Microbial Hydroxylation of (−)-Eburnamonine by *Mucor circinelloides* and *Streptomyces violens*

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(6*R*)-6-Hydroxy-, (6*S*)-6-hydroxy- and (18*S*)-18-hydroxyeburnamonines were obtained by microbial conversion of (−)-eburnamonine using *Mucor circinelloides* and *Streptomyces violens*. Their structures were determined by analyses of the mass, ^{1}H- and ^{13}C-NMR spectra. (−)-Eburnamonine and the three hydroxylated compounds showed cerebral protecting effects against potassium cyanide intoxication in mice.

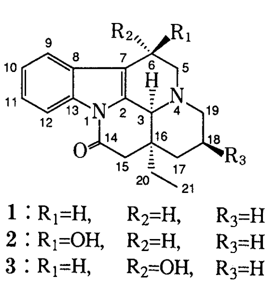
Keywords (−)-eburnamonine; microbial hydroxylation; cerebral protecting effect; *Mucor circinelloides*; *Streptomyces violens*

(−)-Eburnamonine (**1**) is one of the medicinally important indole alkaloids and has been clinically used as a drug for improving cerebral circulation and metabolism.[1] Recently many derivatives of **1** have been synthesized in order to find compounds with superior pharmacological properties. The authors have investigated the microbial conversion of **1** with the aim of preparing new derivatives. We report here the isolation of three hydroxylated derivatives of **1** together with details of their structure determination and cerebral protecting effects.

Several standard strains and the type cultures in our laboratory were tested for converting activity towards (−)-eburnamonine. *Mucor circinelloides* f. *griseo-cyanus* IFO 4563 and *Streptomyces violens* IFO 13486 showed high converting activity, and were used in preparative-scale experiments.

IFO 4563 was cultured in 120 l of media for 24 h, and to which was added 15 g of **1** as the substrate. After 72 h of cultivation, the products were obtained by absorption on Diaion HP-20 and extraction with ethyl acetate. Chromatography on a silica gel column and preparative HPLC on an octadecyl silica (ODS) column afforded 65 mg of (6*R*)-6-hydroxy-(**2**), 1.02 g of (6*S*)-6-hydroxy-(**3**) and 82 mg of (18*S*)-18-hydroxyeburnamonine (**4**). On the other hand, 15 mg of **2** and 17 mg of **3** were obtained from an 1.8 l scale culture of IFO 13486 using 180 mg of **1** after 48 h of cultivation.

The molecular formulae of **2**, **3** and **4** were all determined as $C_{19}H_{22}N_2O_2$ on the basis of elemental analyses, and consideration of the electron impact (EI)-MS and ^{13}C-NMR spectrum, indicating the introduction of one oxygen atom into **1**. The ^{1}H- and ^{13}C-NMR spectra of **2**, **3** and **4** were compared with those of **1**. The chemical shifts of protons and carbons of **1**, **2**, **3** and **4** are listed in Tables I and II.



1 : R$_1$=H, R$_2$=H, R$_3$=H
2 : R$_1$=OH, R$_2$=H, R$_3$=H
3 : R$_1$=H, R$_2$=OH, R$_3$=H
4 : R$_1$=H, R$_2$=H R$_3$=OH

Fig. 1. Structures of (−)-Eburnamonine and Hydroxyeburnamonines

Two H-6 signals in **1** appeared at 2.41 and 2.86 ppm, whereas only one H-6 signal was observed at 4.86 and 5.23 ppm in **2** and **3**, respectively. In the ^{13}C-NMR spectrum, signals assigned to C-6 in **2** and **3** appeared at 62.4 and 61.6 ppm, respectively, which were considerably in lower field than that of **1** (16.5 ppm). The downfield shifts of H-6 and C-6 are clearly due to hydroxylation at C-6, and **2** and **3** were elucidated as a pair of epimers of 6-hydroxyeburnamonine. In the ^{1}H- and ^{13}C-NMR spectrum of **4**, the signals of H-18 and C-18 were observed at 3.99 and 64.2 ppm, respectively, which were in lower field than those of **1** (H-18, 1.38 and 1.72 ppm; C-18, 20.6 ppm). The structure of **4** was therefore elucidated as 18-hydroxyeburnamonine.

