HETEROCYCLES, Vol. 63, No. 11, 2004, pp. 2581 - 2589
Received, 24th August, 2004, Accepted, 21st September, 2004, Published online, 22nd September, 2004

THE ABSOLUTE STRUCTURES OF DIHYDROEPIHEVEADRIDE, AS CHARACTERISTIC ANTIFUNGAL AGENT AGAINST FILAMENTOUS FUNGI, AND ITS RELATED COMPOUNDS FROM UNIDENTIFIED FUNGUS IFM 52672

Tomoo Hosoe[a], Kazutaka Fukushima[b], Takeshi Itabashi[a], Koohei Nozawa[a], Kayoko Takizawa[b], and Ken-ichi Kawai[a]*

[a]Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41, Shinagawa-Ku, Tokyo 142-8501, Japan. E-mail: kawai@hoshi.ac.jp
[b]Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8673, Japan.

Abstract - A new nonadride derivative, deoxoepiheveadride (**2**), was isolated along with dihydroepiheveadride (**1**), as a characteristic antifungal agent, from unidentified fungus IFM 52672. The absolute structures of **1** and **2** were elucidated by the spectroscopic and chemical investigation. The absolute configuration of heveadride (**4**), which had been isolated from *Bipolaris heveae* CBS 241.93, was also determined by X-Ray crystallographic analysis.

INTRODUCTION

The incidence of life-threatening fungal infections has steadily increased in immunocompromised hosts such as HIV infected persons and cancer patients.[1] Invasive pulmonary aspergillosis and *Pneumocystis carinii* pneumonia are a leading cause of deaths in bone marrow transplant recipients and in HIV-infected patients, respectively. Moreover, resistance to the azoles which are the most widely used antifungals today is attracting much attention. Therefore, new antifungal agents are continuously required to overcome the above fungal diseases. In the screening for new antifungal substances from fungal sources against pathogenic filamentous fungi, *Aspergillus fumigatus* FRESENIUS IFM 41362 and *Aspergillus niger* VAN TIEGHEM IFM 41398, and/or pathogenic yeasts, *Candida albicans* (ROBIN) BERKHOUT IFM 40009 and *Cryptococcus neoformans* (SANFELICE) VUILLEMIN ATCC 90112, we reported the isolation and the planar structure of dihydroepiheveadride (**1**), as antifungal agent against *A. fumigatus* and *A. niger*, from unidentified fungus IFM 52672 in the previous paper. [2]

Further investigation of the above fungus brought us the isolation of new nonadride derivative, deoxoepiheveadride (**2**). The absolute stereochemistry of **1** and **2** including heveadride (**4**)[2,3] and the antimicrobial activity of these compounds were discussed in this paper.

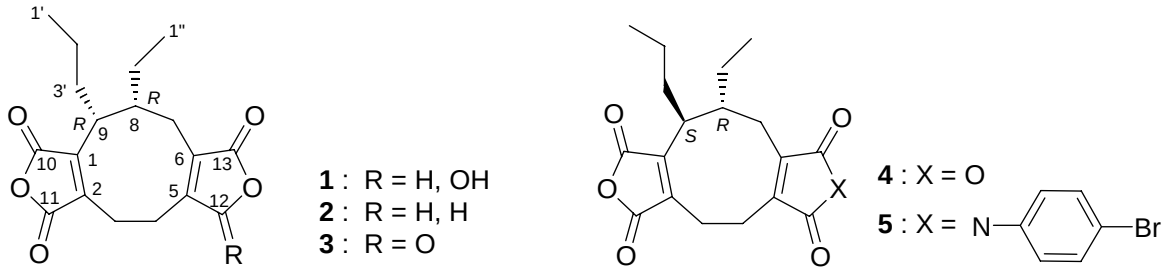


Figure 1. Structures of compounds (**1**- **5**)

