DESERTOMYCIN: PURIFICATION AND PHYSICAL-CHEMICAL PROPERTIES

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Desertomycin was isolated from *Streptomyces macronensis* Dietz sp. nov. UC 8271. Extensive spectroscopic work led us to place desertomycin in the macrocyclic lactone family which contains monazomycin, scopafungin, primycin, azalomycin F_{4a} and niphithricins A and B. The apparent molecular formula was determined by fast atom bombardment mass spectroscopy to be $C_{57}H_{109}NO_{24}$ (MW=1,191). Mild acid hydrolysis yielded mannose but contrary to published reports, glutamic acid is not a constituent of desertomycin.

Our recent soil screening studies resulted in the isolation of an organism characterized as *Streptomyces macronensis* Dietz sp. nov. UC 8271 (NRRL 12566). The new species produced an antibiotic which we ultimately concluded was identical to desertomycin[1] by direct comparison of data.

Desertomycin was first reported in 1958 coproduced with flavofungin by *Streptomyces flavofungini*. Since then, very few papers have been published which describe the chemical nature of desertomycin. In one publication[2] it was claimed that fructose was a hydrolysis product. In a second[3], both mannose and glutamic acid were obtained on hydrolysis. The authors of the second publication also reported that the minimum molecular weight of desertomycin was in the 3,200~3,500 range, based on the amount of mannose obtained after acidic hydrolysis.

We now report an improved isolation scheme for desertomycin, its molecular formula, the NMR spectra, and the fact that there is no glutamic acid present in desertomycin. We conclude from our studies that desertomycin is a large ring macrolide, related to monazomycin[4], scopafungin[5], primycin[6], azalomycin F_{4a}[7~9] and niphithricins[10] A and B.

Microorganisms

The antibiotic producing microorganism was a new soil isolate, classified by A. DIETZ as *Streptomyces macronensis* sp. nov. (UC 8271).

Analytical Methods

The fermentation media were analyzed by thin-layer chromatography on Polygram Cel 400 sheets (Brinkmann Instruments, Inc., Westbury, NY) developed in 95% ethanol - pyridine - acetic acid - water (56: 56: 14: 100, v/v). In this system desertomycin had an Rf value of ~0.9 and was located by bioautography against *Micrococcus luteus* UC 130.

Antibiotic production was determined by the paper disc agar diffusion method employing *Micrococcus luteus* as the test organism. All solvent ratios given are volume: volume (v/v).

Fermentation

All fermentations were conducted under submerged culture conditions in 500 ml Erlenmeyer flasks containing 100 ml of culture medium. Seed cultures of *S. macronensis* UC 8271 were prepared in a

medium composed of glucose monohydrate 1 g/liter, dextrin 3 g/liter, glycerol 20 g/liter, brewers yeast 1 g/liter, corn steep liquor 3 g/liter, Solulac (Grain Processing Co., Muscatine, IA) 4 g/liter, Pharmamedia (Traders Oil Mill Co., Fort Worth, TX) 4 g/liter, NH_4NO_3 2 g/liter, NaCl 5 g/liter, $CaCO_3$ (added after pH adjustment) 4 g/liter, mineral salt solution A 1 ml/liter, mineral salt solution B 1 m/liter. The pH was adjusted to 9.2 before sterilization. Mineral salt solution A contains (g/liter): $MgSO_4 \cdot 7H_2O$ 50, $MnSO_4 \cdot H_2O$ 3, $FeSO_4 \cdot 7H_2O$ 10, $ZnSO_4 \cdot 7H_2O$ 3, $CoCl_2 \cdot 6H_2O$ 1 (adjusted to pH 2 with 1 N H_2SO_4). Mineral salt solution B contains (g/liter): KH_2PO_4 50, KCl 100. The seed flasks were inoculated with a loop of soil stock prepared according to REUSSER[11] and incubated at 28°C for 3 days on a rotary shaker (250 rpm, 6 cm stroke). The fermentation medium contained the same ingredients as the seed medium. These flasks were incubated at 28°C on a rotary shaker. Peak antibiotic titers were obtained after 1~2 days of incubation.

