NOVEL ANTIFUNGAL ANTIBIOTICS OCTACOSAMICINS A AND B

II. THE STRUCTURE ELUCIDATION USING VARIOUS NMR SPECTROSCOPIC METHODS

KAZUYUKI DOBASHI, HIROSHI NAGANAWA, YOSHIKAZU TAKAHASHI, TOMOHISA TAKITA and TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication April 2, 1988)

The structures of octacosamicins A and B, two new antifungal antibiotics, were studied by spectrometries and chemical modifications. The 2D NMR techniques including ^{1}H-detected heteronuclear multiple bond correlation method were successfully applied to this study. These antibiotics have unique linear chain structure possessing *N*-hydroxyguanidyl group at the terminal.

Octacosamicins A and B are new antifungal antibiotics produced by *Amycolatopsis azurea* MG398-hF9. In the preceding paper, we reported the taxonomy of the producing strain, production, isolation, biological properties and physico-chemical properties of these antibiotics.[1] In this paper, we will report the structure determination of octacosamicins A and B.

Structure of Octacosamicin B

The secondary ion mass spectrometry (SI-MS) of octacosamicin B revealed the ion peak at *m/z* 639 (M+H). The elemental analysis showed the molecular formula as $C_{32}H_{54}N_4O_9$ (MW 638).[1] Acidic hydrolysis of octacosamicin B liberated one mol of glycine.

^{13}C NMR data (Table 1) showed the presence of one *C*-methyl, 14 methylenes, 5 sp^3 methines, 8 olefinic methines and 4 C=O or C=N carbons. Since the number of double bond of this substance was found to be 8 from the ^{13}C NMR spectrum, which coincides with the unsaturation number calculated from its molecular formula, the antibiotic was found to be a linear structure.

Octacosamicin B was found to have four methine carbons bearing oxygen atoms from the ^{13}C and ^{1}H NMR spectra. Deuterium-induced isotope shifts in ^{13}C NMR spectrum of octacosamicin B was examined by the use of a dual NMR cell. Deuterium-induced isotope shift values of the four oxymethine carbons were found to be $0.11 \sim 0.14$ ppm suggesting that these oxymethin carbons bear hydroxy group.[2]

^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{13}C-^{1}H COSY spectra of octacosamicin B were measured in acetic acid-d_6. DMSO-d_6 and MeOH-d_4 were also used as the solvents. The poor solubility of the antibiotic to the solvents in general together with overlaps of the methylene and olefin signals in the ^{1}H NMR spectra caused difficulty to elucidate its structure using the above described NMR techniques.

Recently a high sensitive 2D NMR technique, ^{1}H-detected heteronuclear multiple bond correlation (HMBC), was shown to be useful to determine the structure of complex molecules.[3] We showed the correlations of protons and carbons using HMBC method in combination with ^{1}H-^{1}H COSY and

Table 1. ^{13}C NMR and ^{1}H NMR chemical shifts of octacosamicins A and B (CD_3COOD).

Position	Octacosamicin A			Octacosamicin B		
	^{13}C NMR (ppm)		^{1}H NMR (ppm)	^{13}C NMR (ppm)		^{1}H NMR (ppm)
1′	174.8			174.7		
2′	41.8	CH_2[c]	4.12	41.8	CH_2[c]	4.09, 4.15 AB (J=18.4 Hz)
1	176.0			176.0		
2	75.7	CH	4.26	75.7	CH	4.28 d (J=1.8 Hz)
3	70.6	CH	4.40	70.6	CH	4.41
4	40.9	CH_2	1.76, 1.81	40.9	CH_2	1.77, 1.85
5	66.8	CH	4.23	66.7	CH	4.22
6	44.7	CH_2	1.61, 1.70	41.9	CH_2	1.61, 1.69
7	69.9	CH	3.97	72.8	CH	3.97
8	38.2	CH_2	1.48	39.7	CH	1.49
9	26.2	CH_2	1.29, 1.52	33.6	CH_2	1.17, 1.47
10	30.4	CH_2	1.48	28.0	CH_2	1.39
11	33.4	CH_2	2.07	33.7	CH_2	2.05
12	133.6	CH	5.56	133.6	CH	5.57
13	131.6[a]	CH	6.01	131.7[b]	CH	6.02
14	132.6[a]	CH	6.01	132.6[b]	CH	6.02
15	132.1	CH	5.52	132.0	CH	5.53
16	32.9	CH_2	2.07	32.8	CH_2	2.07
17	25.3	CH_2	1.65	25.2	CH_2	1.70
18	40.3	CH_2	2.61	40.3	CH_2	2.62 t (J=7.2 Hz)
19	204.6			204.5		
20	128.9	CH	6.17	128.8	CH	6.17 d (J=15.2 Hz)
21	145.7	CH	7.26	145.7	CH	7.26
22	130.4	CH	6.27	130.3	CH	6.26
23	147.2	CH	6.28	147.2	CH	6.27
24	33.9	CH_2	2.22	33.9	CH_2	2.23
25	29.3	CH_2	1.51	29.3	CH_2	1.48
26	26.9	CH_2	1.34	26.8	CH_2	1.36
27	27.1	CH_2	1.71	26.9	CH_2	1.73
28	52.4	CH_2	3.61	52.3	CH_2	3.62
29				14.7	CH_3	0.75 d (J=6.6 Hz)
30	159.2			159.1		

