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

II. Structure Determination

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The structures of benzastatins A, B, C, and D, new free radical scavengers, were determined by spectroscopic studies. Benzastatins A and B incorporate the *para*-aminobenzamide unit which is rare in fungal metabolites. Benzastatins C and D are unique alkaloids related to virantmycin; they contain the tetrahydroquinoline unit in the molecules.

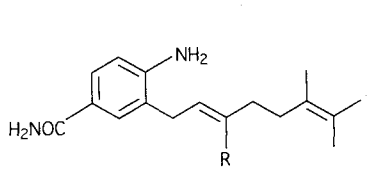
Benzastatins A (**1**), B (**2**), C (**3**), and D (**4**) were isolated as new free radical scavenging substances from the culture broth of *Streptomyces nitrosporeus* 30643. These compounds showed inhibitory activity against glutamate toxicity in N18-RE-105 cells and against lipid peroxidation in rat liver microsomes. In the proceeding paper we described the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological activities of these compounds[1]. In what follows, we present the eludication of the structures of **1**, **2**, **3**, and **4**.

Results

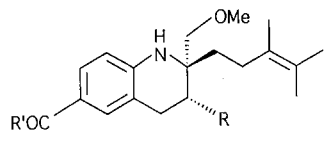
Structure of Benzastatin B (2)

On the basis of HREI-MS and ^{13}C NMR data, the molecular formula of **2** was determined to be $C_{18}H_{26}N_2O$. A close inspection of the ^1H and ^{13}C NMR spectral data (Tables 1 and 2) of **2** by ^1H-^1H COSY, DEPT, and HMBC experiments revealed the presence of a 1,2,4-trisubstituted benzene ring (δ 6.65, d, J=8.2 Hz, δ 7.52, dd, J=8.2, 1.8 Hz, and δ 7.56, d, J=1.8 Hz), a carbonyl carbon (δ 169.4), an olefinic methine (δ 5.20, t, J=6.5 Hz) coupled with a methylene (δ 3.25, d, J=6.5 Hz), two adjacent methylenes (δ 2.05, m and δ 2.15, m), three sp^2 quaternary carbons, and four allylic methyls. One carbonyl carbon (δ 169.4, C-7) and IR absorption at 1639 cm^{-1} suggest the presence of an amide. Two broad exchangeable protons at δ 4.00 and the resonance of the aromatic proton in higher field at δ 6.65 suggest the presence of an NH_2 group on the aromatic carbon at its *ortho*-position. The long range couplings were observed from the aromatic protons of H-2 (δ 7.56) and H-6 (δ 7.52) to the carbonyl carbon of C-7 and from the NH_2 protons to the aromatic carbons of C-3 (δ 125.1) and C-5 (δ 114.6) in the HMBC spectrum (Fig. 2). These couplings indicated the presence of a *p*-aminobenzamide skeleton. In addition, the long range couplings from the methylene protons of H_2-12 and three allylic methyl protons of H-15, H-17, and H-18 to two sp^2 quaternary carbons of C-13 (δ 127.3) and C-14 (δ 124.3), from the methylene protons of H_2-11 to the olefinic carbon of C-9 (δ 120.7) and the sp^2 quaternary carbons of C-10 (δ 138.5), and from the allylic methyl protons of H_3-16 to the carbons of C-9, C-10, and C-11 (δ 38.1) revealed the presence of a 6-methylgeranyl group. Thus the structure of **2** was found to consist of a *p*-aminobenzamide and a 6-methylgeranyl group. The

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, R'=NH_2
4 R=OH, R'=NH_2
Virantmycin R=Cl, R'=OH

Table 1. ^{1}H NMR data for benzastatins A (**1**), B (**2**), C (**3**), and D (**4**) in $CDCl_3$.

