A Novel Bioactive δ lactone FD-211

Taxonomy, Isolation and Characterization

OSAMU NOZAWA, TADAYASU OKAZAKI, NORIYOSHI SAKAI, TOSHI KOMURASAKI, KAZUNORI HANADA, SHIGEO MORIMOTO, ZENG-XIANG CHEN†, BI-MEI HE† and KAZUTOSHI MIZOUE*

Department of Applied Biology, Research Center of Taisho Pharmaceutical Co., Ltd., 1-403, Yoshino-cho, Omiya-shi, Saitama 330, Japan
† Department of New antibiotics, Sichuan Industrial Institute of Antibiotics, Chengdu, P. R. China

(Received for publication September 29, 1994)

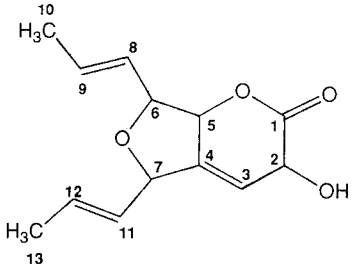
During our screening program for natural product drugs effective against multidrug-resistant mammalian cells, we have discovered a new δ lactone FD-211 from the fermantation broth of *Myceliophthora lutea* TF-0409. FD-211 had a broad spectrum activity against cultured tumor cell lines, including adriamycin-resistant HL-60 cells.

Multidrug resistance remains a serious problem in the chemotherapy of solid tumors[1]. Many tumors are either intrinsically resistant to the chemotherapeutic agent or develop resistance over the course of treatment. Mammalian cells which have acquired resistance to a single cytotoxic natural product drug can become not only resistant to the agent used but also cross-resistant to a wide range of structurally and functionally unrelated antibiotics and alkaloids[2]. As a result, treatment with chemotherapeutic agents generally result in temporary remission of tumor disease in the clinic. For this reason we have been looking for agents effective equally against parent and resistant mammalian cells.

In the course of our screening program for low molecular compounds effective against multidrug-resistant tumor cells using adriamycin-resistant HL-60 (Human promyelocytic leukemia cells), we have discovered a new δ lactone FD-211 in the fermentation broth of *Myceliophthora lutea* TF-0409. The strucuture of FD-211 is shown in Fig.1.

This paper describes the taxonomic study and fementation of strain TF-0409, as well as the fermentation, isolation, physico-chemical properties and biological activities of FD-211.

Fig. 1. Structure of FD-211.



Taxonomy

The strain F-0409 was isolated from a soil sample collected at the state of Guang-Xi in China. The strain TF-0409 grew well on the agar media used except for malt extract agar as shown in Table 1. All of the media used supported good sporulation as shown in Table 1.

Morphological observations were performed under a light microscope. Hyhae possessed septate and branched abundantly. The diameter of aerial hypae was 1.0 to 2.2 μm. Aleuriospore was formed directly from hyhae and at several sites of side branches of which tips were ampulliform swelling. Conidia was pale yellow, comparably thick- and smooth-walled. The feature of conidia was globose to subglobose, and partially ellipisoidal with $4.0 \sim 8.0\ \mu m \times 3.0 \sim 8.0\ \mu m$ in size. There was frequently small scar below 1.0 μm at the base. When the cultivation was prolonged for more three weeks, the formation of sexual spore was not recognized. Macroscopic obsevations for the colors of the aerial mycelium, and the reverse side of agar plates as well as the soluble pigment production were shown in Table 1. The strain TF-0409 could grow in the range of 17 to 41°C in Sabroad liquid medium. The optimal temperature for the growth was 29 to 35°C. The strain TF-0409 could grow at the range of pH 4 to 10 at 26°C in YpSs liqiud medium, and the optimal pH was 5 to 6.

Morphological characteristics and cultural properties of the strain TF-0409 indicated that the strain TF-0409 belongs to *Myceliophthora* in the subphylum of *Fungi*

Table 1. Cultural properties of strain TF-0471.