[a,b] Assignments may be exchanged.
[c] Assigned by DEPT experiments.

^{13}C-^{1}H COSY spectra as follows.

In the ^{1}H NMR spectrum of octacosamicin B in acetic acid-d_4, a pair of signals at 4.09 and 4.15 ppm showed an AB splitting pattern of methylene protons independent from other protons. They were assigned to be CH_2 of glycine moiety. These methylene protons were connected to both carbonyl carbons at 174.7 and 176.0 ppm in the HMBC spectrum (Fig. 1). In the ^{1}H NMR spectrum in DMSO-d_6 (Fig. 2A), an amide proton could be observed at 7.54 ppm (1H, t, J=4.8 Hz) which is correlated to the methylene proton of glycine moiety in the COSY spectrum. This amide proton signal could not be observed in the spectra in CD_3COOD and in CD_3OD.

The carbonyl carbon at 176.0 ppm (C-1) was connected to an oxymethine proton 2-H [the proton

Fig. 1. HMBC spectrum of octacosamicin B in CD_3COOD.

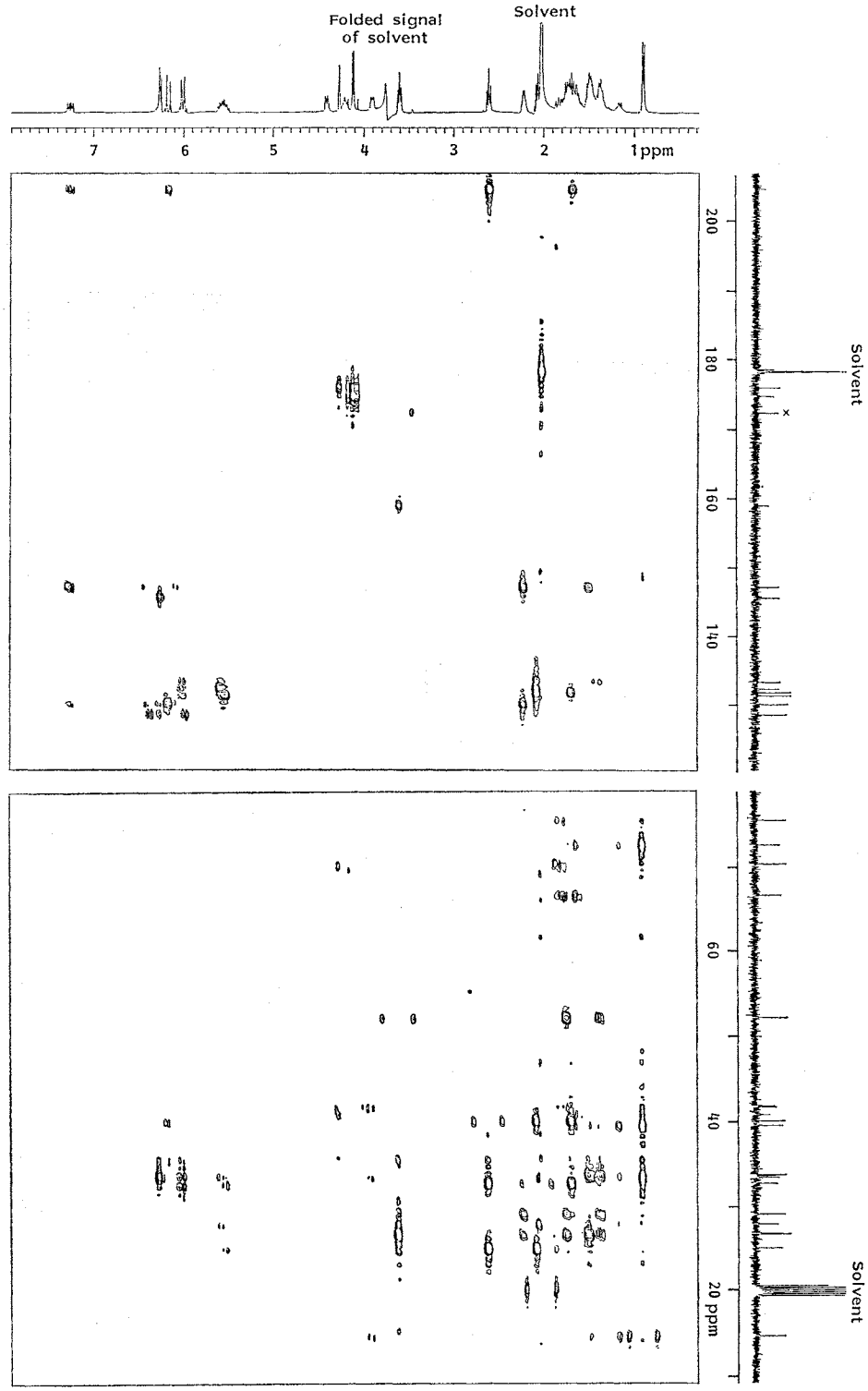
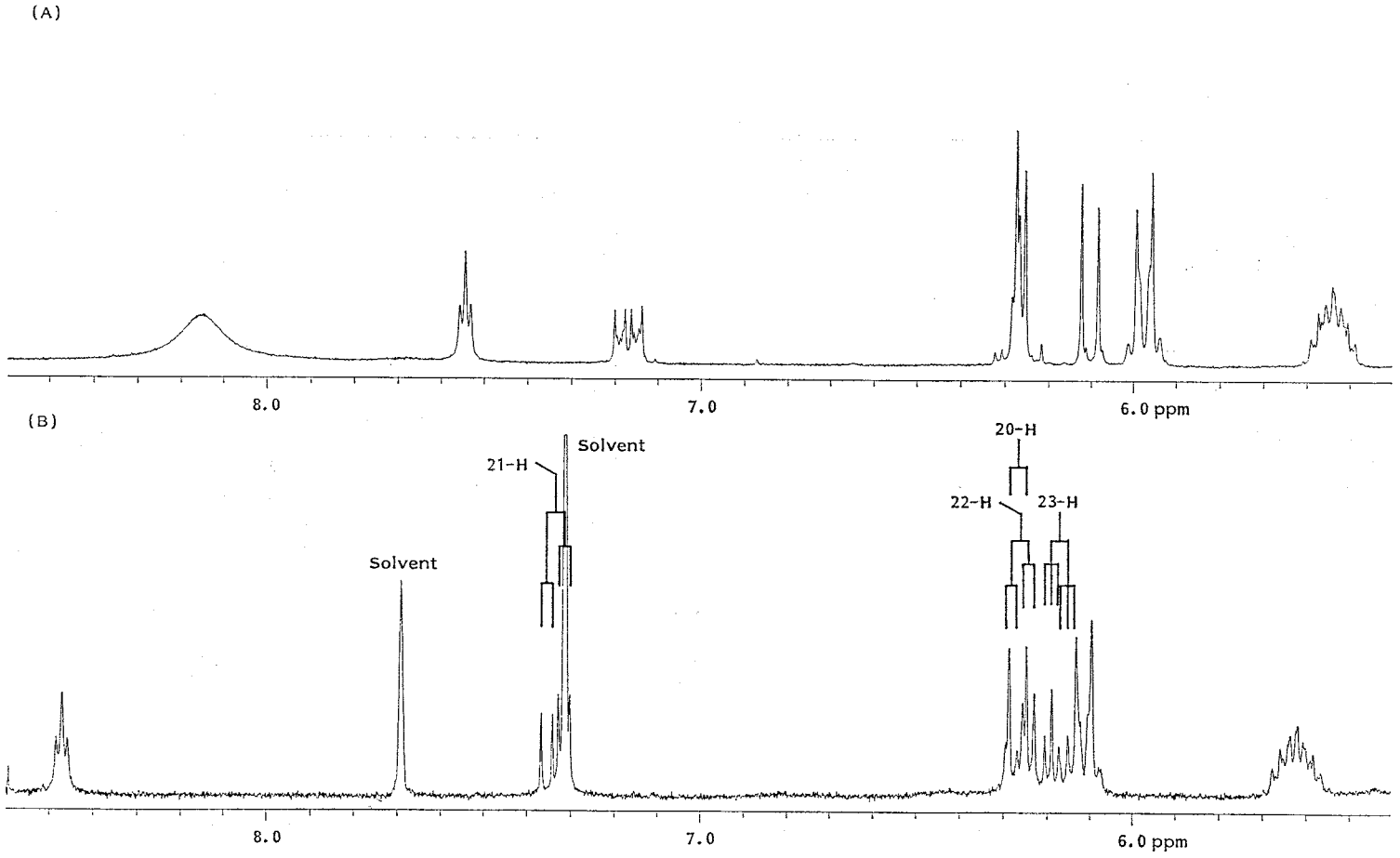