The stereochemistries at C-6 and C-18 of **2**, **3** and **4** were elucidated from the coupling constants. The absolute configurations of C-3 and C-16 in **1** are the same as those in vincamine.[2] Compound **1**, having the same ring system

Table I. ^{1}H-NMR Chemical Shifts of **1**, **2**, **3** and **4** (400 MHz, δ from TMS in $CDCl_3$)

Position	**1**	**2**	**3**	**4**
3	3.85	3.84	3.79	3.94
5_{aq}	3.16	3.30	2.79	3.32
5_{eq}	3.28	3.36	3.50	3.33
6_{ax}	2.41		5.23	2.54
6_{eq}	2.86	4.86		2.91
9	7.40	7.60	7.69	7.43
10	7.24	7.25	7.32	7.29
11	7.28	7.29	7.35	7.33
12	8.35	8.32	8.37	8.36
15_{ax}	2.55	2.56	2.56	2.63
15_{eq}	2.62	2.63	2.61	2.70
17_{ax}	0.98	1.05	0.95	0.93
17_{eq}	1.47	1.49	1.46	1.85
18_{ax}	1.38	1.32	1.33	3.99
18_{eq}	1.72	1.67	1.66	
19_{ax}	2.35	2.76	2.36	2.21
19_{eq}	2.58	2.79	2.70	2.83
20	1.62	1.61	1.56	1.62
20	2.02	2.00	2.00	1.98
21	0.94	0.87	0.91	0.97

Table II. ^{13}C-NMR Chemical Shifts of **1**, **2**, **3** and **4** (100.4 MHz, δ from TMS in $CDCl_3$)

Position	1	2	3	4
2	132.0	133.0	134.3	130.9
3	57.7	57.8	57.6	57.0
5	50.6	60.6	59.7	50.3
6	16.5	62.4	61.6	16.5
7	112.6	114.8	114.9	112.8
8	130.1	128.9	128.9	129.9
9	118.0	119.2	119.4	118.2
10	123.8	124.6	124.3	124.0
11	124.3	125.1	124.6	124.6
12	116.2	116.7	116.3	116.3
13	134.2	134.9	134.4	134.3
14	167.6	168.1	167.8	166.9
15	44.4	44.6	44.0	43.8
16	38.4	39.0	37.9	39.6
17	27.0	27.6	26.8	36.5
18	20.6	21.4	20.6	64.2
19	44.3	47.5	45.6	52.1
20	28.3	28.9	28.3	29.5
21	7.6	8.0	7.6	7.8

Table III. Cerebral Protecting Effects of (−)-Eburnamonine and Hydroxylated Compounds against Potassium Cyanide Intoxication

Compound	Dose (mg/kg)	*n*	Survival time (s) (mean ± S.E.M.)	% of control
Control	—	60	27.65 ± 0.72	
1	100 (*p.o.*[a])	10	40.28 ± 2.18[c]	162.6
	2 (i.v.[b])	10	38.43 ± 1.79	104.2
	5 (i.v.)	10	48.34 ± 1.79[d]	131.1
	10 (i.v.)	10	71.01 ± 5.92[c]	192.6
2	100 (*p.o.*)	8	26.50 ± 1.06	115.8
	2 (i.v.)	9	35.94 ± 3.02	136.0
	5 (i.v.)	9	44.19 ± 2.00[d]	167.3
	10 (i.v.)	10	55.55 ± 5.40[c]	210.3
3	100 (*p.o.*)	8	31.25 ± 1.12[c]	136.5
	2 (i.v.)	10	33.68 ± 2.26	127.5
	5 (i.v.)	10	42.00 ± 1.82[d]	159.0
	10 (i.v.)	9	54.79 ± 3.34[c]	207.4
4	100 (*p.o.*)	10	39.17 ± 2.09[c]	150.7
	2 (i.v.)	10	39.53 ± 2.08[d]	136.6
	5 (i.v.)	10	44.58 ± 3.08[d]	154.1
	10 (i.v.)	10	79.14 ± 8.13[c]	273.6

