VOL. 55 NO. 12, DEC. 2002 THE JOURNAL OF ANTIBIOTICS pp. 1036 — 1041

YM-202204, a New Antifungal Antibiotic Produced by Marine Fungus *Phoma* sp.

KOJI NAGAI[a,*], KAZUMA KAMIGIRI[a], HISAO MATSUMOTO[b], YASUHIRO KAWANO[c], MASAKAZU YAMAOKA[c], HITOSHI SHIMOI[d], MASATO WATANABE[a] and KENICHI SUZUKI[a]

[a] Microbiology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 1-1-8 Azusawa, Itabashi-ku, Tokyo 174-8511, Japan
[b] Analysis & Metabolism Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan
[c] Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan
[d] National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashihiroshima, Hiroshima 739-0046, Japan

(Received for publication July 11, 2002)

A new antifungal antibiotic, YM-202204 (**1**), was found in the culture broth of marine fungus *Phoma* sp. Q60596. The structure of **1** was determined by several spectroscopic experiments as a new lactone compound. This antibiotic exhibited potent antifungal activities against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, and also inhibited glycosyl-phosphatidyl-inositol (GPI)-anchoring in yeast cells.

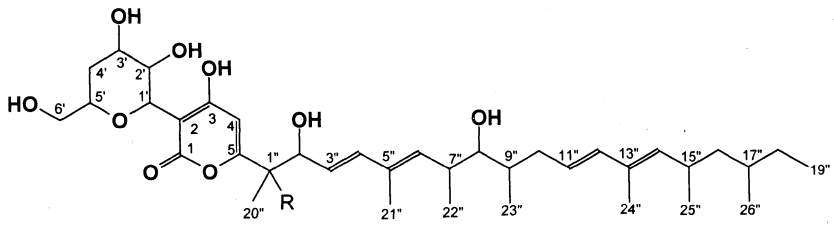
During the course of our screening for new antibiotics from the marine fungi, we found a new antifungal antibiotic YM-202204 (**1**) along with S39163/F-I (**2**) (Fig. 1)[1] from the culture broth of *Phoma* sp. Q60596 which was isolated from the marine sponge as an alkali-tolerant fungus. This antibiotic inhibited the growth of yeasts and fungi including pathogenic strains. Here we describe taxonomy of the producing organism, fermentation, isolation, physicochemical properties, structure elucidation and biological activity of the new antibiotic.

Materials and Methods

Isolation of Producing Organism

Marine sponge *Halichondria japonica* collected at Hoshisuna beach, Iriomote island, Okinawa Prefecture, Japan was used for the isolation of microorganisms. The sponge was cut into pieces *ca.* 5 mm in length. They were successively homogenized and suspended in sterile sea water. Fungi were isolated on cornmeal agar (CMA, pH 6.0; Nissui, Tokyo) and alkaline cornmeal agar (ACMA, pH

Fig. 1. Structures of YM-202204 (**1**) and S39163/F-1 (**2**).



(1) R=H
(2) R=Me

* Corresponding author: nagai@yamanouchi.co.jp

ca. 9.7) by the dilution plate method[2]. The plates were incubated at 24°C for one to three weeks.

Taxonomic Studies

The following media were used for the identification of the fungus: potato dextrose agar (PDA), cornmeal agar (CMA), Miura's medium (LCA) and CZAPEK's agar. Morphological properties were observed under an optical microscope (Nikon OPTIPHOT-2). Growth rates at various pH were examined using the method of NAGAI *et al.*[2].

Fermentation

A loopful of mycelia of strain Q60596 grown on an agar slant was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1.0%, potato starch 2.0%, yeast extract 0.5%, Polypepton (Nihon Pharmaceutical Co., Ltd.) 0.5% and $CaCO_3$ 0.4%. The pH of the medium was adjusted to 7.0 before sterilization. The seed culture was incubated at 24°C for 72 hours on a rotary shaker at 200 rpm. Two ml of the seed culture was transferred to each of thirty 500-ml Erlenmeyer flasks containing buckwheat flour 20.0 g, cellulose 10.0 g, yeast extract 0.05 g, sodium tartrate 0.03 g, K_2HPO_4 0.03 g and 80 ml of distilled water. The pH of the medium was adjusted to 7.0 before sterilization. After inoculation flasks were incubated under static conditions for 15 days at 24°C.

