PYRROINDOMYCINS, NOVEL ANTIBIOTICS PRODUCED BY *Streptomyces rugosporus* sp. LL-42D005

I. ISOLATION AND STRUCTURE DETERMINATION

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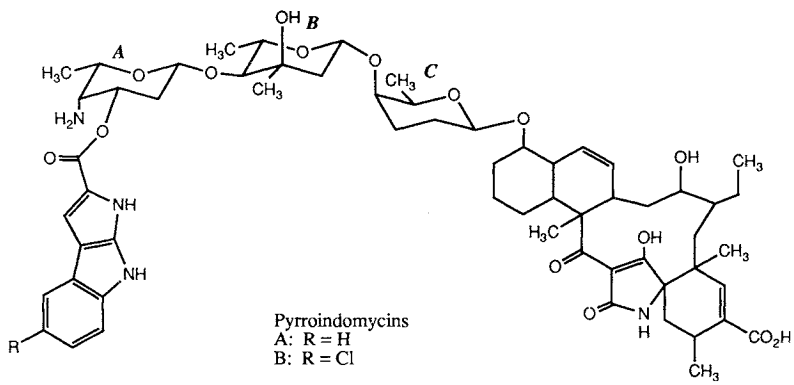
Pyrroindomycins A and B were isolated from fermentations of culture LL-42D005, a strain of *Streptomyces rugosporus*. Pyrroindomycins possess potent antimicrobial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci. Their structures have been determined by using 1- and 2-D NMR, mass spectroscopy and chemical degradations. Pyrroindomycins are the first natural products that contain the highly unsaturated pyrroloindole moiety.

Pyrroindomycins A (MW 1096) and B (MW 1130) are the two principal components of the antibiotic complex isolated from fermentations of culture LL-42D005 during the course of a screening program for agents active against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci[1]. The structures of A and B were determined on the basis of NMR, MS and UV analyses of the intact molecules and their degradation fragments. They are composed of a novel pyrroloindole entity linked *via* an ester bond to an unbranched deoxytrisaccharide. A polyketide macro-ring system containing a tetramic acid functionality is connected to the other end of the trisaccharide through a glycosidic linkage (Fig. 1). Some related, but distinctly different structures have been reported in the literature[2~10].

Fig. 1. Structures of pyrroindomycins A and B. The absolute stereochemistry of the sugars is unknown.



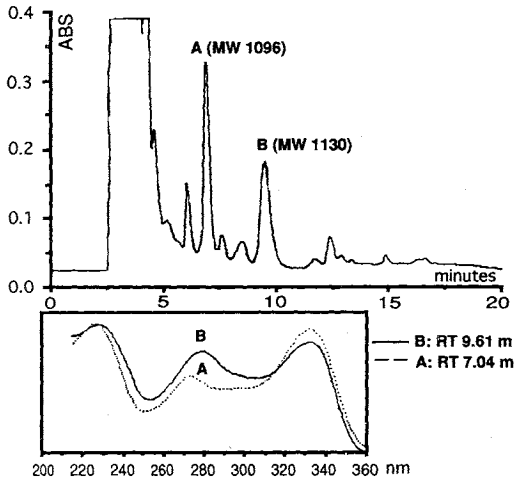
Results and Discussion

Isolation

An analytical HPLC profile of the filtered whole mash is shown in Fig. 2.

For large scale preparation, the whole mash of a 600 liters fermentation was mixed with an equal volume of EtOAc and the pH was adjusted to ~6.5 with H_3PO_4. The broth was filtered using a ceramic filtration system (Ceraflo™). The EtOAc phase was concentrated and the residue was then dissolved in 500 ml of MeOH. A 50 ml portion of the above MeOH solution was chromatographed on a preparative Vydac C18 column. Pyrroindomycins A and B eluted at about 80 and 100 minutes, respectively, using a gradient system (A: CH_3CN; B: 0.4% TFA in H_2O). Ten repetitive runs of this HPLC procedure yielded 0.4 g A and 2.0 g B after desalting and concentration to dryness (Scheme 1). The physico-chemical properties for these purified antibiotics are listed in Table 1.

