Pergamon

SCIENCE DIRECT®

Tetrahedron 59 (2003) 455–459

TETRAHEDRON

Absolute structure, biosynthesis, and anti-microtubule activity of phomopsidin, isolated from a marine-derived fungus *Phomopsis* sp.

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Received 25 September 2002; accepted 29 November 2002

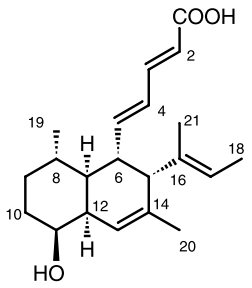
Abstract—The absolute configuration of phomopsidin, a marine-derived fungal metabolite from *Phomopsis* sp. isolated at Pohnpei, was determined by the exciton chirality method as 6*S*, 7*S*, 8*S*, 11*S*, 12*R*, and 15*R*. The biosynthetic study using ^{13}C-labeled precursors revealed the origin of all carbon atoms in phomopsidin, which was built by nine acetates and three methyl groups from L-methionine. Inhibitory activities of phomopsidin and its Me ester derivative against microtubule assembly were examined together with the structurally related compounds MK8383, solanapyrones, and tanzawaic acids. Phomopsidin and its (16*Z*)-isomer (MK8383) showed anti-microtubule activity at IC_{50} of 5.7 and 8.0 μM, respectively, while the Me ester and other compounds were not active at 100 μM.

1. Introduction

Fungi isolated from marine environments have recently been recognized as a rich source of biologically active metabolites.[1] During our systematic screening for anti-mitotic substances from marine-derived fungi,[2] phomopsidin (**1**)[3] was obtained from *Phomopsis* sp. strain TUF 95F47 isolated in Pohnpei as a new inhibitor of microtubule assembly by a screening method using conidia of *Pyricularia oryzae* P-2b.[4] This method observes deformations of mycelia germinated from conidia of *P. oryzae*, and certain antimitotic compounds can be detected by the characteristic curling effect of the mycelia.[4]

Phomopsidin (**1**) showed inhibitory activity against assembly of microtubule proteins purified from porcine brain at IC_{50} of 5.7 μM.[3] Relative stereochemistry of **1** was determined based on the NMR data.[2,3]

This report describes the absolute configuration determined by the exciton chirality method and biosynthesis of **1**, and discusses anti-microtubule activities of **1** together with its Me ester (**2**) and the structurally related compounds MK8383 (**4**),[5] solanapyrones (**5**–**8**),[6] and tanzawaic acids (**9**–**12**).[7]



Phomopsidin (**1**)

2. Results and discussion

2.1. *Phomopsis* sp. and isolation of phomopsidin

Phomopsis sp. strain TUF 95F47 was isolated from a fallen mangrove branch on the bottom of a coral reef in Pohnpei. The fungus grew from the substrate inoculated on an agar plate (1/2 PDA, 80% seawater, see Section 3).[3]

The fungus was cultured in ten 500 mL Erlenmeyer flasks (each 100 mL of 1/2 PD, 50% seawater) for 3 weeks at 20°C. The characteristic curling effect on mycelia from conidia of *P. oryzae* was observed with the broth filtrate. Bioassay-guided separation of the broth filtrate yielded 12.0 mg of phomopsidin (**1**).[2]

2.2. Absolute structure of phomopsidin (1)

Phomopsidin (**1**) did not give an $(M+H)^+$ ion but showed the $(M+Na)^+$ ion at *m/z* 353 in the FAB and ESI mass

Keywords: phomopsidin; marine-derived fungus; absolute stereochemistry; biosynthesis; anti-microtubule activity.

