Production of Brominated Tiacumicin Derivatives

JILL E. HOCHLOWSKI, MARIANNA JACKSON, RONALD R. RASMUSSEN, ALEX M. BUKO, JACOB J. CLEMENT, DAVID N. WHITTERN and JAMES B. MCALPINE†

Pharmaceutical Products Research and Development Abbott Laboratories, Abbott Park, Illinois, 60064, U.S.A.

(Received for publication November 15, 1996)

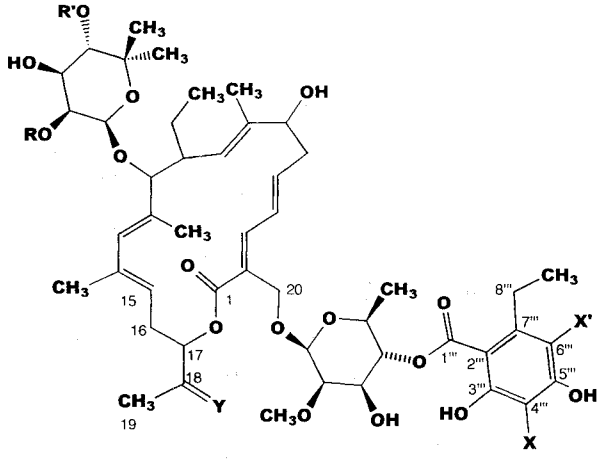
Several novel tiacumicin derivatives containing bromine have been produced by the addition of inorganic bromine to the fermentation broth of *Dactylosporangium aurantiacum* subsp. *hamdenensis*. Structures were elucidated employing mass spectrometry and NMR spectroscopy. Antibacterial activity of the bromotiacumicins is comparable to that of the parent compounds.

The tiacumicins are one of three known complexes of 18-membered macrolides; produced by *Dactylosporangium aurantiacum* subsp. *hamdenensis* (tiacumicins)[1,2] *Actinoplanes deccanensis* (lipiarmycins)[3,4] and *Micromonospora echinospora* subsp. *armeniaca* (clostomicins)[5]. Various members of this class are common to two or all three of the complexes but each such complex contains unique components (see Fig. 1). In an effort to produce novel derivatives of this class, inorganic bromine has been added to the fermentation broth of the tiacumicin producing culture, *Dactylosporangium aurantiacum* subsp. *hamdenensis*. This paper will describe the fermentation conditions under which bromine incorporation was achieved and the isolation, structure determination and biological activities of the novel tiacumicin derivatives so produced.

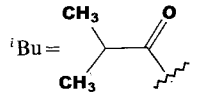
Fermentation

The brominated tiacumicins were produced by submerged fermentation in a 42-liter stainless steel fermenter (LH Fermentation) in a medium consisting of glucose monohydrate 2%, soybean oil 0.1%, soybean flour 1%, beef extract 0.3%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KBr 0.48% and $CaCO_3$ 0.3%. This formulation is similar to that used for the production of tiacumicins[1] with the substitution of KBr for KCl. The fermenter was charged with 30 liters of medium. Sterilization was at 121°C and 1.05 kg/cm^2 for 1 hour. The glucose monohydrate was sterilized separately and added to the fermenter prior to inoculation. The producing organism, *Dactylosporangium aurantiacum* subsp. *hamdenensis* AB 716C-41, was grown on agar slants of ATCC medium 172 for 10 days at 30°C. The slant growth was used to inoculate a seed medium consisting of glucose monohydrate 0.1%, soluble starch 2.4%, yeast extract 0.5%, tryptone 0.5%, beef extract 0.3% and $CaCO_3$ 0.4%. The inoculum was prepared in two steps. The first step, inoculated with slant growth, was incubated for 96 hours. This vegetative growth was used at 5% to inoculate 2-liter Erlenmeyer flasks containing 600 ml of the seed medium. These flasks were incubated for 72 hours. Both steps were incubated at 30°C on a rotary shaker at 225 rpm (5.08 cm stroke). The second step growth was

Fig. 1. Structures of brominated tiacumicins.