Fig. 2. HPLC profile of the pyrroindomycin complex and their UV spectra.



Scheme 1. Isolation and structure determination strategy.

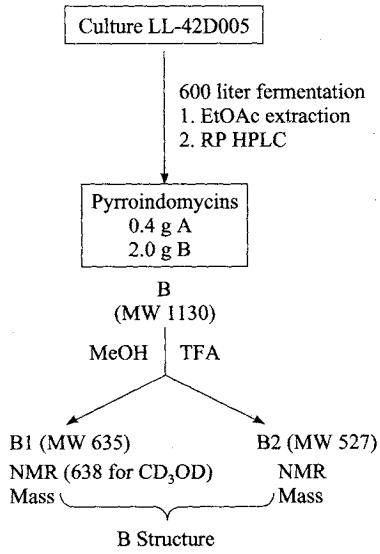


Table 1. Physico-chemical properties of pyrroindomycins.

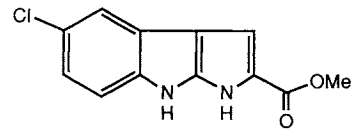
	A	B
Molecular formula:	$C_{60}H_{80}N_4O_{15}$	$C_{60}H_{79}N_4O_{15}Cl$
Mass spectrum ($M+Na^+$) m/z:	1119.5543	1153.5157
FABMS (high resolution)	calc.: 1119.5513	calc.: 1153.5123
Infrared spectrum (cm^{-1})	3380, 3063, 2971, 2878, 1682, 1627, 1598, 1539, 1447, 1382, 1293, 1239, 1206, 1164, 1114, 1091, 1032, 1017, 987, 972, 745	3426, 2972, 2933, 2878, 1684, 1626, 1598, 1567, 1540, 1447, 1405, 1383, 1295, 1270, 1236, 1204, 1163, 1092, 1032, 1017, 987, 971
Ultraviolet spectrum:	226 nm (ε, 26,100), 273 nm (ε, 18,500), 335 nm (ε, 26,200)	229 nm (ε, 26,100), 281 nm (ε, 23,200), 335 nm (ε, 25,600)

Structure Determination

The structural elucidation of pyrroindomycins was carried out using NMR, mass spectroscopy and chemical degradation methods. Carbon and proton chemical shift assignments were based on the analysis of 1- and 2-D NMR data. Because of the large number of carbons and protons of similar chemical shifts, the ^{1}H and ^{13}C NMR signals of pyrroindomycins A and B are not well dispersed. Fortunately, methanolysis of B with TFA and MeOH yielded cleanly two fragments, B1 and B2 with well-resolved NMR spectra. One molecule of MeOH was incorporated into the B1 fragment. The structures of B1 and B2 were determined by a combination of 1- and 2-D NMR analyses and by mass spectroscopic methods (Figs. 3~9) (Tables 2 and 3). B1 exists as a mixture of α and β anomers at the terminal C sugar (2:1), which could not be separated by reverse phase HPLC. However, the excellent signal dispersion in the NMR spectrum allowed the chemical shift assignments to be made for each anomer.

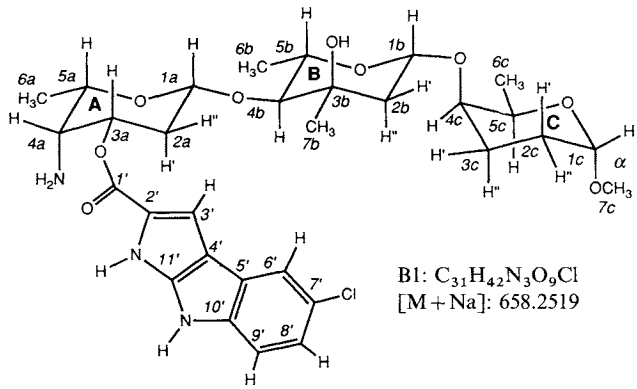
The complete structure of pyrroindomycin B was assembled based on structures of B1 and B2 and derivatization of the intact molecule. The structure

Fig. 3. Pyrroloindole methyl ester.



