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Studies on the Constituents of *Broussonetia* Species. III. Two New Pyrrolidine Alkaloids, Broussonetines G and H, as Inhibitors of Glycosidase, from *Broussonetia kazinoki* SIEB.

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Two new pyrrolidine alkaloids, broussonetines G and H, were isolated from the branches of *Broussonetia kazinoki* SIEB. (Moraceae). Broussonetines G and H were formulated as 2β-hydroxymethyl-3α,4β-dihydroxy-5α-(1-hydroxy-6 : 10;10 : 13-diepoxytridecyl)-pyrrolidine (1) and 2β-hydroxymethyl-3α,4β-dihydroxy-5α-(1-hydroxy-5 : 9;9 : 13-diepoxytridecyl)-pyrrolidine (2), respectively, by spectroscopic methods. 1 and 2 inhibited β-glucosidase, β-galactosidase and β-mannosidase.

Key words pyrrolidine alkaloid; glycosidase inhibitor; *Broussonetia kazinoki*; broussonetine G; broussonetine H; Moraceae

Previously, we reported the structures of eight pyrrolidine alkaloids, broussonetines A—F and broussonetinines A and B as glycosidase inhibitors from *Broussonetia kazinoki* SIEB. (Moraceae).[1,2] In our latest work, we obtained two new pyrrolidine alkaloids, broussonetines G (**1**) and H (**2**) (Fig. 1), from the same tree. The present report deals with the isolation, structural elucidation and inhibitory activity on some glycosidases.

The branches of this tree were extracted with hot water and the alkaloid constituents were concentrated as described in the Experimental section. Compounds **1** and **2** were isolated by preparative HPLC of the concentrated alkaloids.

Compound **1** was obtained as a colorless oil, $[\alpha]_D$ $+17.5°$ (MeOH, $c=0.43$), showing a yellowish spot on TLC when sprayed with ninhydrin reagent followed by heating on a hot plate (ninhydrin reaction). The molecular formula was determined as $C_{18}H_{33}NO_6$ on the basis of positive high resolution secondary ion mass spectroscopy (pos. HR-SI-MS) (*m*/*z*: 360.2393, $[M+H]^+$, error, 0.9 mmu). The IR spectrum showed a strong OH and NH band at $3317\,cm^{-1}$.

The ^{1}H-NMR spectrum of **1** suggested the presence of nine methylene groups [δ 1.13—1.99 (18H, m)], two oxymethylene groups [δ 4.26 (1H, dd, $J=10.8$, 4.3 Hz), δ4.22 (1H, dd, $J=10.8$, 5.5 Hz) and 3.87 (2H, m)], four oxymethine groups [δ 4.95 (1H, t, $J=6.2$ Hz), δ 4.73 (1H, t, $J=6.2$ Hz), δ 4.13 (1H, ddd, $J=6.2$, 4.8, 4.8 Hz), δ 3.82 (1H, m)], and two methine groups attached to a nitrogen atom [δ 3.81 (1H, m), δ 3.65 (1H, t, $J=6.2$ Hz)]. Furthermore, the ^{13}C-NMR spectrum of **1** showed a characteristic carbon signal at δ 105.9, which indicated the presence of an acetal moiety. The ^{1}H- and ^{13}C-NMR signals were assigned by double quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), heteronuclear signal quantum coherence (HSQC), and distortionless enhancement by polarization transfer (DEPT), as shown in the partial structures in Fig. 2 and Table 1.

The linkage among these partial structures was determined from the heteronuclear multiple bond correlation (HMBC) spectrum to establish the complete planar structure of **1**, as shown in Fig. 3.

Compound **2** was obtained as a colorless oil, $[\alpha]_D$ $+15.5°$ (MeOH $c=0.50$), giving a yellow spot on TLC with the ninhydrin reaction, and the molecular formula was determined as $C_{18}H_{33}NO_6$ on the basis of pos. HR-SI-MS (*m*/*z*: 360.2374, $[M+H]^+$, error, 1.0 mmu). The IR spectrum showed a strong OH and NH band at $3320\,cm^{-1}$.

