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Benzastatin J, a New Demethylated Derivative of Benzastatin B Produced by Controlled Fermentation of *Streptomyces nitrosporeus*

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Abstract Feeding experiments of various derivatives of *p*-aminobenzamide to benzastatins-producing *Streptomyces nitrosporeus* were performed to observe whether new biosynthetic analogs of benzastatins were produced. The supplementation of *p*-aminobenzoic acid to the culture medium of *Streptomyces nitrosporeus* led to the production of benzastatin J, a new demethylated derivative of benzastatin B, while production of benzastatins A and B increased and benzastatins C-G were not detected.

Key words: Benzastatin, *p*-aminobenzoic acid, *Streptomyces nitrosporeus*, supplementation, demethylation

A series of *p*-aminobenzamide-incorporated compounds, benzastatins A-I [2-5], produced by *Streptomyces nitrosporeus* 30643 has been isolated as one of our screening for neuronal protecting substances with free radical scavenging activity [6, 9]. Benzastatins A, B, H, and I incorporate *p*-aminobenzamide with a geranyl group. Benzastatins C and D contain a tetrahydroquinoline unit and benzastatins E, F, and G contain an indoline skeleton in the molecule (Fig. 1). Biogenetically, the tetrahydroquinoline and the indoline unit are derived through cyclization by linkage of the NH_2 group of *p*-aminobenzamide moiety to the geranyl chain. *p*-Aminobenzamide and the geranyl group seem to be the biosynthetic precursors of benzastatin B which has been suggested to be the starting material in the biosynthesis of other benzastatins. This hypothesis put forward a possibility of directed biosynthesis study to obtain biosynthetic analogs of benzastatins by feeding *p*-aminobenzamide derivatives to *Streptomyces nitrosporeus*. Among 8 compounds tested for the feeding experiments, only *p*-aminobenzoic acid showed a differential pattern in benzastatins production. By feeding with *p*-aminobenzoic acid, the yield of benzastatins A and B increased, while benzastatin C, D, E, F, and G were not detected. Interestingly, a new demethylated derivative of benzastatin B (**2**), named benzastatin J (**1**), was detected. The production, isolation, physicochemical properties, and determination of structure and biological activity of **1** are reported here.

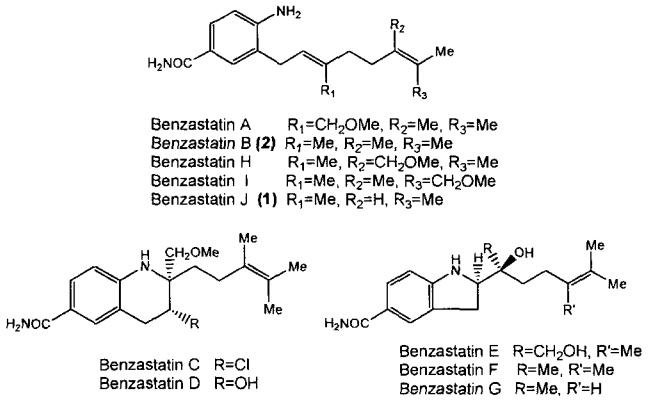

Fig. 1. Structures of benzastatins A-J.

The generation of new analogs of benzastatins was examined by supplementing the culture media with the following *p*-aminobenzamide analogs: *m*-aminobenzoic acid, *p*-aminobenzoic acid, *m*-hydroxybenzoic acid, *m*-aminobenzamide, *p*-hydroxybenzamide, *m*-hydroxybenzaldehyde, *p*-chlorobenzyl alcohol, and *p*-chlorobenzyl amine. Fermentation was carried out in 500-ml Erlenmeyer flasks containing 1% soluble starch, 2% glucose, 2.5% soybean meal, 0.1% beef extract, 0.4% yeast extract, 0.2% NaCl, 0.025% K_2HPO_4, and 0.2% $CaCO_3$ (adjusted to pH 7.2 before sterilization). A piece of *S. nitrosporeus* 30643 from a mature plate culture was inoculated into a 500-ml Erlenmeyer flask containing 80 ml of sterile seed liquid medium with the above composition and cultured on a rotary shaker (150 rpm) at 28°C for 3 days. For the production of benzastatins analogs, 5 ml of

