd, 7.1, 11.8)
8′		3.90 (dd, 3.3, 12.1)		3.82 (dd, 6.7, 10.7)		3.72 (dd, 5.1, 11.9)		3.83 (dd, 4.9, 11.8)
9	42.2	3.48 (dd, 4.9, 13.8)	44.0	3.29 (dd, 4.8, 12.7)	45.5	3.42 (dd, 5.1, 13.3)	46.0	3.38 (dd, 5.3, 13.2)
9′		3.56 (dd, 4.4, 13.8)		3.17 (dd, 6.6, 12.7)		3.13 (dd, 6.7, 13.3)		3.03 (dd, 7.4, 13.2)
11	174.5		173.7		173.5		173.4	
12	22.5	2.01 (s)	22.6	1.99 (s)	22.5	1.98 (s)	22.5	1.97 (s)

[a] data from Ref. 1).

tions of MeOH in water (stepwise gradient, 10, 50, 90, and 100% (v/v)). The desalted and purified active fractions were obtained in 10 and 50% MeOH fractions, which were combined (5300 mL) and evaporated to remove the methanol and then lyophilized. The material (410 mg) was dissolved in 800 μL of 10 mM aqueous ammonium acetate solution and injected (10×80 μL) onto an HPLC using an Asahipak ES502C 20C column (20×100 mm), then isocratically eluted with 10 mM aqueous ammonium acetate solution at a flow rate of 4.8 mL/min and detection at 210 nm to yield 7.06 mg of **2**, 11.18 mg of **3**, and 17.45 mg of **4**. HPLC analysis of compounds **2**–**4** did not provide evidence of any measurable isomerization or degradation products.

Compound **2**: Obtained as colorless syrup; $[\alpha]_D^{16}$ −12.3 (*c* 0.287, MeOH); ^{1}H and ^{13}C NMR data are shown in Table 1 and Figs. S1 and S3; HRESIMS *m*/*z* 245.1498 $[M+H]^+$ (cald. for $C_{11}H_{21}N_2O_4$: 245.1496).

Compound **3**: Obtained as colorless syrup; $[\alpha]_D^{27}$ −25.0 (*c* 0.244, MeOH); ^{1}H and ^{13}C NMR data are shown in Table 1 and Figs. S7 and S9; HRESIMS *m*/*z* 245.1498 $[M+H]^+$ (cald. for $C_{11}H_{21}N_2O_4$: 245.1496).

Compound **4**: Obtained as colorless syrup; $[\alpha]_D^{16}$ −31.5 (*c* 0.880, MeOH); ^{1}H and ^{13}C NMR data are shown in Table 1 and Figs. S13 and S15; HRESIMS *m*/*z* 229.1547 $[M+H]^+$ (cald. for $C_{11}H_{21}N_2O_3$: 229.1547).

5. Assay methods for GlcNAcase inhibitory activity

Inhibitory activity (IC_{50} value) was determined *via* a colorimetric method from our previous work[7,9,10] using 0.5 mM *p*-nitrophenyl β-*N*-acetylglucosaminide as a substrate and 1.13 units/μL of each enzyme. The release of *p*-nitrophenol was measured at 415 nm after the addition of 1.3 M NaOH. IC_{50} values were calculated by plotting the inhibitor concentration *vs.* the rate of inhibition. One unit of enzyme was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per min at 37°C under each assay condition in which H_2O was used instead of the inhibitor solution. The following buffers were used according to the instructions or references: (1) *S. litura*: 100 mM citrate-phosphate-borate buffer (pH 6.0), (2) jack bean: 100 mM citrate buffer (pH 5.0) containing 100 mM NaCl and 0.01% (w/v) bovine serum albumin, (3) human placenta: 100 mM citrate buffer (pH 4.5) containing 100 mM NaCl and 0.01% (w/v) bovine serum albumin.

