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Benzastatins E, F, and G: New Indoline Alkaloids with Neuronal Cell Protecting Activity from *Streptomyces nitrosporeus*

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Abstract : Three new indoline alkaloids, benzastatins E (**5**), F (**6**), and G (**7**), were isolated from the culture broth of *Streptomyces nitrosporeus* 30643 as neuronal cell protecting substances. Their structures were elucidated on the basis of spectral data. **5**, **6**, and **7** suppressed glutamate toxicity in N18-RE-105 cells with EC_{50} values of 1.7, 3.6, and 12.2 μM, respectively. © 1997 Elsevier Science Ltd.

INTRODUCTION

L-Glutamate, a major neurotransmitter in the central nervous system, is extensively released during brain ischemia and induces subsequent neuronal cell death.[1,2] Recent studies indicate that oxygen radicals are produced through a variety of intracellular cascades in such events.[2] It was also reported that blockage of glutamate toxicity by free radical scavengers was effective to ameliorate brain ischemia injury.[3,4] Recently,

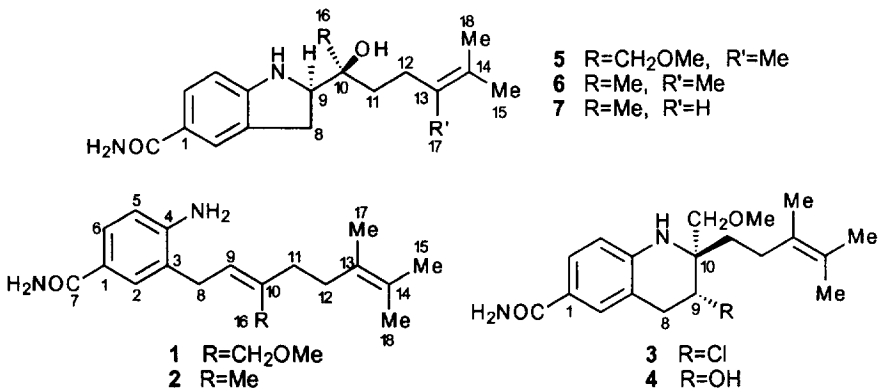

Fig. 1. Structures of benzastatins

some glutamate toxicity inhibitors of microbial origin such as carquinostatin A[5], lavanduquinocin[6], and aestivophoenins A and B[7] have been reported. In the course of our screening for free radical scavengers or inhibitors of glutamate toxicity using the neuronal hybridoma N18-RE-105 cells to prevent the brain ischemia injury, we previously isolated benzastatins A (**1**), B (**2**), C (**3**), D (**4**)[8,9] and phenazostatins A and B[10]. Further studies on metabolites of *Streptomyces nitrosporeus* 30643 which is the producer of benzastatins A-D have been in isolation of three new indoline alkaloids, benzastatins E (**5**), F(**6**), and G(**7**) (Fig. 1). We report here the isolation, structural determination and biological activity of **5**, **6**, and **7**.

RESULTS AND DISCUSSION

The molecular formula of **5** was determined to be $C_{19}H_{28}N_2O_3$ by high resolution EIMS [M^+, *m/z* 332.2105 (+0.5 mmu error)]. A ^{13}C NMR signal at 169.4 ppm and IR absorption at 1646 cm^{-1} suggested the presence of an amide group. A 1,2,4-trisubstituted benzene ring with the amide at C-1 was revealed evidently by the ^1H NMR and ^{13}C NMR spectral data (Tables 1 and 2) together with HMBC data as shown in Fig. 2. The amide and amine groups were identified by PFG ^1H-^{15}N HMQC experiments[11] at -15°C; the amide protons at 6.03 and 5.73 ppm, and the secondary amine proton at 4.77 ppm were correlated to the nitrogens at 72.0 and 55.0 ppm, respectively.[12] One remaining exchangeable proton at 2.73 ppm was assigned to a hydroxyl proton. The ^{13}C NMR (Table 2) spectrum of **5** showed some similarities to that of **4** except C-9 and C-10 carbons (**5**: 66.3 and 72.9 ppm, **4**: 67.4 and 57.5 ppm). In addition to the ^{13}C chemical shift change, typical difference was observed in vicinal coupling constants between H_2-8 and H-9 in the ^1H NMR spectrum. In the case of **5**, the vicinal coupling constants of H-9 at 4.15 ppm (J = 10.1 and 8.8 Hz) were larger than those of **4** (J = 4.4 and 5.9 Hz). From these NMR data, the presence of a five membered ring corresponding to an indoline structure was speculated. In the HMBC spectrum, all correlations were consistent with the proposed indoline structure shown in Fig. 2, but there was no correlation between H-9 and C-4 in the experiments under some conditions (duration for long range coupling $1/2^nJ$ = 60 and 250 msec). The indoline structure was determined by analyses of EIMS fragmentations. In EIMS and high resolution EIMS, the base peak at *m/z* 161.0702 ($C_9H_9N_2O$) was assigned to the fragment caused by cleavage at the C-9 and C-10 bond. Thus the planar structure of **5** was elucidated. The relative stereochemistry of **5** was examined by selective ROESY experiments[13] (Fig. 3). ROEs were observed between H-9 methine proton and both methylene protons of H_b-11 and H_2-16, and between H_2-8 and H_2-11 while no ROE was observed