RESULTS AND DISCUSSION

The molecular formula of **2** was confirmed as $C_{18}H_{22}O_5$ by HREI-MS spectrum. The ^{1}H- and ^{13}C- NMR spectra of **2** was similar to those of **1**, expect for the upfield shift of the carbon at C-12 from δ 98.7 in **1** to δ 71.2 in **2** and the new appearance of methylene signal [δ 4.70 (1H, dd, *J*=17.4, 2.5 Hz) and δ 4.81 (1H, d, *J*=17.4 Hz)] in **2** instead of methine signal [δ 6.04 (1H, s)] in **1** (Table.1) . The analysis of the ^{1}H-^{1}H COSY, HMQC and HMBC spectra supported the planar structure of **2**, as 12-deoxo derivative of **1** (Figure 2) . In order to confirm the relative stereochemistry, the X-Ray crystallographic analysis was undertaken. Compound (**2**), derived from dihydroepiheveadride (**1**) by reduction with $NaBH_4$ followed by oxidation with PCC, was crystallized from acetone as colorless prisms, which is suitable for X-Ray analysis. The crystal structure was solved by direct methods and the final *R* (*R*w) value reached to 0.066 (0.076). The molecular structure was established to be as shown in Figure 3. The bond lengths and angles are not significantly different from the expected ones. Two alkyl side chains were consequently oriented as *cis* configuration. From the above result, the relative stereochemistry of **1**, **2** and **3**, the oxidative of **1** was determined.

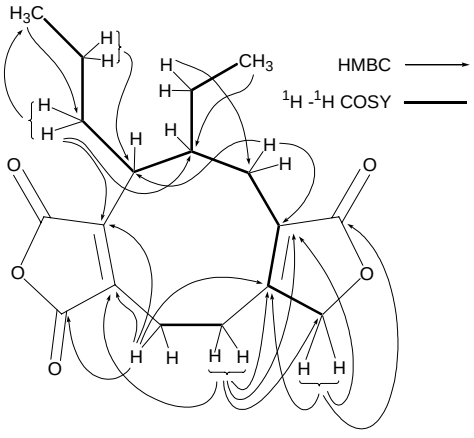


Figure 2. HMBC and ^{1}H - ^{1}H COSY correlations in **2**

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

No.	**1**		**2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	146.5		146.9	
2	146.5		145.6	
3	21.8	2.49 m	22.2	2.20 m
		3.08 ddd (13.7, 10.0, 1.2)		3.11 ddd (13.7, 9.0, 1.4)
4	24.3	2.36 m	24.6	2.48 br t (14.3, 10.7)
		2.73 dd (13.8, 9.0)		2.58 ddd (14.3, 9.0, 1.1)
5	158.8		159.7	
6	130.6		127.8	
7	26.7	1.51m	26.9	1.55 m
		2.64 br d (13.7)		2.68 br d (13.5)
8	46.5	2.04 m	46.4	2.10 m
9	40.1	2.64 m	40.5	2.50 m
10	164.5		164.3	
11	165.8		165.6	
12	98.7	6.04 s	71.2	4.70 dd (17.4, 2.5)
				4.81 br d (17.4)
13	171.8		173.7	
1'	13.9	0.86 t (7.1)	13.9	0.86 t (7.3)
2'	21.7	1.14 m	21.8	1.12 m
		1.14 m		1.12 m
3'	31.5	1.51 m	31.5	1.50 m
		2.14 m		2.10 m
1"	12.9	1.06 t (7.1)	12.9	1.07 t (7.3)
2"	23.2	0.95 m	23.2	0.98 m
		2.04 m		2.00 m

