Confluentic Acid and 2′-*O*-Methylperlatolic Acid, Monoamine Oxidase B Inhibitors in a Brazilian Plant, *Himatanthus sucuuba*

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Monoamine oxidase B (MAO-B) inhibitors were isolated from the bark of a Brazilian plant, *Himatanthus sucuuba* (SPR.). Assignments of the ^{1}H- and ^{13}C-NMR data using two dimensional (2D)-NMR techniques showed the active components to be known lichen depsides, confluentic acid (1) and 2′-*O*-methylperlatolic acid (2). The depside (1) showed selective inhibition of MAO-B with IC_{50} value of 0.22 μM.

Keywords monoamine oxidase inhibitor; *Himatanthus sucuuba*; depside; confluentic acid; 2′-*O*-methylperlatolic acid; Brazilian plant

In the search for pharmacologically active plant constituents, several species of South American plants were evaluated, and a methanolic extract from a Brazilian plant, called Sucuba, was found to have a selective inhibitory activity towards monoamine oxidase B (MAO-B). Sucuba, the bark of *Himatanthus sucuuba* (SPR.) (Apocynaceae), has been used to treat several diseases in Brazil, for example, gastritis, hemorrhoids, and anemia. An antitumoral iridoidal compound was isolated from this plant,[1] and the decoction of its stem bark showed reproductive toxicity in rats.[2] We now report the isolation, and the structure elucidation of the active MAO-B-inhibiting principles.

Isolation The methanolic extract of Sucuba was extracted with ethyl acetate and *n*-butanol, and the ethyl acetate extract was chromatographed on silica gel as shown in Chart 1. The selective MAO-B-inhibitory activity was concentrated in a fraction eluted with ethyl acetate (fr. 5). Repeated chromatographic purification of this fraction with monitoring of the inhibitory activity has led to the isolation of two depsides, confluentic acid (**1**) and 2′-*O*-methylperlatolic acid (**2**), and an iridoid, β-dihydro-