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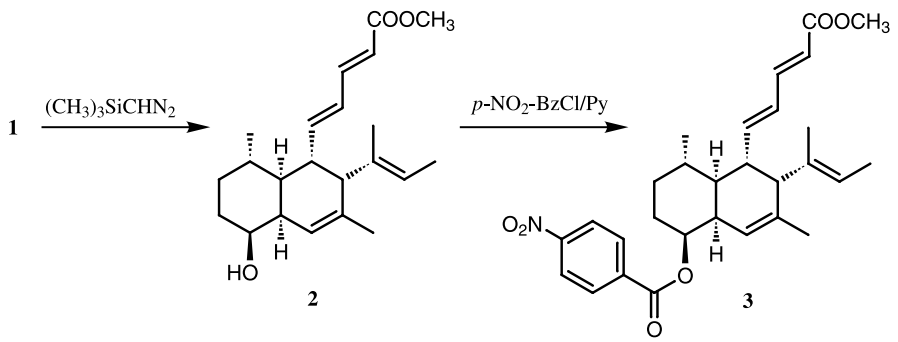
PII: S0040-4020(02)01566-1



Scheme 1. Preparation of *p*-nitrobenzoyl Me ester derivative (**3**) of phomopsidin (**1**).

spectra. The $(M-H)^-$ ion was detected at *m/z* 329 in the negative FABMS. The molecular formula, $C_{21}H_{30}O_3$, was assigned from the negative HRFABMS and NMR data. The structure of **1** including the relative stereochemistry was deduced by the analysis of $^1H-^1H$ COSY, HMQC, HMBC, and NOESY spectra.[2,3]

The absolute stereochemistry of **1** was determined by the exciton chirality method.[8] Phomopsidin (**1**) was treated with (trimethylsilyl)diazomethane to give the Me ester (**2**), which was transformed to the *p*-nitrobenzoyl derivative (**3**) by the reaction with *p*-nitrobenzoyl chloride in pyridine (Scheme 1).

The relative stereochemistry of **3** was determined from ^1H NMR and NOESY data. The ^1H and ^{13}C NMR signals of **3** were assigned by 2D NMR experiments ($^1H-^1H$ COSY, HMQC, HMBC, and NOESY). Signals due to H-10*e* (δ 1.86) and H-12 (δ 2.92) showed a W-shaped long-range coupling. NOEs were observed between H-11/7, H-11/9*a*, H-7/9*a*, and H-7/H_3-19. These data suggested that this ring is a chair form and that H-7, 9*a*, and 11 are *axial*, and the configuration of C-19 is *equatorial*, that is, H-8 is *axial*. The dihedral angle of H-12 and 13 (δ 5.73) was assumed to be ca. 90° ($J_{12,13}$=ca. 0 Hz), and H-13 showed an NOE with H-10*a* (δ 1.69). An NOE was observed between H-15 (δ 2.78) and 8 (δ 1.61). The configuration of H-15 was, therefore, assigned as *pseudaxial*. The dihedral angle of H-15 and H-6 (δ 2.86) was deduced to be ca. 90°, since the coupling constant was small ($J_{6,15}$=ca. 0 Hz). An NOE was observed between H-5 (δ 6.22) and H-12 (δ 2.92). These data revealed that the stereochemistry of **3** in CD_3OD was almost the same as that of **1**, which was shown in Figure 5 in Ref. 3.

Presuming from this relative stereochemistry of **3**, the torsion of one enantiomer (11*S*-isomer) will show a positive Cotton effect and that of another enantiomer (11*R*-isomer) a