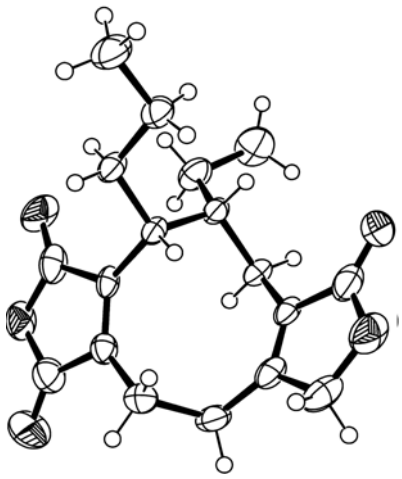


Figure 3. Perspective view of the crystal structure of **2**

with thermal ellipsoids at 50% possibility

Since the stereochemistry of heveadride (**4**) had not been determined at all, the relationship of the relative stereochemistry between heveadride (**4**) and epiheadride (**3**) was determined as followed. When heveadride (**4**), isolated from *Bipolaris heveae* CBS 241.93 as described in the previous paper, [2] was

treated with *p*-TsOH in the mixted solvent of toluene-benzene by heating, the small amount of epiheveadride (**3**), which was synthsized by PCC oxidation of **1** as described in previous paper,[2] appeared. Compound (**3**) was identified on HPLC, showing (-) peak on optical rotation detector as the same as the oxidative product of **1**, naturally occurring one (Figure 4). Therefore the absolute configuration of **3** at C-8 should be as the same as that of **4**, i.e. epiheveadride (**3**) was C-9 epimer of heveadride (**4**).

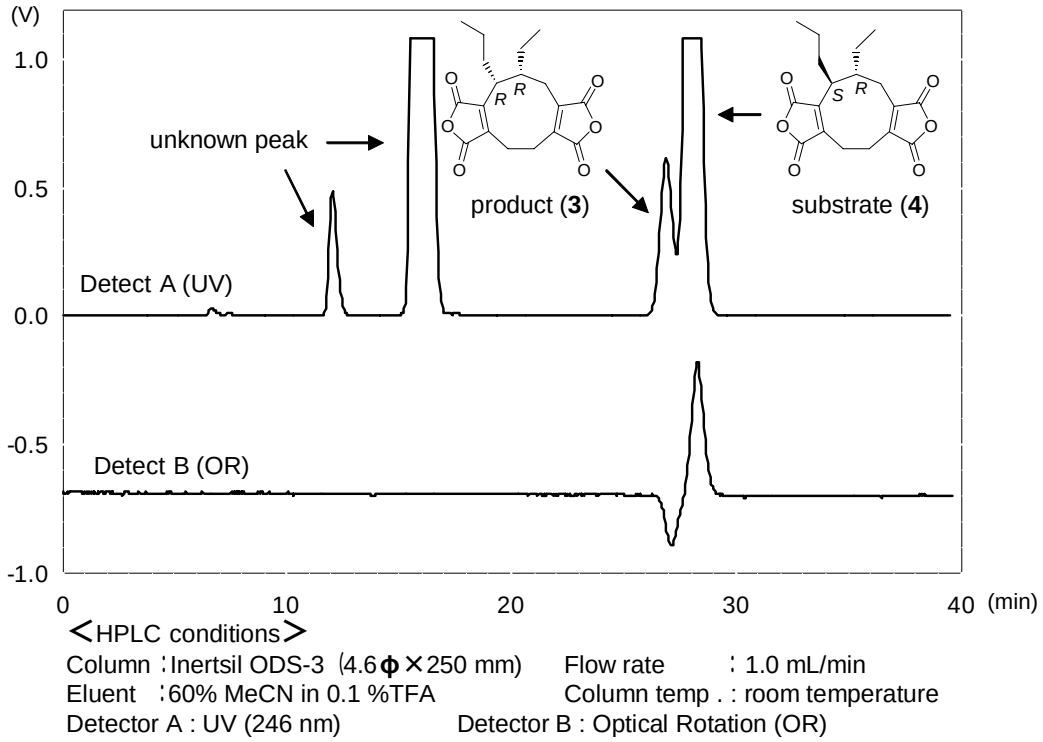
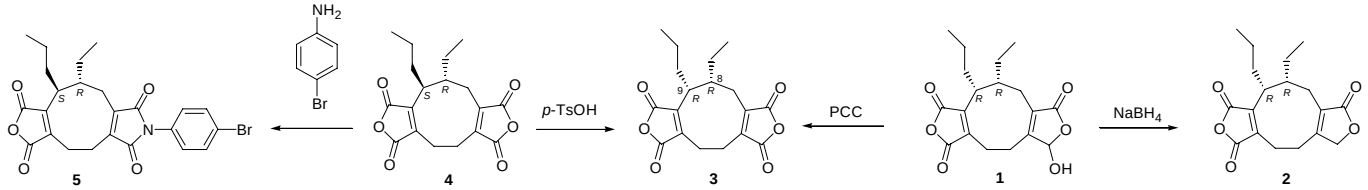