a) The test compounds dissolved in 5% gum arabic solution were administered orally to mice 30 min before the injection of potassium cyanide. *b*) The test compounds dissolved in 2.5% ascorbic acid solution were intravenously administered 5 min before the injection of potassium cyanide. Statistical significance of the differences from the control was assessed by using a one-way analysis of variance followed by Dannett's test. *c*) $p<0.01$. *d*) $p<0.05$.

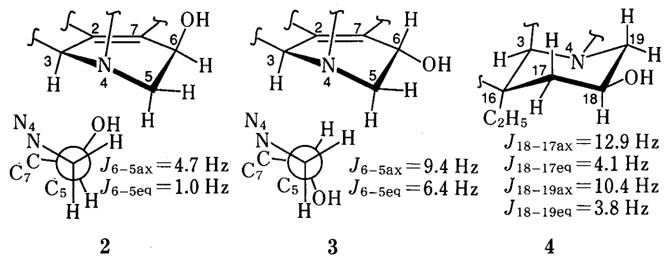


Fig. 2. Stereochemistry of Hydroxyeburnamonines

as vincamine, was expected to have a similar molecular structure to vincamine. The molecular structure of vincamine has been established by X-ray crystallography.[3] Taking into account the torsion angles of vincamine, the partial conformations around C-6 in **2** and **3**, and C-18 in **4** should be as illustrated in Fig. 2. These conformations around C-6 in **2** and **3** were supported by the observation of nuclear Overhauser effect (NOE) enhancement at H-5_{ax} on irradiation of H-3. Judging from the observed coupling constants (Fig. 2), the structures of **2**, **3** and **4** were determined to be (6*R*)-6-hydroxy-, (6*S*)-6-hydroxy- and (18*S*)-18-hydroxyeburnamonine, respectively.

Cerebral protecting effects of **1**, **2**, **3** and **4** against cyanide intoxication were tested in male ICR strain mice (Table III). Histotoxic anoxia was produced by an intravenous injection of potassium cyanide (5 mg/kg). The time between potassium cyanide injection and the last gasp (survival time) was determined. Compounds **1**, **3** and **4** significantly prolonged survival time when administered orally at the dose of 100 mg/kg, but **2** had no effect at the same dose. On the other hand, all the tested compounds prolonged the survival time dose-dependently when administered intravenously.

Discussion

(6*R*)-6-Hydroxyeburnamonine (**2**) was previously reported by Sado *et al.* as an active and a major metabolite of **1** in humans[4] without details of the structure elucidations. We obtained **2** by microbial conversion of **1** and revealed the chemical structure of **2** clearly. In our previous studies on the macrolide antibiotic, a hydroxylated metabolite formed in humans was obtained by microbial conversion using *Mucor circinelloides*.[5] Microbial conversion represents an useful method for preparing the metabolites of medicinal substances.

(−)-Eburnamonine (**1**) and two hydroxylated compounds (**3** and **4**) showed a cerebral protecting effect against cyanide intoxication when administered orally and intravenously. Compound **2**, which was active only when administered intravenously, seems to be less absorbed than **1**, **3** and **4**. Inhibition of energy demand may be effective for preventing cyanide intoxication,[6,7] and the cerebral energy demand in complete ischemia is reduced by RU 24722, a compound structurally related to **1**.[8] The cerebral protecting effects of **1**, **2**, **3** and **4** may be due to the reduction of cerebral energy demand.

