PIPERAZINOMYCIN, A NEW ANTIFUNGAL ANTIBIOTIC

I. FERMENTATION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

SATOSHI TAMAI, MIYUKI KANEDA and SHOSHIRO NAKAMURA

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi, Minami-ku, Hiroshima 734, Japan

(Received for publication May 19, 1982)

A new antifungal antibiotic, named piperazinomycin, was isolated from the cultured broth of *Streptoverticillium olivoreticuli* subsp. *neoenacticus*. The antibiotic was obtained from the mycelial cake by extraction with methanol and also from the broth filtrate by adsorption on Amberlite XAD-2 and subsequent elution with aqueous acetone. The antibiotic is of basic and lipophilic nature and can be extracted with methyl isobutyl ketone at alkaline pH. Its purification was carried out by column chromatography on Sephadex LH-20 and then on Sephadex G-15 followed by preparative thin-layer chromatography on silica gel. The molecular formula of piperazinomycin was determined to be $C_{18}H_{20}N_2O_2$ by high resolution mass spectrum and the spectroscopic and chemical properties were examined. Piperazinomycin showed inhibitory activity against fungi and yeasts, especially against *Trichophyton*.

A new antifungal antibiotic, named piperazinomycin, has been isolated as a minor metabolite of *Streptoverticillium olivoreticuli* subsp. *neoenacticus* from which neo-enactin, an antifungal antibiotic potentiating polyene antifungal antibiotics,[1,2] was previously obtained as a major metabolite. This strain was also reported to produce a tetraene antifungal antibiotic, two bleomycin group antibiotics and two streptothricin-like antibiotics, simultaneously.[1,2]

This paper deals with the fermentation, isolation, characterization and biological properties of piperazinomycin. As described in a following paper,[3] piperazinomycin was named due to its novel, large cyclic structure containing a piperazine ring.

Fermentation

Streptoverticillium olivoreticuli subsp. *neoenacticus* was precultured in 500-ml Sakaguchi flasks each containing 100 ml of an inoculation medium composed of 1.0% maltose, 0.2% yeast extract and 0.2% polypeptone (pH 7.0 before sterilization) at 27°C for 30 hours on a reciprocal shaker (amplitude 7 cm, 130 strokes per minute). The resulting inoculum (2 ml) was transferred into 500-ml Sakaguchi flasks each containing 100 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soy bean meal, 0.5% Ebios (dried yeast, distributed by Tanabe Pharmaceutical Co., Ltd.), 0.25% NaCl and 0.3% $CaCO_3$ (pH 7.6 before sterilization). The culture was grown at 27°C for 36 hours on the reciprocal shaker.

The assay of antibiotic activity during fermentation and isolation was carried out by a paper-disc agar-diffusion method using *Candida albicans* Yu 1200 as the test organism on glucose nutrient agar.

Isolation

The fermentation broth was separated into mycelial cake and broth filtrate by centrifugation at 2,500 rpm for 15 minutes and subsequent filtration. Both piperazinomycin and neo-enactin in the mycelial cake were extracted with methanol, while the antibiotics in the broth filtrate were adsorbed on

Chart 1. Extraction of piperazinomycin.

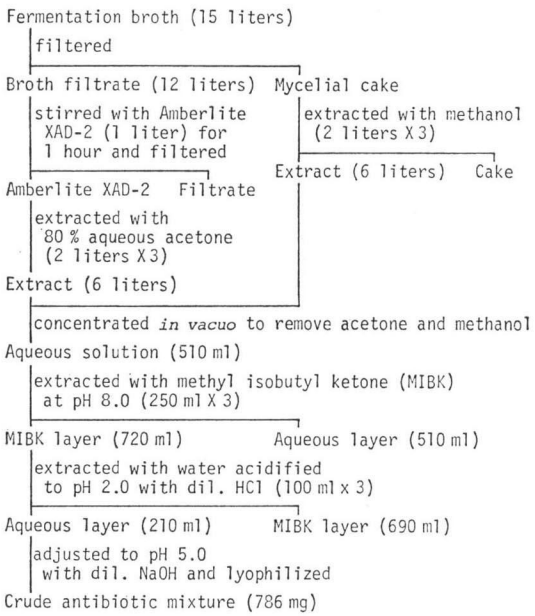
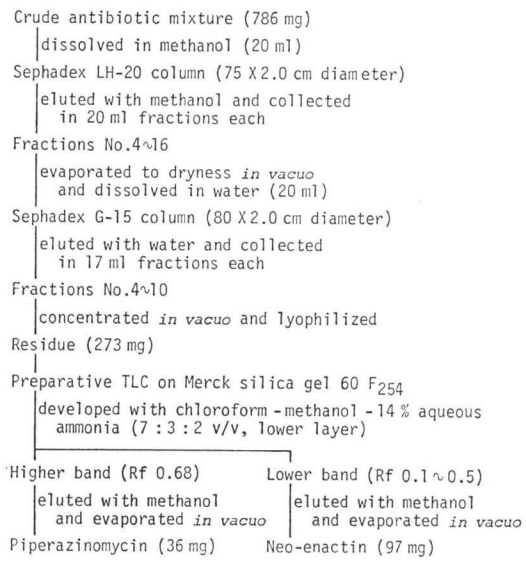


