Studies on the Constituents of *Broussonetia* Species. I. Two New Pyrrolidine Alkaloids, Broussonetines C and D, as β-Galactosidase and β-Mannosidase Inhibitors from *Broussonetia kazinoki* SIEB.[1)]

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Two new pyrrolidine alkaloids called broussonetines C and D were isolated from the branch of *Broussonetia kazinoki* SIEB. (Moraceae). Broussonetines C and D were formulated as (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-(10-oxo-13-hydroxytridecyl)pyrrolidine (1) and (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-(9-oxo-13-hydroxytridecyl)pyrrolidine (2), respectively, by spectroscopic and chemical methods. 1 and 2 showed an inhibition to β-galactosidase and β-mannosidase.

Key words pyrrolidine alkaloid; β-galactosidase inhibitor; β-mannosidase inhibitor; *Broussonetia kazinoki*; broussonetine C; broussonetine D

Several structurally related monocyclic and bicyclic polyhydroxy pyrrolidines have been shown to be biologically active alkaloids: comparative inhibitors of glycosidases,[2–5)] antiviral agents[6)] and acaricides.[7)] In the course of our survey for biologically active constituents extracted from crude drugs with hot water, we found several pyrrolidine alkaloids to be glycosidase inhibitors from *Broussonetia kazinoki* SIEB. (Moraceae).[1)] Now we report the absolute stereostructures of broussonetines C (**1**) and D (**2**), along with their inhibitory activities to β-galactosidase, β-mannosidase and some other glycosidases.

A deciduous tree, *Broussontia kazinoki* (Japanese name "himekouzo"), is distributed throughout China, Taiwan, Korea and Japan. Its cortex is known to be a raw material for the Japanese paper, "washi." Its branches, leaves and fruits have been used as a diuretic, a tonic and a suppressant for edema in Chinese folk medicine. Constituents of this tree are reportedly isoprenylated flavans, isoprenylated 1,3-diphenylpropanes and an isoprenylated spiro-compound.[8–10)] Bipyridine and quinoline alkaloids have been isolated from *B. zeylanica*.[11,12)]

The branches of this tree were extracted with hot water and alkaloidal constituents were concentrated as described in the Experimental section. Two new alkaloids called broussonetines C (**1**) and D (**2**) were isolated by semi-preparative HPLC.

Broussonetine C (**1**) was obtained as a colorless powder, mp 147—149 °C, $[\alpha]_D$ +25.0°, showing a yellowish spot on TLC when sprayed with ninhydrin reagent and heated on a hot plate (ninhydrin reaction); the molecular formula was determined to be $C_{18}H_{35}NO_5$ by a positive high resolution secondary ion mass spectroscopy (pos. HR-SIMS) ($[M+H]^+$, *m/z*: 346.2579). The IR specrum showed a strong hydroxyl band at 3370 cm^{-1} and a carbonyl band at 1706 cm^{-1}.

The ^1H- and ^{13}C-NMR signals were assigned using ^1H–^1H correlated spectroscopy (^1H–^1H COSY), heteronuclear signal quantum coherence (HSQC), and distortionless enhancement by polarization transfer (DEPT) (Table 1). Partial structure A was proposed by the ^1H–^1H shift correlation among the signals in turn due to an oxymethylene [δ 4.28 (1H, dd, J=11.0, 5.0 Hz), δ 4.22 (1H, dd, J=11.0, 5.0 Hz)], a methine [δ 3.85 (1H, m)] adjacent to a nitrogen atom, an oxymethine [δ 4.72 (1H, t, J=6.4 Hz)], another oxymethine [δ 4.44 (1H, t, J=6.4 Hz)], another methine [δ 3.56 (1H, m)] adjacent to a nitrogen atom and a methylene [δ 1.75 (1H, m), 2.04 (1H, m)].