1 X=Cl, X'=Br, R=H, R'=^{i}Bu, Y=OH, H
2 X=Cl, X'=Br, R=H, R'=^{i}Bu, Y=O
3 X=Br, X'=H, R=H, R'=^{i}Bu, Y=OH, H
4 X=Br, X'=H, R=^{i}Bu, R'=H, Y=OH, H
Tiacumicin B X=X'=Cl, R=H, R'=^{i}Bu, Y=OH, H
Tiacumicin C X=X'=Cl, R=^{i}Bu, R'=H, Y=OH, H
Clostomicin D X=X'=Cl, R=H, R'=^{i}Bu, Y=O



† Present address: Phytera Inc., 377 Plantation Street, Worcester, MA. 01605.

used at 5% to inoculate the fermenter. During fermentation the temperature was controlled at 30°C, agitation was 250 rpm, the air flow was 0.7 vol/vol/minute and the head pressure was maintained at 0.35 kg/cm^2. Foam was controlled with a silicone antifoam, XFO 371 (Ivanhoe Industries), added initially at 0.01% and then available on demand. The fermentation was harvested after seven days.

Isolation of the Bromotiacumicins

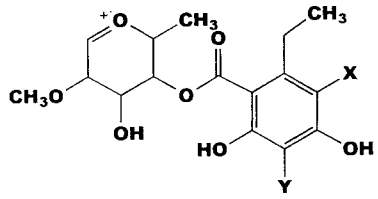
At harvest, whole broth (30 liters) was adjusted to pH 7 and acetone (15 liters) was added. After an hour of agitation, the acetone and fermentation broth mix was extracted with ethyl acetate (3 × 15 liters). The combined extracts were concentrated to dryness, and the residue was partitioned between chloroform-methanol-water (1200 ml of each). The lower layer of this partition was concentrated to leave a pale amorphous solid. This solid was loaded onto a silica gel column and eluted with a stepwise gradient of from 1% to 50% methanol in chloroform. Antimicrobial activity was monitored by an agar disk diffusion assay on plates seeded with *Staphylococcus aureus* ATCC 6538P. Active fractions were combined based upon TLC analysis into three pools. The first pool was loaded onto a silica gel column and eluted with successive step gradients of 10%, 20% and 50% methanol in chloroform. Active fractions from this column were combined and concentrated to leave a white solid. This solid was subjected to countercurrent chromatography on an Ito multi-layered coil planet centrifuge in a solvent system of chloroform - carbon tetrachloride - methanol - water (7 : 3 : 7 : 3) with the lower phase stationary. Active fractions from this column were combined and concentrated to yield 2 mg of pure compound **3**. Similar chromatography of the second pool gave pure tiacumicin B (58 mg) and pure compound **2** (3 mg). The third pool was subjected to countercurrent chromatography under the same conditions to yield pure compound **4** (45 mg), and crude compound **1** which was purified by chromatography on a Sephadex LH-20 column and eluted with methylene chloride - methanol (1 : 1). Active fractions from this LH-20 column were combined and concentrated to yield 42 mg of pure compound **1**.

Structure Determination of the Bromotiacumicins

Structure Determination of 1

The fast atom bombardment (FAB) positive ion mass spectrum of compound **1** had a highest molecular weight ion at $m/z=1123$. As this highest peak could be shifted to $m/z=1139$ by the addition of potassium to the sample, it is assumed that 1123 is the sodium salt of a parent compound which has a molecular weight of 1100 (Mass-Na). The isotope distribution pattern represented by the 1123 ion cluster could be matched to that of a formula containing one bromine and one chlorine atom. As a molecular weight of 1100 corresponds to a difference in molecular weight to the original tiacumicins of 44 mass units (the difference in atomic weight between bromine and chlorine), this is interpreted as representing the substitution of one bromine in compound **1** for one chlorine atom. A substantial fragmentation peak observed in the positive ion mass spectrum at $m/z=437$ (Fig. 2) displays an isotopic distribution pattern corresponding to one bromine and one chlorine atom. This is analogous to the $m/z=393$ peak with a dichloro isotopic pattern observed in the mass spectrum of the tiacumicins and assigned to the sugar fragment along with its attached aromatic ring (**5a, b**).

Fig. 2. Diagnostic mass spectral fragments.