Chart 2. Purification of piperazinomycin.



XAD-2 and desorbed from the resin with 80% aqueous acetone. Both antibiotics are basic and lipophilic and can be extracted with methyl isobutyl ketone at pH 8.0. Extraction procedures of piperazinomycin and neo-enactin are shown in Charts 1 and 2.

Finally, piperazinomycin was separated from neo-enactin by preparative thin-layer chromatography on Merck silica gel 60 F_{254}, as shown in Chart 2.

Characterization

Piperazinomycin (**I**) was obtained as an amorphous powder, mp 102~104°C, $[\alpha]_D^{23}$ +31.1° (*c* 0.74, MeOH). The molecular formula of **I** was established to be $C_{18}H_{20}N_2O_2$ (M^+, *m/z* 296.1531, Calcd. 296.1525) by high resolution mass spectrum. It showed the UV absorption maxima at 214 nm (ε 11,230), 280 nm (ε 2,040) and 290 nm (sh, ε 1,690) in methanol, as shown in Fig. 1. Its IR absorption spectrum suggested the presence of a hydroxyl group or an amino group (3300 cm^{-1}) and an aromatic ring (1600, 1500 and 1430 cm^{-1}), but an absorption band due to an ester or an amide group was absent in the spectrum, as shown in Fig. 2. Piperazinomycin is easily soluble in methanol, soluble in pyridine and methyl isobutyl ketone, slightly soluble in chloroform, ethyl acetate and acetone, and insoluble in water. The presence of a secondary amino group in **I** was suggested by positive ninhydrin reaction and negative fluorescamine reaction, and the presence

Fig. 1. UV spectrum of piperazinomycin (MeOH).

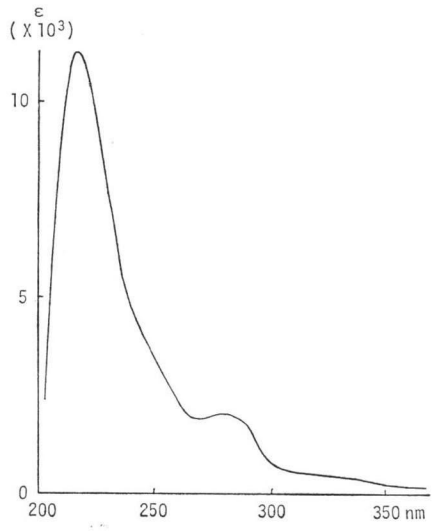


Fig. 2. IR spectrum of piperazinomycin (KBr).