Fig. 2. 400 MHz ^{1}H NMR spectra of octacosamicin B (δ 5.4~8.6) in DMSO-d_6 and in mixed solvent.
(A) DMSO-d_6, (B) DMSO-d_6 - pyridine-d_5 (1 : 4).



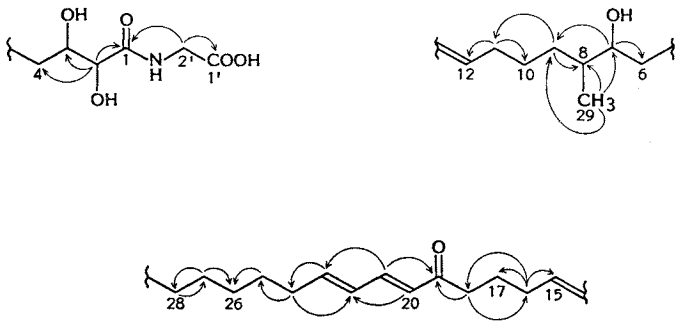
on the C-2 carbon] at 4.28 ppm in the HMBC spectrum (Fig. 1). Following connectivities could be observed in the ^{1}H-^{1}H COSY spectrum: 2-H–3-H (4.41 ppm) –4-H [the high field proton of unequivalent methylene]/4-H′ [the low field proton of unequivalent methylene] (1.77 and 1.85 ppm) –5-H (4.22 ppm) –6-H/6-H′ (1.61 and 1.69 ppm) –7-H (3.97 ppm). The HMBC spectrum showed connectivities of the methine proton 2-H between carbons C-3 (70.6 ppm) and C-4 (40.9 ppm).

An oxymethine proton 7-H at 3.97 ppm was connected with a methyl carbon C-29 (14.7 ppm), a methylene carbon C-6 (41.9 ppm) and another methylene C-9 (33.6 ppm). The 29-H methyl protons (0.75 ppm) were clearly correlated with carbons C-7 (72.8 ppm), C-8 (39.7 ppm) and C-9 in the HMBC spectrum. A proton 9-H (1.17 ppm) and a proton 9-H′ (1.47 ppm) were found to be directly attached to the carbon C-9 in the ^{13}C-^{1}H COSY spectrum. The proton 9-H was connected with carbons C-29, C-8 and C-11 (33.7 ppm) in Fig. 1. The proton 9-H was also correlated with methylene protons 10-H (1.39 ppm) in the COSY spectrum, where the protons 10-H were connected with another methylene protons 11-H (2.06 ppm). The methylene protons 11-H, that were considered to be adjacent to an olefin from their chemical shift, showed HMBC connectivities to the methylene carbon C-10 (28.0 ppm) and olefin carbon C-12 (133.6 ppm).