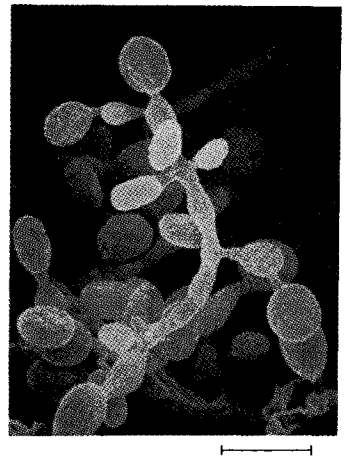
Agar medium	Growth (mm) Features of colony	Color of colony		Sporulation	Soluble pigment
		Surface	Reverse		
Potato - glucose	Moderate and slightly restricted (18~19) Felt like	Very pale yellow green (2.5 GY 9/3) Very pale yellow red (5 YR 9/2) Very pale yellow (10 YR 9/3)	Very dark yellow (5 Y 2.5/2.5) Grayish yellow (10 YR 5/3) Very pale yellow (10 YR 9/3)	Abundant	No
Oat - meal	Good (31~34) Veltinous to felt like	Light grayish yellow (5 Y 8/3) Grayish yellow (5 Y 6.5/3) Grayish red purple (5 RP 5.5/3)	Dark grayish yellow (10 YR 3.5/3) Light grayish yellow (5Y 8/3)	Abundant	No
Malt extract	Poor (14~17) Mainly submerged Hyphae were developed	Very pale yellow (5 Y 9/3) Light grayish yellow (5 Y 8/3)	Light grayish yellow (5 Y 8/3)	Poor	No
YpSs	Good (34~36) Veltinous to felt like	Light grayish yellow (7.5 Y 8/3) Very pale yellow (7.5 Y 9/3)	Dark grayish yellow (5 Y 4.5/3) Dull reddish yellow (10 YR 7/7)	Abundant	No
Czapek - Dox	Good (62~65) Felt like	Very pale yellow (5 Y 9/3)	Very pale yellow (5Y 9/3)	Abundant	No
Sabouraud	Moderate (23~27) Mainly submerged Hyphae were developed	Very pale yellow (10 YR 9/3)	Light grayish yellow (5 Y 8/3)	Poor	No
LCA (Miura)	Good (41~46) Felt like	Very pale yellow (7.5 Y 9/3)	Very pale yellow (7.5 Y 9/3)	Abundant	No

imperfecti. Comparison of data of *Myceliophthoras* reported by C. A. N. VANOORSCHOT[3,4] with those of the strain TF-0409 revealed that the strain TF-0409 very resembled to *Myceliophythola lutea* Cost. Therefore, the strain TF-0409 was identified as *Myceliophthora lutea* TF-0409. The scanning electron micrograph of the strain TF-0409 is shown in Fig. 2.

This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan as the accession No. FERM P-14057.

Fig. 2. Scanning electron micrograph of *Myceliophthora lutea* TF-0409 grown on potato - glucose agar medium.

Bar represents 2.5 μm.

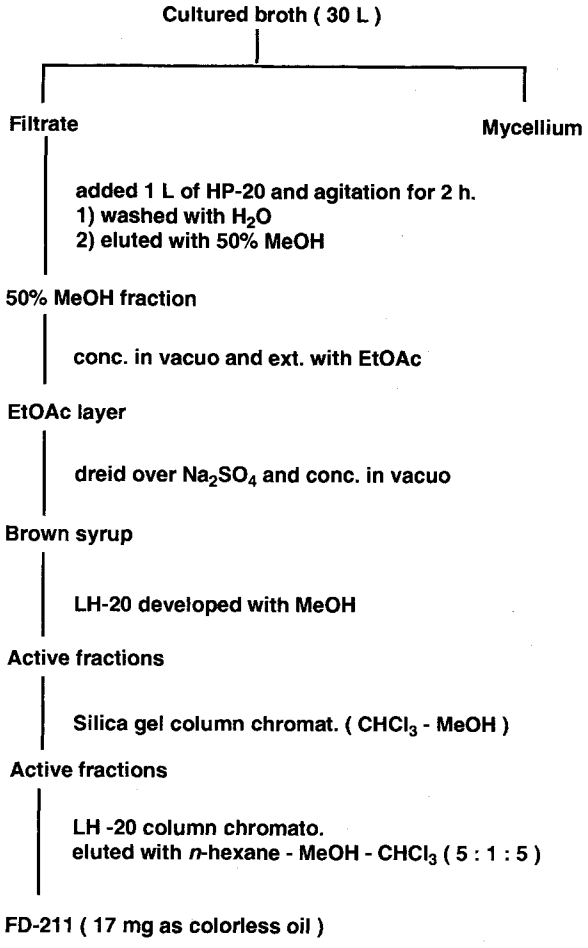


Fermentation and Isolation

A loopful of *Myceliophthora lutea* TF-0409 on oat meal agar slant was inoculated in two 500-ml Erlenmyer flasks each containing 100 ml of the medium consisting of glucose 2%, yeast extract 0.2%, NaCl 0.3%, polypepton 0.5%, Mg_2SO_4 0.05% and $KHPO_4$ 0.1%. The inoculated flasks were cultured at 26°C for 96 hours on the rotary shaker. 400 ml of the cultured broth was transferred into a 50-liter jar fermenter containing 30 liters of the same medium as in the seed culture. The fermentation was carried out at 26°C for 96 hours under airation of 30 liters per minute and agitation speed 150 rpm.