Partial structure C was derived by a similar ^1H–^1H shift correlation among the signals due to an oxymethylene [δ 3.92 (2H, t, J=7.3 Hz)], a methylene [δ 2.12 (2H, quintet, J=7.3 Hz)] and a methylene [δ 2.71 (2H, t, J=7.3 Hz)] adjacent to a carbonyl group, and by the presence of another methylene [δ 2.44 (2H, t, J=7.3 Hz)] adjacent to a carbonyl group and coupled with one of the methylenes. The presence of the carbonyl group (^{13}C: δ 210.81) flanked by the two methylenes in the partial structure C was found by the heteronuclear multiple bond correlation (HMBC) between the carbonyl carbon and the methylene protons. Partial structure B was proposed by the signals of 7 methylene carbons (δ 24.25, 27.34, 29.54, 29.78(2C), 29.94) and 14 protons (δ 1.15—1.62), and by subtracting the partial structures A and C from the molecular formula.

Linkages among the partial structures (A—C) were determined by HMBC experiment as shown in Fig. 1.

The relative stereostructure of the pyrrolidine ring moiety was disclosed by the vicinal coupling constants ($J_{2,3}=J_{3,4}=J_{4,5}$=6.4 Hz) and nuclear Overhauser effects (NOEs) in the nuclear Overhauser enhancement and exchange spectroscopy (NOESY) experiments as shown in Fig. 2.

The absolute stereostructure was determined to be (2*R*,3*R*,4*R*,5*R*) using a benzoate chirality method as follows. A diacetylacetoamide (**1a**) was prepared from broussonetine C (**1**, 43 μmol) selectively by treatment with acetic anhydride (129 μmol) in pyridine at room temperature, and then a dibenzoate (**1b**) was obtained by benzoylation of **1a** and purification of the products in preparative HPLC. The circular dichroism (CD) curve of **1b** showed a negative Cotton effect ($\Delta\varepsilon_{237}$ −15.9) and a positive effect ($\Delta\varepsilon_{223}$ +16.4) to establish the chiral arrangement in a counter-clockwise manner as depicted in the stereochemical drawing of Fig. 3.[13,14)]

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

	Broussonetine C (**1**)			Broussonetine D (**2**)	
	Proton	Carbon		Proton	Carbon
2	3.85 m	65.27	2	3.81 m	65.27
3	4.72 t (6.4)	80.39	3	4.71 t (8.3)	80.53
4	4.44 t (6.4)	84.41	4	4.42 t (8.3)	84.56
5	3.56 m	62.97	5	3.52 m	62.96
1′	2.04,[a] 1.75[a]	35.70	1′	2.02,[a] 1.75[a]	38.86
2′—7′	1.15—1.62[a]	29.94 29.78	2′—6′	1.15—1.70[a]	29.84 29.75
		29.78 29.54			29.53 27.34
		27.34 30.24			30.23
			7′	1.61[a]	24.19
8′	1.62[a]	24.25	8′	2.39 t (8.3)	42.74
9′	2.44 t (7.3)	42.89	9′		210.63
10′		210.81	10′	2.51 t (8.3)	42.64
11′	2.71 t (7.3)	35.70	11′	1.93[a]	21.03
12′	2.12 quintet (7.3)	27.97	12′	1.78[a]	33.19
13′	3.92 t (7.3)	61.41	13′	3.90 t (8.3)	61.82
CH_2OH	4.28 dd (11.0, 5.0)	61.41	CH_2OH	4.27 dd (11.3, 5.0)	63.98
	4.22 dd (11.0, 5.0)			4.21 dd (11.3, 5.0)	

δ in pyridine d_5. ^{1}H-NMR at 300 MHz. ^{13}C-NMR at 75 MHz. ppm (Hz). *a*) Overlapped signals.