Isolation Procedure

Desertomycin could be isolated from *S. macronensis* culture broth by filtration and sorption of the filtrate onto a column of XAD-2 resin (Rohm & Haas Inc., Philadelphia, PA). The column was washed with deionized water and then with water - acetone, 3: 1. The desertomycin was eluted with water - acetone, 1: 1 solution. The pool of eluate fractions containing desertomycin was lyophilized. The dark solid was then percolated over a bed of LH-20 Sephadex (Pharmacia) on which $V_E = 1.6$ Vo in methanol - water, 2: 1. Most of the methanol was stripped from the eluate pool using a rotary evaporator. The residual aqueous solution was percolated over a bed of DEAE cellulose (DE-52, Whatman) to remove the red color. Lyophilization of the now colorless solutions gave pure desertomycin. Ten liters of beer usually gave 300~500 mg of antibiotic.

Butanol extraction is a poor alternative to the XAD-2 process since removal of the butanol leads to decomposition of the antibiotic. Ultrafiltration over UM10 filters in stirred cells (Amicon Corp., Danvers, MA) is a useful alternative to gel chromatography since desertomycin was retained by the UM10 filter. Desertomycin did not stick to either gel or macroreticular cation exchange resins.

Samples obtained as above were further purified by one of several methods.

First, a sample was adsorbed onto a bed of SP-Sephadex (Pharmacia) packed in 0.05 M $(NH_4)_2SO_4$ in MeOH - H_2O, 1: 1. The antibiotic was eluted by gradually adding 0.5 M $(NH_4)_2SO_4$ in MeOH - H_2O, 1: 1 to the 0.05 M solution. The pool of fractions containing desertomycin was desalted using a cycle over XAD-2 resin as described above.

Alternatively, another sample was subjected to 500 tube Craig countercurrent distribution (CCCD) in an all glass CCCD machine. Equal volumes of both phases from *n*-BuOH - MeOH - H_2O, 4: 1: 5 were used in each tube with the upper phase as the mobile phase. The K value found was 0.72 and the product nicely fit the theoretical curve.

Finally, a third sample was subjected to Droplet countercurrent chromatography (DCCC)[12,13]. The best solvent was the 4: 1: 5 system used in CCCD runs above. The upper phase was again the mobile phase and desertomycin was found to emerge just after the appearance of the upper phase from the 300-tube apparatus (Tokyo Rikakikai Co., Ltd.). Systems consisting of chloroform - methanol - water combinations were useless due to the polarity of desertomycin.

Throughout this work, desertomycin was detected by its activity *vs. Micrococcus luteus* UC 130 (agar diffusion assay) or by TLC on silica gel plates using methanol - water, 2: 1 as the solvent and permanganate-periodate spray for visualization. The Rf was 0.3 for salt-free samples. Interestingly, we

found that small amounts of almost any salt (*e.g.* NaCl, $AgNO_3$, $FeSO_4$, $TiCl_3$) raised the Rf to 0.9 on silica gel TLC plates (Analtech Inc., Newark, DE). We know of no precedent for this phenomenon.

Further purification as described above with SP-Sephadex, CCCD, and DCCC techniques removed traces of color from the product but did not alter the spectral characteristics (below). The best product obtained was in the form of a white, fluffy, amorphous solid, mp 158~159°C (dec.). Attempts to crystallize this failed.

Structural Considerations

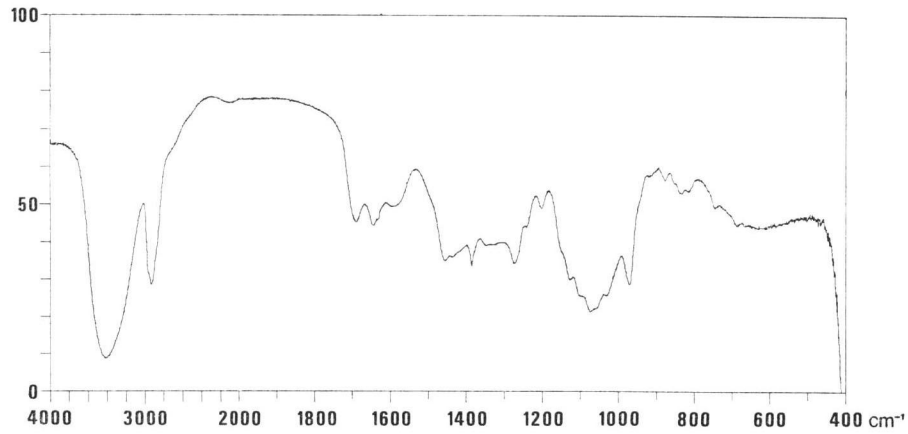
That our antibiotic (produced by *S. macronensis*) was similar to desertomycin was suggested by the similarity of its mobilites on eleven papergram systems[13] to the mobilities of authentic desertomycin in the same systems. The likelihood of correct identification was increased when we matched the infrared spectrum[8] (Fig. 1), the UV spectrum (Fig. 2) and the toxicity in mice[1] (LD_{50} 1.5 mg/kg, s.c.) of our sample with those of desertomycin.