Figure 4. HPLC chromatogram for epimerization of heveadride (**4**)



Scheme

In order to confirm the absolute configuration of epiheveadride (**3**) and heveadride (**4**) etc., the crystallization of various bromo imide derivatives of **3** and **4** was attempted. The imide derivatives of **3** could not be grown to suitable crystals, but the crystals of *p*-bromoanilide (**5**) of **4**, grown from acetone as

colorless plate, were fortunately suitable for X-Ray analysis. The crystal structure of **5** was solved by direct methods and the *R* (*R*w) value reached to 0.093 (0.101). It was clear that two alkyl side chains were oriented as *trans*-configuration. The absolute structure of **5** was determined by the anomalous dispersion method as shown in Figure 5, so the absolute structure of heveadride (**4**) was elucidated as shown in Figure 1. The absolute structure of **3** was therefore confirmed, since **4** was epimerized to **3** by *p*-TsOH . The absolute structure of naturally occurring **1** and **2**, which were correlated with **3** by chemical reactions, was consequently confirmed as shown in Figure 1.

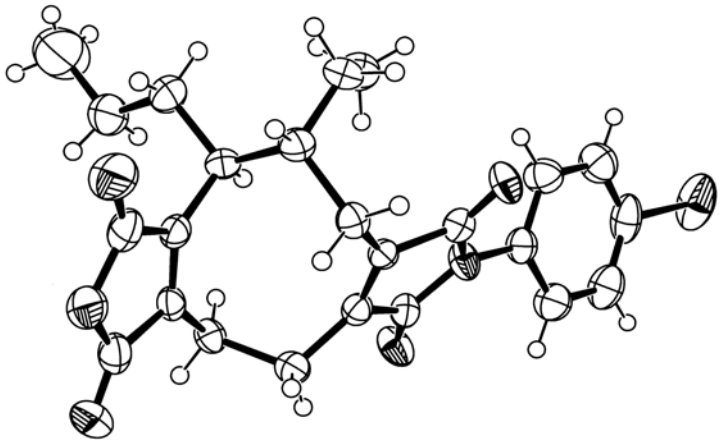


Figure 5. Perspective view of the crystal structure of **5**
with thermal ellipsoids at 50% possibility

Compound (**1**) showed the strong antifungal activity against various filamentous fungi including human pathogens such as *Aspergillus fumigatus, Penicillium marneffei* and *Trichophyton spp*.[2] The antifungal activity of **2** was almost not found against various filamentous fungi and yeasts as the same as those of **3** and **4**. When the hydroxyl group of **1** was methylated, the antifungal activity was extremely reduced. From the above results, it was considered that the hemiacetal structure of **1** was necessary for the expression of the antifungal activity.

EXPERIMENTAL

General. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. EI- and CI-MS spectra were taken with a JEOL JMS-MS600W spectrometer. UV and IR spectra were recorded on a Hitachi U-3210 spectrophotometer and a JASCO IR-810 spectrometer, respectivity. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL Lambda-500 (1H, 500.00 MHz; 13C, 125.25 MHz) spectrometer, using tetramethylsilane as an internal standard. CD curves were determined on a JASCO J-600 spectropolarimeter. Column chromatography was performed using Kieselgel 60 (Art.