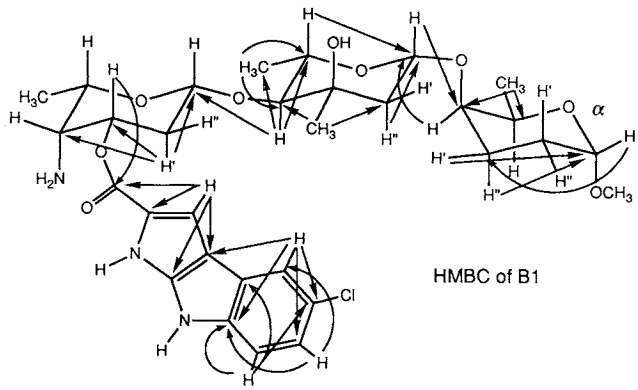
Pyrroindomycin B4 fragment

Fig. 4. Structure of B1 fragment. The absolute stereochemistry of the sugars is unknown.



B1: $C_{31}H_{42}N_3O_9Cl$
[M+Na]: 658.2519

Fig. 5. HMBC of B1 fragment in DMSO. The carbon and proton assignments are based on ^{1}H, ^{13}C, ^{1}H-^{13}C, COSY and HMBC data.



HMBC of B1

Fig. 6. Relative configuration of B1 assigned by analysis of NOE, anomeric ^1J ($^{13}CH_1$) values and decoupling data.

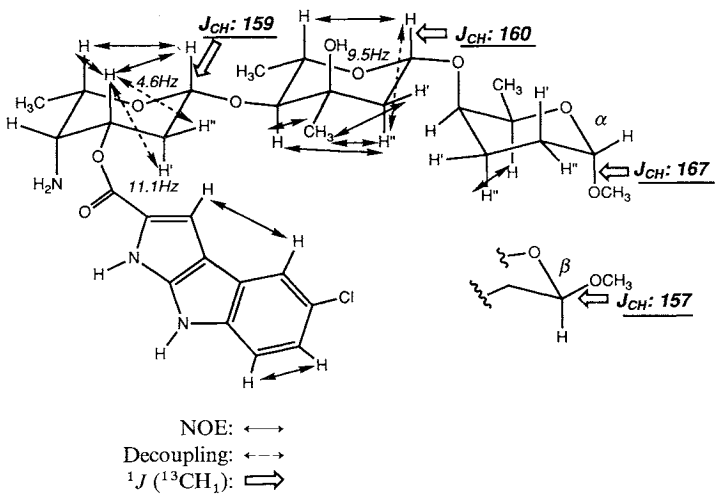


Fig. 7. B2 fragment.

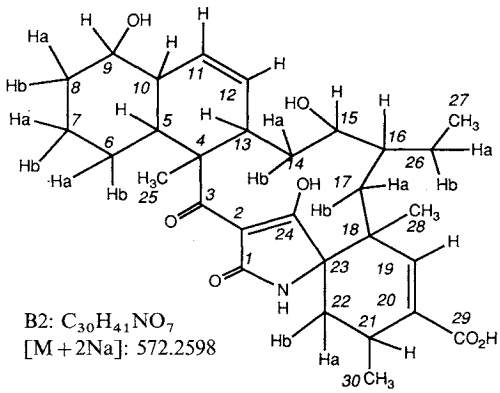


Fig. 8. COSY correlation of B2 fragment (DMSO). Data from ^1H, ^{13}C, ^1H-^{13}C, COSY and HMBC experiments were used to establish the above structure.