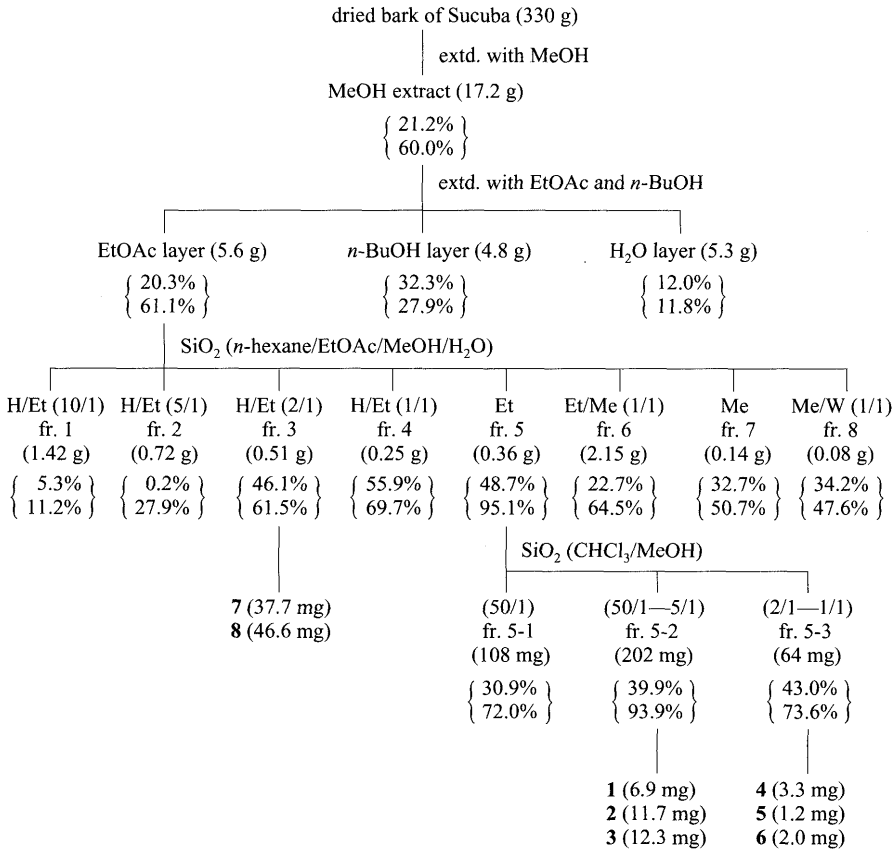


Chart 1. Fractionation of Sucuba Extract

{ }; inhibition % on MAO-A (upper) and MAO-B (lower) at a concentration of 100 μg/ml. Abbreviations: H, *n*-hexane; Et, EtOAc; Me, MeOH, W, H_2O.

plumericinic acid (**3**), together with vanillic acid (**4**), *p*-coumaric acid (**5**), and *p*-hydroxybenzoic acid (**6**). From another fraction eluted with *n*-hexane–ethyl acetate (2 : 1) (fr. 3) two iridoids, plumericin (**7**) and isoplumericin (**8**), were also isolated.

Structures of Isolated Compounds Compound **1**, obtained as colorless needles, mp 156—157 °C, had the molecular formula $C_{28}H_{36}O_8$ as determined by HR-FAB-MS and examination of the ^{13}C-NMR spectrum. The presence of the aromatic ring and carbonyl group was suggested by the UV and IR spectra. The ^{1}H-NMR spectrum presented two pairs of doublet signals (J=2.0, 2.4 Hz, respectively) in the aromatic region due to two *meta*-coupling protons, and a characteristic singlet signal at δ 4.07. The data indicated the presence of two *n*-pentyl units, and suggested that the structure of **1** is similar to that of confluentic acid, a depside isolated from lichen.[3] Although this depside has been known for a long time as a lichen substance,[4] its ^{1}H- and ^{13}C-NMR spectral data have not been reported. So we have assigned the NMR signals unambiguously using various two dimensional (2D)-NMR techniques (Table I, Fig. 1). The identification of **1** as confluentic acid was established unequivocally by the synthesis of this acid according to the procedure of Elix and Ferguson,[5] and comparison of our sample with the authentic sample in respect to physico-chemical and spectral data, as well as HPLC behavior.

Compound **2**, colorless needles, mp 126—127 °C, had the molecular formula $C_{26}H_{34}O_7$ as determined by HR-FAB-MS. The ^{1}H- and ^{13}C-NMR spectra were similar to those of **1**, except for the absence of a methylene signal (δ 4.07) in the ^{1}H-NMR spectrum and a carbonyl signal (δ 207.30) in the ^{13}C-NMR spectrum. The 2D-NMR spectrum of **2** allowed complete assignments of all proton and carbon signals as shown in Table I, and compound **2** was identified as 2′-*O*-methylperlatolic acid, a depside isolated from several lichens.[6] 2′-*O*-Methylperlatolic acid was also synthesized by the method reported by Elix,[7] and the physico-chemical and spectral data as well as HPLC behavior were identical with those of compound **2**. The isolation of these two depsides from a higher plant has not previously been reported, to our knowledge.

Compounds **3**, **7**, and **8** were identified as β-dihydroplumericinic acid, plumericin and isoplumericin, respectively, by comparison of their spectroscopic data with reported values.[8] Plumericin and isoplumericin were the main components of this plant. Vanillic acid (**4**), *p*-

TABLE I. ^{13}C- and ^{1}H-NMR Spectral Data for **1** and **2** in $CDCl_3$[a]