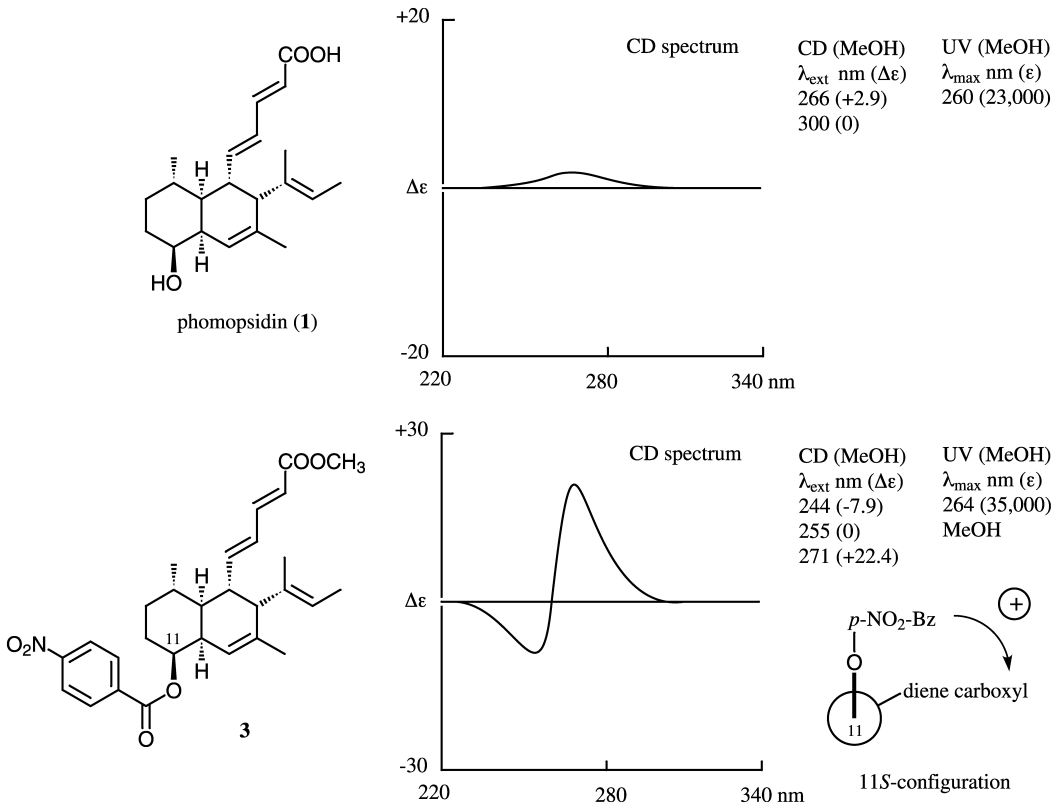


Figure 1. CD and UV spectral data for phomopsidin (**1**) and its *p*-nitrobenzoyl Me ester derivative (**3**).

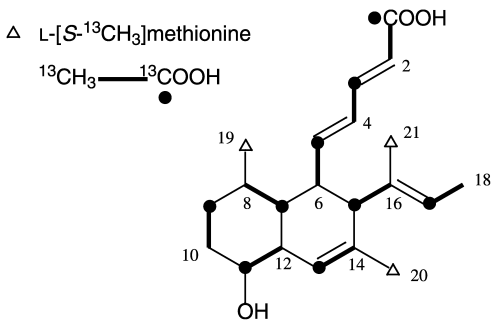


Figure 2. Biosynthetic origins of carbons in phomopsidin (**1**).

negative Cotton effect. The CD spectrum (MeOH) of **3** showed clear Cotton effects of $\Delta e_{271}=+22.4$ and $\Delta e_{244}=-7.9$ (Fig. 1). Therefore, the projection of two chromophores, the *p*-nitrobenzoyl group at C-11 and the diene carboxyl group (C-1 to C-5), should be clockwise as shown in Figure 1, and the absolute configuration at C-11 of **3** was assigned as *S*. Consequently, the absolute stereochemistry of **1** was elucidated as 6*S*, 7*S*, 8*S*, 11*S*, 12*R*, and 15*R*.

2.3. Biosynthesis of phomopsidin (1)

Phomopsis sp. strain TUF 95F47 was cultured respectively with [1-^{13}C]acetate, [2-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate, and L-[*S*-$^{13}CH_3$]methionine as biosynthetic precursors. The ^{13}C signals at C-1, 3, 5, 7, 9, 11, 13, 15, and 17 were enriched by [1-^{13}C]acetate, and [2-^{13}C]acetate increased the intensities of ^{13}C signals at C-2, 4, 6, 8, 10, 12, 14, 16, and 18. ^{13}C–^{13}C couplings were observed between the carbons at C-1/C-2, C-3/C-4, C-5/C-6, C-7/C-8, C-9/C-10, C-11/C-12, C-13/C-14, C-15/C-16, and C-17/C-18 in the ^{13}C NMR spectrum of phomopsidin (**1**) cultured with doubly labeled precursor ([1,2-$^{13}C_2$]acetate). Propionate was not used as a biosynthetic precursor and three methyl signals due to the 19, 20, and 21 positions were enriched when the fungus was cultured with L-[*S*-$^{13}CH_3$]methionine.