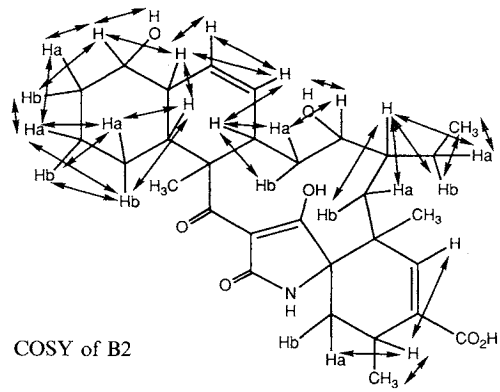
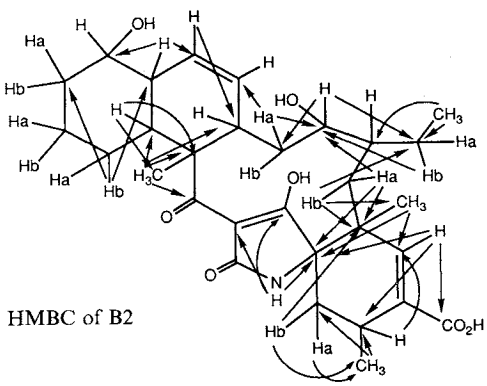


Fig. 9. HMBC of B2 (DMSO).



of pyrroindomycin A was based on mass and NMR spectral comparisons with those of B (Fig. 10).

B1 Fragment

The existence of the pyrroloindole moiety was confirmed by 1- and 2-D NMR data (Table 1 and Figs. 4~6) and by the fact that a methyl ester, B4, (Fig. 3) was formed upon treatment of pyrroindomycin B with NaOMe in MeOH. The molecular weight of this ester was determined by high resolution FABMS to be 248.0351, which is consistent with the proposed molecular formula $C_{12}H_9N_2O_2Cl$ (calc. MW: 248.0353). Its characteristic UV spectrum matches the published spectrum of an

Table 2. ^1H (500 MHz) and ^{13}C (125 MHz) NMR of B1 fragment (δ in ppm in DMSO).

Carbon	δ_C	δ_H	Carbon	δ_C	δ_H
1′	160.0		6a	17.0	1.22
2′	120.4				
3′	107.8	7.25	1b	100.2	4.72
4′	108.9		2b	44.7	H′: 1.83; H″: 1.47
5′	121.9		3b	69.6	
6′	118.5	7.69	4b	84.5	3.07
7′	123.4		5b	68.8	3.69
8′	121.2	7.12	6b	18.1	1.18
9′	112.9	7.35	7b	27.4	1.20
10′	139.7	10′-NH: 11.28[#]			
11′	143.0	11′-NH: 11.96[#]	1c	102.5	4.56
			2c	23.7	H′: 1.80; H″: 1.36
1a	100.0	4.62	3c	24.3	1.80 (2H)
2a	31.1	H′: 2.01; H″:1.79	4c	75.0	3.44
3a	71.0	5.01	5c	65.3	3.72
4a	51.1	2.92	6c	16.8	1.03
5a	69.6	3.66	7c	55.0	3.30

[#] Interchangeable.

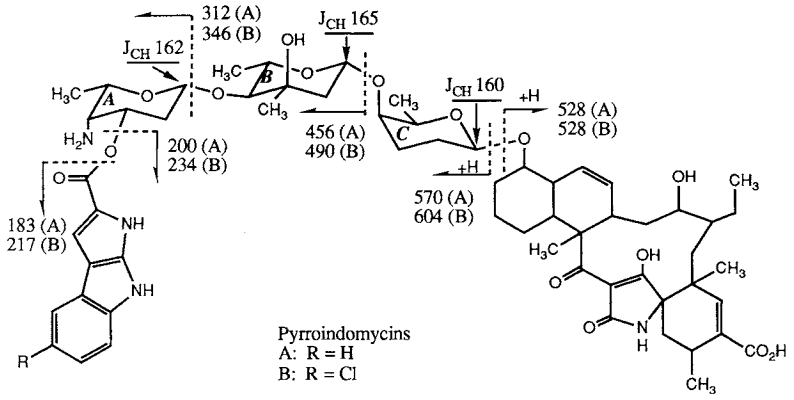
Table 3. ^1H (500 MHz) and ^{13}C (125 MHz) NMR of B2 fragment (δ in ppm in DMSO).