Results and discussion

1. Revision of the specific rotation value for pochonicine

The specific rotation of pochonicine (**1**) was first reported in our previous study as $[\alpha]_D^{17}$ +9.2 (*c* 0.897, MeOH).[1] However, careful measurement of the optical rotation of repurified **1** in a higher concentration than that reported previously indicated that the correct specific rotation of **1** is $[\alpha]_D^{27}$ −3.05 (*c* 2.67, MeOH) . This value is strongly supported by the specific rotation value of the enantiomer of **1**, $[\alpha]_D^{26}$ +3.1 (*c* 1.74, MeOH), reported by Kitamura *et al.*[2] Therefore, the value $[\alpha]_D^{27}$ −3.05 (*c* 2.67, MeOH) was used for the specific rotation of **1**. The incorrect specific value obtained in our previous study was probably due to the presence of a trace amount of base or acid. According to Kitamura *et al.*, the specific rotation value of the free amine was small, and its data were found to change around ±0 due to the pH.[2]

2. Determination of the chemical structure of ***2***

Compound **2** was obtained as an optically active ($[\alpha]_D^{16}$ −12.3, MeOH) colorless syrup from the MeOH extract of the culture broth of *P. suchlasporia* var. *suchlasporia* TAMA 87. The HRESIMS of **2** gave a molecular ion peak at *m*/*z* 245.1498 $[M+H]^+$, which, together with data obtained from the ^{13}C and ^{1}H NMR spectra, indicated that the molecular formula of **2** is $C_{11}H_{20}N_2O_4$. The HSQC and ^{13}C spectral data of **2** revealed the presence of a single methyl, four methylene, and five methine groups and a single carbonyl carbon, as presented in Table 1. Its ^{1}H NMR spectrum showed one singlet signal at δ_H 1.99 (3H, s), which is in agreement with the methyl protons of an acetamido group. The ^{13}C NMR spectrum of **2** also supported the presence of this moiety by the resonance of the carbonyl carbon (δ_C 173.7). Furthermore, the HMBC correlation between the carbonyl carbon (δ_C 173.7) and methylene protons of C-9 (δ_H 3.17, δ_H 3.29) indicated that the acetamido group was attached to the methylene carbon of the C-9 position. The pyrrolizidine ring structure, including the location of its substituents, was demonstrated by ^{1}H, ^{13}C, COSY, HSQC, and HMBC spectra (Figs. 2A and S1–5), which also allowed a complete assignment of the ^{1}H and ^{13}C NMR spectra (Table 1). The relative stereochemistry of **2** was determined using NOE correlations (Figs. 2B and S6) and $^3J_{H,H}$ coupling constants (Table 1). The NOESY spectrum showed cross peaks of H-1/H-2, H-1/H-7′, H-2′/H-3, H-5/H-8, H-5/H-8′, H-6/H-7, and H-7/H-7a, indicating that H-1, H-2, H-5, and H-7′ were on the side opposite to H-2′, H-3, H-6, H-7, and H-7a. The relative configuration at the asymmetric centers of **2** was the same as that of **1**. The same sign of the specific rotation value for **2** (−12.3, *c* 0.287, MeOH) as that of **1** (−3.05, *c* 2.67, MeOH) implied the same configuration. Taking into consideration that **1** and **2** are co-produced in the same fermentation, the absolute configuration of **2** was presumably the same as that of **1**. To our knowledge, this compound has not been reported, so we referred to **2** as 7-deoxypochonicine.

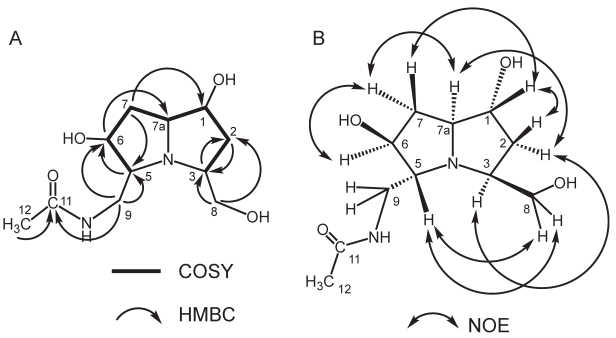

Fig. 2. Key COSY and HMBC correlations (A) and key NOE correlations (B) of **2**.

