Benzastatins H and I, New Benzastatin Derivatives with Neuronal Cell Protecting Activity from *Streptomyces nitrosporeus*

WON-GON KIM, IN-JA RYOO, JI-SEON PARK and ICK-DONG YOO*

Korea Research Institute of Bioscience and Biotechnology, P. O. Box 115, Yusong, Taejon 305-600, Korea

(Received for publication February 13, 2001)

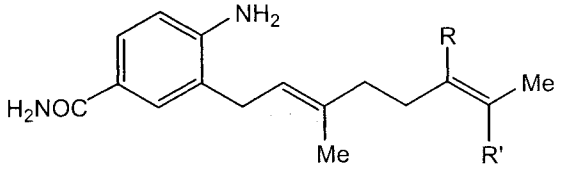
L-Glutamate, a major neurotransmitter in the central nervous system, has been known to be extensively released during brain ischemia and induces subsequent neuronal cell death[1,2]. Recent studies indicate that oxygen radicals are produced through a variety of intracellular cascades in such events[2]. It was also reported that blockage of glutamate toxicity by free radical scavengers was effective to ameliorate brain ischemia injury[3,4]. Recently, 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~G[8~10] and phenazostatins A~C[11~13]. Further investigation on polar metabolites of *Streptomyces nitrosporeus* 30643 which is the producer of benzastatins A~G has resulted in isolation of two hydroxylated derivatives of benzastatin B (**3**), benzastatins H (**1**) and I (**2**) (Fig. 1). We report here the isolation, physico-chemical properties, structure determination, and biological activities of **1** and **2**.

The EtOAc extract from the broth filtrate (26 liters) 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 (1:6) containing 0.5% of conc. NH_4OH. Active fraction dissolved in MeOH was further purified by reverse phase HPLC column (22.6×300 mm, Phenomenex C_{18}, USA) chromatography with a photodiode array detector. The column was eluted with CH_3CN-H_2O (29:71) at a flow rate of 8 ml/minute to afford two structurally related new compounds, benzastatin H (**1**, 1.4 mg) with a retention time of 24 minutes and benzastatin I (**2**, 1.1 mg) at 25 minutes.

The physico-chemical properties of **1** and **2** are summarized in Table 1. They are soluble in methanol and dimethlylsulfoxide, slightly soluble in acetonitrile, and insoluble in water, acetone, ethyl acetate, chloroform, and *n*-hexane. After TLC on silica gel 60 F_{254} (Merck) with chloroform-methanol (10:1) containing 0.5% of conc. NH_4OH, **1** and **2** showed the same Rf value of 0.27 whereas **3** had an Rf value of 0.4. The UV absorption spectra of **1** and **2** showed the same absorption maxima at 206 and 286 nm which was very similar to that of **3**. The IR spectra of **1** and **2** revealed the characteristic absorption bands of an amide carbonyl group (1651 cm^{-1}).

The molecular formula of **1** was determined to be $C_{18}H_{26}N_2O_2$ on the basis of high resolution EI-MS [M^+, *m/z* 302.1997 (+0.3 mmu error)] in combination with ^1H and ^{13}C NMR data. Together with UV and IR spectral data, the ^1H and ^{13}C NMR spectra of **1** were similar to those of **3** (Table 2). Comparison of ^1H and ^{13}C NMR data with HMQC data between **1** and **3** revealed that a methylene signal [δ_H 4.12 (2H, s, H_2-17) and δ_C 61.5 (C-17)] newly appeared instead of the allylic methyl of **3**. The ^1H and ^{13}C chemical shifts of the methylene signal suggest that one of four allylic methyls of **3** was hydroxylated in **1**, which was supported by HREI-MS data showing the molecular formula of **1** with one more oxygen than that of **3**. The position of the hydroxylated methylene was determined by HMBC and NOESY experiments (Fig. 2). Long range couplings were observed from the hydroxylated methylene

Fig. 1. Structures of benzastatins H (**1**), I (**2**), and B (**3**).