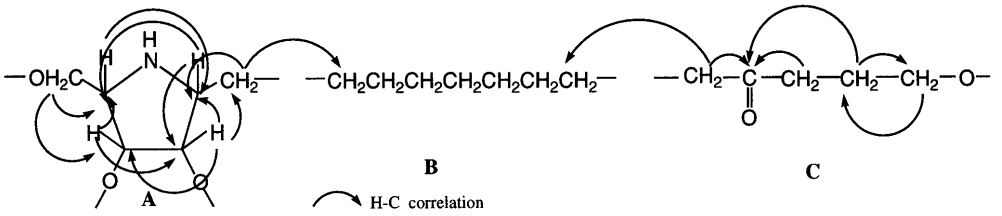


Fig. 1. The Partial Structures (A—C) and HMBC

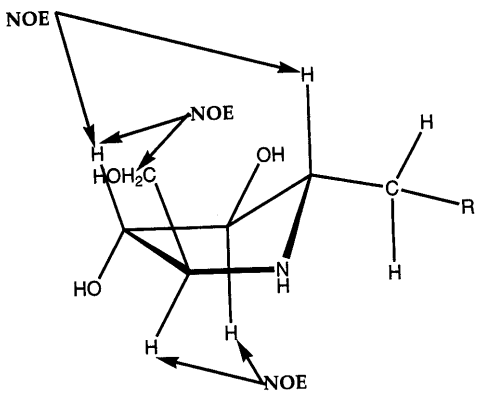


Fig. 2. NOE of Broussonetine C (**1**)

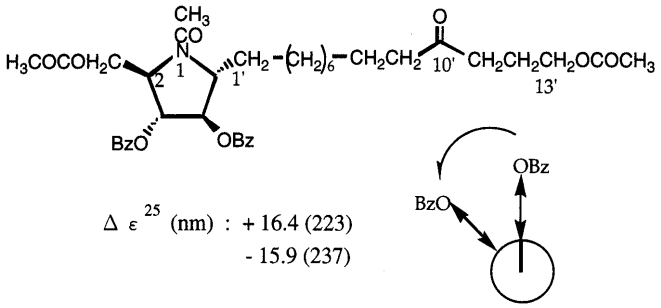


Fig. 3. Absolute Structure of **1b** Clarified by CD Spectrum (in MeOH)

Broussonetine C (**1**) was therefore concluded to be (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-(10-oxo-13-hydroxytridecyl)pyrrolidine as shown in Fig. 5.

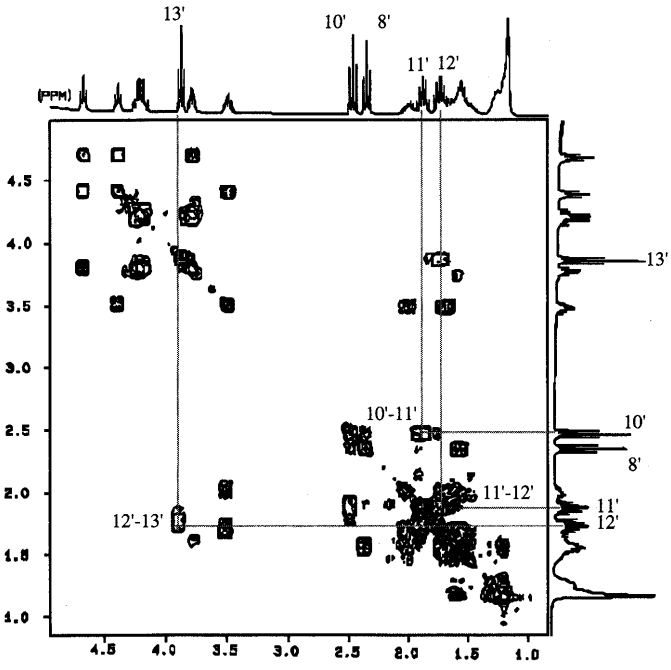
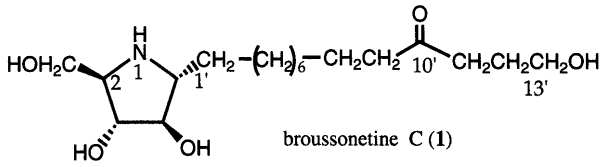
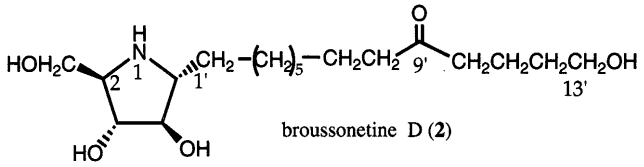