The ^{1}H-NMR spectrum was strikingly similar to that of **1**, except for the signals due to be partial structures shown in Fig. 4. The 13′-H of **1** had correlations with 11′- and 12′-Hs, while that of **2** had correlations with 10′-, 11′- and 12′-Hs in the TOCSY spectra suggesting a tetrahydropyrane ring in place of the tetrahydrofuran ring of **1**. Furthermore, fragment ion peaks in the high resolution electron impact mass spectra (HR-EI-MS) of **1** and **2** characterized their partial structures. (Fig. 5) ^{1}H- and ^{13}C-NMR signals were assinged as in **1** and summarized in Table 1.

The relative stereochemistry of the pyrrolidine ring moieties in **1** and **2** was shown by the vicinal coupling constants ($J_{2,3}=J_{3,4}=J_{4,5}=6.2$ Hz) and nuclear Overhauser effects (NOEs) in the nuclear Overhauser enhancement and exchange spectroscopy (NOESY) experiment. NOEs were observed between 2-H and 4-H, and 3-H and 5-H to confirm the 2β-hydroxymethyl-3α,4β-dihydroxy-5α-alkyl pyrrolidine structures.

Thus the structures containing the partial relative stereostructures were formulated as 2β-hydroxymethyl-3α,4β-dihydroxy-5α-(1-hydroxy-6 : 10;10 : 13-diepoxytridecyl)-pyrrolidine for **1** and 2β-hydroxymethyl-3α,4β-

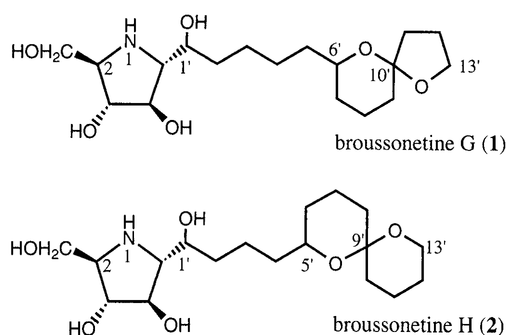

Fig. 1. Structures of **1** and **2**

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Table 1. ^{1}H and ^{13}C-NMR Spectral Data for **1** and **2**

	Broussonetine G (**1**)	
	Proton	Carbon
2	3.81 m[a]	65.88
3	4.73 t (6.2)	80.55
4	4.95 t (6.2)	80.31
5	3.65 t (6.2)	67.53
1′	4.13 ddd (6.2, 4.8, 4.8)	74.03
2′	1.99[a]	35.05
3′	1.86 m, 1.57[a]	26.85
4′	1.46[a]	26.31
5′	1.55,[a] 1.41 m	36.73
6′	3.82[a]	70.31
7′	1.45,[a] 1.13 m	31.38
8′	1.94,[a] 1.57[a]	20.99
9′	1.66[a]	33.39
10′		105.91
11′	1.96,[a] 1.58[a]	38.19
12′	1.95,[a] 1.69[a]	24.17
13′	3.87 m[a]	66.77
CH_2OH	4.22 dd (10.8, 5.5)	63.48
	4.26 dd (10.8, 4.3)	

	Broussonetine H (**2**)	
	Proton	Carbon
2	3.81 m	65.90
3	4.73 t (6.2)	80.57
4	4.96 t (6.2)	80.39
5	3.67 t[a] (6.2)	67.65
1′	4.17 ddd (6.2, 4.8, 4.8)	74.02
2′	2.03 m	35.27
3′	1.95[a]	23.10
4′	1.47,[a] 1.68 m	37.22
5′	3.65 m	69.21
6′	1.46,[a] 1.14 m	31.61
7′	1.92, 1.45[a]	19.36
8′	1.63,[a] 1.32[a]	35.88
9′		95.36
10′	1.63,[a] 1.41[a]	36.27
11′	1.92,[a] 1.40[a]	19.10
12′	1.47,[a] 1.34[a]	25.82
13′	3.71 ddd (10.7, 10.7, 2.2)	60.22
	3.55 br dd (10.7, 4.3)	
CH_2OH	4.21 dd (10.8, 5.5)	63.50
	4.25 dd (10.8, 4.3)	

δ in pyridine-d_5. ^{1}H-NMR at 500 MHz. ^{13}C-NMR at 125 MHz. ppm (Hz). *a*) Overlapped signals.

