# Benzastatins A, B, C, and D : New Free Radical Scavengers from *Streptomyces nitrosporeus* 30643

## I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

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In the course of screening for free radical scavengers, rare metabolites, benzastatins A and B having aminobenzamide skeleton, and benzastatins C and D having tetrahydroquinoline skeleton, were isolated from the culture broth of *Streptomyces nitrosporeus* 30643. They showed inhibitory activity against lipid peroxidation in rat liver microsomes. In the cell assay, benzastatins C and D inhibited glutamate toxicity in N18-RE-105 cells with $EC_{50}$ values of 2.0 and 5.4 $\mu$M, respectively.

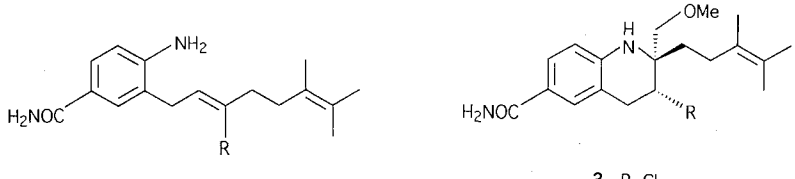
Free radicals have been known to play an important role in the pathogenesis of diseases such as myocardial and cerebral ischemia, atherosclerosis, inflammation, cancer-initiation and aging processes[1~3]. Subsequent peroxidative disintegration by free radicals of cells and organellar membranes has been especially implicated in various pathological processes. Benthophoenin[4], naphterpin[5], and carazostatin[6] *et al.* were reported as substances inhibiting lipid peroxidation. Using the neuroblastoma x retina hybrid cell line N18-RE-105[7] and primary cultures of embryonic cortical neurons[8], J. T. COYLE *et al.* defined a mechanism of quisqualate-type glutamate (Glu) toxicity which results from oxidative stress and suggested that blockade of Glu toxicity by free radical scavengers was expected to be effective against brain ischemia injury. Carquinostatin A was recently reported as an inhibitor of Glu toxicity in N18-RE-105 cells[9]. In the course of screening for free radical scavengers of microbial origin, we isolated 4 metabolites inhibiting lipid peroxidation and Glu toxicity in N18-RE-105 cells from the culture broth of *S. nitrosporeus* 30643 (Fig. 1). The two compounds with an aminobenzamide skeleton were named benzastatins A and B. The other two compounds with a tetrahydroquinoline skeleton were named benzastatins C and D. In this paper we report the taxonomy of the producing strain, fermentation, isolation, physicochemical properties and biological activities of benzastatins A, B, C, and D. The structure determination will be described in the following paper[10].

### Materials and Methods

Taxonomy

Strain 30643 was isolated from a soil sample collected in Sokcho-city, Kangwon-do, Korea. The taxonomic studies were carried out as described by the International *Streptomyces* Project (ISP)[11]. For the evaluation of cultural charateristics, the strain was incubated in ISP media (Difco, U.S.A.) for 14~21 days at 28°C. Physiological properties including utilization of carbon

Fig. 1. Structures of benzastatins A (**1**), B (**2**), C (**3**), and D (**4**).




**1** R=$CH_2OMe$
**2** R=$CH_3$
**3** R=Cl
**4** R=OH

sources were examined by the method of PRIDHAM and GOTTLIEB[12]. The cell wall analysis was performed by the methods of BECKER *et al*[13].

Media and Fermentation

A loopful of strain 30643 from a mature slant culture was inoculated into a 500 ml Erlenmeyer flask containing 80 ml of sterile seed medium consisting of soluble starch 1%, glucose 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, $K_2HPO_4$ 0.025%, and $CaCO_3$ 0.2% (adjusted to pH 7.2 before sterilization) and cultured on a rotary shaker (150 rpm) at 28°C for 2 days. For the production of benzastatins, 3 ml of the seed culture was transferred into one-liter Erlenmeyer flasks containing 150 ml of the above medium, and cultivated for 5 days using the same conditions.