5a X=Y=Cl
$m/z=393, 395, 397$
5b X=Br, Y=Cl
$m/z=437, 439, 441$
5c X=H, Y=Br
$m/z=403, 405$

A ^{1}H NMR spectrum of compound **1** along with a correlation spectroscopy (COSY) experiment suggested a basic structure essentially equivalent to that for tiacumicin B[2] (see Table 1). The only difference of note between the PMR spectra of tiacumicin B and compound **1** lies in the C-8‴ proton signals of the two. In tiacumicin B, these two proton signals appear as a symmetric complex 2H multiplet centered at δ 2.95. In compound **1** the corresponding proton signals appear as 1H pentets at δ 3.04 and δ 2.97. These data would suggest that compound **1** contains a bromine at C-6‴ on the aromatic ring (6‴-dechloro-6‴-bromotiacumicin B).

Structure Determination of 2

The fast atom bombardment (FAB) positive ion mass spectrum of compound **2** had a highest molecular weight

Table 1. ^{1}H NMR assignments for compounds **1**~**4**.[a]

H on C#	1	2	3	4
3	7.21 (d, 11.2)	7.42 (d, 11.6)	7.21 (d, 11.2)	7.18 (d, 11.7)
4	6.59 (dd, 14.8, 11.2)	6.64 (dd, 15.2, 11.6)	6.59 (dd, 14.3, 11.2)	6.58 (dd, 14.8, 11.2)
5	5.94 (ddd, 14.7, 9.8, 5.4)	6.08 (ddd, 15.2, 7.6, 6.7)	5.94 (ddd, 14.3, 9.4, 4.9)	5.92 (ddd, 14.8, 9.8, 5.3)
6	2.70 (mult)	2.70 (mult)	2.66 (mult)	2.65 (mult)
	2.49 (mult)	2.53 (mult)	2.48 (mult)	2.47 (mult)
7	4.21 (br mult)	4.26 (br mult)	4.21 (br mult)	4.20 (br mult)
9	5.13 (br d, 10.3)	5.21 (br d, 10.7)	5.13 (br d, 10.7)	5.10 (dt, 11.2, 1.8)
10	2.70 (mult)	2.70 (mult)	2.68 (mult)	2.55 (ddd, 13.0, 8.4, 3.1)
11	3.69 (d, 10.3)	3.73 (d, 10.2)	3.70 (d, 10.3)	3.65 (d, 9.8)
13	5.82 (br s)	5.89 (br s)	5.82 (br s)	5.84 (br s)
15	5.57 (t, 8.0)	5.34 (dd, 9.4, 7.2)	5.57 (br t, 8.0)	5.57 (br t, 8.0)
16	2.70 (mult)	2.80 (mult)	2.70 (mult)	2.74 (mult)
	2.42 (mult)	2.70 (mult)	2.41 (mult)	2.42 (mult)
17	4.71 (mult)	5.13 (dd, 9.8, 3.6)	4.68 (mult)	4.72 (ddd, 6.4, 4.8, 4.5)
18	4.01 (pent, 6.3)		4.01 (pent, 6.7)	4.03 (pent, 7.0)
19	1.16 (d, 6.7)	2.21 (s)	1.17 (d, 6.7)	1.20 (d, 7.0)
20	4.60 (d, 11.6)	4.58 (d, 11.6)	4.61 (d, 11.6)	4.61 (d, 11.6)
	4.42 (d, 11.6)	4.50 (d, 11.6)	4.42 (d, 11.6)	4.41 (d, 11.6)
21	1.64 (br s)	1.62 (br s)	1.64 (br s)	1.63 (br s)
22	2.00 (mult)	2.02 (mult)	1.99 (mult)	1.82 (mult)
	1.25 (mult)	1.29 (mult)	1.27 (mult)	1.14 (mult)
23	0.87 (t, 7.2)	0.86 (t, 7.6)	0.87 (t, 7.6)	0.80 (t, 7.6)
24	1.80 (br s)	1.87 (br s)	1.80 (br s)	1.76 (br s)
25	1.75 (br s)	1.68 (br s)	1.75 (br s)	1.76 (br s)
1′	4.62 (s)	4.62 (br s)	4.65 (br s)	4.65 (br s)
2′	3.54 (mult)	3.58 (mult)	3.56 (mult)	3.56 (mult)
2′-OCH_3	3.54 (s)	3.58 (s)	3.56 (s)	3.55 (s)
3′	3.72 (dd, 9.8, 3.4)	3.80 (dd, 9.8, 3.6)	3.76 (dd, 9.8, 3.6)	3.76 (dd, 9.8, 3.2)
4′	5.11 (t, 9.8)	5.13 (d, 9.8)	5.13 (t, 9.8)	5.14 (dt, 9.8, 3.1)
5′	3.52 (dq, 9.8, 6.3)	3.56 (mult)	3.58 (mult)	3.56 (mult)
6′	1.30 (d, 6.3)	1.29 (d, 6.2)	1.27 (d, 6.3)	1.22 (d, 6.3)
1″	4.71 (br s)	4.72 (br s)	4.70 (br s)	4.77 (d, 1.4)
2″	3.91 (br d, 2.7)	3.92 (br d, 3.2)	3.91 (br d, 3.1)	5.34 (dd, 3.6, 1.3)
3″	3.69 (dd, 10.3, 2.7)	3.73 (mult)	3.70 (d, 10.3)	3.74 (dd, 10.3, 4.2)
4″	5.01 (d, 10.3)	5.02 (d, 10.3)	5.00 (d, 10.3)	3.45 (d, 10.3)
6″	1.15 (s)	1.16 (s)	1.17 (s)	1.26 (s)
7″	1.12 (s)	1.10 (s)	1.13 (s)	1.08 (s)
6‴			6.35 (s)	6.39 (s)
8‴	3.04 (pentet, 7.2)	3.15 (br mult)	2.83 (mult, 2H)	2.83 (mult, 2H)
	2.97 (pentet, 7.2)	3.03 (pent, 7.2)		
9‴	1.20 (t, 7.2)	1.14 (t, 7.2)	1.19 (mult)	1.20 (t, 7.2)
2⁗	2.58 (hept, 6.7)	2.61 (hept, 6.7)	2.58 (hept, 7.2)	2.68 (hept, 7.2)
3⁗	1.18 (d, 6.7)	1.18 (d, 6.7)	1.18 (d, 7.2)	1.20 (d, 7.2)
4⁗	1.17 (d, 6.7)	1.17 (d, 6.7)	1.16 (d, 7.2)	1.19 (d, 7.2)

