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

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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-deoxy-pochonicine (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 87.1,2 The absolute configuration of 1 was determined to be 1R, 3S, 5R, 6R, 7S, 7αR (Fig. 1) via synthetic studies.3–5 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.1–3 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.
germination, and virus infection. Hence, GlcNAcase inhibitors have potential as research tools and therapeutics.

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

Materials and methods

1. General procedures

Pachonicine (1) was purified using the method previously reported. \(^1\) \(\beta\)-N-acetylglucosaminidase (GlcNAcase) from Spodoptera litura was prepared as described in our previous papers. \(^6,7\) \(\beta\)-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 spectrometer (Varian, Palo Alto, CA, USA) in CD\(_3\)OD; the spectra were referenced according to the solvent peaks (\(\delta\)H 3.35 or 4.00 ppm). 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 preparation. \(^3\)

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 Seibako 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\(_2\)PO\(_4\). The fermentation was performed for 22 days under static conditions at 22°C. This fermentation procedure was repeated 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\(_2\)O followed by elution with increasing concentra-

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**Table 1.** \(^1\)H (600 MHz) and \(^13\)C (150 MHz) NMR data of compounds 1–4 in CD\(_3\)OD (δ in ppm, J in Hz)

| Position | Compound 1 (pachonicine) \(^a\) | 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  | 4.89 (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) | 2.04 (dd, 6.4, 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) | 1.76 (ddd, 2.8, 5.6, 13.2) |
| 3        | 63.9  | 3.70 (ddd, 3.3, 6.2, 6.3, 6.5) | 64.3  | 3.63 (ddd, 5.1, 5.5, 6.7, 10.1) | 62.3  | 3.52 (m) | 64.1  | 3.59 (ddd, 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 (ddd, 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) | 1.74 (ddd, 4.1, 9.6, 13.3) | 1.56 (m) |
| 6'       | 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) | 1.61 (m) |
| 7        | 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) | 3.83 (dd, 4.9, 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) | 3.03 (dd, 7.4, 13.2) |
| 9'       | 3.56 (dd, 4.4, 13.8) | 3.37 (dd, 6.6, 12.7) | 3.13 (dd, 6.7, 13.3) |

\(^a\) 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 E502C 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; [α]D 25 −12.3 (c 0.287, MeOH); 1H and 13C NMR data are shown in Table 1 and Figs. S1 and S3; HRESIMS m/z 245.1498 [M+H]+ (cald. for C11H21N2O4: 245.1496). The incorrect specific rotation value ([α]D 25 −12.3, MeOH) was presumably the same as that reported previously indicated that the specific rotation value of the free amine was small, and its data were found to change around ±0 due to the pH.

2. Determination of the chemical structure of 2

Compound 2 was obtained as an optically active ([α]D 25 −12.3, MeOH) colorless syrup from the MeOH extract of the culture broth of P. subsequa var. subsequa TAM 87. The HRESIMS of 2 gave a molecular ion peak at m/z 245.1498 [M+H]+, which, together with data obtained from the 13C and 1H NMR spectra, indicated that the molecular formula of 2 is C11H20N2O4. The HSQC and 13C 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 1H NMR spectrum showed one singlet signal at δH 1.99 (3H, s), which is in agreement with the methyl protons of an acetylamino group. The 13C NMR spectrum of 2 also supported the presence of this moiety by the resonance of the carbonyl carbon (δc 173.7). Furthermore, the HMBC correlation between the carbonyl carbon (δc 173.7) and methylene protons of C-9 (δH 3.17, δH 3.29) indicated that the acetylamino 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, 13C, COSY, HSQC, and HMBC spectra (Figs. 2A and S1–5), which also allowed a complete assignment of the 1H and 13C NMR spectra (Table 1). The relative stereochemistry of 2 was determined using NOE correlations (Figs. 2B and S6) and 1H-1H coupling constants (Table 1).

5. Assay methods for GlcNAcase inhibitory activity

Inhibitory activity (IC50 value) was determined via a colorimetric method from our previous work25,26 using 0.5 mM p-nitrophenyl β-N-acetylglucosaminidase as a substrate and 1.13 units/µL of each enzyme. The release of 1 µmol of p-nitrophenol per min at 37°C under each assay condition in which H2O 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 [α]D 25 +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 [α]D 25 −3.05 (c 2.67, MeOH). This value is strongly supported by the specific rotation value of the enantiomer of 1, [α]D 25 +3.1 (c 1.74, MeOH), reported by Kitamura et al.2 Therefore, the value [α]D 25 −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 pH21.
3. Determination of the chemical structure of 3

Compound 3 was obtained as an optically active ([α]_20^25 -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 ^1^C and ^1^H NMR spectra, indicated that the molecular formula of 3 is C_{11}H_{20}N_{2}O_{4}. The HSQC and ^1^C spectral data of 3 revealed the presence of a single methyl, five methylene, and four methine groups and a single carbonyl carbon, as presented in Table 1. Its ^1^H NMR spectrum showed one singlet signal at δ_H 1.98 (3H, s), which is in agreement with methyl protons of an acetamido group. The ^1^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 173.4) 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 ^1^H, ^1^C, COSY, HSQC, and HMBC spectra (Figs. 3A and S7–11), which also allowed a complete assignment of the ^1^H and ^1^C NMR spectra (Table 1). The relative stereochemistry of 4 was determined using NOE correlations (Figs. 4B and S18) and ^3^J_HH 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-dideoxyhochonicine.

4. Determination of the chemical structure of 4

Compound 4 was obtained as an optically active ([α]_20^25 -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 ^1^C and ^1^H NMR spectra, indicated that the molecular formula of 4 is C_{11}H_{20}N_{2}O_{3}. The HSQC and ^1^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 ^1^H 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 ^1^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 ^1^H, ^1^C, COSY, HSQC, and HMBC spectra (Figs. 4A and S13–17), which also allowed a complete assignment of the ^1^H and ^1^C NMR spectra (Table 1). The relative stereochemistry of 4 was determined using NOE correlations (Figs. 4B and S18) and ^3^J_HH 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 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-dideoxyhochonicine.
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 difference in sensitivity 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.

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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/.

Table 2. Inhibitory activity (IC₅₀) of pochonicine (1) and its analogs (2–4)

| Origin of enzymes | IC₅₀ (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 |

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