The presence of mannose in our antibiotic was demonstrated by analysis on a Hewlett-Packard Model 5992A desktop GC-MS spectrometer. The antibiotic was hydrolyzed in a sealed tube with 0.5 N HCl at 65±5°C for 16 hours. The dark solution was lyophilized and the residue was treated with *N*-trimethylsilylimidazole in a sealed vial at 90±5°C for 60 minutes. A single peak was detected using total ion abundance on the effluent from a 0.4×80 cm column packed with 3% OV-17 on high efficiency Chromosorb W. The eight possible per-*O*-TMS-hexopyranosides eluted between R_T=10.6 and 13.3 minutes with a thermal gradient of 5°C/minute starting at 124°C. All were well resolved with the exception of the per-*O*-TMS derivatives of gulose, mannose and the unknown sugar (R_T=11.2 minutes). The per-*O*-TMS derivatives of gulose and mannose were distinguishable on the basis of their fragmentation patterns. The peak at *m/z* 191 was always between 24 and 28% of the base peak (*m/z* 204) for gulose and idose but always between 60 and 80% of the (same) base peak for the other six per-*O*-TMS-hexopyranosides[15]. The fragmentation pattern of the unknown sugar derivative (and that from authentic desertomycin) always matched that of mannose.

Analysis of the methyl-per-*O*-TMS-hexopyranosides in the same manner also showed that the sugar present in desertomycin was mannose.

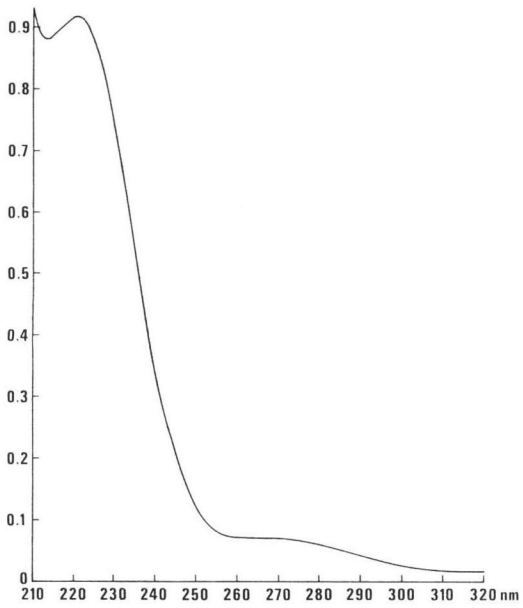
In contrast, the putative presence of glutamic acid in desertomycin could not be confirmed. Desertomycin was hydrolyzed as described[8] and by other methods commonly used in peptide hydrolysis.

Fig. 1. IR spectrum of desertomycin (reflectance plate).



The hydrolysates were lyophilized and the residue was treated sequentially with 3 N butanolic hydrochloric acid and trifluoroacetic anhydride by described methods[16] to give the *N*-trifluoroacetyl-*n*-butyl esters (TAB derivatives). A control sample of glutamic acid was treated in exactly the same manner. The analysis was done on the instrument and with the column used above for the sugar analysis. Under the conditions of the analysis (to 150°C, 5°C/minute gradient) most of the TAB derivatives of protein amino acids were well resolved between 4 and 30 minutes. Detection with total ion abundance was quite sensitive for the controls but in no case could the TAB of glutamic acid (or that of any other amino acid) be detected in the hydrolysate of authentic desertomycin or of the antibiotic isolated from *S. macronensis*. Even selected ion monitoring techniques failed to detect the presence of TAB-glutamic acid. An analysis done on an amino acid analyzer (Dionex Model D500) failed to detect glutamic acid in hydrolysate under conditions where as little as 0.05% was detectable in controls.