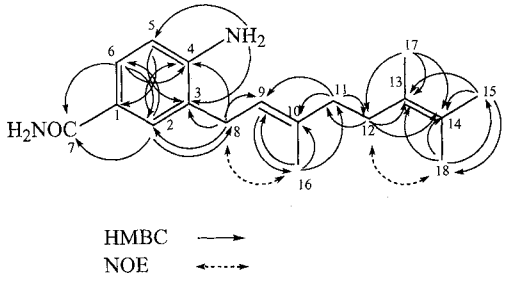
Position	1 (300MHz)	2 (600MHz)	3 (600MHz)	4 (300MHz)
NH			4.52 (1H, brs)	4.35 (1H, brs)
2	7.57 (1H, d, 1.8)	7.56 (1H, d, 1.8)	7.53 (1H, d, 1.6)	7.54 (1H, d,1.6)
5	6.61 (1H, d, 8.3)	6.65 (1H, d, 8.2)	6.54 (1H, d, 8.4)	6.51 (1H, d, 8.4)
6	7.49 (1H, dd, 8.3, 1.8)	7.52 (1H, dd, 8.2, 1.8)	7.46 (1H, dd, 8.4, 1.6)	7.46 (1H, d, 8.4, 1.6)
8	3.35 (2H, d, 7.3)	3.25 (2H, d, 6.5)	H_a 3.36 (1H, dd, 17.1, 4.8)	H_a 3.10 (1H, dd, 16.8, 4.4)
			H_b 3.10 (1H, dd, 17.1, 6.0)	H_b 2.84 (1H, dd, 16.8, 5.9)
9	5.36 (1H, t, 7.3)	5.20 (1H, t, 6.5)	4.36 (1H, dd, 6.0, 4.8)	3.97 (1H, m)
11	2.12 (2H, s)	2.05 (2H, m)	H_a 1.79 (1H, ddd, 12.5, 12.5, 5.0)	H_a 1.78 (1H, m)
			H_b 1.63 (1H, ddd, 12.5, 12.5, 5.0)	H_b 1.54 (1H, m)
12	2.12 (2H, s)	2.15(2H, m)	H_a 2.09 (1H, dt, 12.5, 5.0)	2.05 (2H, m)
			H_b 2.01 (1H, dt, 12.5, 5.0)	
15	1.62 (3H, s)	1.63 (3H, s)	1.61*(3H, s)	1.61*(3H, s)
16	4.02 (2H, s)	1.77 (3H, s)	H_a 3.57 (1H, d, 9.0)	H_a 3.66 (1H, d, 9.2)
			H_b 3.54 (1H, d, 9.0)	H_b 3.48 (1H, d, 9.2)
17	1.62 (3H, s)	1.62 (3H, s)	1.62*(3H, s)	1.62*(3H, s)
18	1.62 (3H, s)	1.64 (3H, s)	1.61*(3H, s)	1.61*(3H, s)
$CONH_2$	5.70 (2H, brs)	5.70 (2H, brs)	5.76 (2H, brs)	5.67 (2H, brs)
OCH_3	3.40 (3H, s)		3.38 (3H, s)	3.40 (3H, s)
NH_2	4.40 (2H, brs)	4.00 (2H, brs)		

Assignments with asterisks may be interchanged.

Table 2. ^{13}C NMR chemical shifts for benzastatins A (**1**), B (**2**), C (**3**), and D (**4**) in $CDCl_3$.

Position	1 (75MHz)	2 (150MHz)	3 (150MHz)	4 (75MHz)
1	122.6 C	122.8 C	121.7 C	121.9 C
2	130.4 CH	129.5 CH	129.7 CH	130.4 CH
3	124.8 C	125.1 C	116.4 C	117.4 C
4	149.5 C	148.6 C	145.7 C	146.0 C
5	114.9 CH	114.6 CH	113.7 CH	113.6 CH
6	127.5 CH	126.9 CH	127.1 CH	127.0 CH
7	169.7 C	169.4 C	169.2 C	169.2 C
8	31.6 CH_2	30.9 CH_2	33.3 CH_2	32.8 CH_2
9	127.1 CH	120.7 CH	56.4 CH	67.4 CH
10	138.4 C	138.5 C	57.9 C	57.5 C
11	35.3 CH_2	38.1 CH_2	33.7 CH_2	33.2 CH_2
12	34.0 CH_2	33.4 CH_2	27.8 CH_2	27.7 CH_2
13	127.5 C	127.3 C	126.6 C	126.9 C
14	124.2 C	124.3 C	124.7 C	124.6 C
15	20.9 CH_3	20.6 CH_3	19.9 CH_3	20.1 CH_3
16	71.1 CH_2	16.4 CH_3	73.9 CH_2	75.0 CH_2
17	18.7 CH_3	18.3 CH_3	18.4 CH_3	18.4 CH_3
18	20.4 CH_3	20.1 CH_3	20.5 CH_3	20.6 CH_3
OCH_3	59.0 CH_3		59.3 CH_3	59.5 CH_3

Fig. 2. NOE and HMBC experiments of benzastatin B (**2**).



HMBC spectrum showed the long range couplings of the methylene protons of H_2-8 in the 6-methylgeranyl group to the aromatic carbons of C-2 (δ 129.5), C-3 (δ 125.1), and C-4 (δ 148.6), indicating that the 6-methylgeranyl group was linked to C-3 in the aminobenzamide unit.