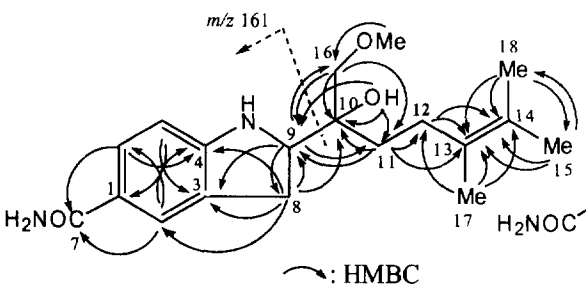

Fig. 2. ^{13}C NMR and HMBC data of **5**.

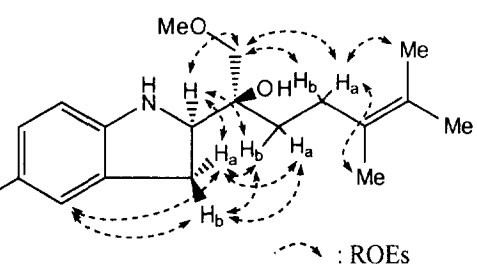

Fig. 3. ROE data of **5**.

Table 1. ^{1}H NMR data for benzastatins D (**4**), E (**5**), F (**6**), and G (**7**) in $CDCl_3$.

Proton	**4**[a] (300MHz)	**5**[b] (600MHz)	**6**[b] (600MHz)	**7**[b] (600MHz)
NH	4.35 (1H, br)	4.67 (1H, br)	4.30 (1H, br)	4.31 (1H, br)
2	7.54 (1H, d,1.6)	7.56 (1H, br. s)	7.56 (1H, br. s)	7.55 (1H, br. s)
5	6.51 (1H, d, 8.4)	6.57 (1H, d, 7.8)	6.60 (1H, d. 8.3)	6.59 (1H, d, 7.8)
6	7.46 (1H, d, 8.4, 1.6)	7.50 (1H, br. d, 7.8)	7.51 (1H, br. d, 8.3)	7.51 (1H, br. d, 7.8)
8	H_a 3.10 (1H, dd, 16.8, 4.4)	3.07 (1H, dd, 15.6, 10.1)	3.08 (1H, dd, 16.1, 9.8)	3.04 (1H, dd, 16.1, 9.8)
	H_b2.84 (1H, dd, 16.8, 5.9)	2.97 (1H, dd, 15.6, 8.8)	3.03 (1H, dd, 16.1, 9.3)	3.02 (1H, dd, 16.1, 9.8)
9	3.97 (1H, m)	4.15 (1H, dd, 10.1, 8.8)	3.98 (1H, dd, 9.8, 9.3)	3.96 (1H, dd, 9.8, 9.8)
11	H_a 1.78 (1H, m)	1.57 (1H, ddd, 14.2, 12.7, 4.9)	1.60 (1H, ddd, 13.2, 12.2, 4.9)	1.60 (1H, ddd, 14.2, 11.2, 5.4)
	H_b 1.54 (1H, m)	1.48 (1H, ddd, 14.2, 12.7, 5.4)	1.44 (1H, ddd, 13.2, 12.2, 4.9)	1.43 (1H, ddd, 14.2, 11.2, 5.9)
12	2.05 (2H, m)	2.15 (1H, ddd, 12.7, 12.7, 4.9)	2.15 (1H, ddd, 12.7, 12.2, 4.9)	2.13 (1H, m)
		2.01 (1H, ddd, 12.7, 12.7, 5.4)	2.07 (1H, ddd, 12.7, 12.2, 4.9)	2.07 (1H, m)
13				5.13 (1H, br. t, 7.3)
15	1.61 (3H, s)	1.64 (3H, s)	1.64 (3H, s)	1.69 (3H, s)
16	H_a 3.66 (1H, d, 9.2)	3.52 (1H, d, 9.3)	1.27 (3H, s)	1.25 (3H, s)
	H_b 3.48 (1H, d, 9.2)	3.42 (1H, d, 9.3)		
17	1.61 (3H, s)	1.64 (3H, s)	1.64 (3H, s)	
18	1.62 (3H, s)	1.66 (3H, s)	1.66 (3H, s)	1.63 (3H, s)
$CONH_2$	5.67 (2H, br)	5.70 (1H, br)	5.79 (1H, br)	5.84 (1H, br)
		5.48 (1H, br)	5.51 (1H, br)	5.45 (1H, br)
OCH_3	3.40 (3H, s)	3.42 (3H, s)		
OH	2.68 (1H, d, 8.7)	2.73 (1H, br. s)	2.02 (1H, br. s)	2.03 (1H, br. s)