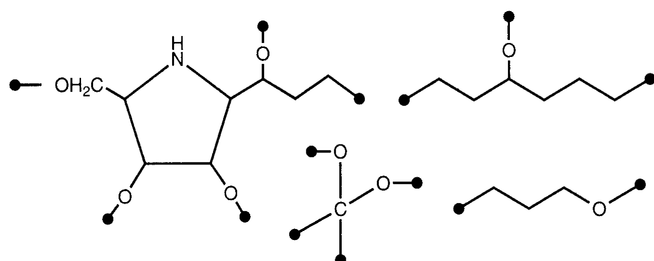

Fig. 2. The Partial Structures of **1**

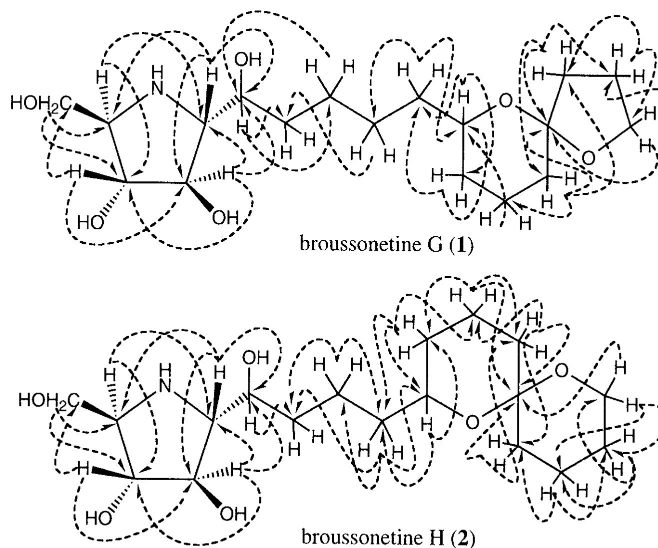

Fig. 3. HMBC of **1** and **2**

dihydroxy-5α-(1-hydroxy-5 : 9;9 : 13-diepoxytridecyl)-pyrrolidine for **2**. Further studies of the absolute stereostructures and conformation of the spiroacetal moieties are now in progress.

The inhibitory activities of **1**, **2** and 1-deoxynojirimycin (DNJ)[3] were assayed with respect to α-glucosidase, β-glucosidase, β-galaclosidase, α-mannosidase and β-mannosidase by the methods described in the Experimental section and the results are summarized in Table 2.

Broussonetines G (**1**) and H (**2**) inhibited β-glucosidase, β-galactosidase and β-mannosidase. Broussonetines E and F,[2] having a hydroxy group on C-1′, inhibited α-glucosidase, but **1** and **2** did not inhibit α-glucosidase, in spite of having the same hydroxy group. These results suggest that the inhibition of α-glucosidase might be attributed to the hydroxy groups on both the C-1′ and C-13′ and the keto groups of C-9′ or C-10′.

Experimental

General The instruments used in this work were: a JASCO digital polarimeter (for specific rotation, measured at 25 °C); a Perkin-Elmer 1720X-FTIR spectrometer (for IR spectra); a Hitachi M-80 spectrometer (for MS spectra); a Varian Mercury 300, unity Inova-500 (for NMR spectra, measured in pyridine-d_5, on the δ scale using tetramethylsilane as an internal standard); a Shimadzu spectrophotometer UV 1200 (for enzyme assay).

Column chromatography was carried out on ion exchange resin (Amberlite CG-50/Orugano Company and Dowex 50W-X4/the Dow Chemical Company), and silica gel (Chromatorex DM1020/Fuji Silysia Chemical Ltd.). HPLC was conducted using a Gilson 305 pump equipped with a JASCO 830-RI detector. Silica gel 60 F_{254} (Merck) precoated TLC plates were used and detection was carried out by spraying with ninhydrin reagent followed by heating.

Isolation of 1 and 2 Dried branches of *Broussonetia kazinoki* (9.5 kg, collected in a mountainous area of Osaka in 1995) were cut finely and then extracted with hot water (40 l × 3) for 2 h. The extracted solution was chromatographed on an Amberlite CG-50 (H^+-form) column (8 l, i.d. 6.5 × 30 cm, repeated 8 times). After washing the column with water and then 50% MeOH, the adsorbed material was eluted with 50% MeOH–28% ammonia solution (9 : 1). The eluted fraction was concentrated in vacuo to give a basic fraction (46.0 g). This fraction was chromatographed on a Dowex 50W-X4 column (200—400 mesh, 500 ml, i.d. 5.0 × 30 cm) pretreated with formic acid-ammonium formate buffer (0.2 M ammonia formate, adjusted to pH 5.7 with 1 N formic acid), with gradient elution (H_2O(2.0 l)→H_2O–28% ammonia solution (9 : 1, 2.0 l)).