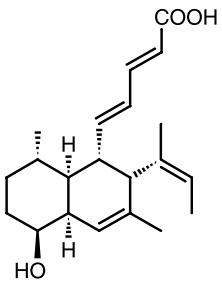
These biosynthetic studies revealed the origin of all carbon atoms in phomopsidin (**1**) as shown in Figure 2, therefore, **1** is biogenetically a trimethylated nonaketide.

Phomopsidin (**1**) would biogenetically be synthesized via a biological Diels–Alder reaction[9] similar to the biosynthesis of solanapyrones and other decalin derivatives.[6,10,11] There may be two possibilities where a biological Diels–Alder reaction takes place in the steps of phomopsidin biosynthesis.[9] One possibility is at the late biosynthetic step as in the case of solanapyrones biosynthesis, in which the biological Diels–Alder reaction occurs after the whole carbon chain was built.[11–13] The other case would be similar to the biosynthesis of lovastatin, in which the biological Diels–Alder reaction occurs in the course of the biogenesis and side chain elongation is continued after the cyclization reaction.[14]

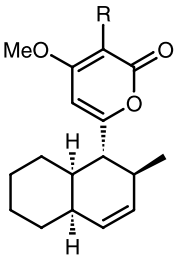
2.4. Anti-microtubule activity of phomopsidin (1) and related compounds

The microtubule assembly assay using purified porcine brain microtubule proteins revealed that **1** is a potent inhibitor against microtubule assembly (IC_{50}, 5.7 μM).[2,3] Colchicine and rhizoxin showed IC_{50} of 10 and 4 μM, respectively, in the same experiment.

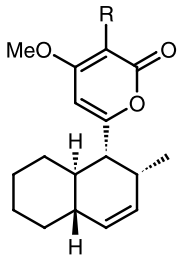
MK8383 (**4**) is a (16*Z*)-isomer of **1** and was isolated from terrestrial *Phoma* sp. as an antifungal component to several phytopathogens.[5] Since **4** showed the anti-microtubule activity (IC_{50}=8.0 μM), the geometry at $\Delta^{16,17}$ did not affect the bioactivity. While the free carboxylic acid at C-1 is essential for the activity as phomopsidin Me ester (**2**) did not inhibit assembly of microtubule proteins at 100 μM.



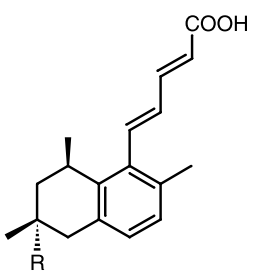
MK8383 (**4**)



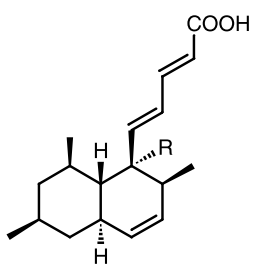
Solanapyrone A: R = CHO (**5**)
Solanapyrone B: R = CH_2OH (**6**)



Solanapyrone D: R = CHO (**7**)
Solanapyrone E: R = CH_2OH (**8**)



Tanzawaic Acid A: R = H (**9**)
Tanzawaic Acid D: R = OH (**12**)



Tanzawaic Acid B: R = H (**10**)
Tanzawaic Acid C: R = OH (**11**)

The structurally related compounds of phomopsidin (**1**) were also tested an anti-microtubule activity. Solanapyrones A (**5**), B (**6**), D (**7**), and E (**8**) were isolated from *Alternaria solani* as phytotoxins, which cause early blight disease of potato and tomato.[6] These four compounds were not active, probably because these compounds do not have a free carboxylic acid moiety at the side chain. Tanzawaic acids A (**9**), B (**10**), C (**11**), and D (**12**) were isolated from *Penicillium citrinum* and inhibited the production of superoxide anion.[7] None of four compounds was active. Compounds **9** and **12** have an aromatic ring, which is totally different from a decalin structure. Tanzawaic acid B (**10**) has the most similar structure to phomopsidin (**1**), but the stereochemistry at the decalin unit is different. It is suggested that the *cis*-decalin structure in phomopsidin (**1**) and MK8383 (**4**) would be important for the anti-microtubule activity. Interestingly, tanzawaic acids A (**9**) and B (**10**) were reported to inhibit superoxide anion production, but C (**11**) and D (**12**) did not show the activity.