Inhibitory Activity against Lipid Peroxidation in Rat Liver Microsomes

Rat liver microsomes were prepared according to the method of YAGI *et al.*[14] with some modifications, and finally suspended in 100 mM Tris-HCl buffer (pH 7.4). Reaction was initiated by the addition of 100 $\mu$M $FeSO_4 \cdot H_2O$. After 30 minutes at 37°C under reciprocal agitation, the reaction was stopped by the addition of 3 M trichloroacetic acid in 2.5 N HCl. Lipid peroxidation was assessed by measuring thiobarbituric acid reactive products (TBAR). Percent inhibition was calculated as follows: $(1-(T-B)/(C-B)) \times 100$ (%), 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.

Inhibitory Activity against Glu Toxicity in N18-RE-105 Cells

The inhibitory activity against Glu toxicity in N18-RE-105 cells was measured according to the method of J. T. COYLE *et al.*[15] with minor modifications. N18-RE-105 cells were maintained at 37°C in 25 $cm^2$ tissue culture flasks in 90% DMEM containing HAT (thymidine 0.14 mM, aminopterin 40 $\mu$M, hypoxanthin 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 $\mu$l media. After culturing for 24 hours, samples containing L-glutamate were added. Cytotoxicity was quantified after treatment for 24 hours by the measurement of the cytosolic enzyme (LDH) which was released into the culture medium from degenerating cells. LDH activity was measured using the commercial kit purchased from Promega. The percentage of cell death was calculated from the following formula: % cell death $= A/(A+B) \times 100$, in which A and B are LDH activity in the culture media (supernatant) and in the cell lysates, respectively.

## Results and Discussion

Taxonomic Studies of the Producing Strain

The strain 30643 was cultured in various ISP media and the characteristics are summarized in Table 1. Substrate mycelia were well developed in all the media. Aerial mycelia were abundantly formed in various media except ISP media 6 and were gray. The spore chains contained more than ten spores per chain and were observed to be *rectiflexibiles* type. Spores were oval in shape with smooth surface, and measured $0.6 \sim 0.7 \times 0.9 \sim 1.0$ $\mu$m in size, as shown in Fig. 2. Sclerotia, sporangia, and zoospores were not observed. The physiological characteristics and the utilization of carbohydrates were observed after cultivation at 28°C for 14 days (Table 2). Melanoid and soluble pigments were not observed. Whole cell hydrolysates of the strain 30643 contained LL-diaminopimelic acid (DAP) and no characteristic sugars, indicating that the strain 30643 could be placed in the type I cell wall group. Microscopic studies and cell wall analysis of strain 30643 indicated that the strain belongs to the genus *Streptomyces*. Compared with the published description[16,17] of various *Streptomyces* species, strain 30643 was determined to belong to

Table 1. Cultural characteristics of strain 30643.

| Medium | Growth | Aerial mycelium | Substrate mycelium | Reverse color | Soluble pigment |
|---|---|---|---|---|---|
| Yeast extract-malt extract agar (ISP medium 2) | Good | Abundant, gray | Black | Dark gray | None |
| Oatmeal agar (ISP medium 3) | Good | Abundant, gray | Yellowish white | Brown gray | None |
| Inorganic salts-starch agar (ISP medium 4) | Good | Abundant, gray | Dark black | Greenish gray | None |
| Glycerol-asparagine agar (ISP medium 5) | Good | Abundant, gray | Black | Greenish gray | None |
| Peptone-yeast extract iron agar (ISP medium 6) | Moderate | Poor, yellowish white | Whitish yellow | Reddish yellow | None |
| Tyrosine agar (ISP medium 7) | Good | Abundant, gray | Black | Grayish black | None |
| Glucose-asparagine agar | Moderate | Moderate, gray | Pale yellow | Pale yellow | None |
| Nutrient agar | Good | Moderate, gray | Pale yellow | Light yellow | None |
| BENNETT agar | Good | Abundant, gray | Yellowish white | Dark gray | None |

Observation after incubation at 27°C for 2 weeks. Color names from Guide to Color Standard, Nihon Shikisai Co., Ltd.