Isolation

Five hundred ml of acetone was added to each of thirty 500-ml flasks of solid fermentation and shaken for 10 minutes. The mixture was centrifuged at 8,000 rpm for 15 minutes and then the supernatant was evaporated *in vacuo*. The aqueous solution was adjusted to pH 7.0 and subjected to solid phase extraction with Diaion HP20. After having washed with 40% (v/v) acetone aq., the column was eluted with 80% (v/v) acetone aq. The elute was evaporated *in vacuo* to remove acetone, and the residual solution was extracted with 1-butanol at pH 7.0. The organic layer was dried, and chromatographed on silica gel using EtOAc-MeOH (30:70) as eluting solvent. Active fractions were collected and evaporated *in vacuo*. The yellow syrup was dissolved in methanol and applied to preparative HPLC (L-column ODS; Simadzu, 20 i.d.×250 mm, flow rate; 8.0 ml/minute). The column was eluted with acetonitrile-distilled water-trifluoroacetic acid (72:28:0.05) to separate the peaks of **1** and **2**. The yield of pure **1** was 27 mg.

Physico-chemical Properties and Structure Elucidation

IR spectra were recorded on a Perkin Elmer microscope FT-IR spectrometer. Optical rotation was determined on Horiba SEPA-200 polarmeter. Fast atom bombardment mass spectra (FAB-MS) were obtained with a JEOL JMS-700T using glycerol as matrix. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-ALPHA500 FT NMR spectrometer.

Bioassays

Antifungal activity (IC_{80}) against *Candida albicans* ATCC10231, *Cryptococcus neoformans* TIMM0362, *Aspergillus fumigatus* TIMM1776 and *Saccharomyces cerevisiae* YFC805 was determined by a micro dilution method of KUME and YAMAZAKI[3].

HeLa S3 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 20 mM HEPES buffer. The cells were incubated in the presence or absence of the antibiotics at 37°C for 3 days in a humidified atmosphere containing 5% CO_2. Cytotoxicity on the cells was determined by a cell counting kit of Wako Pure Chemical Industries, Ltd.

Inhibition of glycosyl-phosphatidyl-inositol (GPI)-anchored protein was measured by the following method. *Saccharomyces cerevisiae* YPH499 (*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*)[4] was used as a host for the expression of the following plasmid. Plasmid pMK1 contains a fusion gene encoding the full length of the Taka-amylase A[5] (TAA) and *C*-terminal 306 amino acid residues of a GPI-anchored protein Sed1p[6] (TAA-Sed1). Releasing of GPI-anchored proteins into the agar medium is followed by a halo formation[5,7]. GPI-anchored proteins released in the liquid medium were separated from cells by centrifugation at 3,000 rpm for 10 minutes. TAA activity of the culture medium was measured with a kit of Kikkoman Co., Ltd.

Results

Taxonomy of the Producing Strain

Cultural and morphological characteristics of the strain Q60596 were as follows: colonies were fast-growing, reaching 35 mm in diameter in 14 days at 24°C on PDA, producing raised wooly mycelium. The color of the colonies was dark olive to dark gray and reverse side of the colonies was dark gray. Soluble pigment was not observed around colonies. The fungus produced dark brownish pycnidia, which were immersed into the medium. Pycnidia were mostly globose to subglobose, 100~250 μm in diameter, with a single ostiole (Fig. 2A). Conidia were single-celled, hyaline, smooth, ellipsoidal to ovoid, 2.5~4×

1.5~2 μm (Fig. 2B). Dark brownish chlamydospores were observed. The pH range for growth was 5.0 to 10.0 with an optimum pH range of 6.0 to 9.0, which indicated this strain as an alkali-tolerant fungi.

Based on cultural and microscopic characteristics described above, strain Q60596 was considered to belong to the genus *Phoma*[8]. The strain has been deposited in the National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, as *Phoma* sp. Q60596, with the accession No. FERM P-17526.

