Tetracyclic Terpenoids from *Dasyscyphus niveus*, Dasyscyphins D and E

Johannes C. Liermann,[†] Heinz Kolshorn,[‡] Heidrun Anke,[§] Eckhard Thines,[§] and Till Opatz*[,†]

Institut für Organische Chemie, Universität Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany, Institut für Organische Chemie, Universität Mainz, Duesbergweg 10-14, D-55128 Mainz, Germany, Institut für Biotechnologie und Wirkstoff-Forschung (IBWF), Erwin-Schrödinger-Strasse 56, D-67663 Kaiserslautern, Germany

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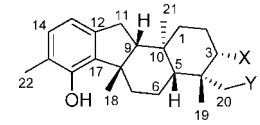
Cultures of the ascomycete *Dasyscyphus niveus* have yielded two new tetracyclic dasyscyphin-type terpenoids (**1** and **2**), and their structures were elucidated by NMR spectroscopy and X-ray crystallography. The absolute configuration of dasyscyphin D (**1**) was determined by synthesis and NMR spectroscopy of diastereomeric MTPA esters. Both compounds inhibited the germination of conidia of *Magnaporthe grisea* at 25 μg/mL.

The tiny, hairy, white, cup-shaped fruiting bodies of the ascomycete *Dasyscyphus niveus* (German: *Schneeweisses Haarbecherchen*) can be found growing on decaying oak or beech wood in European, North American, and New Zealandian forests.[1] Various compounds have recently been isolated from fermentation broths of this fungus. Structure elucidation of these compounds, the dasyscyphins, has been described along with their strong cytotoxic activity.[2,3] Here, we report on the isolation and structure elucidation of two novel structurally related terpenoids (**1** and **2**) from submerged cultures of *D. niveus* collected near Kaiserslautern.

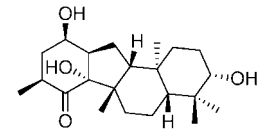
In a screening of extracts obtained from submerged cultures of ascomycetes, strain A79-88 exhibited antifungal activity, no antibacterial activity, and only very low cytotoxic activity. This selective biological activity prompted us to isolate the responsible compounds. The ascomycete was grown in YMG medium until the carbon source was consumed. The culture filtrate was extracted with ethyl acetate, and the resulting crude mixture was subjected to silica gel column chromatography (CC). Further purification by preparative HPLC yielded two nonpolar compounds, **1** (5.5 mg) as colorless crystals (mp 105−106 °C) and **2** (3.3 mg) as a yellow oil.

Analysis of ^{13}C NMR and DEPT data revealed the presence of 22 carbon atoms and 30 C−H protons in either case, suggesting that **1** and **2** were closely related structural isomers. The elemental composition for both compounds was determined to be $C_{22}H_{32}O_2$ by HRMS. This is in accordance with ^{1}H NMR data and proved the presence of 32 protons and thus two protons bound to heteroatoms. Furthermore, ^{13}C and DEPT spectra of compound **1** showed a methine carbon at δ 79.5, whereas the spectra of **2** show a methylene group at δ 65.3. This led to the conclusion that both compounds carry a hydroxy group, which is secondary in **1** and primary in **2**. The UV spectra of methanolic solutions of both compounds exhibited absorption maxima characteristic of a benzenoid system. In combination with a quaternary carbon atom resonating at δ 150.3 in both compounds, this was indicative for a substituted phenol. Tetrasubstitution of the aromatic ring was concluded from two ortho-coupled aromatic protons (AB spin system). The elemental composition of **1** and **2** requires seven double-bond equivalents, four of which are attributed to the benzene ring. As no further evidence for unsaturation was found, the molecule must contain three additional rings.

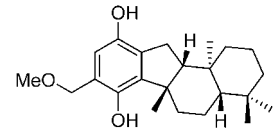
Compound **1** had five methyl groups, all of which appeared as singlets in the ^{1}H NMR spectrum. In contrast, compound **2** shows only four methyl singlets; thus, one of the methyl groups in **1** should be hydroxylated in **2**. Only compound **1** possessed two geminal methyl groups, whereas in both compounds one methyl group resonated at δ 2.20, characteristic for toluene-like aromatics. The substitution pattern of the phenol was established to be that of a 5,6-anellated ortho-cresole by HMBC and NOESY experiments. One of the aromatic protons in **1** showed NOE correlations to geminal methylene protons at δ 3.01 and 2.66, which was confirmed by a corresponding HMBC correlation. Both protons had COSY and NOESY correlations to the multiplet at δ 1.62 (^{1}H), located on a tertiary center. An HMBC correlation of this proton as well as of the methyl protons at δ 1.23 with the ortho-position of the phenol suggested the presence of a five-membered ring with a methylated ring junction fused to the cresole moiety.