a and b were measured at 30 and 22 °C, respectively.

Table 2. ^{13}C NMR chemical shifts for benzastatins D (**4**), Ξ (**5**), F (**6**), and G (**7**) in $CDCl_3$.

Carbon		**4** (75MHz)	**5** (150MHz)	**6** (150MHz)	**7** (150MHz)
1	C	121.9	123.1	123.5	123.4
2	CH	130.4	124.1	124.3	124.3
3	C	117.4	128.6	129.3	129.2
4	C	146.0	154.5	154.3	154.4
5	CH	113.6	107.8	108.1	108.1
6	CH	127.0	127.9	127.9	127.9
7	C	169.2	169.4	169.3	169.4
8	CH_2	32.8	30.0	30.0	30.0
9	CH	67.4	66.3	68.2	68.2
10	C	57.5	72.9	72.8	73.0
11	CH_2	33.2	32.8	35.5	37.0
12	CH_2	27.7	28.2	28.5	22.2
13	C	126.9	127.1	127.2	124.1[a]
14	C	124.6	124.5	124.5	132.2
15	CH_3	20.1	20.6	20.6	25.7
16	CH_2	75.0	78.4	24.6[b]	24.6[b]
17	CH_3	18.4	18.3	18.4	
18	CH_3	20.6	20.0	20.0	17.7
OCH_3		59.5	59.6		

a, CH; b, CH_3.
The assignments were aided by DEPT, HMQC, and HMBC experiments.
The measuring temperatures were the same as those in Table 1.

between H_2-8 and H_2-16. These data indicated that the relative stereochemistries of C-9 and C-10 were *R** and *S**, respectively, with anti configuration for H-9 and OH-10. This configuration was also supported by HMBC. In the HMBC spectra, both carbons of C-11 and C-16 had very weak correlaton with H-9 through small 3-bond coupling constant in the experiment using 250 msec duration time, and these data indicated that H-9 and both C-11 and C-16 carbons are in gauche and H-9 and OH is anti configuration. The structure of benzastatin E (**5**), therefore, was determined to be (2*R**)-2-[(1*S**)-1-hydroxy-1-methoxymethyl-4,5-dimethyl 4-hexenyl]-indoline-5-carboxamide as shown in Fig. 1.

On the basis of HREIMS and ^{13}C NMR data, the molecular formula of benzastatin F (**6**) was determined to be $C_{18}H_{26}N_2O_2$. Together with IR and UV spectral data, the ^1H and ^{13}C NMR spectra of **6** were similar to those of **5** (Tables 1 and 2). The difference between **5** and **6** in ^1H and ^{13}C NMR data was that a methyl signal [δ_H 1.27 (3H, s, H_3-16) and δ_C 24.6 (C-16)] appeared in **6** instead of the methoxymethyl signals of **5**. In the HMBC spectrum, the long range couplings were also observed from H_3-16 to C-9, C-10, and C-11. These spectral data indicated that the methoxymethyl unit in **5** was replaced by a methyl in **6**. Futhermore, ROEs between H-9 methine proton and both methyl protons of H_3-16 and the methylene proton of H_b-11, and between H_2-8 and H_2-11 were observed, indicating that the stereochemistry of **6** is the same as

that of **5**. Thus the structure of **6** was determined to be (2*R**)-2-[(1*S**)-1-hydroxy-1,4,5-trimethyl-4-hexenyl]-indoline-5-carboxamide as shown in Fig. 1.