No.	1		2	
	^{13}C[b]	^{1}H	^{13}C[b]	^{1}H
1	104.41 (s)		103.73 (s)	
2	166.59 (s)		166.54 (s)	
3	100.15 (d)	6.46 (d, 2.4)	99.03 (d)	6.39 (d, 3.2)
4	164.93 (s)		164.88 (s)	
5	113.41 (d)	6.30 (d, 2.4)	111.40 (d)	6.39 (d, 3.2)
6	138.99 (s)		148.44 (s)	
7	169.20 (s)[c]		169.86 (s)[e]	
8	51.28 (t)	4.07 (s)	37.32 (t)	2.98 (dd, 7.8, 7.8)
9	207.30 (s)		32.10 (t)[f]	1.67 (dddd, 7.8, 7.8, 7.8, 7.8)
10	42.50 (t)	2.41 (dd, 7.3, 7.3)	32.11 (t)[f]	1.35 (m)
11	23.38 (t)	1.53 (dddd, 7.3, 7.3, 7.3, 7.3)	22.66 (t)[g]	1.35 (m)
12	31.36 (t)	1.20 (m)	14.05 (q)	0.87 (t, 7.3)
13	22.42 (t)[d]	1.20 (m)		
14	13.82 (q)	0.83 (t, 6.8)		
OCH_3	55.53 (q)	3.84 (s)	55.43 (q)	3.84 (s)
OH		11.29 (br s)		11.37 (br s)
1′	120.07 (s)		119.68 (s)	
2′	157.92 (s)		157.95 (s)	
3′	103.18 (d)	6.54 (d, 2.0)	103.13 (d)	6.66 (d, 2.0)
4′	151.48 (s)		151.92 (s)	
5′	115.24 (d)	6.60 (d, 2.0)	115.23 (d)	6.71 (d, 2.0)
6′	144.75 (s)		144.77 (s)	
7′	169.81 (s)[c]		170.15 (s)[e]	
8′	33.84 (t)	2.73 (dd, 7.8, 7.8)	33.88 (t)	2.77 (dd, 7.8, 7.8)
9′	30.73 (t)	1.63 (dddd, 7.8, 7.8, 7.8, 7.8)	30.71 (t)	1.65 (dddd, 7.8, 7.8, 7.8, 7.8)
10′	31.70 (t)	1.34 (m)	31.67 (t)	1.35 (m)
11′	22.39 (t)[d]	1.34 (m)	22.40 (t)[g]	1.35 (m)
12′	13.96 (q)	0.89 (br t, 6.8)	13.95 (q)	0.89 (t, 7.3)
OCH_3	56.40 (q)	3.87 (s)	56.39 (q)	3.90 (s)

a) ^{13}C and ^{1}H chemical shifts were recorded at 100 and 400 MHz, respectively. Multiplicities and coupling constants (Hz) are given in parentheses. *b*) Multiplicities were determined by means of DEPT (distortionless enhancement by polarization transfer) experiments. *c*—*g*) Assignments in each column may be interchanged.

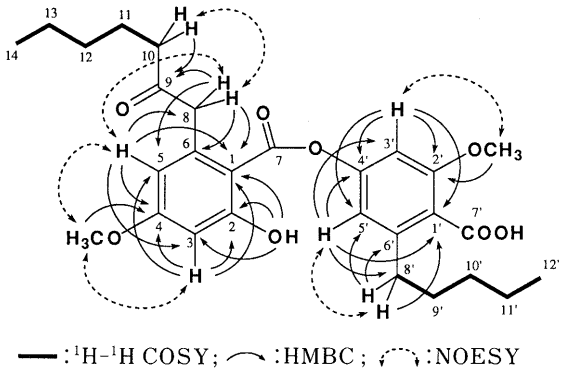
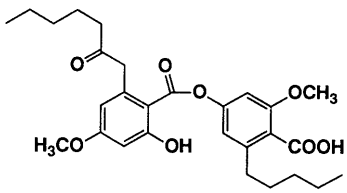


Fig. 1



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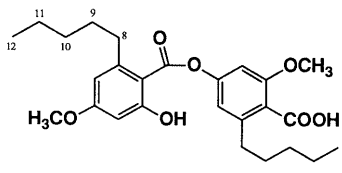

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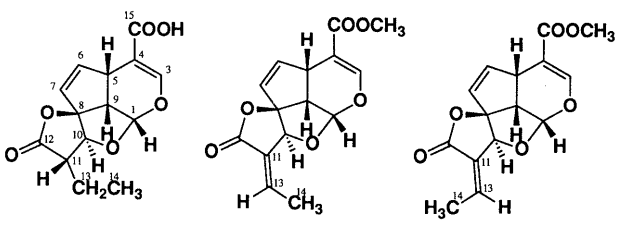