Fig. 2. UV spectrum of desertomycin (0.096 g/liter in pH 7.2 phosphate buffer).



Therefore, the previous report[9] of glutamic acid in desertomycin must be considered artifac-

Fig. 3. ^{13}C NMR spectrum of desertomycin at 20 MHz (Varian CFT-80 spectrophotometer) in methanol-d_4.

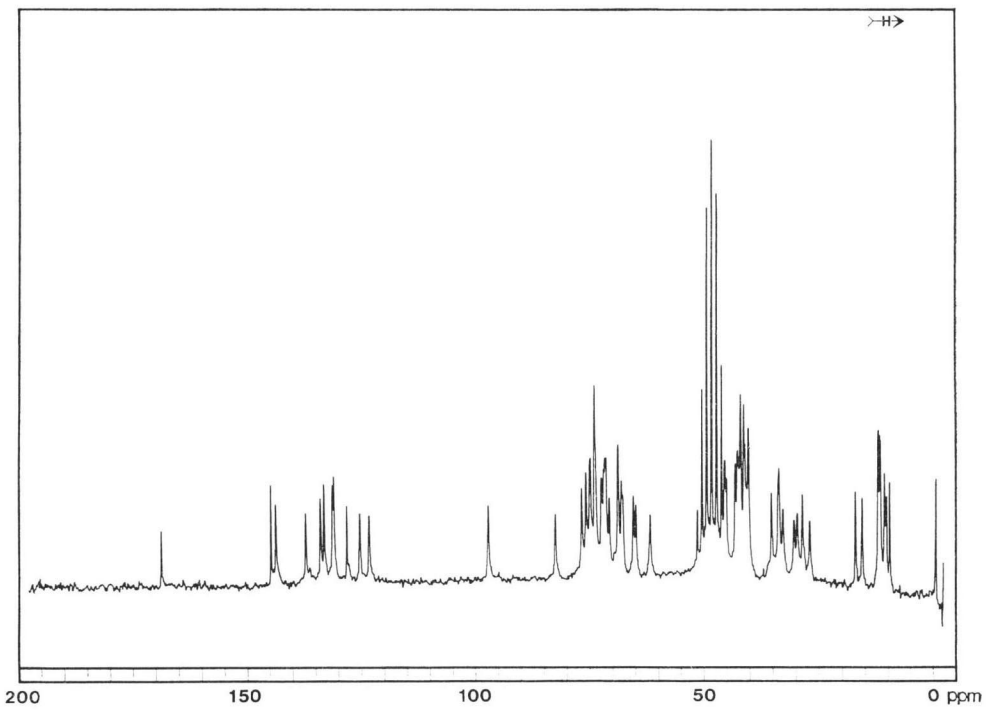


Fig. 4. ^{13}C NMR spectrum of desertomycin at 50.3 MHz (Varian XL-200 instrument) in methanol-d_4.