Protons 11-H showed a cross peak between an olefinic proton 12-H (5.57 ppm) and this olefinic proton had a cross peak with a multiplet signal at 6.02 ppm in the COSY spectrum. This multiplet signal showed the intensity of two protons and was connected to carbon signals at 131.7 and 132.6 ppm in the ^{13}C-^{1}H COSY spectrum. From their chemical shifts, it was considered that the four protons at 5.53 ppm (1H), 5.57 ppm (1H) and 6.02 ppm (2H) were in a conjugated diene moiety. In accord with that cosideration, the olefin proton at 5.57 ppm showed a cross peak with the olefin carbon at 132.6 ppm and the proton at 5.53 ppm was connected with the carbon at 131.7 ppm in the HMBC spectrum. Therefore, the following carbon connectivity was considered to be present: C-12 (133.6 ppm) –C-13 (131.7 ppm) –C-14 (132.6 ppm) –C-15 (132.0 ppm) (assignments of C-13 and C-14 may be exchanged). The olefin proton 15-H (5.53 ppm) showed coupling to protons 16-H (2.07 ppm). These methylene protons 16-H were connected with another methylene, 17-H (1.70 ppm), that were connected with a triplet signal of methylene protons 18-H (2.62 ppm) in the COSY spectrum. As expected, there were HMBC couplings of methylene protons at 16-H between the three carbons C-15, C-17 (25.2 ppm) and C-18 (40.3 ppm).

The antibiotic octacosamicin B showed strong UV absorbance at 283 nm (ε 22,000 in MeOH) indicating the presence of $\alpha,\beta,\gamma,\delta$-unsaturated ketone moiety.[4] A carbonyl carbon at 204.5 ppm

Fig. 3. Partial structures of octacosamicin B.



Arrows indicate a part of HMBC correlations from protons to carbons.

and the four olefinic carbons which were not assigned were attributable for this structure moiety. The simple doublet signal at 6.17 ppm in the ^1H NMR spectrum was deduced to be the olefin proton next to the ketone (20-H), and the neighboring proton (21-H) was followed in the COSY spectrum. The downfield signal at 7.26 ppm is characteristic of a β proton on the carbon of $\alpha,\beta(\gamma,\delta)$-unsaturated carbonyl group. The chemical shifts of these carbons were read from ^{13}C-^1H COSY spectrum: C-20 (128.8 ppm), C-21 (145.7 ppm). As protons 22-H and 23-H were very close in the ^1H NMR spectrum, differentiation of the carbons C-22 and C-23 was difficult in ^{13}C-^1H COSY spectrum. Because the δ-carbon of the $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl compound should be in lower field than the γ-carbon by the electron defficiency,[5,6] we assigned the carbon C-22 as 130.3 ppm and C-23 as 147.2 ppm. The following HMBC results proved their connectivities. The methylene 18-H (2.62 ppm) had a cross peak with the carbonyl C-19 (204.5 ppm), and the olefin proton 21-H (7.26 ppm) had cross peaks with the carbons C-19 and C-23. The olefin proton 20-H (6.17 ppm) showed a connectivity with the olefin carbon C-22.

Olefin attached methylene protons at 2.23 ppm that were assigned to 24-H from ^1H-^1H COSY spectrum, had connectivities with the olefin carbons C-22, C-23 and a methylene carbon C-25 (29.3 ppm) in the HMBC spectrum. Methylene protons of 25-H (1.48 ppm) were connected with carbons C-23, C-24 (33.9 ppm) and C-26 (26.8 ppm) in the HMBC spectrum. The methylene protons of 26-H (1.36 ppm) had a cross peak with another methylene 27-H (1.73 ppm) which was connected to the last methylene at 28-H (3.60 ppm) in the COSY spectrum. The methylene protons of 27-H showed connectivities with the carbons C-26 and C-28 (52.3 ppm), and the methylene protons of 28-H had apparent coupling with carbon C-27 (26.9 ppm) in the HMBC spectrum.

From the above described results, the structure of a linear carbon chain with glycine moiety of octacosamicin B was determined as shown in Fig. 5. The rest of the molecule, CH_4N_8O, is supposed to be *N*-hydroxyguanidine from the ^{13}C chemical shift of the carbon C-30 (159.1 ppm). From the chemical shift of the carbon C-28 (52.3 ppm), it was rationalized that the hydroxylamine nitrogen of *N*-hydroxyguanidyl group is attached to the carbon C-28. This chemical shift is near to the ^{13}C chemical shifts of N(OH)-attached methylene carbons of δ-*N*-hydroxyarginine (53.0 ppm, in H_2O)[7] and miharamycin A (51.2 ppm, in D_2O).[8] The guanidyl carbon C-30 was found to be connected with the methylene protons 28-H in the HMBC spectrum.