Carbon	δ_C	δ_H	Carbon	δ_C	δ_H
1	168.0		16	43.4	0.57
2	107.0		17	45.1	a 1.87; b 0.99
3	202.5		18	44.8	
4	50.2		19	145.0	6.40
5	38.4	1.33	20	132.0	
6	27.9	a 2.08; b 0.94	21	26.1	2.75
7	24.2	a 1.71; b 1.36	22	35.0	a 2.56; b 1.42
8	36.4	a 1.91; b 1.21	23	63.0	
9	72.3	3.05	23-NH		8.10
9-OH		4.68	24	202.2	
10	45.0	1.57	25	14.7	1.63
11	123.0	5.81	26	25.1	a 1.43; b 1.07
12	131.0	5.67	27	13.5	0.74
13	44.7	2.83	28	22.3	1.14
14	44.9	a 1.24; b 1.09	29	167.0	
15	81.0	3.41	30	20.6	1.20
15-OH		4.50			

authentic sample[11]. The strong NOE between H3′ and H6′ established the position of the chlorine at 7′ ($J_{H8'\sim9'}=8.55$; $J_{H6'\sim8'}=2.14$). An HMBC correlation between H3a and C1′ allowed the connection of the pyrroloindole carbonyl to C3a of sugar A.

The assignments of the carbon atoms of the B1 fragment are based on extensive ^1H-^1H COSY, ^1H-^{13}C HETCOR, HMBC and NOE data (See Table 2 and Figs. 4~6). The absolute stereochemistry of the sugars, however, has not been assigned. Carbon-carbon connections were made mostly through ^1H-^1H COSY, ^1H-^{13}C HETCOR except for sugar B, which has a quaternary carbon. However, HMBC correlations between H7b of the methyl group and C4b and C2b indicate that this quaternary carbon is at 3b. The position of methyl group (7b) on C3b is equatorial because of the NOEs between H7b and H′2b, H7b and H″2b, and H7b and H4b. HMBC correlations between H4b and C1a,

Fig. 10. Construction of pyrroindomycins based on evidence from NMR, mass spectroscopy and chemical derivatization.



H4c and C1b, and H1b and C4c established the positions for the sugar-sugar linkage as shown in Fig. 5. The primary amino group of sugar A is easily acetylated.

Strong NOEs between H1a, H3a and H5a, and between H1b and H5b, and the large coupling constants of 9.45 Hz for H1a and H'2a of sugar A and 9.5 Hz for H1b and H'2b of sugar B indicate that both these sugars contain a β glycosidic linkage. This is supported by the low $^1J(^{13}CH_1)$ coupling constants of 159 and 160 Hz for sugars A and B, compared with 157 for the β anomer and 167 for the α anomer of terminal sugar C in the B1 fragment (Fig. 6). It should be noted that the anomeric $^1J(^{13}CH_1)$ values of sugars A and B obtained for the intact pyrroindomycin B are larger than those in the B1 fragment by 3~5 ppm (163 and 165 for A and B, respectively). However, we believe the type of glycosidic linkage between A and B, or B and C should be the same in both B1 and the intact antibiotic since B1 was produced by methanolysis, which did not affect these glycosidic bonds. The glycosidic linkage connecting sugar C to the B2 fragment for the intact pyrroindomycins is most likely to be β because of the low anomeric $^1J(^{13}CH_1)$ value (160.3 Hz, Fig. 10). A coupling constant between H1c and H2c for sugar C of the intact antibiotic would give a straightforward answer to this, but it could not be obtained due to the complexity of proton NMR signals in this region of the intact molecule. The proposed structure of the B1 fragment is consistent with the mass spectroscopic data summarized in Fig. 10.

B2 Fragment

Structural assignments were made difficult due to the high number of quaternary carbons (nine) present in B2, which interrupt COSY correlations. A combination of COSY and HMBC analysis was, therefore, employed to establish the B2 structure as shown in Fig. 7.