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Fig. 2. Structures of Isolated Compounds

Table II. Inhibitory Effects of Isolated Compounds on MAO-A and MAO-B

Compounds	MAO-A	MAO-B
	10 μM	IC_{50} (μM)[a]
1	6.5%	0.22
2	1.8%	81
3	0	0.4%[b]
4	4.4%	4.7%[b]
5	11.1%	7.4%[b]
6	3.4%	0.9%[b]
7	3.0%	0.4%[b]
8	2.8%	3.8%[b]

a) The IC_{50} value of *l*-deprenyl was 0.025 μM in our study. *b*) Per cent inhibition at 10 μM.

coumaric acid (**5**), and *p*-hydroxybenzoic acid (**6**) were identified by direct comparison with authentic samples and by spectral comparison.

Biological Activity Rat brain MAO-A or MAO-B inhibition assay was performed by the modified method of Yamazaki *et al.*[9] using kynuramine as a substrate. The inhibitory activities of the isolated compounds against MAO-A and MAO-B are shown in Table II. Compound **1** exhibited inhibitory activities of 6.5% and 87.9% on MAO-A and MAO-B at 10 μM, respectively. Among the eight isolated compounds, compound **1** showed the most potent inhibitory effects on MAO-B with an IC_{50} value of 0.22 μM. Though compound **2** was also active against MAO-B, its IC_{50} value (81 μM) was much less than that of compound **1.** This suggested that the presence of the benzyl ketone moiety is essential for potent activity. The other four compounds isolated from the active fraction, **3** to **6**, as well as **7** and **8**, showed no significant effect. It appears that the selective MAO-B inhibitory activity in the original methanol extract of Sucuba was attributable to confluentic acid. Several depsides from lichen and fungi have MAO-B-inhibitory activity,[10] and interestingly, confluentic acid, a depside first isolated from lichen, was found to inhibit MAO-B highly preferentially. MAO catalyzes the oxidative deamination of neurotransmitter monoamines and exogenous bioactive monoamines, and MAO-B-specific inhibitors are expected to be clinically useful for the treatment of Parkinson's disease. More detailed evaluation of the MAO-inhibitory activity and structure–activity relationship of confluentic acid are in progress.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and without correction. UV spectra were recorded on a Hitachi U-3200 spectrophotometer and IR spectra were recorded on a Hitachi 270-30 IR spectrometer. ^{1}H- and ^{13}C-NMR spectra were determined at 400 MHz and 100 MHz, respectively, on a JEOL FX-400 spectrometer with tetramethylsilane as the internal standard. Chemical shift are given in δ (ppm) and multiplicities are indicated as follows: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br=broad. Coupling constants (J) are given in hertz (Hz). MS were recorded using JEOL DX-300 and Shimadzu Concept 1H mass spectrometers. Column chromatographies were performed on Kieselgel 60 (70—230 mesh; Merck), Sephadex LH-20 (25—10 μ; Pharmacia Fine Chemicals), and CIG columns (10 μ; Kusano Scientific Co.). TLC was carried out with precoated Kieselgel 60 F_{254} plates (Merck) and spots were determined under UV irradiation and by heating on a hot plate after spraying dilute sulfuric acid reagent. HPLC was run on a Shimadzu LC-9A pump equipped with a Shimadzu SPD-6A UV detector set at 250 nm. Preparative HPLC was carried out on a Toyo Soda TSK-gel ODS-80$_{TM}$ (2.15 cm i.d. × 30 cm) at the flow rate of 3.0 ml/min.