This *N*-hydroxyguanidyl structure was confirmed by a derivatization experiment. Reaction of octacosamicin B (**2**) with 2,4-dinitrofluorobenzene in MeOH in the presence of triethylamine

Fig. 4. Chemical shifts (ppm) of aromatic protons in **4** (MeOH-d_4).

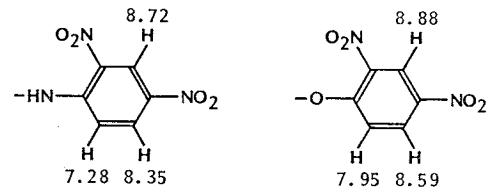
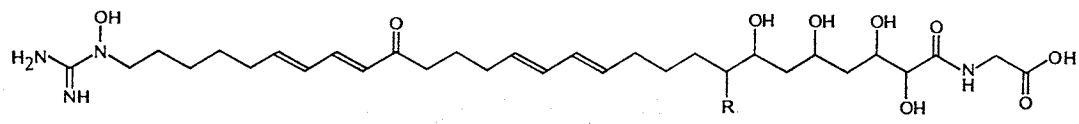


Fig. 5. Structures of octacosamicins A and B.



Octacosamicin A (**1**) R=H
Octacosamicin B (**2**) R=CH_3

followed by esterification with dry HCl - MeOH gave the bis(dinitrophenyl)octacosamicin B methyl ester (4). From its ^{1}H NMR spectrum in MeOH-d_4 (Fig. 4), it was found that one dinitrophenyl group is on a nitrogen atom and the other is on an oxygen atom.[9] From the above described data, we propose the structure of octacosamicin B as shown in Fig. 5.

The Structure of Octacosamicin A

The results of SI-MS and analysis of octacosamicin A showed the formula of this antibiotic as $C_{31}H_{52}N_4O_9$ (MW 624).[1] ^{13}C NMR data in Table 1 showed it is constructed of 31 carbons classified to 15 methylenes, 4 sp^3 methines, 8 olefinic methines and 4 sp^2 C=O or C=N carbons. Octacosamicin A was less soluble than octacosamicin B in all NMR solvents tested.

The ^{1}H and ^{13}C NMR spectra indicated that octacosamicin A is different from octacosamicin B only by the lack of the *C*-methyl group (Fig. 5). The ^{13}C NMR chemical shifts in Table 1 showed no discrepancy with the structure shown in Fig. 5.[10]

The Geometry of the Diene and the Dienone

In the ^{1}H NMR spectrum of octacosamicin B, the signals of the diene overlaped each other. The ^{13}C chemical shifts are useful to show the geometry of the diene moiety.[11] The ^{13}C NMR chemical shifts of C-12, C-13, C-14 and C-15 in octacosamicin B were 133.6, 131.7, 132.6 and 132.0 ppm respectively, indicating the (12*E*,14*E*) geometry. The ^{13}C NMR chemical shifts of both olefin-attached methylene carbons (C-11; 33.7 ppm, C-16; 32.8 ppm) are also in good agreement with this configuration.[11]

When the ^{1}H NMR spectrum was measured in a mixed solvent DMSO-d_6 - pyridine-d_5 (1 : 4), the signals of the dienone moiety were less overlapped as shown in Fig. 2B. Their coupling constants were read from the spectrum with the aid of ^{1}H-^{1}H spin decoupling experiments; J_{20-21}=15.4 Hz, J_{21-22}=10.4 Hz, J_{23-24}=15.0 Hz. Therefore, the both olefin geometries at C-20 and C-22 were determined as *E* configurations.

Octacosamicin A showed similar pattern of ^{1}H NMR in the olefin region to octacosamicin B. Therefore, it is also suggested to have (12*E*,14*E*,20*E*,22*E*) geometry.

Experimental

General

NMR spectra were recorded with a Jeol JNM-GX400 spectrometer. SI-MS spectra and high resolution (HR)-MS spectrum were measured on a Hitachi M-80H mass spectrometer.