7734, Merck) and Wakogel C-200 (Art. 237-00071, Wako). Low-pressure liquid chromatography (LPLC) was performed with a Chemco Low-Prep 81-M-2 pump and glass column (200 × 10 mm) packed with silica gel CQ-3 (30-50 μm, Wako). TLC were detected by UV light on 254 nm and/or by spraying with 5%-H_2SO_4 and then heating. HPLC was performed with a Shimadzu LC-9A pump (flow rate 1.0 mL/min) and a Inertsil ODS-3 (5 μm, 4.6 × 250 mm) column, equipped with a JASCO 875-UV detector and a JASCO OR-1590 chiral detector.

Fermetation and isolation of 2. The fungus IFM 52672 was cultivated on rice (2000 g) for 28 days at 25℃ as described in the previous paper.[2] The cultivated rice was extracted with $CHCl_3$-MeOH 4 L (1:1) , and the evaporated extract (28 g) was suspended with water, and extracted with hexane (350 mL), benzene (350 mL), $CHCl_3$ (350 mL), acetone (350 mL), and methanol (350 mL), in turn. The combined residue of the hexane and benzene extracts was chromatographed on silica gel with CH_2Cl_2-acetone using stepwise gradient system (CH_2Cl_2-acetone 100:1→50:1→20:1→10:1→5:1 and MeOH) to yield Fr. 1 to Fr. 12. Fr. 2 was repeatedly chromatographed on a silica gel column by using n-hexane-acetone (4:1) followed by recrystallization from acetone to give **2** (12 mg).

Deoxoepiheveadride (**2**) Colorless crystal ; mp 202-203℃; $[\alpha]_D^{25}$ -83.0 ° (*c* 1.04, $CHCl_3$); HREIMS Found: 318.1480 (M^+), Calcd: 318.1467 (for $C_{18}H_{22}O_5$); UV λ_{max} (MeOH) nm (log ε) 247 (3.56), 216 (4.11); IR ν_{max} (KBr) 1840, 1770 (anhydride) cm^{-1}; CD λ_{max} (EtOH) nm ($\Delta\varepsilon$) 261 (-5.21), 224 (-3.77). The assignments of ^{1}H- and ^{13}C-NMR spectral signals are summarized in Table 1.

Synthesis of 2 from dihydroepiheveadride (1) : $NaBH_4$ (150 mg, 1.1 mmol) was added to a solution of **1** (300 mg, 4.0 mmol) in MeOH (10 mL) and the mixture was stirred at rt for 1 h. The reaction mixture was acidified with 4N-HCl and extracted with CH_2Cl_2, and the solvent was evaporated in *vacuo*. A solution of the residue (274 mg) in CH_2Cl_2 10 mL was treated with PCC (450 mg) and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was filtered after addition of anhydrous $MgSO_4$ and ether, the filtrate was evaporated in *vacuo*. The residue was purified by LPLC [CH_2Cl_2-MeOH (100:1)] to give **2** (167 mg), which was identical with naturally occurring deoxoepiheveadride (**2**) on basis of comparisons of the ^{1}H-NMR, HREIMS, UV and CD spectra, mp, and TLC and HPLC behavior.

X-Ray structure analysis of 2. Compound (**2**) were grown slowly from acetone to give as colorless prisms. Diffraction intensities were collected from a crystal of dimensions 0.60 × 0.50 × 0.25 mm on a Rigaku AFC7R diffractometer with filtered Cu-*K*α radiation. Of a total of 1762 reflections, 1708 unique

reflections within a 2θ range of 135.9° were used in the solution and refinement of the structure. The data were collected for Lorenz and polarization effects.