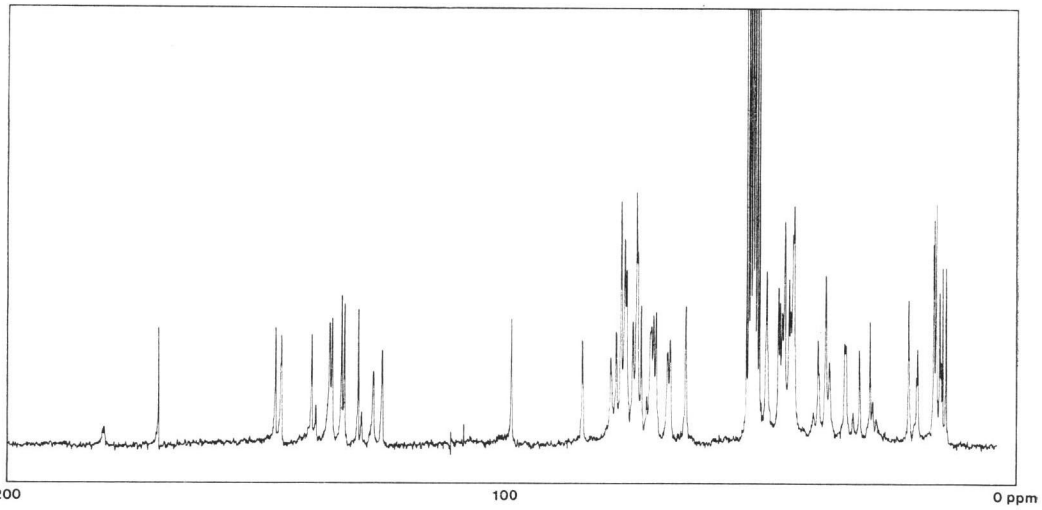


Table 1. The chemical shifts of desertomycin at 50.3 MHz (Fig. 4).

Sp^2 carbons	169.5, s; 145.5, s; 144.5, 137.9, 134.6, 133.7, 131.9, 131.6, (all d); 128.7, s; 128.6, s; 125.8, d; 123.8, d
C–O region	97.6, 83.3, 83.1, 77.4, 77.3, 76.7, 76.3, 75.8, 75.5, 74.6 (2~5 carbons), 73.1, 72.7, 72.4, 72.2, 71.5, 70.5, 69.5, 68.7, 68.5, 66.3, 66.1, 65.6 (all d); 62.7, t
Alkyls	46.2, t; 43.9, 43.8, 49.3 (all d); 43.2, t; 42.7, o; 41.9, t; 41.7, d; 41.4, t; 40.9, d; 40.8, d; 36.4, t; 35.8, d; 35.6, d; 34.3, 33.3, 30.8, 30.3, 29.0, 28.7, 27.5, 27.4 (all t)
Quartets	17.6, 17.5, 16.4, 16.3, 12.7, 12.6, 12.5, 12.3, 12.1, 11.9, 11.2, 11.0, 10.8, 10.0

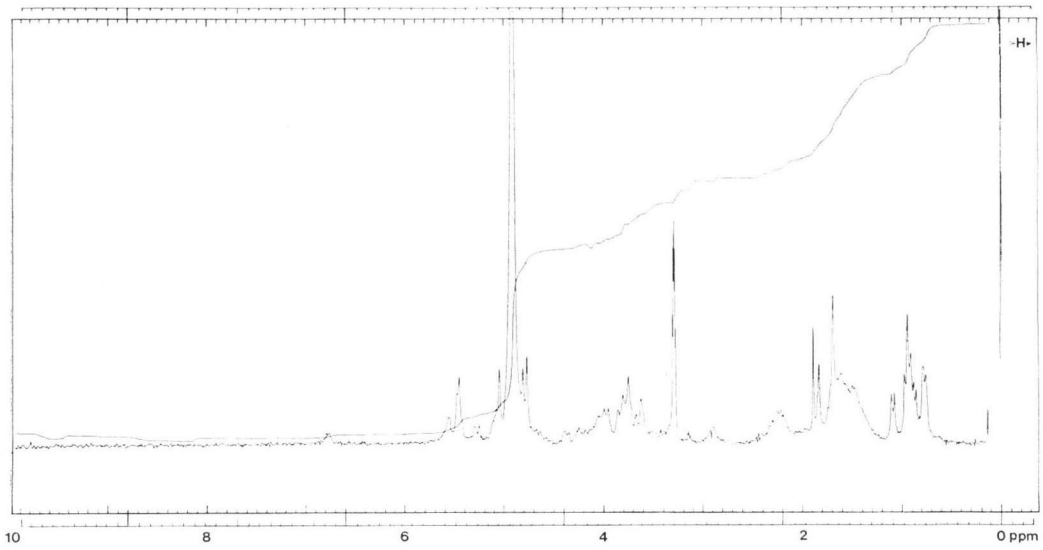
tual or due to a purification problem.

The ^{13}C NMR spectrum of desertomycin at 20 MHz is shown in Fig. 3. There are 53 lines resolved, some of which may represent more than one carbon atom. There is a close similarity between the ^{13}C NMR spectra of desertomycin and of niphithricin[10]. At 50.3 MHz (Varian XL-200 spectrometer) the spectrum is much more complex (Fig. 4). A total of 72 lines is observable. The multiplicities were determined using the INEPT technique.[17] The chemical shift data, multiplicities and assignments are presented in Table 1.