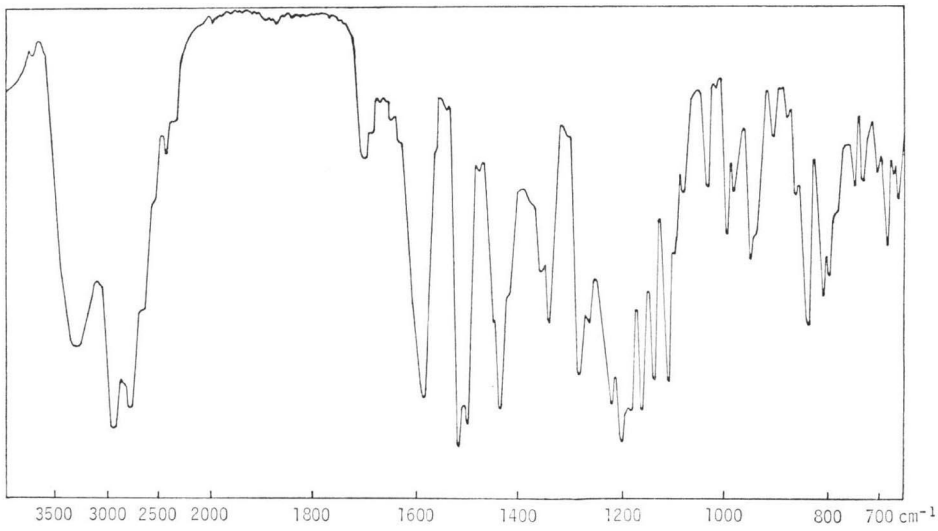
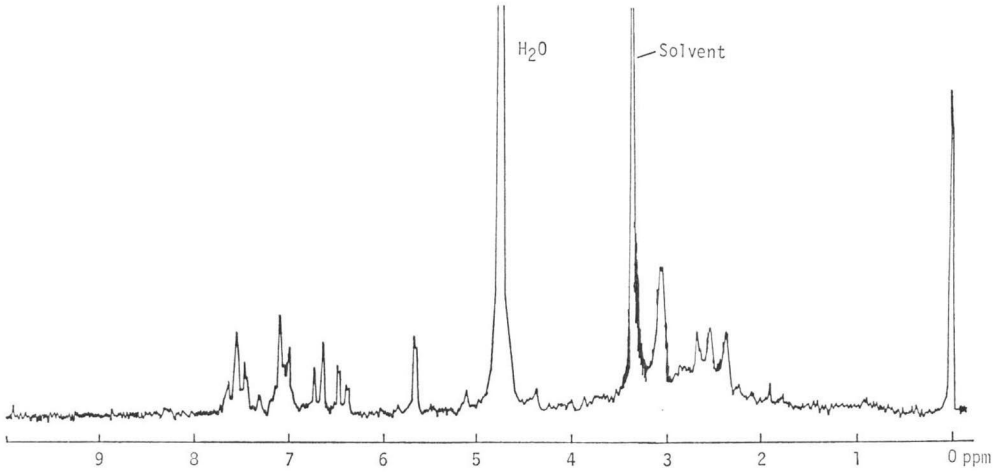


Fig. 3. PMR spectrum of piperazinomycin (CD_3OD).