Fig. 2. Scanning electron micrograph of spore chains of strain 30643 on ISP-2 agar incubated at 27°C for 2 weeks (×20,000).

Bar represents 1 μm.

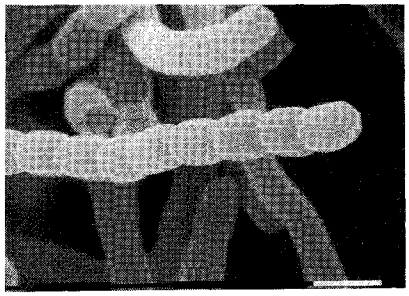


Table 2. Taxonomical characteristics of strain 30643.

| | |
|---|---|
| DAP type | LL |
| Spore chain | *Rectiflexibiles* |
| Spore surface | Smooth |
| Aerial mass color | Gray |
| Soluble pigments | None |
| Melanoid pigments | None |
| Growth temperature (°C) | 15~34 |
| Optimum temperature (°C) | 27~30 |
| NaCl tolerance (%) | 7 |
| Starch hydrolysis | Positive |
| Nitrate reduction | Positive |
| Gelatin liquefaction | Negative |
| Milk peptonization | Positive |
| Hydrolysis of skim milk | Positive |
| Carbon utilization[a] | |
| D-Glucose | + |
| D-Fructose | − |
| Cellulose | + |
| Inositol | − |
| D-Mannitol | − |
| Raffinose | − |
| D-Xylose | + |
| L-Arabinose | + |
| Cellobiose | + |
| D-Galactose | + |
| Inulin | − |
| Melibiose | − |
| L-Rhamnose | + |
| Sucrose | − |

[a] +, Positive utilization; −, no utilization.

*S. nitrosporeus*. Therefore the strain was named *S. nitrosporeus* strain 30643.

## Fermentation

The production of benzastatins was carried out in 1-liter Erlenmeyer flasks as described in the Materials and Methods section; lower yields of benzastatins were produced in jar fermentor culture. A typical time course of fermentation in one-liter Erlenmeyer flask is shown in Fig. 3. The production of benzastatins A, B, and D began at day 2 and the maximum production was observed after 6 days of cultivation and the yields of

Fig. 3. Fermentation profiles for the production of benzastatins A (**1**), B (**2**), C (**3**), and D (**4**).

□ **1**, ■ **2**, ○ **3**, ● **4**, △ PCV (packed cell volume), ▲ pH.

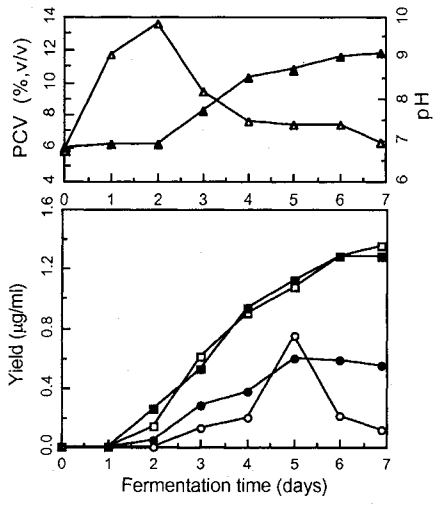
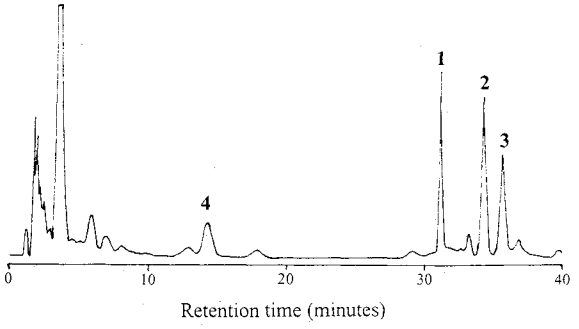



Fig. 4. HPLC chromatogram of a broth supernatant after the cultivation for 5 days.