Deuterium Induced Isotope Shift Value

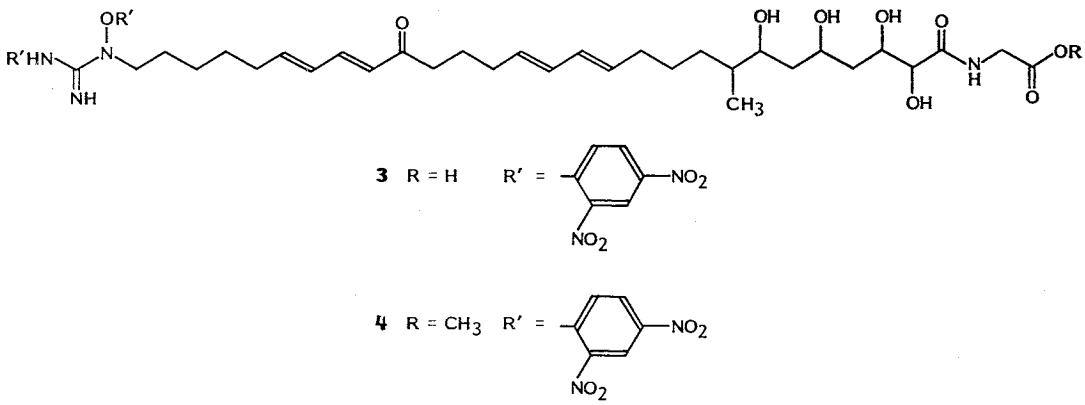
Deuterium induced isotope shift values in ^{13}C NMR of octacosamicin B were measured using dual cell NMR tube. The inner tube contained 1.0 mg of octacosamicin B in 0.2 ml of CD_3OH and the outer tube contained 1.0 mg of the antibiotic in 0.2 ml of CD_3OD. The differential spectrum of proton decoupled ^{13}C NMR was obtained using this dual tube at 24°C and the isotope shift value of each carbon was read from the spectrum.[12]

Acid Hydrolysis of Octacosamicin B

Octacosamicin B (14.0 mg, 21.9 μmol) was hydrolyzed with 1 M HCl at 105°C for 3 hours. The hydrolysate was diluted with water and passed through Diaion CHP-20P column (4 ml). To the passed solution was added Dowex WGR resin to neutralize the solution. The resin was removed and the solution was lyophilized to give 1.6 mg of glycine (yield 97%).

TLC: Merck Kieselgel 60 F_{254} No. 5715, BuOH - AcOH - H_2O (12 : 3 : 5); Rf 0.37. High voltage

Fig. 6. Structures of derivatives **3** and **4**.



electrophoresis: Avicell SF-1010, pH 1.6, 800 V, 8 minutes; Rm 1.17 (Ala 1.0). HR-MS *m/z* (M^+): Calcd for $C_2H_5NO_2$: 75.0320. Found: 75.0329.

Preparation of *N,O*-Bis(dinitrophenyl)octacosamicin B Methyl Ester (**4**)

To an emulsion of octacosamicin B (**2**) in dry MeOH (30 mg/1 ml, 0.047 mmol) was added 1 ml of 1% triethylamine in MeOH. To this solution was added 2,4-dinitrophenylfluorobenzene (52 mg, 0.28 mmol) with stirring. After the reaction mixture was stirred for 3.5 hours at room temperature, 20 g of ice was added to the solution. MeOH was removed by evaporation from the mixture and the remaining aqueous solution was washed with ethyl ether (20 ml×2), followed by extraction with BuOH (30 ml×3) at pH 2.5. After washing with water, the BuOH layer was concentrated and subjected to the chromatography on a column of Sephadex LH-20 in MeOH. Major eluate of the chromatography gave bis(dinitrophenyl)octacosamicin B (**3**): 18.1 mg (39.6%); ^{1}H NMR (MeOH-d_4) δ 0.88 (3H), 1.1~1.9 (17H), 2.06 (4H), 2.22 (2H), 2.57 (2H), 3.54 (2H), 3.76 (1H), 3.8~4.05 (4H), 4.18 (1H), 5.53 (2H), 5.98 (2H), 6.07 (1H), 6.24 (2H), 7.19 (1H), 7.27 (1H), 7.95 (1H), 8.33 (1H), 8.58 (1H), 8.71 (1H), 8.88 (1H).