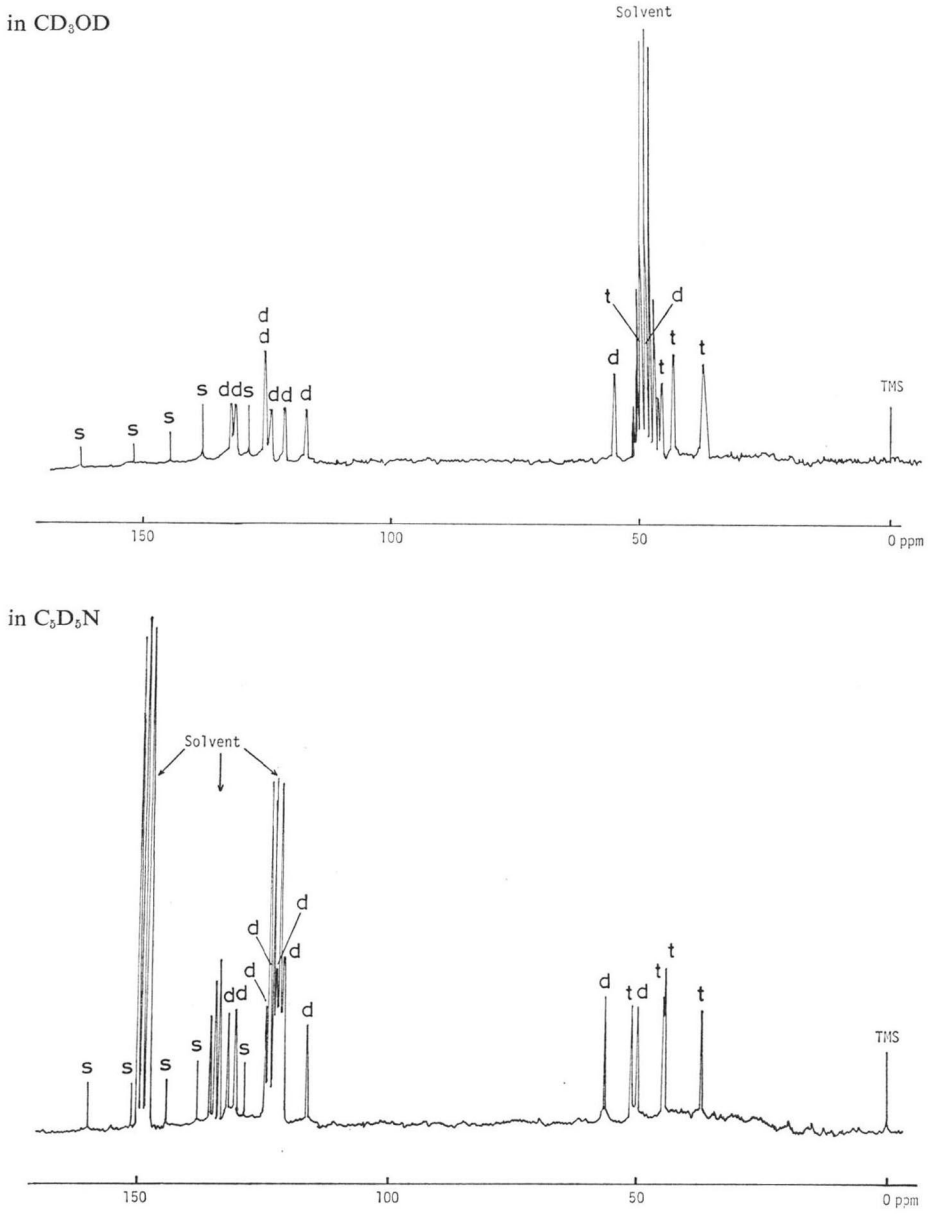


of a phenolic hydroxyl group was suggested by positive $K_3Fe(CN)_6$-$FeCl_3$ reaction and diazo reaction.

In the PMR spectrum of **I** in CD_3OD (Fig. 3), the signals at δ 6.65 (1H, d, J=7.5 Hz), δ 6.40 (1H, dd, J=7.5, 2.0 Hz) and δ 5.76 (1H, d, J=2.0 Hz) indicated the presence of a 1,2,4-trisubstituted benzene ring and the signals at δ 7.55 (2H) and δ 7.04 (2H), which were both ambiguously split, indicated the presence of a disubstituted benzene ring. Broad multiplet signals were observed at δ 2.20~3.30 indicating the presence of 8~10 aliphatic protons, but none of signals assignable to olefinic, *O*-methyl, *N*-methyl or *C*-methyl protons were observed in the spectrum.

In the CMR spectra of **I** in CD_3OD and C_5D_5N (Fig. 4 and Table 1), the signals of all eighteen carbons of **I** were observed and among them twelve sp^2 carbon signals were assigned to the carbons of two benzene rings. Out of these aromatic carbon signals, five were singlets and seven were doublets in the single-frequency off-resonance decoupled spectra of **I**. These data are consistent with those of

Fig. 4. CMR spectra of piperazinomycin in CD_3OD and in C_5D_5N.



PMR of **I**. The six sp^3 carbon signals due to four methylene and two methine carbons were observed in the high field region in the spectra, and no signals due to aliphatic carbons bound to an oxygen function were detected. In addition, three singlet signals, δ 163.3, 152.6 and 145.4, in the spectrum in CD_3OD were easily attributed to aromatic carbons bound to oxygen atoms. From these CMR data, it might be concluded that seventeen out of the twenty hydrogen atoms of **I** were bound to carbon atoms and that three remaining hydrogen atoms existed as two secondary amino and one phenolic hydroxyl groups. Furthermore, one of the two oxygen atoms of **I** was deduced to form a phenolic hydroxyl group as described above and the other to exist as a diphenyl ether.

In order to confirm the number of secondary amino group and that of phenolic hydroxyl group in **I**, selective acetylation and exhaustive acetylation were carried out as follows to yield an *N*-diacetyl and an *N,O*-triacetyl derivative, respectively.

To a solution of **I** (40 mg) in absolute methanol (2 ml), acetic anhydride (0.2 ml) was added at 0°C and the mixture was allowed to stand at 0~5°C for half an hour and then at room temperature for 5 hours. The solution was concentrated *in vacuo* and the residue was chromatographed on a silica gel column using a mixture of chloroform and methanol (40: 1) as the solvent to give an *N*-diacetate (**II**, 33 mg), amorphous powder, $C_{22}H_{24}N_2O_4$ (M^+, *m/z* 380), IR ν_{max}^{KBr} 3300 (hydroxyl group) and 1640 cm^{-1} (amide group). In the PMR spectrum of **II** in $CDCl_3$, two *N*-acetyl groups were apparent from the signals at δ 2.02 (3H, s) and 2.07 (3H,s).