The molecular formula of benzastatin F (**7**) was determined to be $C_{17}H_{24}N_2O_2$ by HREIMS and ^{13}C NMR data. The ^1H and ^{13}C NMR spectra revealed that **7** was demethyl derivative of **6**. Comparison of the ^1H and ^{13}C NMR data together with ^1H-^1H COSY data between **6** and **7** revealed that an olefinic methine [δ_H 5.13 (1H, br.t, J=7.3 Hz, H-13) and δ_C 124.1 (C-13)] coupled with H_2-12, H_3-15, and H_3-18 was newly appeared in **7** instead of an allylic methyl and an sp^2 quaternary carbon of **6**. Long range couplings were observed from the olefinic methine (H-13) to C-12, C-14, C-15, and C-18 in the HMBC spectrum. These spectral data indicate that **7** is a derivative demethylated at C-13 of **6**, this conculsion being supported by the observation of *m/z* 69 peak in **7** instead of *m/z* 83 peak corresponding to the trimethylallyl unit of **6** in EIMS spectrum. The assignments of C-15 and C-18 were made by the NOE observed between H-13 and H_3-15, and between H_2-12 and H_3-18. NOEs observed from H-9 to H_a-8, , H_b-11, and H_3-16 indicated the presence of the same stereochmistry with **6**. Thus the structure of **7** was determined to be (2*R**)-2-[(1*S**)-1-hydroxy-1,5-dimethyl-4-hexenyl]-indoline-5-carboxamide as shown in Fig. 1.

5, **6**, and **7** protected neuronal N18-RE-105 cells from glutamate toxicity in a dose dependant fashion with EC_{50} values of 1.7, 3.6, and 12.2 μM, respectively, as shown in Table 3. Idebenone[14], a known brain protective agent, which was used as a positive control, showed EC_{50} value of 0.7 μM. **5**, **6**, and **7** did not show cytotoxicity at 200 μM while idebenone exhibited a strong cytotoxicity with an IC_{50} value of 4.9 μM in this assay system. In addition, **5**, **6**, and **7** inhibited lipid peroxidation induced by free radicals in rat liver microsomes with EC_{50} values of 4.7, 5.3, and 15.7 μM, respectively. Interestingly, the relative potency of inhibitory activity of **1**-**7** on glutamate toxicity well correlated with that of their lipid peroxidation inhibitory activity in rat liver microsomes (Table 3).

Table 3. Preventive effects (EC_{50}) on glutamate toxicity in N18-RE-105 cells, cytotoxicity (IC_{50}), and free radical scavenging activity (EC_{50}) of benzastatins.

Compounds	Rat liver microsomes	N18-RE-105		
	EC_{50}(μM)	EC_{50}(μM)	IC_{50}(μM)	IC_{50}/EC_{50}
1	37.9	47.6	>100	>2.1
2	16.9	18.4	84.3	4.6
3	3.3	2.0	38.1	19.1
4	4.2	5.4	>200	>37.0
5	4.7	1.7	>200	>117.6
6	5.3	3.6	>200	>55.6
7	15.7	12.2	>200	>16.4
Vitamin E	0.4	3.5	>200	>57.1
Idebenone	4.1	0.7	4.9	7

Biosynthesis of benzastatin E (**5**) together with benzastatin D (**4**) could be speculated as follows; benzastatin A (**1**) is oxygenated at C-9 double bond to form a common possible intermediate epoxide at first, and then the intermediate cyclized by attack of the amino group to the C-9 or C-10 of the trans epoxide to give benzastatin E (**5**) and D(**4**), respectively. Natural products such as benzastatins E-G that contain an indoline skeleton without a ring system at C-8 and C-9 are not common. The only cyclodopa glucoside[15], a biosynthetic intermediate of betanin, which is derived from tyrosine has been reported as far as we know.[16]

EXPERIMENTAL SECTION

General Experimental Procedures.

Optical rotations were measured on a Polartronic polarimeter. Melting points were determined on a MEL-TEMP II capillary melting point apparatus. UV-visible spectra were recorded on a Shimadzu UV-260 spectrometer in MeOH. Infrared spectra were obtained on a Laser Precision Analect RFX-65 FT-IR spectrometer. HREIMS spectra were measured on a JEOL JMS-HX 110/100A spectrometer. NMR spectra were recorded on a JEOL Alpha 600 spectrometer in chloroform-*d*. Chemical shifts are given in ppm using 0.03% tetramethylsilane (TMS) as an internal reference.

Inhibitory Activity against Glutamate Toxicity in N18-RE-105 Cells.