The isolation procedure is shown in Fig. 3. The whole fractionation was guided by a bioassay for cytocidal activities against adriamycin-resistant HL-60 cells. The cultured broth was separated into supernatant and mycelium by centrifugation. To 30 liters of supernatnat

Fig. 3. Isolation procedure of FD-211.



was added 1 liter of HP-20 resins and then mixed for 2 hours to absorb active material on HP-20 resins. The HP-20 resins were washed with water and then eluted with 5 liters of 50% MeOH. After removal of MeOH *in vacuo*, the aqueous layer was extracted with 2 liter of ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to give *ca.* 3.2 g of brownish syrup. The resultant material was chromatographed over a LH-20 column prepared with MeOH. Active fractions were collected and concentrated *in vacuo* to yield *ca.* 1.3 g of brownish oil. This material was applied to a silica gel column charged with $CHCl_3$. The column was eluted with $CHCl_3$ - MeOH by a stepwise of 0.05% increase in MeOH concentration from $0 \sim 1.0\%$. The active material was eluted with 0.2% of MeOH in $CHCl_3$. The active fraction was concentrated *in vacuo* and chromatographed over a Sephadex LH-20 column with $CHCl_3$ - MeOH - *n*-hexane (5 : 1 : 5) to yield about 100 mg of colorless oil. This material was further purified by Sephadex LH 20 column chromatography and developed with MeOH. The active fractions were combined and concentrated to obtain 17 mg of FD-211 as colorless oil.

Table 2. Physico-chemical properties of FD-211.

Appearance	Colorless oil
$[\alpha]_D^{26}$	$-170°$ (*c* 0.01, MeOH)
EI-MS	*m/z* 236 (M^+)
FAB-MS (+)	*m/z* 237 $(M+H)^+$
FAB-MS (−)	*m/z* 235 $(M-H)^-$
HREI-MS	*m/z* 236.1050 (found)
	m/z 236.1049 Calcd for $C_{13}H_{16}O_4$
MF	$C_{13}H_{16}O_4$ (u.s. 6)
MW	236
UV λ_{max}^{MeOH} nm (ε)	End
IR ν_{max}^{KBr} cm^{-1}	3450, 1767

Table 3. The ^1H and ^{13}C NMR data measured in $CDCl_3$.

No.	^1H (δ_H)	^{13}C (δ_C)
1		166.4
2	4.10	64.1
3	6.90 dd, J=2.4, 3.8 Hz	133.6
4		132.9
5	4.38 ddd, J=7.7, 2.4, 2.4 Hz	78.6
6	4.60 dd, J=7.5, 7.7 Hz	82.9
7	4.05 m	79.8
8	5.61 ddq, J=15.4, 7.5, 1.6 Hz	125.9
9	6.02 dq, J=15.4, 6.5 Hz	134.0
10	1.80 d, J=6.5 Hz	17.9
11	5.70 ddq, J=15.4, 7.5, 1.6 Hz	125.7
12	5.91 dq, J=15.3, 6.5 Hz	131.2
13	1.80 d, J=6.5 Hz	18.1
2-OH	2.06 br s	

Physico-chemical Properties of FD-211

The physico-chemical properties of FD-211 are described in Table 2. FD-211 is lipophilic, neutral in nature and gave positive color response to iodine, H_2SO_4 and vanillin-H_2SO_4, but negative to ninhydrin. The UV spectrum showed end absorption. The bands at 3450 cm^{-1} and 1767 cm^{-1} in the IR spectrum were ascribed to a hydroxy group and a lactone carbonyl, respectively. The presence of the latter was confirmed by the signal at 166.4 ppm in a ^{13}C NMR spectrum. The positive and negative FAB mass spectra showed pseudo-molecular ions at 237 $(M+H)^+$ and 235 $(M-H)^-$, respectively. The molecular weight was determined to be 236 by the observation of its molecular ion in the EI mass spectrum. The molecular formula of FD-211 was established as $C_{13}H_{16}O_4$ by its molecular ion measurement (M^+) at *m/z* 236.1050 (calcd. 236.1049 for $C_{13}H_{16}O_4$) in the high resolution EI mass spectrum in combination with the ^1H and ^{13}C NMR spectra. The degree of unsaturation was estimated to be 6 by its molecular formula. Three unsaturations were assigned to three double bonds and one to a carbonyl groups, leaving the final two unsaturations to accommodate two rings. The ^1H and ^{13}C NMR data of FD-211 are shown in Table 3. The