3. Experimental

NMR spectra were measured on a JEOL JNM A-500 NMR spectrometer. Mass spectra were obtained by either a JEOL HX-110 mass spectrometer (FAB mode, *m*-nitrobenzyl-alcohol as matrix) or a Finnigan TSQ 700 triple quadrupole mass spectrometer (ESI mode). UV and IR spectra were recorded on a Shimadzu UV-300 and on a JASCO A-102, respectively. Optical rotations and CD spectra were taken in MeOH solution on a JASCO DIP-130 polarimeter and on a JASCO J-20A recording spectropolarimeter, respectively.

3.1. Isolation and fermentation of *Phomopsis* sp. strain TUF 95F47

The filamentous fungus, TUF 95F47 was isolated from a fallen mangrove branch on the bottom (−3 m) of a coral reef in Pohnpei during the Pohnpei/Palau expedition by the research vessel Sohgen-maru operated by Marine Biotechnology Institute in 1995. The substrate was sealed in a sterile plastic bag in the water. The treatment of substrate was performed within three hours in a laboratory of the research vessel. Five small pieces of the substrate were cut into a sterilized counting vial (20 mL) and washed three times with sterilized seawater. Three pieces of the substrate were applied on an agar plate of a half nutrient Potato Dextrose Agar with 80% seawater containing 100 ppm of chloramphenicol (1/2 PDA, 80% seawater) (hot water (200 mL) extract of potato (100 g), 10 g dextrose, 800 mL natural seawater, and 100 mg chloramphenicol), and the plate was placed in a research room (25°C) of the ship.

The fungus was isolated from a colony formed from the substrate and inoculated on a slant (1/2 PDA, 50% seawater) as a stock culture. Identification of the fungus was done in the usual manner using PDA and OatMeal Agar as media, and the strain TUF 95F47 was identified as a *Phomopsis* sp.

Phomopsis sp. strain TUF 95F47 was cultured in a plastic plate for the screening bioassay with 15 mL of 1/2 PD medium (50% seawater) for 3 weeks at 20°C. MeOH (8 mL) was added to the broth and stored for the conidia assay.

3.2. Conidia assay

The assay was performed as described previously.[4] Briefly, the suspension (50 μL) of conidia of *P. oryzae* P-2b in sterile water containing 0.2% yeast extract was taken into each well of 96-well assay plates, and the sample solution (50 μL) was added to the first well. The suspension was mixed and taken 50 μL to the second well. The procedure was repeated to the last well of the column. The assay plates were incubated for 16 h at 27°C, and the shape of mycelia germinated from conidia was observed and compared with controls (negative: water, positive: rhizoxin) under an inverted microscope.

3.3. Microtubule assembly assay

The assay was performed as reported.[15] In brief, fresh porcine brains were homogenized at 0°C in a buffer solution (100 mM 4-morphorineethansurfonic acid, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, and 1 mM 2-mercaptoethanol, pH 6.5) and centrifuged at 50,000***g*** at 4°C. A glycerol buffer (8 M glycerol in the above buffer solution, pH 6.5) was added to the supernatant, and the mixture was incubated at 37°C for 30 min and centrifuged at 100,000***g*** to afford the precipitate (microtubule). The depolymerization and polymerization procedure was further performed twice to purify the microtubule proteins. The concentration of proteins was quantified using the Coomassie Protein Assay Kit® (Pierce).

Polymerization and depolymerization were observed by the turbidity at 400 nm in a glass UV cell at 37°C with a Shimadzu model U-3000 spectrophotometer equipped with an electronic temperature controller. Each sample was dissolved in DMSO and added to the suspension of microtubule proteins in buffer solution (1.3 mg in 1 mL). The final concentration of DMSO was less than 2%. DMSO and rhizoxin[15] were used as negative and positive controls, respectively.