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-GX400 spectrometer (^{1}H 400 MHz, ^{13}C 100.4 MHz). The ^{1}H- and ^{13}C-NMR signals were assigned with aid of the ^{1}H–^{1}H and ^{1}H–^{13}C 2D COSY NMR spectra. Melting points were measured using a Yanaco micromelting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on E. Merck Silica gel 60 F_{254} plates using $CHCl_3$–MeOH–concentrated NH_4OH (20 : 1 : 0.1, v/v) as a developing solvent.

Hydroxylation of 1 by *Mucor circinelloides* *Mucor circinelloides* f. *griseo-cyanus* IFO 4563 was cultured for 24 h at 28 °C with agitation (400 rpm) and aeration (120 l/min) in 120 l of medium (pH 7.0) containing glucose (3%), molasses (0.5%), whole yeast (0.5%), peptone (1%) and $CaCO_3$ (0.1%). Compound **1** (15 g) in EtOH (2.4 l) was added to the above solution, and cultivation was continued for 72 h. The culture broth was separated to obtain the mycelium and the filtrate. The mycelium was extracted with acetone (10 l × 2), and the extract was concentrated. The resulting concentrate and the above filtrate were combined and applied to a column of Diaion HP-20 (10 l, Mitsubishi Chemical Ind. Ltd.). After washing of the column with 50% MeOH–H_2O, the products were eluted with MeOH (30 l). After evaporation of the MeOH, the residue was diluted with 750 ml of water and extracted with AcOEt (1 l × 2). The organic layer was washed with saturated NaCl, dried ($MgSO_4$) and evaporated. The crude residue was chromatographed on a column of silica gel (Wako gel C200) with $CHCl_3$–MeOH (40 : 1, v/v) as an eluent. The products were further purified by preparative HPLC (column, YMC-Pack, ODS-5 (20 mm i.d. × 28 cm, Yamamoto Chem. Co. Ltd.); eluent, MeOH–H_2O–monoethanolamine (50 : 50 : 0.01, v/v)) to give 65 mg of **2** ($Rf = 0.20$, mp 158—159 °C (crystallized from diethyl ether), $[\alpha]_D^{30}$ $-67.0°$ ($c = 0.25$, EtOH); UV λ_{max}^{EtOH} nm (ε): 240 (18000), 263 (9300), 291 (4460), 299 (4330); IR (KBr): 3430, 1715 cm^{-1}; MS *m/z*: 310 (M^+)), 1.02 g of **3** ($Rf = 0.14$, mp 103—104 °C (crystallized from diethyl ether), $[\alpha]_D^{30}$ $-113.4°$ ($c = 0.25$, EtOH); UV λ_{max}^{EtOH} nm (ε): 240 (17300), 264 (8300), 293 (4660), 299 (4620); IR (KBr): 3401, 1713 cm^{-1}; MS *m/z*: 310 (M^+)), and 82 mg of **4** ($Rf = 0.16$, mp 217—218 °C (glass), $[\alpha]_D^{30}$ $-102.5°$ ($c = 0.25$, EtOH); UV λ_{max}^{EtOH} nm (ε): 241 (16100), 265 (7980), 293 (3770), 301 (3730); IR (KBr): 3425, 1707 cm^{-1}; MS *m/z*: 310 (M^+)), respectively.

Hydroxylation of 1 by *Streptomyces violens* *Streptomyces violens* IFO 13486 was cultured for 72 h at 28 °C with agitation (220 rpm) in 1.8 l of medium (pH 7.0) containing glucose (1.5%), soybean meal (1.5%), corn steep liquor (0.5%), NaCl (0.5%) and $CaCO_3$ (0.2%). Compound **1** (180 mg) in EtOH (36 ml) was added to the above solution, and the cultivation was continued for 48 h. The culture broth was adjusted to pH 7.0, and extracted with AcOEt (1.8 l × 2). The organic layer was washed with saturated NaCl, dried ($MgSO_4$) and evaporated. The crude residue was chromatographed on a column of silica gel (Silica gel 60, E. Merck) with $CHCl_3$–MeOH (30 : 1, v/v) to give 15 mg of **2** and 17 mg of **3**.

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