The assignment of H_3-18 was based on the significantly larger NOE from the methylene protons of H_2-12 to the methyl protons of H_3-18 rather than to H_3-15 (Fig. 2). The high field chemical shift of the methyl protons of H_3-16 appears to be due to a γ-effect[2], suggesting that the configuration of the double bond at C-9 is *E*. This configuration was confirmed by the NOE experiment in which the H_3-16 resonance was enhanced when H_2-8 was irradiated as shown in Fig 2. From these spectroscopic data, the structure of **2** was determined as shown in Fig. 1.

Structure of Benzastatin A (1)

HREI-MS and ^{13}C NMR data of compound **1** assigned the molecular formula as $C_{19}H_{28}N_2O_2$. The ^{1}H and ^{13}C NMR data of 1 were similar to those of **2**. The ^{1}H NMR and ^{13}C NMR assignments for **1**, which were executed by comparison with the data of **2** and analysis of DEPT, HMQC, and HMBC data, are listed in Tables 1 and 2, respectively. In the ^{1}H and ^{13}C NMR spectra, methoxy signals at δ 3.4 and δ 59.0 and oxygenated singlet methylene signals at δ 4.02 and δ 71.1 were observed in **1** instead of the allylic methyl of C-16 in **2**. In addition, the carbon resonances of C-9 and C-11 were shifted from δ 120.7 and δ 38.1 to δ 127.1 and δ 35.3, respectively. The long range couplings were observed from the methylene protons of H_2-16 to the carbons of C-9, C-10 (δ 138.4), and C-11 (δ 35.3) in the HMBC spectrum. These evidences indicated that the methyl

group of C-16 in **2** was replaced by a methoxymethylene in **1**. The NOE effect between H_3-16 and H_2-8 showed that the configuration of the double bond at C-9 was *Z*. Thus the structure of **1** was determined as shown in Fig. 1.

Structure of Benzastatin C (3)

The molecular formula of **3** was determined to be $C_{19}H_{27}N_2O_2Cl$ on the basis of HREI-MS and ^{13}C NMR data. The ^1H and ^{13}C NMR data of **3** is similar to those of **2** (Tables 1 and 2). The ^1H and ^{13}C NMR spectral data of **3** with ^1H-^1H COSY, DEPT, and HMQC data suggest that **3** contain the *p*-aminobenzamide unit and the alkenyl side chain of the geranyl group in **2**. The major difference between **2** and **3** is that an sp^3 methine (δ 4.36, dd, J=6.0, 4.8 Hz, δ 56.4) coupled with a methylene (δ 3.10, dd, J=17.1, 6.0 Hz and δ 3.36, dd, J=17.1, 4.8 Hz, δ 33.3), an sp^3 quaternary carbon (δ 57.9), a methoxymethylene, and one broad exchangeable proton were observed in **3** instead of the olefinic methine of C-9, the sp^2 quaternary carbon of C-10, the allylic methyl of C-16, and the NH_2 group in **2**. One carbonyl carbon (δ 169.2) and IR absorption at 1651 cm^{-1} suggest the presence of an amide. As shown in Fig. 3, the long range couplings were observed from the NH proton to the aromatic carbon of C-3 (δ 116.4) and the methine carbon of C-9 (δ 56.4), from the methylene protons of H_2-8 to the aromatic carbon of C-4 (δ 145.7) and the sp^3 quaternary carbon of C-10 (δ 57.9), and from the methine proton of H-9 to the aromatic carbon of C-3. This evidence indicated that a piperidine ring was formed in **3** through the linkage of the NH_2 group to C-10 in **2**. The long range couplings between the oxymethylene protons of H_2-16 and the carbons of C-9 and C-10, and between the methylene protons of H_2-11 and the carbons of C-9 and C-10 revealed that the alkenyl side chain and the methoxymethylene were linked to C-10. From these spectroscopic data, the structure of **3** was deduced except for the substituent at C-9. Together with HREI-MS data, the M+2 peak having approximately one-third intensity of the molecular ion peak indicated the presence of a chlorine atom. This evidence and the chemical shift (δ 56.4) of C-9 indicated a chlorine atom to be attached to C-9. The structure of **3** was supported by the mass spectrum having *m/z* 352 (3.9%), 350 (11%, M^+), 315 (1.2%, M^+-Cl), 307 (34%), 305 (100%, $M^+-CH_2OCH_3$), 269 (21%, $M^+-CH_2OCH_3-HCl$), 255 (5.8%), 253 (17%, $M^+-C_7H_{13}$), and 83 (66%, C_6H_{11}). Compound **3** is an amide derivative of virantmycin[3].