To a solution of **3** in dry MeOH (9.0 mg/1 ml) was added 15 μl of 1% HCl - MeOH. The mixture was refluxed for 30 minutes, cooled on ice and was added 20 ml of $CHCl_3$. It was reacted with Ag_2CO_3 (50 mg) for 30 minutes under agitation. After removal of AgCl and Ag_2CO_3 by filtration, the filtrate was concentrated and purified with preparative TLC (Merck Kieselgel 60F No. 13794, $CHCl_3$ - MeOH, 6:1, Rf 0.6). It was further purified with a column of Sephadex LH-20 in MeOH to give *N,O*-bis(dinitrophenyl)octacosamicin B methyl ester (**4**): 3.8 mg (42%); ^{1}H NMR (MeOH-d_4) δ 0.88 (3H), 1.1~1.9 (17H), 2.06 (4H), 2.22 (2H), 2.58 (2H), 3.73 (3H), 3.75~3.81 (3H), 3.85~4.12 (3H), 4.17 (1H), 5.53 (2H), 5.98 (2H), 6.09 (1H), 6.25 (2H), 7.20 (1H), 7.28 (1H), 7.96 (1H), 8.33 (1H), 8.58 (1H), 8.72 (1H), 8.88 (1H).

References

1) Dobashi, K.; N. Matsuda, M. Hamada, H. Naganawa, T. Takita & T. Takeuchi: Novel antifungal antibiotics octacosamicins A and B. I. Taxonomy, fermentation and isolation, physico-chemical properties and biological activities. J. Antibiotics 41: 1525~1532, 1988
2) Hansen, P. E.: Isotope effects on nuclear shielding. Annu. Rep. NMR Spectrosc. 15: 105~234, 1983
3) Bax, A.; A. Aszalos, Z. Dinya & K. Sudo: Structure elucidation of the antibiotic desertomycin through the use of new two-dimensional NMR technique. J. Am. Chem. Soc. 108: 8056~8063, 1986
4) Stern, E. S. & C. J. Timmons (*Ed.*): Electric Absorption Spectroscopy in Organic Chemistry, Third Ed., pp. 78~88, Edward Arnold Ltd., London, 1970
5) Marr, D. H. & J. B. Stothers: ^{13}C NMR studies. Part VI. Carbon-13 spectra of α,β-unsaturated carbonyl compounds. Can. J. Chem. 43: 596~607, 1965

6) KAMANO, Y.; J. E. LEET, C. L. HERALD & G. R. PETTIT: Structure of the new antineoplastic bryostatins, 12 and 13, from coastal California *Bugula neritina*. Symposium Paper of 28th Symposium on the Chemistry of Natural Products, pp. 176~183, Sendai, Oct. 7~10, 1986
7) FISCHER, B.; W. KELLER-SCHIERLEIN, H. KNEIFEL, W. A. KÖNIG, W. LOEFLER, A. MÜLLER, R. MUNTWYLER & H. ZÄHNER: Stoffwechselprodukte von Mikroorganismen 118. Mitteilung. δ-*N*-Hydroxy-L-arginin, ein Aminosäure-Antagonist aus *Nannizzia gypsea*. Arch. Mikrobiol. 91: 203~220, 1973
8) SETO, H.; M. KOYAMA, H. OGINO, T. TSUKUOKA, S. INOUE & N. ŌTAKE: The structure of novel nucleoside antibiotics, miharamycin A and miharamycin B. Tetrahedron Lett. 24: 1805~1808, 1983
9) ROY, S. K.; S. NAKAMURA, J. FURUKAWA & S. OKUDA: The structure of neo-enactin A, a new antifungal antibiotic potentiating polyene antifungal antibiotics. J. Antibiotics 39: 717~720, 1986
10) BREITMAIER, E. & W. VOELTER (*Ed.*): ^{13}C NMR Spectroscopy. 2nd Ed., pp. 131~137, Verlag Chemie, Weinheim, 1978
11) ANDO, T.; K. KUSA, M. UCHIYAMA, S. YOSHIDA & N. TAKAHASHI: ^{13}C NMR analyses on conjugated dienic pheromones of lepidoptera. Agric. Biol. Chem. 47: 2849~2853, 1983
12) PFEFFER, P. E.; K. M. VALENTINE & F. W. PARRISH: Deuterium-induced isotope shift ^{13}C NMR. 1. Resonance reassignments of mono- and disaccharides. J. Am. Chem. Soc. 101: 1265~1274, 1979