Fig. 4. ^{1}H–^{1}H COSY Spectrum of **2** in Pyridine-d_5

Broussonetine D (**2**) was obtained as a colorless powder, mp 136—138 °C, $[\alpha]_D$ +22.9°, a yellow TLC spot by ninhydrin reaction, and the molecular formula was determined to be $C_{18}H_{35}NO_5$ by pos. HR-SI-MS ($[M+H]^+$, *m/z* 346.2586). The IR spectrum showed strong hydroxyl band at 3405 cm^{-1} and a carbonyl band at 1704 cm^{-1}. The ^{1}H-NMR and ^{13}C-NMR spectra (pyridine-d_5) were



broussonetine C (**1**)



broussonetine D (**2**)

Fig. 5. Structure of **1** and **2**

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)	NI	NI	0.58
β-Galactosidase (from bovine liver)	0.036	0.029	NI
α-Mannosidase (from jack beans)	NI	NI	NI
β-Mannosidase (from snail acetone powder)	0.32	0.34	NI

NI: up to 100 μM or no inhibition.

strikingly similar to those of **1**. These signals were assigned using ^{1}H–^{1}H COSY, HSQC, HMBC and DEPT as shown in Table 1. Especially, the ^{1}H–^{1}H COSY spectrum (Fig. 4) suggested the presence of a partial structure, $COCH_2CH_2CH_2CH_2OH$ instead of $COCH_2CH_2CH_2OH$ in **1**. These results led to the conclusion that **2** was a structural 9-oxo isomer to **1** (10-oxo compound).

The ablolute configuration of the asymmetric carbons, (2*R*,3*R*,4*R*,5*R*), was derived from the value of $[\alpha]_D$ +22.9°, by comparison with those of **1** (+25.0°), of (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-methylpyrrolidine (+26.2°),[5] of (2*R*,3*R*,4*R*,5*R*)-2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (+56.4°),[15] and of (2*S*,3*S*,4*S*)-1,4-dideoxy-1,4-imino-L-arabinitol (−34.6°).[16] Thus, **2** should be formulated as (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-(9-oxo-13-hydroxytridecyl)pyrrolidine as shown in Fig. 5.

The inhibitory activities of **1**, **2** and 1-deoxynojirimycin[17] were assayed toward α-glucosidase, β-glucosidase, β-galactosidase, α-mannosidase and β-mannosidase by the methods described in the experimental section and the results are summarized in Table 2. Broussonetines C (**1**) and D (**2**) were unique in inhibiting β-galactosidase and β-mannosidase, while their congeners isolated from the same tree inhibited some other glycosidases as will be reported in subsequent papers. The inhibition of β-mannosidase with **1** and **2** is similar to a report that (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxy-5-methylpyrrolidine isolated from *Angylocalyx pyraertii* DeWild has been a selective inhibitor of β-mannosidase.[5] It is interesting that the long side chains of **1** and **2** (β-galactosidase and β-mannosidase inhibitors) might modify some biological properties due to (2*R*,3*R*,4*R*,5*R*)-2-hydroxymethyl-3,4-dihydroxypyrrolidine moiety as the active center.

Experimental

General The instruments used in this work were: Yanagimoto micromelting apparatus (for melting points, uncorrected); JASCO digital polarimeter (for specific rotation, measured at 25 °C) and JASCO J-20A spectrometer (for CD, measured at 25 °C); Perkin–Elmer 1720X-FTIR spectrometer (for IR spectra); Hitachi M-80 spectrometer (for MS spectra); Varian Gemini-200, Varian XL-300, General Electric DMX-500 (for NMR spectra, measured in pyridine-d_5, on the δ scale using tetramethylsilane as an internal standard); 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 on a Gilson 305 pump or a JASCO PU 980 equipped with a JASCO 830-RI or 875-UV/VIS as a detector. Silica gel 60 F_{254} (Merck) precoated TLC plates were used and detection was carried out by ninhydrin reagent followed by heating.

Isolation of 1 and 2 Dried branches of *B. 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 each. 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), eluted with gradient elution [H_2O(2.0 l)→H_2O–28% ammonia solution (9 : 1, 2.0 l)]. 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** (65 mg) and **2** (55 mg) were finally obtained.