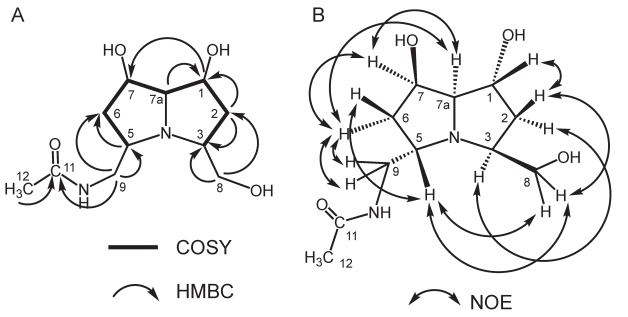

Fig. 3. Key COSY and HMBC correlations (A) and key NOE correlations (B) of **3**.

*3. Determination of the chemical structure of **3***

Compound **3** was obtained as an optically active ($[\alpha]_D^{27}$ −25.0, MeOH) colorless syrup from the MeOH extract of the culture broth of *P. suchlasporia* var. *suchlasporia* TAMA 87. The HRESIMS of **3** gave a molecular ion peak at *m*/*z* 245.1498 $[M+H]^+$, which, together with data obtained from the ^{13}C and ^1H NMR spectra, indicated that the molecular formula of **3** is $C_{11}H_{20}N_2O_4$. The HSQC and ^{13}C spectral data of **3** revealed the presence of a single methyl, four methylene, and five methine groups and a single carbonyl carbon, as presented in Table 1. Its ^1H NMR spectrum showed one singlet signal at δ_H 1.98 (3H, s), which is in agreement with the methyl protons of an acetamido group. The ^{13}C NMR spectrum of **3** also supported the presence of this moiety by the resonance of the carbonyl carbon (δ_C 173.5). Furthermore, the HMBC correlation between the carbonyl carbon (δ_C 173.5) and methylene protons of C-9 (δ_H 3.13, δ_H 3.42) indicated that the acetamido group was attached to the methylene carbon of the C-9 position. The pyrrolizidine ring structure, including the location of its substituents, was demonstrated by ^1H, ^{13}C, COSY, HSQC, and HMBC spectra (Figs. 3A and S7–11), which also allowed a complete assignment of the ^1H and ^{13}C NMR spectra (Table 1). The relative stereochemistry of **3** was determined using NOE correlations (Figs. 3B and S12) and $^3J_{H,H}$ coupling constants (Table 1). The NOESY spectrum showed cross peaks of H-1/H-2, H-2/H-8′, H-2′/H-3, H-5/H-8, H-5/H-8′, H-5/H-6, H-6′/H-7, H-6′/H-7a, H-6′/H-9, H6′/H-9′, and H-7/H-7a, indicating that H-1, H-2, H-5, and H-7′ were on the side opposite to H-2′, H-3, H-6′, H-7, and H-7a. The relative configuration at the asymmetric centers of **3** was the same as that of **1**. The same sign of the specific rotation value for **3** (−25.0, *c* 0.244, MeOH) as that of **1** (−3.05, *c* 2.67, MeOH) implied the same configuration. Taking into consideration that **1** and **3** are co-produced in the same fermentation, the absolute configuration of **3** was presumably the same as that of **1**. To our knowledge, this compound has not been reported, so we referred to **3** as 6-deoxypochonicine.