N18-RE-105 cells[17] (mouse neuroblastoma clone N18TG-2 x Fisher rat 18-day embryonic neural retina) were maintained at 37°C in 25 cm^2 tissue culture flasks in 90% DMEM containing HAT (thymidine 0.14 mM, aminopterin 40 μM, hypoxanthine 0.1 mM) and 10% fetal calf serum under a humidified atmosphere of 5% CO_2, 95% air. Cells were plated in 96 well microplates at a density of 20,000 cells per well with 100 μl media. After culturing for 24 hours, the medium was removed and replaced with a medium containing 10 mM L-glutamate and/or drugs. Cytotoxicity was quantified after treatment for 24 hours by the measurement of the cytosolic enzyme, lactate dehydrogenase (LDH), which was released into the culture medium from degenerating cells. LDH activity was measured using commercial kit purchased from Promega. The percentage of cell death was calculated from the following formula: % cell death = A/(A+B)100, in which A and B are LDH activity in the culture media (supernatant) and in the cell lysates, respectively. EC_{50} value is the drug concentration necessary to reduce glutamate-induced cell death by 50%.[18]

Inhibitory Activity against Lipid Peroxidation in Rat Liver Microsomes.

According to the method of Ohkawa *et al.*[19], rat liver microsomes were prepared and suspended in 100 mM Tris-HCl buffer (pH 7.4). Lipid peroxidation was initiated by adding 100 μM $FeSO_4 \cdot H_2O$. After 30 minutes at 37 °C, the reaction was stopped by adding 3 M trichloroacetic acid in 2.5 N HCl. Lipid peroxidation was assessed by measuring thiobarbituric acid reactive products. Percent inhibition was calculated as follows: (1-(T-B)/(C-B)) x 100(%), in which T, C, and B are absorbance values at 530 nm of the

drug treatment, the control (peroxidation without a drug) and the 0 time control (no peroxidation), respectively.

Isolation of 5-7.

The ethyl acetate extract from the broth filtrate (26 L) of *S. nitrosporeus* 30643 was subjected to SiO_2 (Merck Art No. 7734.9025) column chromatography followed by elution with hexane-EtOAc (1:2) containing 0.5% of conc. NH_4OH. The active fractions were pooled and concentrated *in vacuo* to give an oily residue. The residue was applied again to a SiO_2 column and then eluted with hexane-EtOAc (2:3) containing 0.5% of conc. NH_4OH. Active fraction dissolved in MeOH was further purified by reverse phase HPLC column (22.6 x 300 mm, Phenomenex C_{18}, USA) chromatography with a photodiode array detector. The column was eluted with CH_3CN - H_2O (57:43) at a flow rate of 8 ml/min to afford 3 related compounds, benzastatin E (**5**, 3.3 mg) with a retention time of 17 min, benzastatin F (**6**, 3.7 mg) at 15 min, and benzastatin G (**7**, 2.7 mg) at 13 min.

Benzastatin E (**5**). - White powder; $[\alpha]_D^{18}$ = +17 ° (*c* = 0.1, MeOH) ; IR (KBr) : 3413, 1646, 1606, 1380 cm^{-1}; UV λ_{max} nm (ε) in MeOH : 207 (21300), 230 (sh), 303(11000); EIMS (m/z) 332 (5%), 287 (8%, M^+-CH_2OCH_3), 161 (100%), 118 (84%, 161-$CONH_2$), 83 (16%, C_6H_{11}); HREIMS found (M^+) 332.2105 ($C_{19}H_{28}N_2O_3$ requires 332. 2100); ^{1}H-NMR and ^{13}C- NMR see Tables 1 and 2.

Benzastatin F (**6**). - White powder; $[\alpha]_D^{18}$ = +18 ° (*c* = 0.1, MeOH) ; IR (KBr) : 3403, 1644, 1608, 1378 cm^{-1}; UV λ_{max} nm (ε) in MeOH : 206 (19500), 230 (sh), 303(11000); EIMS (m/z) 302 (6%), 161 (68%), 118 (100%, 161-$CONH_2$), 83 (9%, C_6H_{11}); HREIMS found (M^+) 302.1986 ($C_{18}H_{26}N_2O_2$ requires 302. 1994); ^{1}H-NMR and ^{13}C- NMR see Tables 1 and 2.

Benzastatin G (**7**). - White powder; $[\alpha]_D^{18}$ = +23 ° (*c* = 0.1, MeOH) ; IR (KBr) : 3332, 1650, 1606, 1376 cm^{-1}; UV λ_{max} nm (ε) in MeOH : 206 (18000), 230 (sh), 301(8900); EIMS (m/z) 288 (11%), 161 (63%), 118 (100%, 161-$CONH_2$), 69 (13%, C_6H_{11}-CH_3+H); HREIMS found (M^+) 288.1831 ($C_{17}H_{24}N_2O_2$ requires 288.1838); ^{1}H-NMR and ^{13}C- NMR see Tables 1 and 2.

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