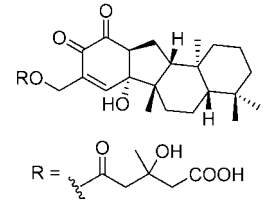
1: X = OH, Y = H, Dasyscyphin D
2: X = H, Y = OH, Dasyscyphin E



Dasyscyphin A



Dasyscyphin B



Dasyscyphin C

Analysis of further HMBC, COSY, and NOESY correlations revealed the presence of a fused trans-decalin system carrying an angular methyl group. In compound **1**, the carbinol center is bound to a quaternary carbon carrying the two remaining methyl groups. In its isomer **2**, the aliphatic OH group is instead located on one of the geminal methyl groups. The structures of **1** and **2** are based on a drimane-type sesquiterpene anellated to an indane ring system, a

* To whom correspondence should be addressed. Tel: +49-(0)40-42838-4239. E-mail: opatz@chemie.uni-hamburg.de.
[†] Universität Hamburg.
[‡] Universität Mainz.
[§] Institut für Biotechnologie und Wirkstoff-Forschung (IBWF).

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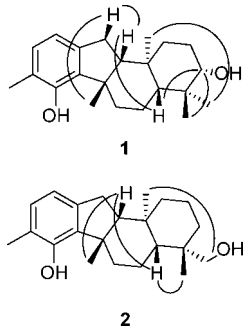


Figure 1. Characteristic NOE contacts in **1** and **2**.

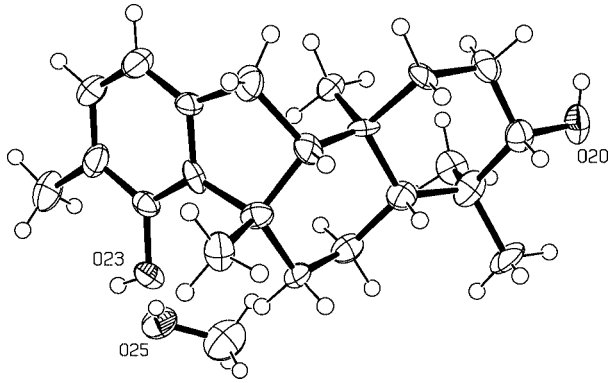
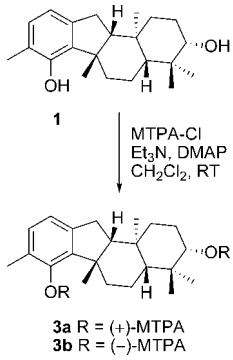


Figure 2. Crystal structure of **1**·CH_3OH (ORTEP, ellipsoids drawn at 50% probability).

Scheme 1. Synthesis of (+)- and (−)-MTPA Esters



moiety which is closely related to the skeleton of the dasyscyphins A−C that were isolated from another strain of *Dasyscyphus niveus*.[2,3] These three compounds were also present in crude extracts of strain A79-88. Dasyscyphin B features a similarly substituted aromatic ring, which is modified in dasyscyphins A and C. Therefore, **1** and **2** were given the names dasyscyphins D and E. The tetracyclic framework of the dasyscyphins is also found in akaol,[4] while pelorol[5,6] has an epimeric skeleton. Synthetic analogues of these marine sesquiterpene derivatives have recently been reported to be modulator prodrugs for the SHIP-1 enzyme.[7,8]

The relative configuration of both compounds was determined by NOESY experiments. Characteristic NOE contacts are depicted in Figure 1. For compound **1**, the structure and relative configuration were confirmed by X-ray crystallography (Figure 2).

Unfortunately, the collected diffraction data did not allow the determination of its absolute configuration. Instead, this was achieved using the well-established Mosher method.[9−11] The diastereomeric MTPA-diesters **3a** and **3b** were formed by reaction of **1** (1 mg each) with (+)- and (−)-MTPA chloride, triethylamine, and catalytic amounts of DMAP in dry CH_2Cl_2. Although full characterization of the esters was hampered by the small amounts obtained after chromatographic purification, characteristic ^{1}H NMR shifts were evaluated by analysis of COSY and NOESY experiments (Figure 3). By comparison of the data with those of vitetrifolin A[12] and similar compounds reported in the literature[11,13−15] the absolute configuration of **1** was determined to be *S* at C-3, being in accordance with that of dasyscyphins A−C. Since compound **2** does not carry a secondary OH group, the Mosher method is not suitable in this case. Nevertheless, the close structural relation between all known dasyscyphins suggests that **2** should have the same absolute configuration.