Crystal Data $C_{18}H_{22}O_5$, M= 318.37, orthorhombic, space group $P2_12_12_1$, a = 11.311 (4), b = 17.941 (5), c = 8.127 (2) Å, V = 1649.2 (9) Å^3, Z = 4, Dc = 1.282 g·cm^{-3}, F(000)= 680, μ(Cu-Kα) = 7.67 cm^{-1}, Cu-$K\alpha$ X-radiation (filtered), λ = 1.54178 Å

The structure was solved by direct method using SIR92[4] and expanded with Fourier techniques using DIRDIF99[5] and refined by the full-matrix least-squares method. Although most of the hydrogen atoms were found from the difference Fourier synthesis, all of the hydrogen atoms used were calculated. In the final refinement, anisotropic thermal parameters were used for non-hydrogen atoms and the fractional and isotropic thermal parameters for hydrogen atoms were fixed. All calculations were performed using the Crystal Strucrure crystallographic software package.[6] The final R and R_w values were 0.066 and 0.076, respectively, for 1708 independent reflections.[7]

***p*-Bromoanilide (5) of 4.** *p*-Bromoaniline (400 mg, 2.3 mmol) was added to a solution of **4** (200 mg, 0.6 mmol), isolated from *B. heveae* CBS 241.93 as described in the previous paper,[2] in toluene 10 mL and the mixture was stirred on refluxing for 6 h. The reaction mixture was acidified with 4N-HCl and extracted with CH_2Cl_2, and the extract was evaporated in *vacuo*. The residue was purified by LPLC using hexane-acetone (4:1) to obtain a monoimide derivative (**5**) (80 mg, 27.5 %), along with diimide derivative (10 mg) and **4** (60 mg).

Compound (**5**) : Colorless crystalline powder (EtOH); mp 178-189 ℃; HREIMS Found: 485.0813 (M^+), Calcd: 485.0837 (for $C_{24}H_{24}NO_5Br$); UV λ_{max} (MeOH) nm (log ε) 246 (4.49); IR ν_{max} (KBr) 1840, 1770 (anhydride) cm^{-1}; CD λ_{max} (EtOH) nm ($\Delta\varepsilon$) 249 (+1.77).

X-Ray structure analysis of 5. *p*-Bromoanilide (**5**) of heveadride (**4**) was grown slowly from acetone to give as colorless plates. Diffraction intensities were collected from a crystal of dimensions 0.60 × 0.30 × 0.05 mm on a Rigaku AFC7R diffractometer with filtered Cu-$K\alpha$ radiation. Of a total of 4349 reflections, 4068 unique reflections within a 2θ range of 135.9° were used in the solution and refinement of the structure. The data were collected for Lorenz and polarization effects.

Crystal Data $C_{24}H_{24}O_5NBr$, M= 486.36, monoclinic, space group $C2$, a= 18.506 (5), b= 11.563 (2), c= 10.706 (2) Å, b= 103.96 (2) °,V = 2223.3 (8) Å^3, Z= 4, D_C= 1.453 g·cm^{-3}, F(000)= 1000, μ(Cu-$K\alpha$) = 28.22 cm^{-1}, Cu-$K\alpha$ X-radiation (filtered), λ = 1.54178 Å.

The structure was solved by direct method using SIR92[4] and expanded with Fourier techniques using DIRDIF99,[5] and refined by the full-matrix least-squares method. All of the hydrogen atoms used were

calculated. In the final refinement, anisotropic thermal parameters were used for non-hydrogen atoms and the fractional and isotropic thermal parameters for hydrogen atoms were fixed. All calculations were performed using the Crystal Strucrure crystallographic software package.[6] The final *R* and R_w values were 0.093 and 0.101, respectively, for 4068 independent reflections. Since the observed intensity ratios of the Friedel pairs compared with those of the calculated ones were incorrect for the original structure, the antipodal structure should be correct.[7] The absolute structure of **5** was determined as shown in Figure 5.