The $CHCl_3$ extract of a broth supernatant was dissolved in MeOH and analyzed by a Cosmosil $C_{18}$ column (4.6×150 mm, 1.2 ml/minute, UV at 280 nm) eluted with $CH_3CN$-$H_2O$ (37:63 for 20 minutes, then 50:50). **1**=benzastatin A, **2**=benzastatin B, **3**=benzastatin C, **4**=benzastatin D.

benzastatins A, B, and D were 1.36, 1.29, and 0.58 μg/ml, respectively. However, benzastatin C production began at day 3, and reached a maximum (0.75 μg/ml) at about 5 days and decreased beyond 6 days. Fig. 4 shows the HPLC chromatogram for a chloroform extract from the culture supernatant after 5 days of cultivation.

## Isolation

The procedure for isolation of benzastatins A, B, C, and D is shown in Scheme 1. The culture supernatant obtained from the culture broth (28 liters) was extracted with an equal volume of EtOAc three times and the EtOAc layer was concentrated *in vacuo*. The resultant residue was extracted again with one liter of $CHCl_3$-

MeOH-$H_2O$ (1:1:1) and the organic layer was concentrated *in vacuo*. Then the crude extract was subjected to silica gel (Merck art No 7734.9025) column chromatography followed by stepwise elution with hexane-EtOAc (3:1, 3:2, 1:1, 1:2, 1:3) containing 0.5% of conc. $NH_4OH$. The column was eluted with 3 liters of hexane-EtOAc (3:2~1:1) to give the first active fraction (fraction I) and subsequently eluted with 1.25 liters of hexane-EtOAc (1:3) to give the second active fraction (fraction II). The fraction I was concentrated *in vacuo* to give an oily residue, which was redissolved in MeOH. The solution was applied to a reverse phase HPLC column (Phenomenex $C_{18}$ 22.6×300 mm, flow rate 8 ml/minute) with photodiode array detector. Elution with $CH_3CN$-$H_2O$ (65:35) gave **3** related pure compounds, benzastatins A (11 mg), B (33 mg), and C (10.5 mg), as white powders. The fraction II was also concentrated *in vacuo* to give an oily residue. The oily residue was subjected to silica gel column and the column was eluted with hexane-EtOAc (1:2) containing 0.5% of conc. $NH_4OH$. The active fraction, dissolved in MeOH, was further purified on Phenomenex $C_{18}$ HPLC column. Pure benzastatin D (8.1 mg) was obtained as a white powder by elution with $CH_3CN$-$H_2O$ (53:47).

## Physico-chemical Properties

Benzastatins A, B, and C are soluble in chloroform, ethyl acetate, acetone, methanol, and dimethyl sulfoxide, and insoluble in water. Benzastatin D is soluble in methanol, acetone, and dimethyl sulfoxide, slightly soluble in chloroform, and insoluble in water. The physico-chemical properties of benzastatins A, B, C, and D are summarized in Table 3. The mass spectral data indicated that only benzastatin C contains a chlorine atom in the molecule. The IR spectra of all these compounds showed characteristic absorption bands of an amide carbonyl group (1620~1650 $cm^{-1}$).

Scheme 1. Isolation procedure of benzastatins A, B, C and D.