1 R=CH_2OH, R'=Me
2 R=Me, R'=CH_2OH
3 R=Me, R'=Me

* Corresponding author: idyoo@mail.kribb.re.kr

Table 1. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	white powder	white powder
EI-MS (*m/z*)	302 $(M)^+$	302 $(M)^+$
HREI-MS (*m/z*)		
found	302.1997	302.1991
calcd.	302.1994	302.1994
Molecular formula	$C_{18}H_{26}N_2O_2$	$C_{18}H_{26}N_2O_2$
UV λ_{max} nm (log ε)	206(4.27), 286 (4.11)	206(4.32), 286 (4.14)
IR (KBr)ν cm^{-1}	3385, 2926, 1651, 1601, 1383	3356, 2925, 1651, 1601, 1383

Table 2. ^1H and ^{13}C NMR spectral data for **1**, **2** and **3**.

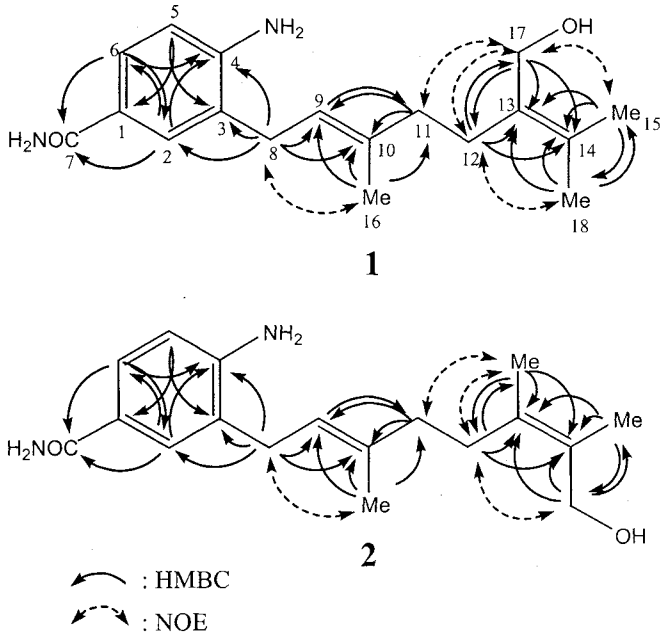
Position	**1** (CD_3OD)		**2** (CD_3OD)		**3** ($CDCl_3$)[9]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		122.5		122.4		122.8
2	7.62 (1H, d, 2.1)[a]	130.0	7.57 (1H, d, 2.0)	129.8	7.56 (1H, d, 1.8)	129.5
3		125.1		125.4		125.1
4		150.5		150.7		148.6
5	6.73 (1H, d, 8.3)	114.6	6.68 (1H, d, 8.3)	114.8	6.65 (1H, d, 8.2)	114.6
6	7.56 (1H, dd, 8.3, 2.1)	127.6	7.52 (1H, dd, 8.3, 2.0)	127.6	7.52 (1H, dd, 8.2, 1.8)	126.9
7		172.5		172.6		169.4
8	3.28 (2H, d, 7.2)	30.6	3.23 (2H, d, 7.2)	30.5	3.25 (2H, d, 6.5)	30.9
9	5.36 (1H, t, 7.2)	121.8	5.31 (1H, t, 7.2)	122.6	5.20 (1H, t, 6.5)	120.7
10		138.4		138.0		138.5
11	2.17 (2H, m)	39.1	2.15 (2H, m)	39.7	2.05 (2H, m)	38.1
12	2.33 (2H, m)	30.2	2.25 (2H, m)	33.5	2.15 (2H, m)	33.4
13		132.7		133.5		127.3
14		130.2		128.8		124.3
15	1.77 (3H, s)	19.9	1.70 (3H, s)	16.2	1.63 (3H, s)	20.6
16	1.84 (3H, s)	16.0	1.77 (3H, s)	15.9	1.77 (3H, s)	16.4
17	4.12 (2H, s)	61.5	1.69 (3H, s)	18.6	1.62 (3H, s)	18.3
18	1.74 (3H, s)	20.5	4.03 (2H, s)	61.8	1.64 (3H, s)	20.1