Table 1. ^{13}C Chemical shifts of piperazinomycin.

Chemical shift*, multiplicity**		Carbon number
in CD_3OD	in C_5D_5N	
37.2, t	36.9, t	3 } or
43.4, t	44.5,[a] t	3′ } or
45.8, t	44.8,[a] t	1
48.9, d	49.7, d	2′
49.7, t	51.1, t	1′
55.5, d	56.3, d	2
117.6, d	116.6, d	8
122.0, d	121.8, d	5 }
124.7, d	122.8, d	6′ } or
126.0, d	124.2, d	8′ }
126.0, d	125.0, d	9
129.8, s	129.3, s	4
132.2, d	131.1, d	5′ } or
133.1, d	132.6, d	9′ } or
138.9, s	138.8, s	4′
145.4, s	144.8, s	7
152.6, s	151.2, s	6
163.3, s	160.1, s	7′

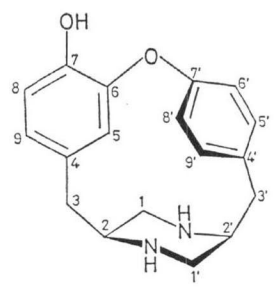
* δ in ppm from internal TMS.
** s=singlet, d=doublet, t=triplet.
[a] Values may be interchanged.

To a solution of **I**(6.0 mg) in absolute pyridine (0.5 ml), acetic anhydride (0.5 ml) was added at 0°C and the mixture was allowed to stand at 0~5°C for half an hour and then at room temperature for 17 hours. The solution was concentrated *in vacuo* and the residue was purified on a preparative TLC (silica gel) developed with chloroform - methanol (20: 1) to give an *N,O*-triacetate (**III**, 4.3 mg), amorphous powder, mp 228~231°C, IR ν_{max}^{KBr} 1765 (ester group) and 1640 cm^{-1} (amide group). Its molecular formula, $C_{24}H_{26}N_2O_5$ (M^+, *m/z* 422.1839, Calcd. 422.1839), established by high resolution mass spectrum, indicated the increase by there acetyl units ($CH_2CO \times 3$) on going from **I** to **III**. The presence of two *N*-acetyl groups and one phenolic *O*-acetyl group in **III** were also evident by the signals at δ 2.02, 2.07 (3H each, both s, *N*-Ac) and 2.44 (3H, s, phenolic *O*-Ac) observed in the PMR spectrum of **III** in $CDCl_3$.

From the above findings, it was deduced that **I** had four ring systems in all including one 1,2,4-trisubstituted and one disubstituted benzene rings which were forming a diphenyl ether, and that **I** also possessed one phenolic hydroxyl, two secondary amino, four methylene and two methine groups in it.

Finally, the complete structure and the absolute configuration of piperazinomycin were established as shown in Fig. 5 by X-ray crystallographic analysis of its monohydrobromide as described in a following paper.[3] The name of piperazinomycin was derived from its novel, large

Fig. 5. Chemical structure of piperazinomycin.



I

cyclic structure having a piperazine ring. After the structure elucidation by X-ray analysis, the CMR signals in CD_3OD and C_5D_5N were assigned, though incompletely, as shown in Table 1 by examination of the single-frequency off-resonance decoupled spectra and by consideration of known chemical shift rules[4,5,6] with the aid of the reported data for acerogenin A[7,8] whose structure contains the same aromatic system as piperazinomycin.

Biological Properties

Piperazinomycin inhibits the growth of fungi and yeasts, but, unlike neo-enactin, does not potentiate polyene antifungal antibiotics. Piperazinomycin also shows inhibitory activity against some species of *Mycobacterium*, but shows no activity against other Gram-positive or Gram-negative bacteria. Antimicrobial spectrum of piperazinomycin is shown in Table 2.

Table 2. Antimicrobial spectrum of piperazinomycin.