The configuration of **3** was examined by the NOE experiments. As shown in Fig. 4, NOEs between H_a-8 and H_a-11 as well as between H_b-8 and H_b-16 were observed in the NOE experiments of **3**. It showed that the piperidine ring was conformationally flexible. Together with J values ($J_{8a,9}$=4.8 Hz, $J_{8b,9}$=6.0 Hz) between H_2-8 and H-9, the presence of equal NOEs from the aromatic H-2 proton to H_a-8 and H_b-8 lead to ambiguity for the assignment of each proton of H_2-8 to equatorial or axial orientation. These facts cause ambiguity in elucidation of the relative stereochemistry of **3** like the case of virantmycin[4,5]. But, the ^1H chemical shifts and coupling constants of the substituents at C-9 and C-10 of **3** matched closely to those of the reported virantmycin rather than its diastereomer[6] as shown in Table 3, suggesting that compound **3** have the same relative stereochemistry as virantmycin. The absolute configuration of **3** was determined by comparing the CD spectra of **3** and virantmycin. The CD spectrum (λ_{ext} (nm) ([θ]) 315 (+4100), 304 (0), 285 (−7400), in MeOH) of **3** was very similar to that of virantmycin (λ_{ext} (nm) ([θ]) 316 (+5800), 301 (0), 286 (−4900), in MeOH). Thus the absolute configuration of **3** was assigned to be 9*R*, 10*R* at the two chiral centers as shown in Fig. 1.

Fig. 3. HMBC experiment of benzastatin C (**3**).

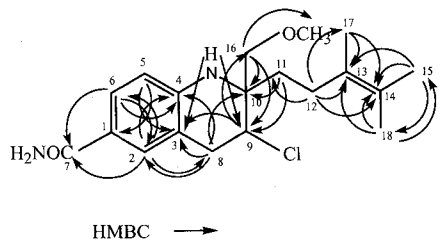
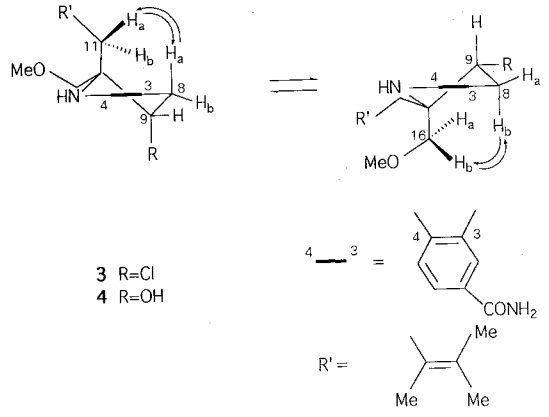


Fig. 4. NOEs observed in benzastatins C (**3**) and D (**4**).



Structure of Benzastatin D (4)

The molecular formula of **4** was determined to be $C_{19}H_{28}N_2O_3$ on the basis of HREI-MS and ^{13}C NMR

Table 3. Comparison of ^{1}H NMR data of the substituents at C-9 and C-10 of benzastatin C (**3**) with those of virantmycin and its diastereomer[6].

Position*	**3** (500MHz, in $CDCl_3$)	virantmycin (400MHz, in $CDCl_3$)	diastereomer of virantmycin (400MHz, in $CDCl_3$)
8	H_a 3.36 (1H, dd, 17.1, 4.8)	H_a 3.37 (1H, dd, 17.1, 4.7)	H_a 3.28 (1H, dd, 17.1, 4.9)
	H_b 3.10 (1H, dd, 17.1, 6.0)	H_b 3.11 (1H, dd, 17.1, 6.1)	H_b 3.13 (1H, dd, 17.1, 4.9)
9	4.36 (1H, dd, 6.0, 4.8)	4.36 (1H, dd, 6.1, 4.7)	4.42 (1H, dd, 6.6, 4.9)
11	H_a 1.79(1H, ddd, 12.5, 12.5, 5.0)	H_a 1.81 (1H, ddd, 13.9, 12.4, 5.0)	H_a 1.85 (1H, m)
	H_b 1.63 (1H, ddd, 12.5, 12.5, 5.0)	H_b 1.63 (1H, ddd, 13.9, 12.4, 5.0)	H_b 1.72 (1H, m)
12	H_a 2.01 (1H, dt, 12.5, 5.0)	H_a 2.00 (1H, dt, 12.4, 5.0)	2.09 (2H, t, 8.5)
	H_b 2.09 (1H, dt, 12.5, 5.0)	H_b 2.09 (1H, dt, 12.4, 5.0)	
16	H_a 3.57 (1H, d, 9.0)	H_a 3.58 (1H, d, 9.2)	H_a 3.47 (1H, d, 9.3)
	H_b 3.54 (1H, d, 9.0)	H_b 3.55 (1H, d, 9.2)	H_b 3.42 (1H, d, 9.3)

* The numbering differs from the reported virantmycin.