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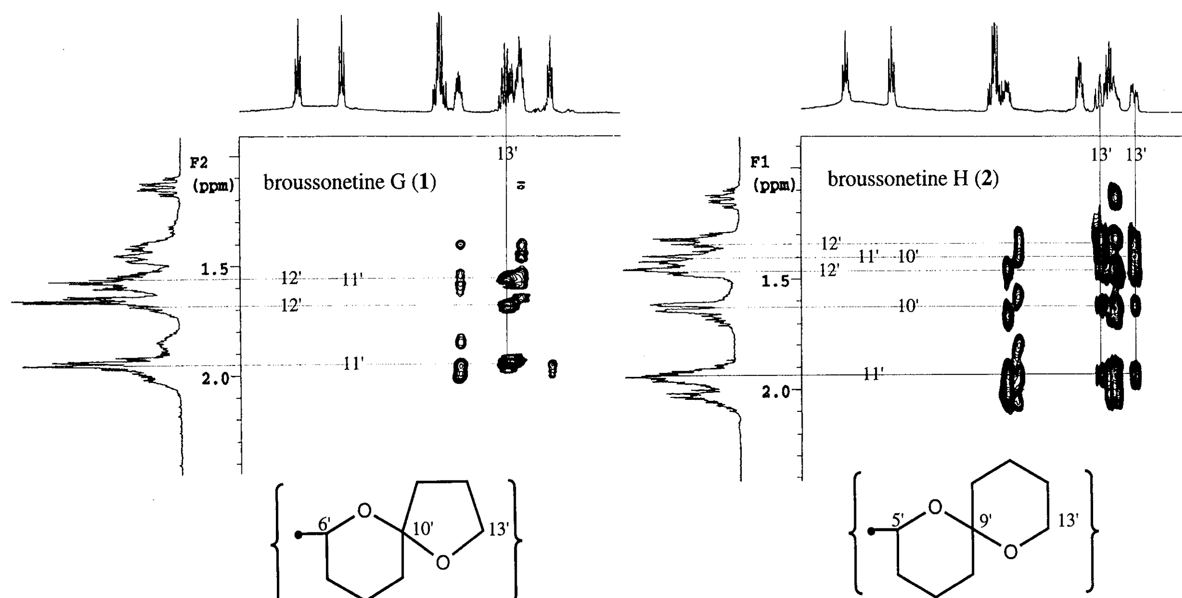

Fig. 4. Partial TOCSY Spectra of **1** and **2**

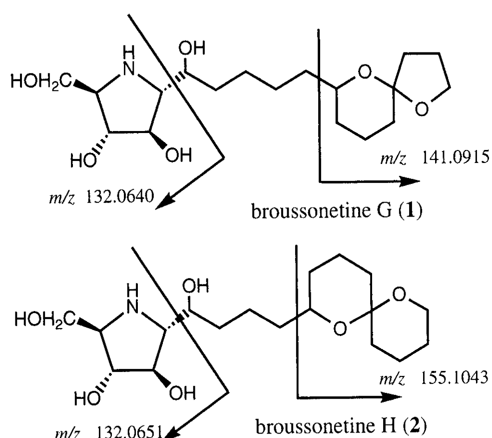

Fig. 5. HR-EI-MS Fragment Ions of **1** and **2**

The fraction containing **1** and **2** was rechromatographed on silica gel (Chromatorex DM1020) using $CHCl_3$ and MeOH, followed by preparative HPLC [column: Asahipak ODP 5E (i.d. 10 × 250 mm); solvent: CH_3CN–H_2O (17 : 83), adjusted to pH 12.0 with ammonia solution; flow rate: 1.5 ml/min; column temperature: ambient]. **1** (70 mg) and **2** (75 mg) were finally obtained.