All spectra of **1** and **2** were recorded at 300 MHz for ^1H and 125 MHz for ^{13}C.
[a]Proton resonance multiplicity and coupling constant (J = Hz) are in parenthesis.
The assignments were aided by NOESY, HMQC, and HMBC.

protons (H_2-17) to one methylene carbon at δ 30.2 (C-12) and two sp^2 quaternary carbons at δ 132.7 (C-13) and δ 130.2 (C-14). In addition, NOEs were observed from the hydroxylated methylene protons (H_2-17) to H_2-11, H_2-12, and H_3-15. These spectral data indicate that the hydroxylated methylene should be attached at C-13. The remaining structure of **1** was also confirmed by the HMBC spectral data as shown in Fig. 2. Thus, **1** was determined to be a derivative hydroxylated at C-17 of **3**.

The molecular formula of **2** was determined to be $C_{18}H_{26}N_2O_2$ on the basis of high resolution EI-MS [M^+, *m/z* 302.1991 (−0.3 mmu error)] in combination with ^1H and ^{13}C NMR data. The molecular formula of **2** was the same as that of **1**. Together with UV and IR spectral data, the ^1H and ^{13}C NMR spectra of **2** were very similar to those of **1** (Table 2). The only difference in ^1H and ^{13}C NMR data with

Fig. 2. HMBC and NOE data of **1** and **2**.



1

2

HMQC data was that ^{1}H and ^{13}C chemical shifts of three allylic methyls in **2** were a little bit different from those of **1**, suggesting that another allylic methyl may be hydroxylated in **2**. The position of the hydroxylated methylene was determined by HMBC and NOESY experiments. Long range couplings were observed from the hydroxylated methylene protons (H_2-18) to two sp^2 quaternary carbons at δ 133.5 (C-13) and δ 128.8 (C-14) and one allylic methyl carbon at 16.2 (C-15), not the methylene carbon at 30.2 (C-12). Instead, one allylic methyl at δ 18.6 (C-17) was long range coupled to C-12, C-13, and C-14. In addition, NOEs were observed from H_3-17 to H_2-11 and H_2-12, and from the hydroxylated methylene protons (H_2-18) to H_2-12 and H_3-15. These spectral data indicate that **2** is a derivative hydroxylated at C-18 of **3**. The remaining structure of **2** was also confirmed by the HMBC spectral data in Fig. 2.

The protective effect of **1** and **2** on glutamate toxicity in neuronal N18-RE-105 cells[14,15] was examined. **1** and **2** protected the cells from glutamate toxicity in a dose dependant fashion with EC_{50} values of 30.3 and 21.6 μM, respectively. The inhibition activity of **1** and **2** was similar to that of **3**. Idebenone[16], a known brain protective agent with free radical scavenging activity, which was used as a positive control, showed EC_{50} value of 0.7 μM. **1** and **2** did not show cytotoxicity at 200 μM while idebenone exhibited a strong cytotoxicity with an IC_{50} value of 4.0 μM in this assay system.