functionalities of the carbon signals of FD-211 were determined by the DEPT spectra. Consistent with its molecular formula, the ^{13}C NMR spectrum of FD-211 gave 13 lines, which were classified into $-CH_3 \times 2$, $-CHO- \times 4$, $-CH= \times 5$, $>C= \times 1$ and $>C=O \times 1$. It was revealed that 15 protons were bonded to 11 carbons with the existence of one hydroxy group in the molecule. The ^1H NMR spectrum showed 16 proton signals. Among them the line at δ_H 2.06 was exchangable by the addition of D_2O, which was assigned to a hydroxyl. The ^1H-^1H COSY spectrum showed two correlation peaks from the signal at δ_H 4.10 (H-2) to the lines at δ_H 4.90 (H-3) and at δ_H 2.06 (2-OH), respectively. The resonance at δ_H 4.60 (H-6) made up two cross peaks with the signals at δ_H 4.38 (H-5) and at δ_H 5.61 (H-8), respectively. The latter was further correlated with the line at δ_H 6.02 (H-9), which in turn coupled to the methyl signal at δ_H 1.80 (H-10). Similarly by tracing a spin network from the line at δ_H 4.05 (H-7) in the ^1H-^1H COSY spectrum, the fragment of H-7 to H-13 was deduced. These three spin systems were assembled together with a quartenary carbon at δ_C 132.9 and an ester carbonyl group at δ_C 166.4 by the HMBC experiment and the observation of N. O. E. to establish the structure of FD-211 as discussed below. The signal at δ_H 6.90 (H-3) showed long range correlations with the carbon lines at δ_C 166.4 (C-1) and at δ_C 132.9 (C-4), respectively. The latter resonance in turn coupled to two proton signals at δ_H 4.38 (H-5) and at δ_H 5.70 (H-11), respectively. The NOESY spectrum showed cross peaks from the signal at δ_H 4.05 (H-7) to those at δ_H 4.38 (H-5) and at δ_H 4.60 (H-6), respectively, indicating that these protons are spatically near to each other and on the same side as shown in Fig. 4. Taking into consideration the existence of a δ lactone and the unsaturated degrees, the structure of FD-211 was elucidated as shown in Fig. 1. The coupling constants of 15.4 Hz between H-8 and H-9 and 15.3 Hz between H-11 and H-13 indicated that their geometrical relationships were all trans. The obsevation of N.O.Es between H-7 and H-6 and between H-7 and H-5 indicated that these protons have the same configuration. The stereostructure of FD-211 will be described in detail elsewhere.

Biological Activities of FD-211

As shown in Table 4, FD-211 exerted eight to ten times weaker cytocidal activities against various cultured cell lines than adriamycin. FD-211 showed two times higher

Fig. 4. Long range couplings and N.O.E.

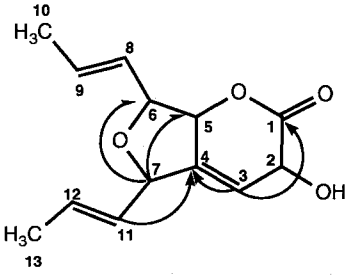


Table 4. Activities of FD-211 and adriamycin against various cultured tumor cells.

	IC_{50} (μg/ml)	
	FD-211	Adriamycin
HL-60/ADR*	0.1	2.0
HL-60	0.2	0.02
P388	4.0	0.03
T-24	0.5	0.08
Hela	1.0	0.08
A549	1.0	0.08

* Adriamycin-resistant cells.

Table 5. Inhibition of macromolecule biosynthesis by FD-211 in Hela cells.

IC_{50} (μg/ml)		
DNA	RNA	Protein
1.25	1.25	1.25

Table 6. Antimicrobial activities of FD-211, amphotericin B and clarithromycin.