Epimerization of heveadride (4). *p*-TsOH monohydrate (60 mg) was added to a solution of **4** (6 mg) in toluene-benzene (1:1) 2 mL and the solution was stirred at reflux for 40 h. The reaction mixture was poured into water and extracted with ether. The organic layer was dried over Na_2SO_4 and then evaporated in *vacuo*. Acetonitrile (400 μL) was added to the resulting residue and 10 μL of solution was analyzed on HPLC. The HPLC chromatogram was shown in Figure 4.

Methylation of 1. *p*-TsOH monohydrate (30 mg) and a small amount of MeOH were added to a solution of **1** (47 mg) in toluene-benzene (1:1) 10 mL and the mixture was refluxed for 16 h. The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and then evaporated in *vacuo*. The residue was purified on HPLC using hexane-acetone (1:1) to obtain the mixture of 12α- and 12β- methylate of **1** (27 mg), along with **1** (3 mg). The abundant ratio in the mixture was determined about 2:1 from the intensity ratio of ^{1}H-NMR. ^{1}H-NMR ($CDCl_3$) : δ 0.86 (3H, t, J = 7.2 Hz), δ 1.06 (3H, t, J = 7.1 Hz), δ 3.02* (1H, dd, J = 8.1, 13.5 Hz) / 3.10 (1H, dd, J = 10.0, 13.5 Hz), δ 3.67* (3H, s, 12-OMe) / 3.57 (3H, s, 12-OMe), δ 5.56* (1H, d, J = 1.7 Hz, 12-H) / 5.71 (1H, s, 12-H). *The ^{1}H-NMR signals were expressed in major / minor.

Antimicrobial activity assay. Antibactrial and antifungal activites were semi-quantitatively determined using the agar diffusion method with paper disc (6 mm in diameter), as described in the previous paper,[8] loaded with 5 and/or 100 μg of compound on test plate.

ACKNOWLEDGEMENTS

We are grateful to Dr. H. Kasai and Miss N. Kobayashi of Hoshi University for NMR and mass measurements. This study was supported in part by Hoshi University Science/Technology Frontier Research Base from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by a Cooperative Research Program of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University (04-23). This study was also performed as part of the program “Frontier Studies and International Networking of Genetic Resources in Pathogenic Fungi and Actinomycetes (FN-GRPF)”

through Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, Japan 2003.

REFERENCES

1. N. H. Georgopapadakou and T. J. Walsh, *J. Antimicrob. Agents Chemother*., 1996, **40**, 279.
2. T. Hosoe, K. Fukushima, T. Itabashi, K. Nozawa, K. Takizawa, K. Okada, G. M. de Campos Takaki, and K. Kawai, *J. Antibiotics*, 2004, accepted.
3. R. I. Crane, P. Hedden, J. MacMillan, and W. B. Turner, *J. Chem. Soc., Perkin Trans. 1*, 1973, 194.
4. A. Altomare, G. Cascarano, C. Giacovazzo, and A. Guagliardi, *J. Appl. Cryst.*, 1994, **27**, 1045.
5. P. T. Beurskens, G. Admiraal, G. Beurskens, W. P. Bosman, R. de Gelder, R. Israel, and J. M. M. Smits, The DIRDIF-99 Program System, Technical Report of the Crystallographic Laboratory, University of Nijmegen, The Netherlands, 1999.
6. Crystal Structure 3.6.0: Crystal Structure Analysis Package, Rigaku and Rigaku/MSC (2000-2004), 9009 New Trails Dr., The Woodlands TX 77381 U. S. A.
7. Lists of atomic parameters, bond length, and bond angles will be deposited to the Cambridge Crystallographic Data Centre.
8. S. Komai, T. Hosoe, K. Nozawa, K. Okada, G. M. de Campos Takaki, K. Fukushima, M. Miyaji, Y. Horie, and K. Kawai, *Mycotoxins*, 2003, **53**, 11.