Table 4. Comparison of ^{1}H NMR data of the substituents at C-9 and C-10 of benzastatin D (**4**) with those of the alcohol derivative of virantmycin[7].

Position*	**4** (300 MHz, in $CDCl_3$)	alcohol derivative of virantmycin (300 MHz, in $CDCl_3$)
8	H_a 3.10 (1H, dd, 16.8, 4.4)	H_a 3.08(1H, dd, 16.6, 4.3)
	H_b 2.84 (1H, dd, 16.8, 5.9)	H_b 2.82 (1H, dd, 16.6, 5.8)
9	3.97 (1H, m)	3.95 (1H, m)
11	H_a 1.78 (1H, m)	H_a 1.78 (1H, ddd, 13.8, 10.6, 6.4)
	H_b 1.54 (1H, m)	H_b 1.54 (1H, m)
12	2.05 (2H, m)	2.05 (2H, m)
16	H_a 3.66 (1H, d, 9.2)	H_a 3.64 (1H, d, 9.6)
	H_b 3.48 (1H, d, 9.2)	H_b 3.46 (1H, d, 9.6)

* The numbering differs from the reported alcohol derivative of virantmycin.

data. The ^{1}H and ^{13}C NMR data revealed similarity between **3** and **4**. The ^{1}H NMR and ^{13}C NMR assignments for **4**, which were executed by comparison with the data of **3**, are listed in Tables 1 and 2, respectively. In the ^{1}H and ^{13}C NMR spectra, the difference between **3** and **4** is that proton and carbon resonances of C-9 is shifted from δ 4.35 to δ 3.97 and from δ 56.4 to δ 67.4, respectively. Together with these NMR data, the absence of M+2 isotope peak and the molecular weight of *m/z* 332 indicated that the chlorinated methine carbon (δ 56.4) of **3** was replaced by a hydroxylated methine carbon (δ 67.4) in **4**. The structure of **4** was supported by the mass spectrum pattern of *m/z* 332 (M^+), 287 ($M^+-CH_2OCH_3$), 269 ($M^+-CH_2OCH_3-H_2O$), 235 ($M^+-C_7H_{13}$), and 83 (C_6H_{11}). The stereochemistry of **4** deduced from NOE experiments as shown in Fig. 4 is ambiguous like the case of **3**. The ^{1}H chemical shifts and coupling constants of the substituents at C-9 and C-10 of **4**, however, matched closely to those of the reported alcohol derivative of virantmycin[7] as shown in Table 4. In addition, the CD spectrum (λ_{ext} (nm) ([θ]) 318 (+6400), 304 (0), 286 (−10100), in MeOH) of **4** resembles markedly that of virantmycin like the case of **3**. Thus the structure of **4** was determined as shown in Fig. 1.

Discussion

The structures of benzastatins A, B, C, and D, which were isolated from the culture broth of *S. nitrosporeus* 30643, were determined by spectroscopic analysis. Benzastatins A and B contain the *para*-aminobenzamide unit. A precedents for the aminobenzamide unit was the *ortho*-aminobenzamide unit in the 2-pyruvoylaminobenzamide isolated from *Penicillium chrysogenum*, *P. notatum*[8] and *Colletotrichum lagenarium*[9]. Benzastatins C and D are unique alkaloids related to virantmycin which is the only reported metabolite with the tetrahydroquinoline unit in the molecule. It has been found to exhibit antifungal and prominent antiviral activities[10,11]. Benzastatins C and D appear to have the same relative stereostructure as virantmycin since there are the closely similar chemical shifts and coupling constants of the substituents of the chiral carbons. The absolute configuration of benzastatins C and D was assigned to be 9*R*, 10*R* from the direct comparison of their CD spectra with that of virantmycin. We supposed that benzastatin D appeared to be biogenetically derived from benzastatin A through epoxide formation followed by

cyclization.

Experimental

^{1}H and ^{13}C NMR spectra were obtained on Bruker DRX 300, Bruker DMX 600, or Varian UNITY 300 spectrometers. EI-MS and HREI-MS spectra were measured on Hewlett Packard HP 5989A and JEOL JMS-HX 110/110A spectrometers, respectively. UV and IR spectra were recorded on Shimadzu UV-260 and Laser Precision Analect RFX-65 spectrometers, respectively. CD spetra were recorded on a JASCO J-720 spectropolarimeter.

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