3.3.1. Isolation of phomopsidin (1). *Phomopsis* sp. 95F47 was cultured in ten 500 mL flasks (each 100 mL of 1/2 PD, 50% natural seawater) for 3 weeks at 20°C. Acetone was added to the cultured broth and filtered. The filtrate was extracted with benzene, and the extract was separated by a silica gel column (benzene, benzene–acetone=2:1, and then MeOH). The bioactive fraction (benzene–acetone eluate) was subjected to silica gel column chromatography (benzene–acetone=4:1) followed by HPLC (ODS, CH_3CN–H_2O=1:1) to afford 12.0 mg of **1**, white powder (mp=94–98°C); $[\alpha]_D^{27}$=+31° (*c* 0.1, MeOH); HRFABMS [$(M-H)^-$, *m/z* 329.2121, Calcd for $C_{21}H_{29}O_3$, 329.2125]; CD and UV spectral data are shown in Figure 1; IR ν_{max} (KBr) cm^{-1}: 3430, 2940, 2860, 1688, 1634, 1380, 1300, 1265, 1052, 1003; ^1H NMR (500 MHz, $CDCl_3$): δ 5.77 (d, *J*=15.2 Hz, H-2), 7.18 (dd, *J*=15.2, 10.2 Hz, H-3), 6.15 (dd, *J*=15.0, 10.2 Hz, H-4), 6.26 (dd, *J*=15.0, 9.0 Hz, H-5), 2.78 (m, H-6), 1.25 (m, H-7), 1.42 (m, H-8), 1.00 (m, H-9*a*), 1.65 (m, H-9*e*), 1.38 (m, H-10*a*), 1.63 (m, H-10*e*), 3.57 (m, *J*=11.2, 4.6, 4.6 Hz, H-11), 2.65 (m, H-12), 5.73 (s, $J_{12,13}$=ca. 0 Hz, H-13), 2.76 (s, $J_{6,15}$=ca. 0 Hz, H-15), 5.22 (q, *J*=6.5 Hz, H-17), 1.55 (dd, *J*=6.5, 0.8 Hz, H_3-18), 0.94 (d, *J*=6.5 Hz, H_3-19), 1.60 (s, H_3-20), 1.48 (s, H_3-21);

^{13}C NMR (125 MHz, $CDCl_3$): δ 170.9 (C-1), 120.6 (C-2), 146.9 (C-3), 129.0 (C-4), 148.8 (C-5), 45.3 (C-6), 49.3 (C-7), 29.9 (C-8), 34.0 (C-9), 31.1 (C-10), 73.3 (C-11), 39.1 (C-12), 124.3 (C-13), 136.1 (C-14), 51.3 (C-15), 136.4 (C-16), 123.5 (C-17), 13.5 (C-18), 19.4 (C-19), 22.3 (C-20), 16.8 (C-21).

3.4. Fermentation of *Phomopsis* sp. strain TUF 95F47 with ^{13}C-labeled precursors

Phomopsis sp. was culture in ten plastic plates per a precursor with each 20 mL of 1/2 PD medium for 3 weeks at 20°C. [1-^{13}C]Sodium acetate (^{13}C: 99 atom%, ICON), [2-^{13}C]sodium acetate (^{13}C: 99.1 atom%, ISOTEC), [1,2-^{13}C]sodium acetate (^{13}C: 99 atom%, ISOTEC), [3-^{13}C]sodium propionate (^{13}C: 90 atom%, MSD), and L-[*S*-$^{13}CH_3$]methionine (^{13}C: 99 atom%, CEA) were each dissolved in water (50 μg/μL) and 200 μL were added to a plate after inoculation of the fungus. Phomopsidin (**1**) was isolated from each culture as above.

The positions of enrichment by ^{13}C-labeled precursors were determined from respective ^{13}C NMR spectra.