	MIC (μg/ml)		
	FD-211	Amphotericin B	Clarithromycin
Candida albicans TIMM0239	>100	0.39	N.D.
Cryptococcus neoformans TIMM0354	>100	0.78	N.D.
Aspergillus fumigatus TIMM0063	>100	3.13	N.D.
Trichophyton mentagrophytes #81028	>100	1.56	N.D.
Staphylococcus aureus 209P-JC	100	N.D.	0.10
Escherichia coli NIHJ JC-2	>100	N.D.	100

N.D.: Not determined.

50% inhibitory concentration against resistant cells of HL-60 than parent cells. As depicted in Table 5, FD-211 inhibited the incorporation of thymidine and uridine labeled with ^{3}H, and leucine labeled with ^{14}C into the macromolecules, however no significant differences of the IC_{50}'s were observed. FD-211 did not prolong the survival time of mice transplanted with P388 leukemia cells (i.p.-i.p.). Among test microbes tested, FD-211 only showed a very weak activity against *Staphyloccocus aureus* Smith (Table 6).

Discussion

We have isolated a new bioactive δ lactone FD-211 from the fermentation broth of *Mycetophthora lutea* TF-0409. FD-211 possesses a, β, γ unsaturated δ lactone. Bioactive δ lactones such as LL-p880a,b[5,6], pestalotin[7], asperlin[8] and phomalactone[9~11] have been reported. However, all of these compounds have an α, β unsaturated δ lactone. Although many β, γ unsaturated δ lactones with some ring fused at Δ 3,4 have been known, δ lactones such as FD-211 include only carapolides A and B[12], which were isolated from the seeds of the plant capara procera. As far as we know, FD-211 is the first 3-hydro, Δ 3, 4 δ lactone isolated from microbial fermentation. Like other δ lactone compounds[13,14], FD-211 is expected to be synthesized from polyketide. However, the structure of FD-211 suggests that the biosynthetic pathway of FD-211 may significantly differ from that of the others. Our next purpose is to elucidate biosynthesis of FD-211. FD-211 showed moderate cytocidal activities against various cultured cancer cell lines including adriamycin-resistant HL-60. No significant differences were observed for its inhibitory effect on the incorporation of the labeled precursors into the macromolecules. FD-211 did not show any antitumor effect on P388 leukemia in mice. The mode of action of FD-211 is under study in detail.

Materials and Methods

General

Melting point was determined with a Yanagimoto micro-melting point apparatus and uncorrected. Optical rotation was measured on a Jasco DIP-360 polarimeter in 10 cm tube. IR spectrum was recorded on a Perkin-Elmer 1760 FT-IR spectrophotometer. UV spectrum measured on a Hitachi 220 spectrophotometer. EI-MS, HR-EI and FAB-MS spectra were determined with a Jeol JMX-SX 102 mass spectrometer. NMR spectra were obtained with a Jeol JMN-GX400 at ambient temperature with ^{1}H NMR at 400 MHz and ^{13}C at 100 MHz using solvent peaks as internal references downfield of TMS at 0 ppm.

Taxonomy

The media used for the identification of the fungus were; CZAPEK yeast extract agar, Czapek-Dox agar, YpSs agar, oat meal agar, potato-glucose agar, MIURA agar, SABROUD agar and malt extract agar. Morphological observations were made on potato-glucose agar after 7 days incubation at 26°C unless stated otherwise.

Color names and hue numbers indicated in Table 1 are adopted from the Book of JIS (Japanese Industrial Standard) Color Standards (JISZ 8721).

Isolation and Maintenance of Adriamycin-resistant HL-60 cell.

Adriamycin-resistant cells were isolated by stepwise selection in increasing concentrations of adriamicin starting with $2 \times IC_{50}$ (0.02 μg/ml). Cells were grown and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 1 μg/ml of adriamycin.

Cytocidal Activity

HL-60, HL-60/ADR, P388 and L1210 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. A549 lung adenocarcinoma, Hela and T-24 renal carcinoma were grown in DULBECCO's modified EAGLE's medium supplemented with 10% calf serum. All cells were maintained at 37°C in a humidified 5% CO_2 atmospher. Cells were seeded into 96-well microtiter plates (2×10^4 cells/well) and incubated for 24 hours. The test sample, dissolved in MeOH, was added in serial dilutions. After addition, the plates were incubated for 72 hours. For the evaluation of *in vitro* cytocidal activity, a microculture tetrazolium assay (MTT assay)[15] method was used. The IC_{50} value was calculated with PROBIT's method.