[a] Values in parentheses indicate coupling constants in Hz.

ion at $m/z=1121$. This was shifted to $m/z=1137$ by the addition of potassium and the molecular weight of compound **2** was therefore assumed to be 1098 or two mass units lower than compound **1**. The isotopic distribution pattern of the sodium adduct of compound **2** suggested that this structure contained one bromine and one chlorine atom.

A comparison of the ^{1}H NMR and COSY spectra of compounds **1** and **2** revealed that the two differed in the C-15 to C-19 region. The series of coupled proton signals which define atoms C-15 to C-19 in compound **1** are terminated at C-17 in compound **2**, where the δ 5.13 proton signal shows coupling only to the methylene protons on C-16. Further, the doublet methyl proton signal for C-19 in compound **1** at δ 1.16 has been replaced with a singlet methyl proton signal at δ 2.21. These data are interpreted as an oxidation at C-18 in compound **2** relative to compound **1**. This structure therefore could be described as 6‴-dechloro-6‴-bromoclostomicin D[5] (*i.e.* 6‴-dechloro-6‴-bromo-18-ketotiacumicin B).

Structure Determination of 3

The molecular weight of compound **3** was established as 1064. The isotopic distribution pattern observed in

Table 2. Antimicrobial potency of compounds **1**~**4**.

Organism	MIC (μg/ml)				
	Tiacumicin B	**1**	**2**	**3**	**4**
Staphylococcus aureus ATCC 6538P	0.78	6.2	50	6.2	50
Staphylococcus epidermidis 3519	1.56	6.2	100	12.5	50
Enterococcus faecium ATCC 8043	1.56	6.2	50	12.5	25
Streptococcus pyogenes EES61	6.2	6.2	50	25	50
Escherichia coli JUHL	>200	>200	>200	>200	>200
Bacteroides fragilis ATCC 25285	>128	>128	>128	>128	>128
Bacteriodes thetaiotaomicron ATCC 29741	>128	>128	>128	>128	>128
Clostridium perfringens ATCC 13124	0.06	0.03	0.015	≦0.06	0.06
Clostridium difficile ATCC 9689	0.06	0.06	0.25	0.12	0.5
Clostridium difficile ATCC 17857	0.12	0.06	0.25	0.25	1
Clostridium difficile 2532	0.12	0.06	1	0.5	2