Production and Isolation

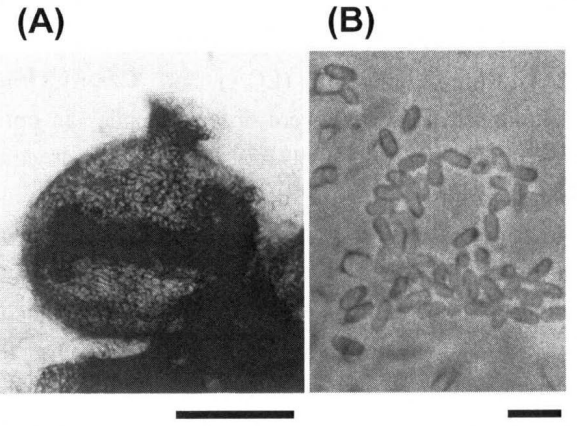
The production of **1** in the solid medium containing buckwheat flour and cellulose peaked after 15 days incubation at a titer of 1.2 mg/flask. The pH of the media (pH 6, 7, 8 and 9) didn't affect the production of **1** (data not shown).

Solid culture (thirty 500-ml flasks) was extracted with acetone, then the aqueous solution was subjected to Diaion HP20 extraction and eluted with 80% (v/v) acetone aq. After the eluate was extracted with 1-butanol, the extract was chromatographed on a silica gel column to give a crude syrup. The syrup was purified by preparative HPLC using a column of ODS to give **1** as a yellow syrup.

Physico-chemical Properties

The molecular formula of **1** was determined to be $C_{37}H_{58}O_9$ on the basis of positive-ion high resolution FAB-MS data ($(M+H)^+$ *m/z* calcd: 647.4157, found: 647.4158). The IR spectral data had an absorption band at 3221 cm^{-1} indicating the presence of a hydroxyl group, and absorption bands at 1667 cm^{-1}, 1199 cm^{-1} and 1138 cm^{-1} indicating presence of an ester group. The physico-chemical properties of **1** were shown in Table 1.

Fig. 2. Optical micrograph of strain Q60596.



(A) Pycnidium. Bar represents 100 μm. (B) Conidia. Bar represents 5 μm.

Structure Elucidation

The ^{13}C NMR spectrum of **1** showed 37 carbon signals, which were assigned to eight methyl, five methylene, eighteen methine and six quaternary carbons by a DEPT experiment. The ^{1}H and ^{13}C NMR spectral data of **1** are summarized in Table 2. A ^{1}H-^{1}H DQF COSY experiment of **1** revealed four spin networks, from 1′-H to 6′-H, from 4″-H to 20″-H, from 6″-H to 12″-H and from 14″-H to 19″-H as shown in Fig. 3. From analysis of the heteronuclear multiple-bond correlation (HMBC)[9] spectrum, ^{1}H-^{13}C long-range observed from 1′-H to C-5′, C-1, C-2 and C-3, from 4′-H to C-2, C-3, C-5 and C-1″, from 21″-H to C-4″,

Table 1. Pysico-chemical properties of YM-202204 (**1**).

Appearance	Yellow syrup
$[\alpha]_D^{25}$	-16 (*c* 0.1, MeOH)
Molecular formula	$C_{37}H_{58}O_9$
FAB-MS (*m/z*)	647 $(M+H)^+$
HRFAB-MS (*m/z*)	
calcd:	647.4157 $(M+H)^+$
Found:	647.4158 $(M+H)^+$
UV λ_{max} nm (ε) (in MeOH)	289 (5,400), 236 (32,000), 208 (17,000)
IR ν_{max} cm^{-1}	3221, 2962, 2926, 1667, 1583, 1433, 1199, 1138, 964, 885, 840, 800
Solubility	
soluble in	EtOAc, MeOH, DMSO
insoluble in	H_2O

Table 2. ^{1}H NMR and ^{13}C NMR data of YM-202204 (**1**).