Test organisms	MIC (μg/ml)	Test organisms	MIC (μg/ml)
1*. *Candida tropicalis* NI 7495	>100	13. *Gloeosporium laeticolor*	>100
2. *Candida pseudotropicalis* NI 7494	100	14. *Elsinoe fawcettii* Bitancourt et Jenkins	>100
3. *Candida albicans* 3147	100	15. *Trichophyton mentagrophytes* (833)	3.12
4. *Candida albicans* Yu 1200	100	16. *Trichophyton asteroides* 429	6.25
5. *Candida krusei* NI 7492	>100	17. *Aspergillus niger* F-16	>100
6. *Saccharomyces cerevisiae*	50	18. *Piricularia oryzae*	>100
7. *Alternaria kikuchiana*	100	19. *Helminthosporium oryzae*	25
8. *Glomerella cingulata*	>100	20. *Cryptococcus neoformans* (Sanfelice) Vuillemin	50
9. *Glomerella cingulata* No. 3	>100	21. *Xanthomonas oryzae* N 5824	25
10. *Colletotrichum lindemuthianum* No. 1	100	22. *Pseudomonas phaseolicola*	>100
11. *Colletorichum gloeosporioides* Penzig	>100		
12. *Colletotrichum lagenarium*	>100		

* No. 1~6: Glucose nutrient agar medium. No. 7~22: Potato sucrose agar medium.

Discussion

Piperazinomycin is an ansa-type compound containing *m*- and *p*-bridged benzene rings and the two benzene rings are approximately perpendicular to each other as was revealed by the X-ray analysis of its monohydrobromide.[3] Piperazinomycin has an aliphatic ansa bridge containing a piperazine ring, but an amide group which is usually contained in ansamycin group antibiotics is missing in its structure.[9] This point and the physico-chemical and biological properties of piperazinomycin above described differentiate it from the known ansamycin group antibiotics.

Acknowledgement

The authors are grateful to Dr. I. SAKAMOTO and Mr. H. KANAMORI, Hiroshima Prefectural Institute of Public Health, for the measurements of high resolution mass spectra, and to Mr. T. TANAKA of this institute for the measurements of CMR spectra. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, which is gratefully acknowledged.

References

1) KONDO, H.; H. SUMOMOGI, T. OTANI & S. NAKAMURA: Neo-enactin, a new antifungal antibiotic potentiating polyene antifungal antibiotics. I. Fermentation, extraction, purification and physicochemical and biological properties. J. Antibiotics 32: 13~17, 1979

2) OTANI, T.; K. ISHIMARU, Y. KAWAKAMI, H. YOSHIYAMA, H. KONDO & S. NAKAMURA: Neo-enactin, a new antifungal antibiotic potentiating polyene antifungal antibiotics. II. Taxonomic studies of the producing microorganism and simultaneous production of bleomycin group and streptothricin-like antibiotics. Jap. J. Antibiotics 32: 720~728, 1979
3) KANEDA, M.; S. TAMAI, S. NAKAMURA, T. HIRATA, Y. KUSHI & T. SUGA: Piperazinomycin, a new antifungal antibiotic. II. Structure determination by X-ray crystallography. J. Antibiotics 35: 1137~1140, 1982
4) BEIERBECK, H.; J. K. SANDERS & J. W. APSIMON: The semiempirical derivation of ^{13}C nuclear magnetic resonance chemical shifts. Hydrocarbons, alcohols, amines, ketones, and olefins. Can. J. Chem. 55: 2813~2828, 1977
5) STOTHERS, J. B.: "Carbon-13 NMR Spectroscopy". Academic Press, New York, 1972
6) LEVY, G. C. & G. L. NELSON: ^{13}C Nuclear Magnetic Resonance for Organic Chemists. Wiley, Interscience, New York, 1972
7) NAGAI, M.; M. KUBO, M. FUJITA, T. INOUE & M. MATSUO: Studies on the constituents of Aceraceae plants. II. Structure of aceroside I, a glucoside of a novel cyclic diarylheptanoid from *Acer nikoense* Maxim. Chem. Pharm. Bull. 26: 2805~2810, 1978
8) KUBO, M.; K. TAKAHASHI, T. INOUE & M. NAGAI: Two new heterosides from *Acer nikoense* Maxim. Apiosylglucosides of diarylheptanol and arylbutanol. 22nd Symposium on the Chemistry of Natural Products, Abstract Papers pp. 283~290, Fukuoka, Japan, 1979
9) RINEHART, Jr., K. L.: Antibiotics with ansa rings. Accounts Chem. Res. 5: 57~64, 1972