Broussonetine G (1) Colorless oil, ninhydrin reaction: positive (a yellow spot on TLC), $[\alpha]_D$ +17.5° (MeOH c=0.43), $C_{18}H_{33}NO_6$, pos. HR-SI-MS *m/z*: 360.2393 ($[M+H]^+$) error: 0.9 mmu, pos. EI-MS *m/z* (%): 359 (M^+, 1.8) 328 (23.0) 227 (11.1) 141 (12.1) 132 (100), pos. HR-EI-MS: *m/z* 132.0640 ($C_5H_{10}NO_3$) error −2.0 mmu, *m/z* 141.0915 ($C_8H_{13}O_2$) error 0.0 mmu, IR ν (KBr) cm^{-1}: 3317 (OH, NH), ^1H- and ^{13}C-NMR (pyridine-d_5): Table 1.

Broussonetine H (2) Colorless oil, ninhydrin reaction: positive (a yellow spot on TLC), $[\alpha]_D$ +15.5° (MeOH c=0.50), $C_{18}H_{33}NO_6$, pos. HR-SI-MS *m/z*: 360.2374 ($[M+H]^+$) error: 1.0 mmu, pos. EI-MS *m/z* (%): 359 (M^+, 1.8) 328 (26.8) 227 (17.0) 155 (10.2) 132 (100), pos. HR-EI-MS: *m/z* 132.0651 ($C_5H_{10}NO_3$) error −0.9 mmu, *m/z* 155.1043 ($C_9H_{15}O_2$) error −2.8 mmu, IR ν (KBr) cm^{-1}: 3320 (OH, NH), ^1H- and ^{13}C-NMR (pyridine-d_5): Table 1.

Enzyme Assays Materials α-Glucosidase (from Bakers yeast, lot 83H8000), β-galactosidase (from bovine liver, lot 54H7025), α-mannosidase (from Jack beans, lot 48F95454), and β-mannosidase (from snail acetone powder, lot 45H3826) were obtained from Sigma Chemical Company (St.Louis, U.S.A.), and β-glucosidase (from sweet almond, lot 34552) was obtained from Toyobo Company (Osaka, Japan). *p*-Nitrophenyl-α-D-glucopyranoside, -β-D-glucopyra-noside, -α-D-mannopyranoside, and -β-D-galactopyranoside were obtained Nacalai Tesque, Inc. (Osaka, Japan), *p*-nitrophenyl-β-D-mannopyranoside from Sigma Chemical Company, and DNJ from Funakoshi Company (Tokyo, Japan).

Table 2. Concentration of Inhibitor Required to Produce 50% Inhibition of Enzyme Activity

	Inhibitor (μM)		
	1	**2**	DNJ
α-Glucosidase (from yeast)	NI	NI	0.93
β-Glucosidase (from sweet almond)	0.024	0.036	0.58
β-Galactosidase (from bovine liver)	0.003	0.002	NI
α-Mannosidase (from Jack beans)	NI	NI	NI
β-Mannosidase (from snail acetone powder)	0.76	0.32	NI

NI: up to 100 μM or no inhibition.

Assay of β-Glucosidase Inhibition The reaction mixture consisted of 475 μl 0.1 M acetate buffer (pH 5.0), 250 μl 250 mM *p*-nitrophenyl-β-D-glucopyranoside and 250 μl β-glucosidase solution (a stock solution of 1.0 mg/ml in 50 mM Tris–HCl buffer, pH 7.8 was diluted 200 times with the 10 mM phosphate buffer, pH 7.0, just before assay), with the substrates **1**, **2** or DNJ (25 μl solution, concentration: 20—0.1 mg/ml). After incubation for 20 min at 37 °C, the reaction was interrupted by the addition of 1 ml 0.5 M sodium carbonate, and the amount of *p*-nitrophenol liberated was measured colorimetrically at 400 nm (ODtest). The inhibition rates (%) were calculated from the formula 100 − 100 × (ODtest − ODblank)/(control ODtest − control ODblank) and the IC_{50} values were obtained from the inhibition curves. The IC_{50} values were 0.024 μM for **1** and 0.036 μM for **2**, while that of DNJ was 0.58 μM. Assays for α-glucosidase, β-galactosidase, β-mannosidase and α-mannosidase were carried out as above using *p*-nitrophenyl-α-D-glucopyranoside, -β-D-galactopyranoside, -β-D-mannopyranoside and -α-D-mannopyranoside as substrates. The IC_{50} values are shown in Table 2.

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