the sodium adduct of the mass spectrum suggested that compound **3** contained one bromine atom and no chlorine atoms. A substantial fragmentation peak observed in the positive ion mass spectrum at $m/z=403$ displays an isotopic distribution pattern corresponding to one bromine. This is analogous to the $m/z=393$ peak with a dichloro isotopic pattern observed for the sugar fragment (5a, c) in the mass spectrum of the tiacumicins. A comparison of the proton and COSY spectra of compounds **1** and **3** revealed that these two differed only in the aromatic ring portion of their structures. In particular, compound **3** contained a singlet methine proton signal at δ 6.35 which showed one-bond coupling in an HMQC experiment[6] to a carbon signal at δ 111.5. This same proton signal (δ 6.35) showed three-bond coupling in an HMBC experiment[7] to the 8‴ methylene carbon signal at δ 27.7 as well as to two nonprotonated aromatic carbon signals at δ 98.0 and δ 107.6. These data indicate that this proton must be on carbon 6‴ in compound **3** and established the structure as 4‴,6‴-didechloro-4‴-bromotiacumicin B.

Structure Determination of 4

The molecular weight and isotopic distribution pattern of the parent peak of compound **4** was identical to that of compound **3** with a molecular weight of 1064 and one bromine as the only halogen. The same $m/z=403$ (5c) fragmentation peak with isotopic pattern indicative of a single bromine atom is observed in the mass spectrum of compound **4**. A comparison of the proton and COSY spectra of compound **4** to tiacumicin C revealed that these two differed only in the aromatic ring portion of their structures. A comparison of the proton and COSY spectra of compound **4** to compound **3** revealed that these two differed only in the sugar moieties attached at C-11 in these two. Compound **4** contained a singlet methine proton signal at δ 6.39 which showed one-bond coupling to a carbon signal at δ 111.8. This same proton signal (δ 6.39) showed three-bond coupling to the 8‴ methylene carbon signal at δ 31.0 These data would support a structure of 4‴,6‴-didechloro-4‴-bromotiacumicin C for compound **4**.

Antimicrobial Activity of the Bromotiacumicins

The brominated tiacumicins retain excellent activity against *Clostridium* strains, but are less active than tiacumicin B against *Staphylococcus* and *Enterococcus*. Compound **1**, 6‴-dechloro-6‴-bromotiacumicin B, is the most potent of the compounds described (see Table 2) in this paper and is quite comparable to tiacumicin B.

Experimental

General Procedures

Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter in a 10 cm cell. Fast atom bombardment mass spectra were measured on a Kratos MS-50 mass spectrometer. Ultraviolet spectra were recorded on a Perkin-Elmer Lambda 3B UV-visible spectrophotometer and infrared spectra on a Nicolet model 60SX FT-IR attached to a Nicolet computer. NMR spectra were acquired on either a General Electric GN500 or GN300 spectrometer. NMR spectral data are reported in Table 1 within the text. Rf values reported were acquired on Analtech TLC plates developed with chloroform : methanol (9 : 1, v/v) and were visualized using ceric sulfate spray reagent. Melting points were determined on a Hoover Unimelt and are reported uncorrected. Minimal inhibitory concentrations were determined by twofold agar dilution. Brain heart agar was used for aerobes and Wilkins-Chalgren agar for anaerobes.

1: $C_{52}H_{74}O_{18}BrCl$ $[\alpha]_D^{25} = +3°$ ($c=0.33$, MeOH), white amorphous solid, mp 145~151°C, Rf=0.34. MW 1123 (sodium salt). UV (MeOH): λ_{max} 224 nm (ε 9,900), 232 (shoulder) (9,700), 266 (shoulder) (5,900), 314 (2,400), in acidic methanol λ_{max} 206 nm (shoulder) (ε 9,400), 222 (11,000), 230 (shoulder) (10,800), 266 (6,500), in basic methanol at λ_{max} 206 nm (ε 13,500), 226 (shoulder) (10,300), 232 (10,800), 312 (10,100). IR ν_{max} ($CDCl_3$): 3690, 3665, 3605, 3497, 2976, 2935, 2876, 1733, 1684, 1601, 1575, 1468, 1456, 1411, 1385, 1371, 1322, 1312, 1244, 1196, 1159, 1143, 1113, 1087 and 1023 cm^{-1}.