References

1) Choi, D. W.: Cerebral hypoxia: some approaches and unanswered questions. J. Neurosci. 10: 2493~2501, 1990
2) Coyle, J. T. & P. Puttfarcken: Oxidative stress, glutamate, and neurodenegerative disorders. Science 262: 689~695, 1993
3) Jacoben E. J.; F. J. Vandoornik, D. A. Ayer, K. L. Belonga, J. M. Braughler, E. D. Hall & D. J. Houser: 2-(Aminomethyl)chromans that inhibit iron-dependent lipid peroxidation and protect against central nervous system trauma and ischemia. J. Med. Chem. 35: 4464~4472, 1992
4) Kinouchi, H.; C. J. Epstein, T. Mizui, E. Carlson, S. F. Chen & P. H. Chan: Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. Proc. Natl. Acad. Sci. USA 88: 11158~11162, 1991
5) Shin-ya, K.; M. Tanaka, K. Furihata, Y. Hayakawa & H. Seto: Structure of carquinostatin A, a new neuronal cell protecting substance produced by *Streptomyces exfoliatus*. Tetrahadron Lett. 34: 4943~4944, 1993
6) Shin-ya, K.; S. Shimizu, T. Kunigami, K. Furihata, Y. Hayakawa & H. Seto: A new neuronal cell protecting substance, lavanduquinocin, produced by *Streptomyces viridochromogenes*. J. Antibiotics 48: 574~578, 1995
7) Shin-ya, K.; S. Shimizu, T. Kunigami, K. Furihata, Y.

HAYAKAWA & H. SETO: Novel neuronal cell protecting substances, aestivophoenins A and B, produced by *Streptomyces purpeofuscus*. J. Antibiotics 48: 1378~1381, 1995

8) KIM, W. G.; J. P. KIM, C. J. KIM, K. H. LEE & I. D. YOO: Benzastatins A, B, C, and D : new free radical scavengers from *Streptomyces nitrosporeus* 30643. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. J. Antibiotics 49: 20~25, 1996

9) KIM, W. G.; J. P. KIM & I. D. YOO: Benzastatins A, B, C, and D: new free radical scavengers from *Streptomyces nitrosporeus* 30643. II. Structure determination. J. Antibiotics 49: 26~30, 1996

10) KIM, W. G.; J. P. KIM, H. KOSHINO, K. SHIN-YA, H. SETO & I. D. YOO: Benzastatins E, F, and G: new indoline alkaloids with neuronal cell protecting activity from *Streptomyces nitrosporeus*. Tetrahedron 52: 4309~4316, 1997

11) YUN, B. S.; I. J. RYOO, W. G. KIM, J. P. KIM, H. KOSHINO, H. SETO & I. D. YOO: Structures of phenazostatins A and B, neuronal cell protecting substatnces of microbial origin. Tetrahedron Lett. 37: 8529~8530, 1996

12) KIM, W. G.; I. J. RYOO, B. S. YUN, K. SHIN-YA, H. SETO & I. D. YOO: New diphenazines with neuronal cell protecting activity, phenazostatins A and B, produced by *Streptomyces* sp. J. Antibiotics 50: 715~721, 1997

13) KIM, W. G.; I. J. RYOO, B. S. YUN, K. SHIN-YA, H. SETO & I. D. YOO: Phenazostatins C, a new diphenazine with neuronal cell protecting activity from *Streptomyces* sp. J. Antibiotics 52: 758~761, 1999

14) MIYAMOTO, M.; T. H. MURPHY, R. L. SCHNAAR & J. T. COYLE: Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. J. Pharmacol. Exp. Ther. 250: 1132~1140, 1989

15) MURPHY, T. H.; M. MIYAMOTO, A. SASTRE, R. L. SCHAAR & J. T. COYLE: Glutamate toxicity in a neuronal cell involves inhibition of cystine transport leading to oxidative stress. Neuron 2: 1547~1558, 1989

16) SEKIMOTO, H.; I. NAKADA, T. NAKANO, N. FUSE, K. HASEDA, K. YASUMOTO, T. SHINAGAWA, T. NAGAI, T. OHKA, S. UCHIYAMA & T. TAKEKOSHI: Efficacy and safety of CV-2619 (idebenone) in multiple cerebral infarction, cerebrovascular dementia and senile dementia. Ther. Res. 2: 957~972, 1985