Isolation The crushed, dried bark of Sucuba (330 g) was extracted five times with methanol at room temperature and the extract was concentrated *in vacuo*. The residue (17.2 g) was dissolved in water and successively extracted with ethyl acetate and *n*-butanol. The ethyl acetate extract (5.6 g) was chromatographed on silica gel with mixtures of *n*-hexane and ethyl acetate of increasing polarity, and divided into eight fractions (fr. 1 to fr. 8). Fraction 5 (0.36 g), which was eluted with ethyl acetate, was subjected to silica gel chromatography with chloroform–methanol (50 : 1 to 1 : 1) and the active fraction was eluted with chloroform–methanol (50 : 1 to 5 : 1, 202 mg). This fraction (fr. 5-2) was chromatographed again on silica gel with chloroform–methanol (20 : 1) and successively purified by HPLC with acetonitrile–water (90 : 10) and on Sephadex LH-20 with chloroform–ethanol (3 : 1) to give **1** (6.9 mg), **2** (11.7 mg), and **3** (12.3 mg). Fraction 5-3 (52 mg) eluted with chloroform–methanol (2 : 1 to 1 : 1) was chromatographed on silica gel with chloroform–methanol (5 : 1) and further purified by preparative TLC with chloroform–methanol (5 : 1) to afford **4** (3.3 mg), **5** (1.2 mg), and **6** (2.0 mg). Fraction 3 (512 mg) eluted with *n*-hexane–ethyl acetate (2 : 1) was subjected repeatedly to silica gel chromatography with chloroform and *n*-hexane–ethyl acetate (4 : 1), and further purified by CIG with chloroform to give **7** (37.7 mg) and **8** (46.6 mg).

Confluentic Acid (1) Colorless needles from methanol, mp 156—157 °C (lit.[3] mp 157 °C).UV λ_{max}^{MeOH} nm (log ε): 217 sh. (4.59), 230 sh. (4.32), 267 (4.27), 305 (3.98). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3150 (OH), 1716, 1708, 1668 (C=O), 1620, 1594 (aromatic). ^{1}H- and ^{13}C-NMR: Table I. EI-MS *m/z* (%): 262 (72), 238 (34), 221 (10), 206 (44), 182 (70), 177 (64), 164 (100). Positive ion FAB-MS *m/z*: 501 $(M+1)^+$, 412, 263. HR-FAB-MS *m/z*: 501.24755 $(M+1)^+$, Calcd for $C_{28}H_{37}O_8$: 501.24884.

2′-*O*-Methylperlatolic Acid (2) Colorless needles from chloroform, mp 126—127 °C (lit.[6] mp 126—127 °C). UV λ_{max}^{MeOH}nm (log ε): 218 sh. (4.60), 268 (4.27), 308 (3.91). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3100 (OH), 1702, 1660 (C=O), 1618, 1596 (aromatic). ^{1}H- and ^{13}C-NMR: Table I. EI-MS *m/z* (%): 238 (49), 221 (28), 182 (100), 177 (64), 138 (59). Positive ion FAB-MS *m/z*: 459 $(M+1)^+$, 412, 221. HR-FAB-MS *m/z*: 459.23778 $(M+1)^+$, Calcd for $C_{26}H_{35}O_7$: 459.23828.

Assay of MAO Activity. Enzyme Preparation The brains of male Wister rats weighing 300 g were homogenized with 10 volumes of ice-cold 0.25 M sucrose. The homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C to remove cell debris. The supernatants were taken and stored at −20 °C until use. The protein content was determined with a Bio-Rad protein assay kit.

Assay Methods MAO-A or MAO-B activity was assayed by a slight modification of the method described by Yamazaki *et al.*[9] Briefly, the reaction mixture (1.0 ml) contained kynuramine (100 μM), sodium phosphate buffer (20 μM, pH 7.4), enzyme preparation (42 μg protein), and *l*-deprenyl (0.1 μM, for MAO-A assay) or clorgyline (0.1 μM, for MAO-B assay), and test sample solution. Test samples were dissolved in distilled water or dimethylsulfoxide (10 μl). The reaction mixture was preincubated at 37 °C for 5 min and the reaction was started by the addition of a substrate, carried out for 30 min at 37 °C, and terminated by the addition of 1 N HCl (500 μl). Then 1 N NaCl was added to the mixture and fluorescence was measured at 380 nm with excitation at 315 nm.

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