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the seed culture was transferred into 500-ml Erlenmeyer flasks containing 100 ml of the above medium supplemented with 2 mM *p*-aminobenzamide analogs and cultivated for 6 days under the same conditions. The EtOAc extracts from the culture broths were dissolved in MeOH and analyzed by a Cosmosil C_{18} column (4.6×150 mm, 1.5 ml/min, UV at 280 nm) with a photodiode array detector eluted with CH_3CN-H_2O (50:50). The results showed that a new compound, the demethylated derivative (**1**) of benzastatin B, was isolated only when *p*-aminobenzoic acid was supplied. For the large production of **1**, fermentation was performed in 500-ml Erlenmeyer flasks (20 flasks) containing 100 ml of the above medium supplemented with 2 mM *p*-aminobenzoic acid, and cultivated for 6 days under the same conditions. The culture supernatant obtained from the culture broth (2 liter) was partitioned with an equal volume of EtOAc three times following $CHCl_3$, and the $CHCl_3$ layer was concentrated *in vacuo*. The resultant residue was subjected to C_{18} (YMC-gel ODS-A Lot No. 51252) column chromatography followed by stepwise elution with CH_3CN-H_2O (2:8, 4:6, 6:4, 8:2, 1:0). The fractions of CH_3CN-H_2O (6:4) were pooled and concentrated *in vacuo*. The residue dissolved in MeOH was further purified by reverse phase HPLC column (Phenomenex C_{18}, 22.6×300 mm) chromatography. The column was eluted with CH_3CN-H_2O (50:50) at a flow rate of 1.5 ml/min to yield benzastatin A (3.9 mg), **1** (1.9 mg), and **2** (8.3 mg) at retention time of 4.2, 4.5, and 6.1 min, respectively, and appeared as white powder.

The physicochemical properties of **1** are summarized in Table 1. The UV absorption spectrum of **1** showed the absorption maxima at 207 and 282 nm which was the same as those of **2**. The IR spectrum of **1** revealed the characteristic absorption bands of an amide carbonyl group (1,651 cm^{-1}) which was very similar to that of **2**.

The molecular formula of **1** was determined to be $C_{17}H_{24}N_2O$ on the basis of high resolution EI-MS [M^+, *m/z* 272.1886 (+0.2 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 found to be similar to those of **2** (Table 2). The major differences of ^1H and ^{13}C NMR data with HMQC data between **1** and **2** were that an olefinic methine [δ_H 5.01 (1H, t, 7.3) and δ_C 123.9] appeared in **1** instead of an olefinic methyl and an sp^2 quaternary carbon of **2**. This spectral data suggested that one of four olefinic methyls in **2** was demethylated in **1**. The position of the demethylated carbon was determined by ^1H-^1H COSY (Correlated Spectroscopy), HMBC (Heteronuclear Multiple Quantum Coherence Experiment), and NOESY (Nuclear Overhauser and Exchange Spectroscopy) experiments (Fig. 2). The new olefinic proton at δ_H 5.01 (H-13) was coupled to the methylene protons at δ_H 2.11 (H_2-12) in ^1H-^1H COSY spectrum. The multiplicity (triplet) and coupling constant (7.3 Hz) of the new olefinic proton signal (H-13) in the ^1H NMR spectrum suggested that the new olefinic

Table 1. Physicochemical properties of **1**.

Appearance	white powder
EI-MS (*m/z*)	272 $(M)^+$
HREI-MS (*m/z*)	
found	272.1886
calcd.	272.1884
Molecular formula	$C_{17}H_{24}N_2O$
UV λ_{max} nm (log ε)	206 (4.23), 282 (3.98)
IR (KBr) νcm^{-1}	3363, 2925, 1652, 1603, 1380

Table 2. ^1H and ^{13}C NMR spectral data for **1** and **2** in $CDCl_3$.

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1		122.5		122.8
2	7.56 (1H, d, 2.1)[a]	129.3	7.56 (1H, d, 18)	129.5
3		125.0		125.1
4		148.6		148.6
5	6.65 (1H, d, 8.4)	114.1	6.65 (1H, d, 8.2)	114.6
6	7.52 (1H, dd, 8.4, 2.1)	127.0	7.52 (1H, dd, 8.2, 1.8)	126.9
7		169.2		169.4
8	3.27 (2H, d, 6.6)	30.8	3.25 (2H, d, 6.5)	30.9
9	5.20 (1H, t, 6.6)	121.1	5.20 (1H, t, 6.5)	120.7
10		138.0		138.5
11	2.07 (2H, m)	39.5	2.05 (2H, m)	38.1
12	2.11 (2H, m)	26.3	2.15 (2H, m)	33.4
13	5.01 (1H, t, 7.3)	123.9		127.3
14		131.8		124.3
15	1.67 (3H, s)	25.5	1.63 (3H, s)	20.6
16	1.59 (3H, s)	17.4	1.64 (3H, s)	20.1
17	1.75 (3H, s)	15.9	1.77 (3H, s)	16.4
13-Me			1.62 (3H, s)	18.3

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.