Position	δ_C[a]	δ_H[b]
1	167.2	
2	100.4	
3	170.9	
4	102.3	6.08 (s)
5	168.9	
1'	75.7	4.49 (d, *J*=9.8 Hz)
2'	73.5	4.01 (t, *J*=9.8 Hz)
3'	74.1	3.65 (m)
4'	36.6	1.95 (ddd, *J*=12.8, 4.9, 1.4 Hz), 1.56 (m)
5'	78.4	3.62 (m)
6'	65.7	3.57 (2H, m)
1"	46.5	2.63 (m)
2"	76.0	4.22 (t, *J*=8.2 Hz)
3"	127.7	5.54 (m)
4"	139.3	6.31 (d, *J*=15.9 Hz)
5"	133.8	
6"	136.1	5.60 (d, *J*=9.8 Hz)
7"	36.7	2.77 (m)
8"	80.3	3.20 (dd, *J*=7.9, 3.7 Hz)
9"	38.8	1.50 (m)
10"	37.0	2.46 (m), 1.90 (dt, *J*=14.0, 8.5 Hz)
11"	126.7	5.53 (m)
12"	137.9	6.03 (d, *J*=15.9 Hz)
13"	133.3	
14"	138.2	5.06 (d, *J*=9.8 Hz)
15"	31.3	2.59 (m)
16"	46.2	1.29, 1.09 (m)
17"	33.6	1.26 (m)
18"	31.3	1.30, 1.14 (m)
19"	11.7	0.86 (3H, t, *J*=7.0 Hz)
20"	15.3	1.15 (3H, d, *J*=7.3 Hz)
21"	13.0	1.79 (3H, d, *J*=1.2 Hz)
22"	18.7	1.03 (3H, d, *J*=7.3 Hz)
23"	16.4	0.81 (3H, d, *J*=6.7 Hz)
24"	13.0	1.73 (3H, s)
25"	22.1	0.93 (3H, d, *J*=6.7 Hz)
26"	19.6	0.84 (3H, d, *J*=6.1 Hz)

[a] 125 MHz, CD_3OD as solvent.
[b] 500 MHz CD_3OD as solvent.

C-5" and C-6", from 24"-H to C-12", C-13" and C-14" as shown in Fig. 3. The geometrical configuration of C-5" was determined to be 5"*E* by NOE observed between 7"-H and 21"-H. The 13"*E* configuration was confirmed by NOEs observed between 12"-H and 14"-H and observed between 15"-H and 24"-H. The 3"*E* and 11"*E* configuration were confirmed by coupling constants (J=15.9 Hz). Presence of an α-pyrone was determined by comparison with the chemical shifts of carbons (C-1, C-2, C-3, C-4 and C-5) of dactylfungins A and B[10].

Biological Activities

Antifungal activity (IC_{80}) was determined by the micro dilution method[3]. The IC_{80}s of **1** against *Candida albicans* ATCC10231, *Cryptococcus neoformans* TIMM0362, *Aspergillus fumigatus* TIMM1776 and *Saccharomyces cerevisiae* YFÇ805 were 6.25 μg/ml, 1.56 μg/ml, 12.5 μg/ml and 1.56 μg/ml, respectively. Cytotoxicity of **1** was examined against HeLa S3 cells *in vitro*, and the IC_{50} value was >100 μg/ml.

We found **1** as the inhibitor of glycosyl-phosphatidyl-inositol (GPI)-anchoring. Inhibition of GPI-anchored

Fig. 3. ^{1}H-^{1}H DQF COSY, HMBC and NOE experiments of YM-202204 (**1**).