Compound **1** completely inhibited conidial germination in *Magnaporthe grisea* 70-15 at 20 μg/mL; compound **2** was slightly less active with 100% inhibition being observed at 25 μg/mL. Under minimal conditions, no germ tubes were observed at these concentrations. In the disk diffusion assay with *Penicillium chrysogenum* (DSM 848), *Paecilomyces variotii* (DSM 1961), *Rhizomucor miehei* (Tü 284), or *Nematospora coylii* (ATTC 10647), both compounds showed no inhibition at 50 μg/disk. Cytotoxic activity toward HeLA S3 cells was not observed up to concentrations of 50 μg/mL. For dasyscyphins B and C weak antibacterial and antifungal but strong cytotoxic activities have been reported, whereas dasyscyphin A was devoid of activity.[3] In the conidia germination assay, dasyscyphins B and C exhibited similar activities to those of D and E. Therefore, we conclude that the antifungal activities detected during the screening were due to the presence of dasyscyphins B−E.

Experimental Section

General Experimental Procedures. Melting points were determined with a Dr. Tottoli apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 546 and 578 nm and were extrapolated to 589 nm. UV and IR spectra were measured with a Perkin-Elmer Lambda-16 spectrophotometer and a Bruker IFS48 FTIR spectrometer, respectively. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) were recorded with a Bruker Avance-II spectrometer. NOESY spectra (500 MHz) were measured with a Bruker DRX-500 spectrometer. The spectra were measured in $CDCl_3$, and the chemical shifts were referenced to the residual solvent signal ($CDCl_3$: $\delta_H = 7.26$ ppm, $\delta_C = 77.16$ ppm).[16] APCI-MS spectra were measured with a Hewlett-Packard MSD1100. ESIMS spectra were measured with a Finnigan MAT95 spectrometer; HREIMS spectra were measured with a direct inlet VG70SE spectrometer using PFK as internal reference. Reactions were monitored on Merck TLC aluminum sheets (silica gel 60 F_{254}). Diethyl ether was distilled and stored over KOH pellets. CH_2Cl_2 was dried by distillation from CaH_2. Triethylamine was dried by distillation from $LiAlH_4$. MTPA chlorides were prepared from the commercially available acids as described in the literature.[17]

Producing Organism. *Dasyscyphus niveus* strain A79-88 was isolated from spore prints of fruiting bodies growing on a dead twig of beech wood collected near Kaiserslautern, Germany. The specimen showed all characteristics of the genus and the species.[1,18] The strain was maintained at 4 °C on agar slants on yeast malt glucose medium (YMG) composed of (g/liter) yeast extract (4, Hartge Ingredients, Hamburg), malt extract (10, Fränkle & Eck, Fellbach), glucose (4), and agar (20), pH 5.5. Mycelial cultures of strain A79-88 are deposited in the culture collection of the Institute of Biotechnology and Drug Research (IBWF e.V.).

Fermentation and Isolation. Fermentations were carried out in YMG medium in a 20 L fermentor (Biostat A-20, Braun Melsungen) at 22 °C with aeration (3.0 L/min) and agitation (120 rpm). A well-grown culture (200 mL) in the same medium was used as inoculum, and the antifungal activity was measured with samples withdrawn every day in a spore germination assay using *Magnaporthe grisea* as test organism. After 13 days of fermentation, when the antifungal activity

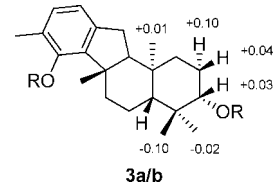


Figure 3. Characteristic shift differences in **3a** and **3b**. R = (+)/(−)-MTPA, $\Delta\delta = \delta(+) - \delta(-)$.

had reached the maximum and the glucose in the medium was consumed, the culture fluid (14.5 L) was separated from the mycelia and extracted with EtOAc (8 L). The mycelium containing no active compounds was discarded. The organic extract was dried with Na_2SO_4 and concentrated to yield 4.2 g of crude product, which was further purified by silica gel chromatography (silica gel 60, Merck Darmstadt, 107 g, column size 17 cm × 4.5 cm). Elution with cyclohexane−EtOAc (7:3) yielded 98.1 mg of a product containing compounds **1** and **2**. The final purification by preparative HPLC (Merck LiChroSpher RP18, 5 μm, column 25 × 250 mm, flow 20 mL/min; isocratic mode in MeCN−H_2O, 7:3 v/v) resulted in 5.5 mg of pure dasyscyphin D (**1**) and 3.3 mg of dasyscyphin E (**2**).