Since monazomycin[18] has exactly 72 carbons, we compared the two antibiotics in detail. The spectrum of desertomycin has more lines in the δ 123~138 region and fewer lines in the δ 26~43 region than does the ^{13}C NMR spectrum of monazomycin. This suggests that desertomycin has more carbon-carbon double bonds than does monazomycin. One of the additional double bonds in desertomycin is probably α,β to the lactone carbonyl since the UV spectrum of desertomycin shows a maximum at 223 nm while that of monazomycin shows no maximum above 200 nm. The ^{13}C NMR spectra of both compounds show one anomeric doublet, one carbonyl, and about the same number of lines due to oxygenbearing carbons. The spectrum of desertomycin shows one extra line in the *C*-methyl region compared to that of monazomycin.

The molecular formula of desertomycin was determined by the fast atom bombardment (FAB)[19] technique (by J. CARTER COOK of the University of Illinois). Both positive and negative ion spectra led to the conclusion that the molecular formula was $C_{57}H_{109}NO_{24}$. The exact mass observed for

Fig. 5. ^{1}H NMR spectrum of desertomycin at 200 MHz (Varian XL-200 instrument) in methanol-d_4.



$(M+H)^+$ was 1,192.7480 (theory 1,192.7548).

Low resolution FAB studies in our laboratory showed that authentic desertomycin and the antibiotic produced by *S. macronensis* gave identical mass spectra with the $(M+H)^+$ ion at *m/z* 1,192. We were able to differentiate desertomycin from monazomycin since we observed a $(M+H+Na)^+$ ion at *m/z* 1,388 for monazomycin[18] (MW 1,364).

The elemental analysis of desertomycin supports the suggested molecular formula.

Found:	C 57.76, H 8.62, N 1.04
Calcd. for $C_{57}H_{109}NO_{24}$:	C 57.41, H 9.21, N 1.17

The presence of only one nitrogen atom differentiates desertomycin (and monazomycin) from related antibiotics such as scopafungin, the niphithricins and primycin, each of which contain three nitrogen atoms in a guanidyl group.

The 200 MHz NMR spectrum (Varian XL-200) of desertomycin is shown in Fig. 5. The pattern of broad bands is consistent with what has been said above.

We suspect that the extra lines observed in the 50.3 MHz ^{13}C NMR spectrum are due to a subtle type of isomerism in the molecule. This isomerism cannot be due to a hemiketal function as it is in the niphithricin case since only one line is seen near δ 99 (the anomeric doublet of the mannosidyl group). The molecular formula of desertomycin requires four degrees of unsaturation. Since we suggest one carbon-oxygen double bond, one mannose ring and one lactone ring, there can only be one carbon-carbon doublebond. The ^{13}C NMR spectrum shows 10 lines in the carbon-carbon double bond region, implying 5 carbon-carbon double bonds.

It is conceivable (but not likely) that the molecular formula as determined by FAB-MS is not correct. We find this possibility to be unsatisfying since we were able to see ions related to the molecular ion of the 72-carbon monazomycin. There is no hint of ions in this range for desertomycin. We suspect that we have isolated a mixture of isomers corresponding to the $C_{57}H_{109}NO_{24}$ formula. Further work will be necessary to fully explain this dichotomy.