No COSY correlation was observed for H15-H16 (Fig. 8, Table 2). However, the HMBC correlations between H26b-C15, H17a-C15 and H15-C26 connected C15 with C16 (Fig. 9, see Fig. 7 for numbering). The HMBCs between 23-NH and carbons C2, C23 and C24 were essential for establishing the presence of the acyltetramic acid moiety, the ^{13}C chemical shifts of which are similar to those reported in the literature[12]. An acyltetramic acid residue was further supported by the characteristic UV spectrum with an intense peak at 290 nm which, upon addition of NH_4OH, blue-shifted to 285 nm with increased intensity along with the emergence of a new peak at 245 nm[13].

Consistent with the presence of a free carboxylic acid grouping was the ease of formation of amides

and esters (data not shown).

The Intact Antibiotics

Since HMBC data on the whole molecule failed to provide connectivity between B1 and B2, we were forced to rely on the results from chemical derivatization to make this assignment (Fig. 10). The following experimental observations provided important clues: i) Upon methanolysis, MeOH is incorporated into the terminal deoxysugar at the anomeric position of sugar C, ii) the 15-OH group on the 11-membered ring of intact pyrroindomycin can be acetylated (δ_{H15}: 3.44→4.51 ppm), iii) the carboxyl group is free and can be reacted to form esters and amides, iv) the tetramic acid functionality can be reduced without breaking the polyketide linkage, and v) the low $^1J(^{13}CH_1)$ value for the anomeric carbon and proton of sugar C in the intact antibiotics indicates a β glycosidic linkage as mentioned above (Fig. 10).

The structure of pyrroindomycin A was deduced from that of B since: (1) methanolysis of A yielded B2 (identical HPLC retention time, molecular weight and spectral properties) and (2) pyrroindomycin A lacks the chlorine atom on the pyrroloindole ring. These structural assignments were supported by mass spectral analysis (Fig. 10).

Experimental

HPLC Methods

(a) Analytical: Vydac C18 p and p Column, 5μ, 0.46×25 cm. Flow rate: 1 ml/minute. Detection: 280 nm. Gradient: A: CH_3CN; B: 0.1% TFA in H_2O; A: 50→90%: 10 minutes. (b) Preparative: Column: Vydac C18 protein and peptide, 55×250 mm, 10~15μ, equipped with a 55×55 mm Vydac C18 guard column. Detection: 280 and 330 nm. Gradient system: A: CH_3CN; B: 0.4% TFA in H_2O. A: 50%: 40 minutes; 51.2%: 25 minutes; 52.5%: 45 minutes; 55%: 15 minutes; 85%: 25 minutes. Flow rate: 40 ml/minute. Desalting was carried out with C18 cartridges and the MeOH eluate contained the antibiotics.

Formation and Purification of B1, B2 and B4

Methanolysis of pyrroindomycin B (100 mg in 25 ml MeOH containing 10% CH_3CN) in a water bath at 55°C for 1 hour led to the complete disappearance of the starting material and the formation of B1 and B2. HPLC Purification: column, solvents, flow rate and detection conditions are the same as described above [Gradient: A: 37%: 10 minutes; 38.5%: 20 minutes; 40%: 30 minutes; 43%: 15 minutes. Retention time (Rt): 43 minutes for B1 and 63 minutes for B2]. B4 was formed upon treatment of B with saturated NaOMe in MeOH at room temperature for 16 hours. HPLC conditions were the same as above except for the gradient [Gradient: A: 31%: 15 minutes; 34%: 20 minutes; 37%: 20 minutes; 38.5%: 20 minutes; 40%: 15 minutes; 55%: 20 minutes. Rt: 83 minutes].

NMR

GE Omega-500. ^1H: 500 MHz; ^{13}C: 125 MHz.

Mass Spectrum

For FABMS (low resolution): VG ZAB-SE mass spectrometer; magic bullet (Dithiothreitol/DET). For FABMS (high resolution): resolution=8000, using CAA87 surfactant as reference.

Infrared Spectrum

Hewlett-Packard FTIR (KBr).