Inhibition of Macromolecule Biosynthesis in the Cultured Hela Cells

Hela cells grown in RPMI-160 medium containing 10% fetal bovine serum for 24 hours at 37°C in CO_2 incubator were collected by centrifugation at 1,000 rpm for 2 minutes. The cells were resuspended in a warm fresh medium (5×10^4 cells/ml) and 0.8 ml of the cell suspension was incubated with 0.1 ml of the medium containing the test compound at various concentrations at 37°C for 15 minutes prior to the addition of 0.1 ml of ^{3}H-labeled uridine and thymidine (0.25 mCi/ml) and ^{14}C-labeled leucine (0.25 mCi/ml). After incubation for 48 hours, cells were harvested, treated with 1.0 ml of cold trichroloacetic acid (TCA) was added and centrifuged at 2,000 rpm for 5 minutes. The radioactivity remained in the acid insoluble fraction was determined with a Pharmacia Betaplate liquid scintillation counter

Antimicrobial Activities

The MIC values of anti microbial activities against several yeast, fungi and bacteria were determined by an agar dilution method. Mueller-Hinton agar was used for the test at 37°C for 18 hours. Sabroad agar was used for the evaluation of antifungal activities. The test organisms and the temperature are used; *C. albicans*,

37°C for 48 hours; *C. rubidus*, 26°C for 48 hours; and *T. phyton*, 26°C for 72 hours.

References

1) GOTTESMAN, M. M. & I. PASTAN: Biochemistry of multidrug resistance mediated by the multidrug transport. Annu. Rev. Biochem. 62: 385~427, 1993
2) BIELDER, J. L.: Genetic aspects of multidrug resistance. Cancer 70: 1749~1809, 1992
3) VANOORSCHOT, C. A. N.: The genus of Myceliophthora. Persoonia 9: 401~408, 1977
4) VANOORSCHOT, C. A. N.: A revision of *Chrysosporium* and allie genera. Studies in Mycology 20: 46~56, 1980
5) ELLESTAD, G. A.; W. J. MACGAHREN & M. P. KUNSTMANN: Structure of a new fungal lactone, LL-p880a from an unidentified *Penicillium* sp. J. Org. Chem. 37: 2045~2047, 1972
6) WILLIAM, J.; G. MACGAHRAN, A. ELLESTAD, G. O. MORTON & M. P. KUNSTMANN: A new fungal lactone, LL-p880b, and a new pyrone LL-880c, from a *Penicillium* sp. J. Org. Chem. 38: 3542~3544, 1972
7) KIMURA, Y.; K. KATAGIRI, T. INOUE & S. TAMURA: Isolation and biological activity of pestalotin, a gibberellin synergist from *Pestalitia cryptomeriaecoea*. Agri. Biol. Chem. 35: 1313~1314, 1971
8) ARGOUDELIS, A. P. & J. F. ZIESERL: The structure of U-13,933, a new antibiotic. Tetrahed Lett 1969~1973, 1969
9) YAMANO, T.; S. HEMMMI, I. YAMAMOTO & K. TSUBAKI: Fermentative production of antibiotic phomalactone. Chemical Abst. 76: 12827, 1972
10) MIZUBA, S.; K. LEE & J. JIU: Three antimicrobial metabolites from aspergillus caespitosus. Can. J. Microbiol. 21: 1781~1787, 1975
11) STRUNZ, G. M.; C. J. HEISSER, M. KAKUSHIMA & M. A. STILLWELL: Metabolites of an unidentified fungus. New 5,6-dihydro-2-pyrone related to pestalotin Can. J. Chem. 52: 825~826, 1974
12) KIMBU, S. F.; J. F. AYAFOR, B. L. SONDENGAN, J. D. CONNOLLY & D. S. PYCROFT: Carapolide A, a novel hexanortriterpenoide and carapolides B and C, novel tetranorterpenpids from the seeds of capara procera (Meliaceae) Tetrahed Lett.: 1613~1616, 1984
13) TANABE, M.; T. HAMASAKI & D. THOMAS: Biosynthetic studies with carbon-13; Asperlin. J. Amer. Chem. Soc. 93: 273~274, 1971
14) TURNER, W. B. & D. C. ALDRIGE: Fungal Metabolites II. pp. 55~109, Academic Press, 1983
15) MOSMANN, T.: Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assay. J. Immunol. Methods 65: 55~63, 1983