3.4.1. *p*-Nitrobenzoyl Me ester derivative of phomopsidin (3). Phomopsidin (**1**, 42 mg) was dissolved in a mixture of benzene and MeOH (4:1, 1 mL), and (trimethylsilyl)diazomethane (10% in hexane, 0.5 mL) was added to the solution. The mixture was stirred at room temperature for 30 min and evaporated to dryness. The residue was chromatographed on silica gel (5% acetone in benzene) to give 24 mg of Me ester (**2**), HRFABMS [$(M+H)^+$, *m/z* 345.2478, Calcd for $C_{22}H_{33}O_3$, 345.2430]; ^{1}H NMR (500 MHz, CD_3OD): δ 5.86 (H-2), 7.20 (H-3), 6.15 (H-4), 6.28 (H-5), 2.76 (H-6), 1.25 (H-7), 1.42 (H-8), 1.00 (H-9*a*), 1.63 (H-9*e*), 1.36 (H-10*a*), 1.41 (H-10*e*), 3.57 (H-11), 2.64 (H-12), 5.73 (H-13), 2.76 (H-15), 5.22 (H-17), 1.55 (H_3-18), 0.96 (H_3-19), 1.60 (H_3-20), 1.47 (H_3-21), 3.70 ($COOCH_3$).

p-Nitrobenzoyl chloride (7 mg) and 4-dimethylaminopyridine (0.1 mg) were added to a solution of Me ester (**2**, 8 mg) in pyridine (79 μL) at 0°C, and the mixture was stirred at room temperature. After reaction was completed (monitored by TLC with benzene–acetone=9:1), water was added to the reaction mixture and extracted with $CHCl_3$. The organic extract was evaporated, and the residue was separated by a silica gel column with benzene–acetone (20:1) followed by HPLC (silica gel, 3.5% 2-PrOH in hexane) to give **3**, HRFABMS [$(M+H)^+$, 494.2545, Calcd for $C_{29}H_{36}NO_6$, 494.2543]; ^{1}H NMR (500 MHz, CD_3OD): δ 5.83 (d, *J*=15.2 Hz, H-2), 7.21 (dd, *J*=15.2, 10.2 Hz, H-3), 6.20 (m, H-4), 6.22 (m, H-5), 2.86 (m, H-6), 1.44 (m, H-7), 1.61 (m, H-8), 1.16 (m, H-9*a*), 1.77 (m, H-9*e*), 1.69 (m, H-10*a*), 1.86 (m, H-10*e*), 5.05 (m, H-11), 2.92 (m, H-12), 5.73 (m, H-13), 2.78 (m, H-15), 5.23 (q, *J*=6.5 Hz, H-17), 1.55 (d, *J*=6.5 Hz, H_3-18), 1.01 (d, *J*=6.5 Hz, H_3-19), 1.61 (m, H_3-20), 1.48 (s, H_3-21), 3.70 (s, $COOCH_3$), 8.24 (d, *J*=8.5 Hz, H-2′ and 6′), 8.35 (d, *J*=8.5 Hz, H-3′ and 5′); ^{13}C NMR (125 MHz, CD_3OD): δ 169.1 (C-1), 120.0 (C-2), 146.7 (C-3), 129.5 (C-4), 148.5 (C-5), 45.2 (C-6), 46.2 (C-7), 34.7 (C-8), 29.8 (C-9), 28.0 (C-10), 77.8 (C-11), 36.7 (C-12), 123.5 (C-13), 137.5 (C-14), 51.4 (C-15), 136.0 (C-16), 123.9 (C-17), 13.5 (C-18), 19.3 (C-19), 22.3 (C-20), 17.0 (C-21), 52.0 ($COOCH_3$), 165.4 (COO), 137.3 (C-1′), 131.7 (C-2′ and 6′), 124.7 (C-3′ and 5′), 152.1 (C-4′); CD and UV spectral data are shown in Figure 1.

Acknowledgements

This study was supported in part by grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (07680625, 08680628, and 08266220) to M. Namikoshi and the Sasakawa Scientific Research Grant from The Japan Science Society to T. Yoshimoto. We thank Dr T. Hosoya of Sankyo Co., Ltd. for identification of the fungus, and Drs S. Miyachi, M. Endo, and Y. Shizuri of Marine Biotechnology Institute, Inc. for our participation in the Pohnpei/Palau Expedition by the research vessel Sohgen-maru. We are grateful to Meiji Seika Kaisha, Ltd., Dr H. Oikawa (Hokkaido University), and Drs D. Uemura (Nagoya University) and M. Kuramoto (Ehime University) for the authentic samples of MK8383, solanapyrones, and tanzawaic acids, respectively.

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