UV spectrum

Hewlett-Packard 8450A UV/Vis Diode array spectrophotometer. Sample concentration: 10 μg/ml in MeOH.

References

1) ABBANAT, D. R.; V. S. BERNAN, D. A. MONTENEGRO, D. A. STEINBERG, C. J. PEARCE, J. D. KORSHALLA, M. R. ALLURI, G. T. CARTER, W. M. MAIESE & M. GREENSTEIN: Pyrroindomicins, novel antibiotics produced by *Kitasatosporia* sp. LL-42D005: I. Taxonomy and fermentation. Program and Abstracts of 33rd Intersci. Conf. on Antimicrob. Agents Chemother., No. 449, p. 201, New Orleans, Oct. 17~20, 1993
2) KELLER-SCHIERLEIN, W.; R. MUNTWYLER, W. PACHE & H. ZAHNER: Chlorothricin und Des-chlorothricin. Helv. 52: 127~142, 1969
3) KOBINATA, K.; M. URAMOTO, T. MIZUNO & K. ISONO: A new antibiotic, antlermicin A. J. Antibiotics 33: 244~246, 1980
4) TOMITA, F.; T. TAMAOKI, K. SHIRAHATA, M. KASAI, M. MORIMOTO, S. OHKUBO, K. MINEURA & S. ISHII: Novel antitumor antibiotics, tetracarcins. J. Antibiotics 33: 668~670, 1980
5) TOMITA, F. & T. TAMAOKI: Tetracarcins, novel antitumor antibiotics. I. Producing organism, fermentation and antimicrobial activity. J. Antibiotics 33: 940~945, 1980
6) TAMAOKI, T.; M. KASAI, K. SHIRAHATA, S. OHKUBO, M. MORIMOTO, K. MINEURA, S. ISHII & F. TOMITA: Novel antitumor antibiotics, tetracarcins. J. Antibiotics 33: 946~951, 1980
7) WAITZ, J. A.; A. C. HORAN, M. KALYANPUR, B. K. LEE, D. LOEBENBERG, J. A. MARQUEZ, G. MILLER & M. G. PATEL: Kijanimicin (Sch 25663), a novel antibiotic produced by *Actinomadura kijaniata* SCC 1256: Fermentation, isolation, characterization and biological properties. J. Antibiotics 34: 1101~1106, 1980
8) ASHTON, R. J.; M. D. KENIG, K. LUK, D. N. PLANTEROSE & G. SCOTT-WOOD: MM 46115, a new antiviral antibiotic from *Actinomadura pelletieri*. J. Antibiotics. 42: 1387~1393, 1990
9) KAWASHIMA, A.; Y. NAKAMURA, Y. OHTA, T. AKAMA, M. YAMAGISHI & K. HANADA: New cholesterol biosynthesis inhibitors MC-031 (O-demethylchlorothricin), -032 (O-demethylhydroxychlorothricin), -033 and -034. J. Antibiotics 45: 207~212, 1992
10) SCHROEDER, D.; K. S. LAM, G. A. HESLER, D. R. GUSTAVSON, K. TOMITA & R. L. BERRY (Bristol-Myers Squibb Co., New York, N.Y.): Antitumor antibiotic BMY-42448. U.S. 5,082,933, Jan. 21, 1992
11) OHNO, M.; T. F. SPANDE & B. WITKOP: Cyclization of tryptophan and tryptamine derivatives to 2,3-dihydropyrrolo[2,3-b]indole. J. Am. Chem. Soc. 92: 343~348, 1970
12) LEE, V.; A. R. BRANFMAN, T. R. HERRIN & K. L. RINEHART, Jr.: Synthesis of 3-dienoyl tetramic acids related to streptolydigin and tirandamycin. J. Am. Chem. Soc. 100: 4225~4236, 1978
13) RINEHART, K. L.; J. R. BECK, D. B. BORDERS, T. H. KINSTLE & D. KRAUSS: Streptolydigin. III. Chromophore and structure. J. Am. Chem. Soc. 85: 4038~4039, 1963