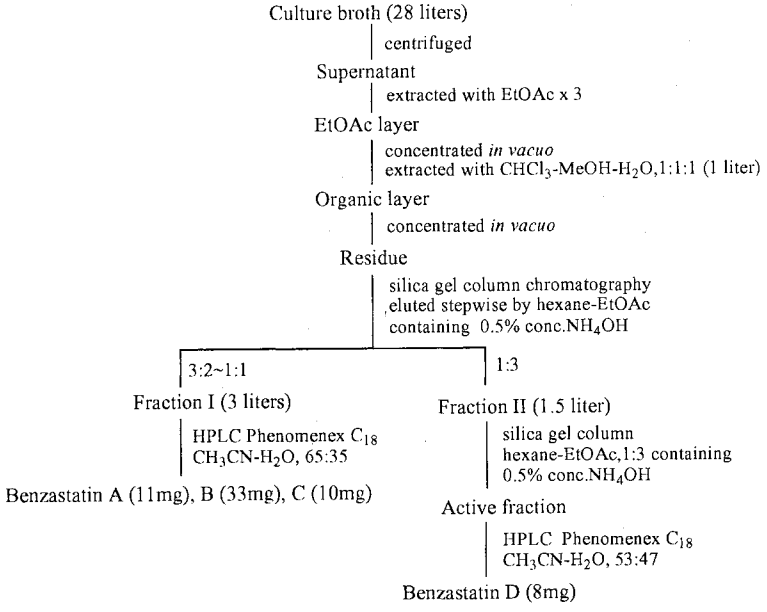



Table 3. Physico-chemical properties of benzastatins A (**1**), B (**2**), C (**3**), and D (**4**).

| | **1** | **2** | **3** | **4** |
|---|---|---|---|---|
| Appearance | White powder | White powder | White powder | White powder |
| MP (°C) | 74 | 98 | 152 | 220 |
| $[\alpha]_D^{18}$ (MeOH) | — | — | −23° (*c* 0.1) | −30° (*c* 0.1) |
| EI-MS (*m/z*) | 316 $(M)^+$ | 286 $(M)^+$ | 350 $(M)^+$ | 332 $(M)^+$ |
| HREI-MS (*m/z*) | | | | |
| Found | 316.2156 | 286.2052 | 350.1746 | 332.2133 |
| Calcd. | 316.2151 | 286.2045 | 350.1761 | 332.2100 |
| Molecular formula | $C_{19}H_{28}N_2O_2$ | $C_{18}H_{26}N_2O$ | $C_{19}H_{27}N_2O_2Cl$ | $C_{19}H_{28}N_2O_3$ |
| UV $\lambda_{max}^{MeOH}$ nm (log ε) | 206 (4.53), 284 (4.11) | 207 (4.51), 282 (4.13) | 206 (4.55), 230 (sh), 301 (4.19) | 208 (4.34), 230 (sh), 305 (4.20) |
| IR (KBr) $\nu$ $cm^{-1}$ | 3359, 1655, 1605, 1383 | 3379, 1639, 1604, 1390 | 3431, 1651, 1612, 1385 | 3301, 1619, 1597, 1367 |

## Biological Activities

### Inhibition of Lipid Peroxidation in Rat Liver Microsomes

Benzastatins A, B, C, and D inhibited lipid peroxidation in rat liver microsomes as shown in Fig. 5.

Fig. 5. Inhibitory activity of benzastatins A (**1**), B (**2**), C (**3**), and D (**4**) against lipid peroxidation in rat liver microsomes.

□ **1**, ■ **2**, ○ **3**, ● **4**, △ Vitamin E, ▲ Idebenone.

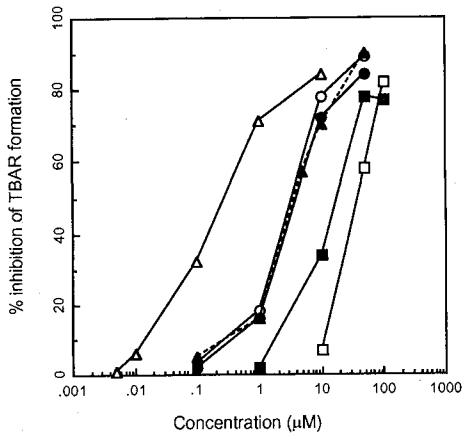



Fig. 6. Effects of benzastatins C (**3**) and D (**4**) on Glu toxicity in N18-RE-105 cells and cell viability.