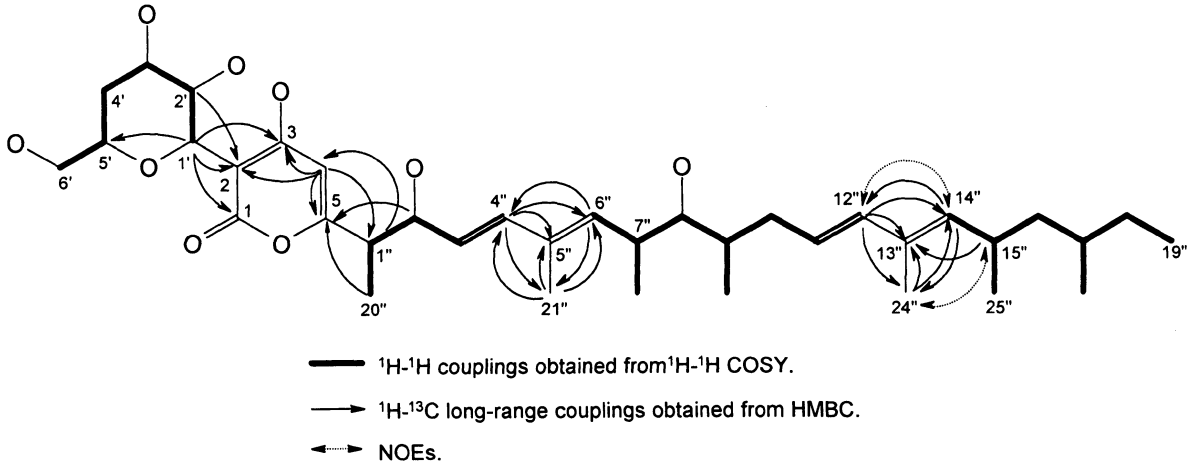


Table 3. TAA activity in the culture medium treated with YM-202204 (**1**).

	Concentration (μg/ml)		
	3	1	0
Time (hrs)	(x 10^{-3} U/ml)		
0	0.597	0.597	0.597
1	8.95	4.30	0.537
2	8.41	9.49	1.79
4	14.0	11.8	4.83
8	45.8	28.1	11.8

proteins was measured by the method described in Materials and Methods. When *S. cerevisiae* YPH499 harboring pMK1 was cultured without the antibiotic, TAA-Sed1 fusion proteins were localized in the cell wall. When *S. cerevisiae* YPH499 harboring pMK1 was cultured with **1** at 1.0 μg/ml for one hour, TAA activity of the culture medium containing **1** is 8-fold higher than that of the liquid medium without **1** as shown in Table 3. This result suggested that **1** inhibited retention of TAA-Sed1 fusion protein in the cell wall and released it into the culture medium.

Discussion

In this study, YM-202204 was isolated from the culture broth of marine fungus *Phoma* sp. Q60596. YM-202204 was shown to be a new member of the family of fungal products, which contain an α-pyrone substituted with a polyalcohol and a long side chain. Examples of previously reported congeners are S39163/F-I (**2**), dactylfungins, fusapyrone[11], deoxyfusapyrone, *etc.*[12,13]. YM-202204 exhibited a relatively wide range of antifungal activities *in vitro* and inhibition of fungal GPI-anchoring activity. GPI-anchoring represents a mechanism for attaching proteins to membranes that is used among all eucaryotic cells[14]. Since GPI-anchored protein is essential for the growth of yeasts and fungi[15,16], this may be one of the unique targets for antifungal chemotherapy. Recently novel GPI-anchoring inhibitors, isolated from fungal broth, have been reported by the Novartis group[17,18]. One of these compounds was known to block the addition of the third mannose to the

intermediate structure Man2-GlcN-acylPI and inhibit GPI-synthesis in both *C. albicans* and in animal cells. Additional studies related the mechanism of action of YM-202204 are being pursued.

Acknowledgement

This study was carried out as a part of the project for Technological Development of Biological Resources in Bioconsortia on R&D of New Industrial Science and Technology Frontiers which was performed by Industrial Science, Technology and Environmental Policy Bureau, Ministry of Economy, Trade & Industry, and entrusted by New Energy and Industrial Technology Development Organization (NEDO).