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References

1) URI, J.; R. BOGNAR, I. BEKESI & B. VARGA: Desertomycin, a new crystalline antibiotic with antibacterial and cytostatic action. Nature 182: 401, 1958
2) SZTARICSKAI, F. & J. URI: Characteristic chemical reactions of desertomycin. Mikrochim. Ichnoanal. Acta 1963: 431~441, 1963 (Chem. Abstr. 59: 8007a)
3) BOGNAR, R.; F. SZTARICSKAI, L. SOMOGYI, M. PUSKAS & S. MAKLEIT: Investigation of the hydrolysis products of desertomycin, Acta Chim. Acad. Sci. Hung. 56: 53~60, 1968 (Chem. Abstr. 69: 19455y)
4) AKASAKI, K.; K. KARASAWA, M. WATANABE, H. YONEHARA & H. UMEZAWA: Monazomycin, a new antibiotic produced by a *Streptomyces*. J. Antibiotics 26: 127~131, 1963
5) SAMAIN, D.; J. C. COOK & K. L. RINEHART: Structure of scopafungin, a potent nonpolyene antifungal antibiotic. J. Am. Chem. Soc. 104: 4120~4141, 1982
6) ABERHART, J.; T. FEHR, R. C. JAIN, P. DEMAYO, O. MOTL, L. BACZYNSKYJ, D. E. F. GRACEY, D. B. MACLEAN & I. SZILAGYI: Primycin. J. Am. Chem. Soc. 92: 5816~5817, 1970
7) NAMIKOSHI, M.; K. SASAKI, Y. KOISO, K. FUKUSHIMA, S. IWASAKI, S. NOZOE & S. OKUDA: Studies on macrocyclic lactone antibiotics. I. Physicochemical properties of azalomycin F_{4a}. Chem. Pharm. Bull. 30: 1653~1657, 1982
8) NAMIKOSHI, M.; S. IWASAKI, K. SASAKI, M. YANO, K. FUKUSHIMA, S. NOZOE & S. OKUDA: Studies on macrocyclic lactone antibiotics. II. Partial structures of azalomycin F_{4a}. Chem. Pharm. Bull. 30: 1658~1668, 1982
9) IWASAKI, S.; M. NAMIKOSHI, K. SASAKI, M. YANO, K. FUKUSHIMA, S. NOZOE & S. OKUDA: Studies on macrocyclic lactone antibiotics. III. Skeletal structure of azalomycin F_{4a}. Chem. Pharm. Bull. 30: 1669~1673, 1982
10) FIEDLER, H.-P.; W. WÖRNER, H. ZÄHNER, H. P. KAISER, W. KELLER-SCHIERLEIN & A. MÜLLER: Metabolic products of microorganisms. 200. Isolation and characterization of niphithricins A, B, and elaiophylin, antibiotics produced by *Streptomyces violaceoniger*. J. Antibiotics 34: 1107~1118, 1981
11) REUSSER, F.: Stability and degeneration of microbial cultures on repeated transfer. Adv. Appl. Microbiol. 5: 189~215, 1963
12) OGIHARA, Y.; O. INOUE, H. OTSUKA, K. KAWAI, T. TANIMURA & S. SHIBATA: Droplet counter-current chromatography for the separation of plant products. J. Chromatog. 128: 218~223, 1976
13) HOSTETTMANN, K.; M. H. KALDAS & K. NAKANISHI: Droplet counter-current chromatography for the preparative isolation of various glycosides. J. Chromatog. 170: 355~361, 1979
14) ARGOUDELIS, A. D.; T. A. BRINKLEY, T. F. BRODASKY, J. A. BUEGE, H. F. MEYER & S. A. MIZSAK: Paulomycins A and B, isolation and characterization. J. Antibiotics 35: 285~294, 1982
15) DEJONGH, D. C.; T. RADFORD, J. D. HRIBAR, S. HANESSIAN, M. BIEBER, G. DAWSON & C. C. SWEELEY: Analysis of trimethylsilyl derivatives of carbohydrates by gas chromatography and mass spectrometry. J. Am. Chem. Soc. 91: 1728~1740, 1969
16) LEIMER, K. R.; R. H. RICE & C. W. GEHRKE: Complete mass spectra of *N*-trifluoroacetyl-*n*-butyl esters of amino acids. J. Chromatog. 141: 121~144, 1977
17) DODDRELL, D. M. & D. T. PEGG: Assignment of proton-coupled carbon-13 spectra of complex molecules by using polarization transfer spectroscopy. A superior method to off-resonance decoupling. J. Am. Chem. Soc. 102: 6388~6390, 1980
18) NAKAYAMA, H.; K. FURIHATA, H. SETO & N. ŌTAKE: Structure of monazomycin, a new ionophorous antibiotic. Tetrahedron Lett. 22: 5217~5220, 1981
19) BARBER, M.; R. S. BORDOLI, R. D. SEDGWICK & A. N. TYLER: Fast atom bombardment of solids (F.A.B.): A new ion source for mass spectrometry. J. Chem. Soc., Chem. Comm. 1981: 325~327, 1981