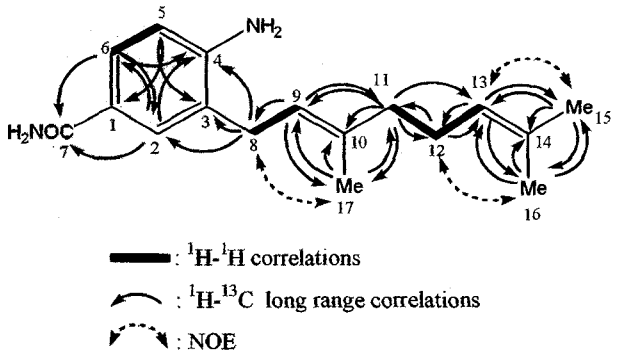

Fig. 2. ^1H-^1H correlations, ^1H-^{13}C long range correlations, and NOE effects of **1**.

The ^1H-^1H correlations, ^1H-^{13}C long range correlations, and NOE effects were measured by ^1H-^1H COSY, HMBC, and NOESY experiments, respectively, which were operated at 600 MHz (^1H) or 125 MHz (^{13}C) in $CDCl_3$.

proton was adjacent to H_2-12. In HMBC spectrum, the methylene protons at δ_H 2.07 (H_2-11) adjacent to H_2-12 were long range coupled to δ 121.1 (C-9), δ 138.0 (C-10), δ 26.3 (C-12), δ 123.9 (C-13), and δ 15.9 (C-17). Also, long range couplings were observed from the olefinic proton at δ_H 5.01 to the methylene carbon at δ 26.3 (C-12) and two olefinic methyl carbons at δ 25.5 (C-15) and δ 17.4 (C-16). In addition, NOEs were observed from the olefinic proton to H_3-15 and from H_2-12 to H_3-16. The above spectral data indicated that the demethylated carbon should be C-13. The remaining structure of **1** was also confirmed by HMBC spectral data, as shown in Fig. 2. Thus, **1** was determined to be a derivative demethylated at C-13 of **2**.

The protective effect of **1** on glutamate toxicity in neuronal N18-RE-105 cells [7] was examined. Analog **1** protected the cells from glutamate toxicity in a dose-dependant fashion with an EC_{50} value of 20.8 μM. The inhibition activity of **1** was similar to that of **2**. Idebenone, a known brain protective agent with free radical scavenging activity and used as a positive control, showed an EC_{50} value of 0.7 μM. Compound **1** 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.

In this study, any substituted analogs of various derivatives of *p*-aminobenzamide were not observed, when *p*-aminobenzoic acid derivatives were supplemented into the culture media of benzastatins-producing *Streptomyces nitrosporeus*. Instead, benzastatin J, the demethylated derivative of benzastatin B, was found when *p*-aminobenzoic acid was supplied in the culture media. Interestingly, the production of benzastatins A and B increased while benzastatins C-G were not detected, compared with those in the control without *p*-aminobenzoic acid.

The effect of aminobenzoic acid compounds on antibiotics biosynthesis during methylation and biosynthesis of phenazine pigments in *Pseudomonas aeruginosa* has been reported [1]. *p*-Aminobenzoic acid has been reported to inhibit the formation of all phenazine antibiotics. Since the phenazine antibiotics have been known to be biosynthesized through the linkage of NH_2 group at one chorismic acid to the other [8], *p*-aminobenzoic acid seems to block the early step of phenazine biosynthesis. On the other hand, *m*-aminobenzoic acid inhibited an N-methylation step of phenazine 1-carboxylic acid to pyocyanine [1] without inhibition of the production of phenazine 1-carboxylic acid, indicating that *m*-aminobenzoic acid did not affect the linkage of NH_2 group of one chorismic acid to the other. Similarily, these results suggest a possibility that *p*-aminobenzoic acid may inhibit the C-methylation of the geranyl chain to produce benzastatin J. Also, no (or decreased) production of benzastatins C-G by *p*-aminobenzoic acid suggests that *p*-aminobenzoic acid inhibits the linkage of the NH_2 group to the geranyl chain, resulting in the inhibition of biosynthesis of 2-ring compounds, benzastatins C-G, from 1-ring compounds, benzastatins A and B. It is interesting that *p*-aminobenzoic acid might inhibit C-methylation and biosynthetic linkage of the NH_2 group in an aromatic moiety to a carbon atom in the other group. In conclusion, *p*-aminobenzoic acid induces demethylation of benzastatin B as well as inhibition of benzastatins E-G production, suggesting that *p*-aminobenzoic acid is a useful agent to alter biosynthetic pathways.

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