References

1) TSCHER, H.; H. HOFMAN, R. EWALD & M. M. DREYFUSS (Sandoz A. G.): Antibiotic lactone compound. U. S. 4,753,959, June 28, 1988
2) NAGAI, K.; T. SAKAI, R. M. RANTIATMODJO, K. SUZUKI, W. GAMS & G. OKADA: Studies on the distribution of alkalophilic and alkali-tolerant soil fungi I. Mycoscience 36: 247~256, 1995
3) KUME, H. & T. YAMAZAKI: Practical measurement of antifungal activity. (in Japanese) Clinical Microbiology (JAPAN) 21: 572~580, 1994
4) SIKORSKI, R. S. & P. HIETER: A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19~28, 1989
5) NAGASHIMA, T.; S. TADA, K. KITAMOTO, K. GOMI, C. KUMAGAI & H. TODA: Site-directed mutagenesis of catalytic active-site residues of taka-amylase A. Biosci. Biotech. Biochem. 56: 207~210, 1992
6) SHIMOI, H.; H. KITAGAKI, H. OHMORI, Y. IIMURA & K. ITO: Sed1p is a major cell wall protein of *Saccharomyces cerevisiae* in the stationary phase and is involved in lytic enzyme resistance. J. Bacteriol. 180: 3381~3387, 1998
7) KAMBE-HONJOH, H.; K. OHSUMI, H. SHIMOI, H. NAKAJIMA & K. KITAMOTO: Molecular breeding of yeast with higher metal-adsorption capacity by expression of histidine-repeat insertion in the protein anchored to the cell wall. J. Gen. Appl. Microbiol. 46: 113~117, 2000
8) DOMSCH, K. H.; W. GAMS & T-H. ANDERSON: Compendium of Soil Fungi. Vol. 1. pp. 630~643, IHW-Verlag, 1993
9) BAX, A. & M. F. SUMMERS: ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiplebond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2390, 1975
10) XAIO, J.; S. KUMAZAWA, N. YOSHIKAWA, T. MILAWA & Y. SATO: Dactylfungins, novel antifungal antibiotics produced by *Dactylaria parvispora*. J. Antibiotics 46: 48~55, 1993
11) ALTOMARE, C.; G. PERRONE, M. C. ZONNO, A. EVIDENTE, R. PENGUE, F. FANTI & L. POLONELLI: Biological characterization of fusapyrone and deoxyfusapyrone, two bioactive secondary metabolites of *Fusarium semitectum*. J. Nat. Prod. 63: 1131~1135, 2000
12) BREINHOLT, J.; C. N. ROSENDAHL & H. DEMUTH (Novo Nordisk A/S): Fungicidally active compounds, their manufacture with *Fusarium*, and their use in timber, cosmetics, and feed or food. W. O. 9,322,444, November 11, 1993
13) CHAIET, L.; S. ZIMMERMAN, B. SHELDON, R. L. MONAGHAN & G. M. GARRITY (Merck and Co., Inc.): Antifungal antibiotic from *Fusarium*. U. S. 5,008,187, April 16, 1991
14) MCCONVILLE M. J. & M. A. J. FERGUSON: The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem. J. 294: 305~324, 1993
15) LEIDICH, S. D.; D. A. DRAPP & P. ORLEAN: A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. J. Biol. Chem. 269: 10193~10196, 1994
16) LEIDICH, S. D.; Z. KOSTOVA, R. R. LATEK, L. C. COSTELLO, D. A. DRAPP, W. GRAY, J. S. FASSLER & P. ORLEAN: Temperature-sensitive yeast GPI anchoring mutants gpi2 and gpi3 are defective in the synthesis of *N*-acetylglucosaminyl phosphatidylinositol: cloning of the GPI2 gene. J. Biol. Chem. 270: 13029~13035, 1995
17) SÜTTERLIN, C.; A. HORVATH, P. GEROLD, R. T. SCHWARZ, Y. WANG, M. DREYFUSS & H. RIEZMAN: Identification of a species-specific inhibitor of glycosylphosphatidylinositol synthesis. Embo J. 16: 6374~6383, 1997
18) WANG, Y.; M. DREYFUSS, M. PONELLE, L. OBERER & H. RIEZMAN: A glycosylphosphatidylinositol-anchoring inhibitor with an unusual tetracarbocyclic sesterterpene skelton from the fungus *Codinaea simplex*. Tetrahedron 54: 6415~6426, 1998