○ **3**, ● **4**, △ Vitamin E, ▲ Idebenone.

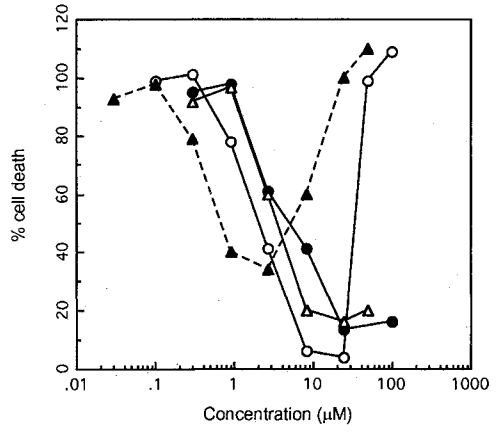



Table 4. Free radical scavenging activity ($EC_{50}$) and cytotoxicity ($IC_{50}$) of benzastatins A (**1**), B (**2**), C (**3**), and D (**4**).

| Compound | Rat liver microsome | N18-RE-105 | |
|---|---|---|---|
| | $EC_{50}$ ($\mu$M) | $EC_{50}$ ($\mu$M) | $IC_{50}$ ($\mu$M) |
| **1** | 37.9 | 47.6 | >100 |
| **2** | 16.9 | 18.4 | 84.3 |
| **3** | 3.3 | 2.0 | 38.1 |
| **4** | 4.2 | 5.4 | >100 |
| Vitamin E | 0.4 | 3.5 | >100 |
| Idebenone | 4.1 | 0.7 | 4.9 |

The inhibition was dose-dependant. Benzastatins C and D each showed about the same activity with $IC_{50}$ values of 3.3 and 4.2 $\mu$M, respectively; they showed stronger activity than benzastatins A (37.9) and B (16.9 $\mu$M) without the piperidine ring in their structures. Although the activity of benzastatins C and D was weaker than vitamin E, it is similar to that of idebenone which is now being used as a brain protective agent[18].

### Inhibition of Glu Toxicity in N18-RE-105 Cells

The free radical scavenging activities of benzastatins A, B, C, and D were evaluated in N18-RE-105 cell assays by analyzing inhibitory activity against Glu toxicity. Fig. 6 shows the effects of benzastatins C and D on Glu toxicity and cell viability. Benzastatins C and D inhibited Glu toxicity in a dose-dependant fashion with $EC_{50}$ values of 2.0 and 5.4 $\mu$M, respectively. The inhibition activity of benzastatins C and D was similar to that of vitamin E. The $EC_{50}$ and $IC_{50}$ values in the cell assay are summarized in Table 4. Benzastatin C showed cytotoxicity with an $IC_{50}$ value of 38.1 $\mu$M, but benzastatin D was inactive even at 100 $\mu$M. Idebenone showed strong cytotoxicity with an $IC_{50}$ value of 4.9 $\mu$M in this assay system.

## Conclusion

In the course of screening for free radical scavengers, we discovered the rare metabolites, benzastatins A, B, C, and D, which were isolated from the culture broth of *S. nitrosporeus* 30643. Benzastatins A and B contain an aminobenzamide unit which is unusual for microbial metabolites. The only metabolite reported to contain an aminobenzamide unit was 2-pyruvoylaminobenzamide isolated from *Penicillium chrysogenum*, *P. notatum*[19] and *Colletotrichum lagenarium*[20]. Benzastatins C and D contain an unique tetrahydroquinoline skeleton which is only found in the antibiotic virantmycin[21]. Benzastatins A, B, C, and D showed weaker inhibitory activity than vitamin E against lipid peroxidation in rat liver microsomes. In the cell culture system using N18-RE-105 cells, however, benzastatins C and D showed comparable activity to that of vitamin E.

### Acknowledgments

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