2: $C_{52}H_{72}O_{18}BrCl$ $[\alpha]_D^{25} = +13°$ ($c=0.21$, MeOH), white amorphous solid, mp 139~144°C, Rf=0.35. MW 1121 (sodium salt). UV (MeOH): λ_{max} 222 nm (ε 10,300), 266 (6,800), in acidic methanol λ_{max} 222 nm (ε 8,800), 230 (shoulder) (8,500), 240 (shoulder) (8,200), 316 (shoulder) (5,500) in basic methanol at λ_{max} 206 nm (ε 13,600), 238 (10,800), 270 (shoulder) (6,700), 316 (4,600). IR ν_{max} ($CDCl_3$): 3690, 3608, 2974, 2931, 2874, 1705, 1601, 1457, 1383, 1376, 1312, 1251, 1196, 1162, 1147, 1136, 1111, 1068 and 1022 cm^{-1}.

3: $C_{52}H_{75}O_{18}Br$ $[\alpha]_D^{25} = +3°$ ($c=0.16$, MeOH), white amorphous solid, mp 138~140°C , Rf=0.42. MW 1087 (sodium salt). UV (MeOH): λ_{max} 226 nm (ε 6,000), 266 (3,800), in acidic methanol at λ_{max} 226 nm (ε 6,700), 266 (4,200) in basic methanol at λ_{max} 206 nm (ε 7,700), 236 (6,300), 278 (shoulder) (3,800), 304 (3,600), 312 (shoulder) (3,500). IR ν_{max} ($CDCl_3$): 3690, 3606, 3501, 2987, 2935, 2875, 1731, 1692, 1653, 1603, 1570, 1469, 1456, 1423, 1386, 1323, 1312, 1295, 1256, 1233, 1187, 1162, 1116, 1069, 1022 and 789 cm^{-1}.

4: $C_{52}H_{75}O_{18}Br$ $[\alpha]_D^{25} = -5°$ ($c=0.42$, MeOH), white amorphous solid, mp 143~150°C, Rf=0.30. MW 1087 (sodium salt). UV (MeOH): λ_{max} 226 nm (ε 11,500), 266 (7,600), 306 (shoulder) (2,300), in acidic methanol at λ_{max} 226 nm (ε 11,800), 266 (7,800), 306 (shoulder) (2,000) in basic methanol at λ_{max} 204 nm (ε 11,500), 226 (shoulder) (10,400), 238 (11,800), 270 (6,500), 306 (7,200). IR ν_{max} ($CDCl_3$): 3619, 2976, 2935, 2895, 1733, 1700, 1652, 1646, 1603, 1447, 1388, 1371, 1323, 1311, 125 , 1188, 1160, 1145, 1114, 1068 and 1047 cm^{-1}.

References

1) THERIAULT, R. J.; J. P. KARWOWSKI, M. JACKSON, R. L. GIROLAMI, G. N. SUNGA, C. M. VOJTKO & L. J. COEN: Tiacumicins, a novel complex of 18-membered macrolide antibiotics. I. Taxonomy, fermentation and antibacterial activity. J. Antibiotics 40: 567~574, 1987
2) HOCHLOWSKI, J. E.; S. J. SWANSON, L. M. RANFRANZ, D. N. WHITTERN, A. M. BUKO & J. B. MCALPINE: Tiacumicins, a novel complex of 18-membered macrolides. II. Isolation and structure determination. J. Antibiotics 40: 575~588, 1987
3) ARNONE, A.; G. NASINI & B. CAVALLERI: Structure elucidation of the macrocyclic antibiotic lipiarmycin. J. Chem. Soc. Perkin Trans. I. 1987: 1353~1359, 1987
4) CAVALLERI, B.; A. ARNONE, E. MODUGNO, G. NASINI & B. P. GOLDSTEIN: Structure and biological activity of lipiarmycin B. J. Antibiotics 41: 308~315, 1988
5) ŌMURA, S.; N. IMAMURA, R. OIWA, H. KUGA, R. IWATA, R. MASUMA & Y. IWAI: Clostomicins, new antibiotics produced by *Micromonospora echinospora* subsp. *armeniaca* subsp. Nov. I. Production, isolation, and physicochemical and biological properties. J. Antibiotics 39: 1407~1412, 1986
6) MULLER, L.: Sensitivity enhanced detection of weak nuclei using heteronuclear multiple quantum coherence. J. Am. Chem. Soc. 101: 4481~4484, 1979
7) BAX, A. & M. F. SUMMERS: ^1H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986