Broussonetine C (**1**): Colorless powder, ninhydrin reaction: positive (a yellow spot on TLC), mp 147—149 °C, $[\alpha]_D$ +25.0° (c = 0.96, MeOH). $C_{18}H_{36}NO_5$. pos. HR-SI-MS m/z: 346.2579 ($[M+H]^+$), error: −0.5 m mass. IR ν (KBr) cm^{-1}: 3370 (OH, NH), 1706 (C=O). ^{1}H- and ^{13}C-NMR (pyridine-d_5): Table 1.

Broussonetine D (**2**): Colorless powder, mp 136—138 °C, $[\alpha]_D$ +22.9° (c = 0.31, MeOH), ninhydrin reaction: a yellow spot on TLC, $C_{18}H_{36}NO_5$. pos. HR-SI-MS m/z: 346.2586 ($[M+H]^+$), error: −0.5 m mass. IR ν (KBr) cm^{-1}: 3405 (OH, NH), 1704 (C=O). ^{1}H- and ^{13}C-NMR (pyridine-d_5): Table 1.

Broussonetine C Dibenzoate (1b) Compound **1** (43 μmol) was treated with 129 μmol acetic anhydride in pyridine at room temperature, and after usual treatment and HPLC, diacetylacetoamide (**1a**) was obtained as colorless oil (11.4 mg), ^{1}H-NMR (pyridine-d_5): 1.85, 1.95, 1.98 (3H each, s, 3 × $COCH_3$), 4.10 (2H, t, J = 5.7 Hz, 13′-H), 4.50 (2H, m, 2-, 5-H), 4.65—5.00 (4H, m, 3-, 4-H, $C\underline{H}_2OCOCH_3$). Acetoamide **1a** (11.4 mg) was dissolved to pyridine (5.0 ml) and benzoylchloride (6 drops) was added and the solution was stirred at room temperature overnight. After usual treatment, the products were subjected to HPLC [column, CrestPak C18T-5 (i.d. 7.5 × 250 mm); flow rate, 1.5 ml; column temperature, ambient; solvent, CH_3CN–H_2O (75 : 25); detection, 254 nm]. Dibenzoate (**1b**) was obtained as colorless oil (3.3 mg). pos. SI-MS m/z: 680 ($[M+H]^+$, 2%), 207 (base peak). ^{1}H-NMR ($CDCl_3$) δ: 1.95, 2.10, 2.12 (3H each, s, $COCH_3$), 3.95 (2H, t, J = 6.1 Hz, 13′-H), 4.24 (1H, m, 5-H), 4.40 (1H, t, J = 10.8 Hz, 2-$C\underline{H}_2$OH), 4.65 (1H, dd, J = 10.8, 3.2 Hz, 2-$C\underline{H}_2$OH), 4.55 (1H, m, 2-H), 5.45 (1H, d, J = 4.0 Hz, 4-H), 5.56 (1H, d, J = 2.0 Hz, 3-H), 7.40—7.60 (6H, m, phenyl H), 7.95 (4H, m, phenyl H). CD (c = 5.89 × 10^{-5}, MeOH) $\Delta\varepsilon^{25}$ (nm): +16.4 (223), −15.9 (237).

Enzyme Assays Materials α-Glucosidase (from Bakers yeast, lot 83H8000), β-galactosidase (from bovine liver, lot 54H7025), α-mannosidase (from Jack beans, lot 48F95 454), 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 252 40) was obtained from Toyobo Company (Osaka, Japan).

p-Nitrophenyl α-D-glucopyranoside, β-D-glucopyranoside, α-D-mannopyranoside, and β-D-galactopyranoside were obtained from Nacalai Tesque, Inc., (Osaka, Japan), *p*-nitrophenyl β-D-mannopyranoside from Sigma Chemical Company, and 1-deoxynojirimycin (DNJ) from Funakoshi Company (Tokyo, Japan).