*4. Determination of the chemical structure of **4***

Compound **4** was obtained as an optically active ($[\alpha]_D^{16}$ −31.5, MeOH) colorless syrup from the MeOH extract of the culture broth of *Pochonia suchlasporia* var. *suchlasporia* TAMA 87. The HRESIMS of **4** gave a molecular ion peak at *m*/*z* 229.1547 $[M+H]^+$, which, together with data obtained from the ^{13}C and ^1H NMR spectra, indicated that the molecular formula of **4** is $C_{11}H_{20}N_2O_3$. The HSQC and ^{13}C spectral data of **4** revealed the presence of a single methyl, five methylene, and four methine groups and a single carbonyl carbon, as presented in Table 1. Its ^1H NMR spectrum showed one singlet signal at δ_H 1.97 (3H, s), which is in agreement with methyl protons of an acetamido group. The ^{13}C NMR spectrum of **4** also supported the presence of this moiety by the resonance of the carbonyl carbon (δ_C 173.4). Furthermore, the HMBC correlation between the carbonyl carbon (δ_C 173.4) and methylene protons of C-9 (δ_H 3.03, δ_H 3.38) indicated that the acetamido group was attached to the methylene carbon of the C-9 position. The pyrrolizidine ring structure, including the location of its substituents, was demonstrated by ^1H, ^{13}C, COSY, HSQC, and HMBC spectra (Figs. 4A and S13-17), which also allowed a complete assignment of the ^1H and ^{13}C NMR spectra (Table 1). The relative stereochemistry of **4** was determined using NOE correlations (Figs. 4B and S18) and $^3J_{H,H}$ coupling constants (Table 1). The NOESY spectrum showed cross peaks of H-1/H-2, H-1/H-7′, H-2/H-5, H-2′/H-3, H-5/H-6, H-5/H-8, H-5/H-8′, H-6′/H-7, H-6′/H-9′, and H-7/H-7a, indicating that H-1, H-2, H-5, H-6 and H-7′ were on the side opposite to H-2′, H-3, H-6′, H-7, and H-7a. The relative configuration at the asymmetric centers of **4** was the same as that of **1**. The same sign of the specific rotation value for **4** (−31.5, *c* 0.880, MeOH) as that of **1** (−3.05, *c* 2.67, MeOH) implied the same configuration. Taking into consideration that **1** and **4** are co-produced in the same fermentation, the absolute configuration of **4** was presumably the same as that of **1**. To our knowledge, this compound has not been reported, so we referred to **4** as 6,7-dideoxypochonicine.

*5. Enzyme inhibitory activity of pochonicine analogs **2**–**4***

Pochonicine analogs **2**–**4** showed inhibition of the GlcNAcases from an insect, a mammal, and a plant (Table 2). However their inhibitory activities were weaker than those of pochonicine (**1**). The order of their GlcNAcase inhibitory activities was **1**>**2**>**3**>**4**, indicating that the presence of hydroxy substituents at C-6 and C-7 is necessary for the potent GlcNAcase inhibitory

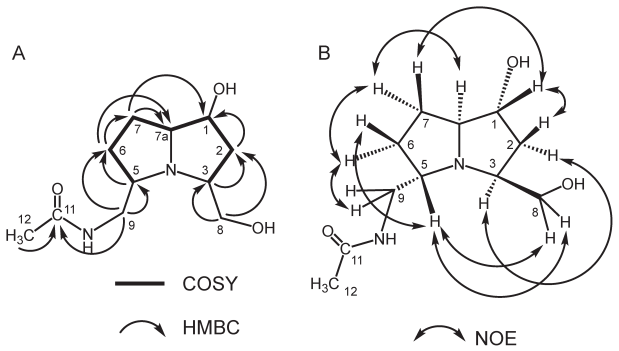

Fig. 4. Key COSY and HMBC correlations (A) and key NOE correlations (B) of **4**.

Table 2. Inhibitory activity (IC_{50}) of pochonicine (**1**) and its analogs (**2**–**4**)

Origin of enzymes		IC_{50} (nM)			
		1	**2**	**3**	**4**
Spodoptera litura (crude enzyme)	insect	13.0	471	6,400	71,900
Jack bean	plant	0.678	11.2	190	8,670
Human placenta	mammal	6.00	63.9	2,350	5,190

activity of **1**. In addition, the 6-OH group of **1** is considered to be more important for the GlcNAcase inhibitory activity than the 7-OH group. The structure–activity relationship observed in the present study would be helpful in understanding the key structural factors affecting the GlcNAcase inhibitory activity of **1** and related compounds. The sensitivity of the used enzymes to inhibition by compounds **1**–**3** was in the following order: jack bean GlcNAcase>human placenta GlcNAcase>*Spodoptera litura* GlcNAcase (crude). Sensitivity to inhibition by compound **4** was in the following order: human placenta GlcNAcase>jack bean GlcNAcase>*S. litura* GlcNAcase (crude). The diference in sensitybity of these enzymes to compounds **1**–**4** was considered to be due to the difference in the interaction between substrate and enzyme, especially that for the region involved in substrate specificity, where the hydroxy groups at C-6 and/or C-7 of compounds **1**–**3** may interact with the key residues.

Acknowledgements

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Electronic supplementary materials

The online version of this article contains supplementary materials (Supplemental Figs. S1–S18, Tables S1–S12), which are available at http://www.jstage.jst.go.jp/browse/jpestics/.

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