Assay on Inhibition of β-Mannosidase The reaction mixture consisted of 475 μl of 0.1 M acetate buffer (pH 5.0), 250 μl of 250 mM *p*-nitrophenyl-β-D-mannopyranoside and 250 μl of β-mannosidase solution (A stock solution of 7.0 units/ml in 3.0 M $(NH_4)_2SO_4$ containing 10 mM sodium acetate, pH 4.0 was diluted 100 times with the same buffer, pH 5.0, just before the assay), with the substrates **1**, **2** or DNJ (25 μl of solutions, concentrations: 20—0.1 mg/ml). After incubation for 20 min at 37 °C and interrupting the reaction by addition of 1 ml of 0.5 M sodium carbonate, the amount of *p*-nitrophenol liberated was measured colorimetrically at 400 nm (OD_{test}). The inhibition rates (%) were calculated from a formula $100-100\times(OD_{test}-OD_{blank})/(\text{control } OD_{test}-\text{control } OD_{blank})$ and IC_{50} values were found from the inhibition curves. The IC_{50} values were 0.32 μM for **1** and 0.34 μM for **2**, while DNJ showed no inhibition. Assays for α-glucosidase, β-glucosidase and, β-galactosidase and α-mannosidase were carried out as above using *p*-nitrophenyl α-D-glucopyranoside, β-D-glucopyranoside, β-D-galactopyranoside, and α-D-mannopyranoside as the substrates. The IC_{50} values are shown in Table 2.

References

1) Shibano M., Kitagawa S., Kusano G., Symposium Papers, 37th Symposium on the Chemistry of Natural Products, Tokushima, 1995, pp. 433—438.
2) Saul R., Chambers J. P., Molyneux R. J., Elbein A. D., *Arch. Biochem. Biophys.*, **221**, 593—597 (1983).
3) Saul R., Molyneux R. J., Elbein A. D., *Arch. Biochem. Biophys.*, **230**, 668—675 (1984).
4) Evans S. V., Fellows L. E., Shing T. K. M., Fleet G. W. J., *Phytochemistry*, **24**, 1953—1955 (1985).
5) Molyneux R. J., Pan Y. T., Tropea J. E., Elbein A. D., Lawyer C. H., *J. Nat. Prod*, **56**, 1356—1364 (1993).
6) *a*) Grusters R. A., Neefjet J. J., Tersmette M., de Goede R. E. Y., Tulp A., Huisman H. G., Miedeman F., Ploegh H. L., *Nature* (London), **330**, 74—77 (1987); *b*) Elbein A. D., *Ann. Rev. Biochem.*, **56**, 497—534 (1987); *c*) Look G. C., Fotsch C. H., Wong C. H., *Acc. Chem. Res.*, **26**, 182—190 (1993).
7) Tatsuta K., Kitagawa M., Symposium Papers, 37th Symposium on the Chemistry of Natural Products, Tokushima, 1995, pp. 97—102.
8) Ikuta J., Hano Y., Nomura T., Kawakami Y., Sato T., *Chem. Pharm. Bull.*, **34**, 1968—1979 (1986).
9) Kato S., Hano Y., Fukai T., Kosuge Y., Nomura T., *Heterocycles*, **24**, 2141—2145 (1986).
10) Kato S., Fukai T., Ikuta J., Nomura T., *Chem. Pharm. Bull.*, **34**, 2448—2455 (1986).
11) Gunatilaka A. A. L., Sultanbawa M. U. S., Surendrakumar S., Somanathan R., *Phytochemistry*, **22**, 2847—2850 (1983).
12) Gunatilaka A. A. L., Surendrakumar S., Thomson R. H., *Phytochemistry*, **23**, 921—931 (1984).
13) Koreeda M., Harada N., Nakanishi K., *Chem. Commun.*, **1969**, 548—549.
14) Harada N., Sato H., Nakanishi K., *Chem. Commun.*, **1970**, 1691—1693.
15) Welter A., Jadot J., Dardenne G., Marlier M., Casimir J., *Phytochemistry*, **15**, 747—749 (1976).
16) Jone D. W. C.,Nash R. J., Bell E. A., Williams J. M., *Tetrahedron Lett.*, **26**, 3125—3126 (1985).
17) Hughes A. B., Rudge A. J., *Nat. Prod. Reps.*, **1994**, 135—162.