Biological Assays. Hela S3 (ATCC CCL 2.2 human cervix carcinoma) cell lines were grown in DMEM medium with 65 μg/mL penicillin G and 100 μg/mL streptomycin sulfate. The cells (10^5/mL) were incubated in microtiter plates with the compounds at 37 °C in a humidified atmosphere containing 5% CO_2. Viable cells were counted under the microscope after 24 and 48 h.

The antifungal activity was measured in a spore germination assay with *M. grisea* 70-15 conidia in water (5 × 10^4/mL). Germination was evaluated after 18 h under the microscope and compared to the control containing no inhibitor. The number of ungerminated conidia or conidia with significantly shorter germ tubes (germ tube length <30% of the control) was assessed. A total of 3 × 100 conidia were counted. The antifungal spectrum of the compounds was evaluated using the conventional agar diffusion assay.

Dasyscyphin D (1): Colorless crystals (5.5 mg); mp 105−106 °C; $[\alpha]^{25}_D$ +2.92 (*c* 0.49, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 205 (4.53), 275 (2.91) nm; IR (KBr) ν_{max} 3436, 2934, 1628, 1586, 1473, 1388, 1221, 1106, 1031, 798, 558 cm^{-1}; ^1H NMR, COSY, NOESY (400 MHz, $CDCl_3$) δ 6.87 (1H, d, J = 7.5 Hz, H-14), 6.62 (1H, d, J = 7.5 Hz, H-13), 4.53 (1H, s, OH-16), 3.26 (1H, dd, J = 11.3, 4.6 Hz, H-3), 3.01 (1H, dd, J = 16.6, 8.2 Hz, H-11a), 2.68 (1H, dt, J = 13.6, 6.2 Hz, H-7a), 2.66 (1H, br d, J = 16.6 Hz, H-11b), 2.20 (3H, s, H_3-22), 1.78 (1H, mc, H-7b), 1.73 (1H, dd, J = 13.2, 3.5 Hz, H-1a), 1.71−1.58 (3H, m, H-2a, H-6a, H-9), 1.54 (1H, ddd, J = 12.8, 3.5, 1.5 Hz, H-2b), 1.42 (1H, dddd, J = 13.5, 11.0, 8.8, 4.8 Hz, H-6b), 1.31 (1H, br s, OH-3), 1.23 (3H, s, H_3-18), 1.06 (1H, dd, J = 13.2, 4.3 Hz, H-1b), 1.01 (3H, s, H_3-19), 0.99 (1H, dd, J = 11.0, 4.9 Hz, H-5), 0.78 (3H, s, H_3-20), 0.52 (3H, s, H_3-21); ^{13}C NMR, HSQC, HMBC (100.6 MHz, $CDCl_3$) δ 150.3 (C, C-16), 143.8 (C, C-12), 136.0 (C, C-17), 129.1 (CH, C-14), 120.8 (C, C-15), 116.5 (CH, C-13), 79.5 (CH, C-3) 62.2 (CH, C-9), 51.2 (CH, C-5), 47.6 (C, C-8), 39.6 (CH_2, C-1), 39.2 (C, C-4), 37.2 (C, C-10), 33.9 (CH_2, C-7), 32.4 (CH_2, C-11), 30.7 (CH_3, C-18), 28.3 (CH_3, C-19), 27.3 (CH_2, C-2), 19.5 (CH_2, C-6), 15.9 (CH_3, C-21), 15.5 (CH_3, C-20), 15.5 (CH_3, C-22); APCIMS *m/z* pos. 311.2 $[M - OH]^+$ (100) neg. 309.2 $[M - H_3O]^-$ (10), 327.2 $[M - H]^-$ (100); HREIMS *m/z* 328.2397 (calcd for $C_{22}H_{32}O_2$, 328.2402).

X-ray Crystallographic Data of 1·CH_3OH. Colorless single crystals of **1**·CH_3OH were grown by slow evaporation of a methanolic solution of **1**. Data were obtained at 193 K on a Turbo CAD4 diffractometer with graphite-monochromated Cu Kα radiation. Formula $C_{22}H_{32}O_2$·CH_3OH, crystal size 0.032 × 0.064 × 0.265 mm^3, orthorhombic, space group $P2_12_12$, a = 16.491(2) Å, b = 19.995(4) Å, c = 6.399(1) Å, V = 2110.0(6) $Å^3$, Z = 4, D = 1.135 g cm^{-3}, R = 0.0978, R_w = 0.3326. CCDC-695149 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (internat.) +44-1223/336-033; e-mail: deposit@ccdc.cam.ac.uk].

Dasyscyphin E (2): Yellow oil (3.3 mg); $[\alpha]^{25}_D$ +1.07 (*c* 0.33, $CHCl_3$); UV (MeOH) λ_{max} (log ε) = 204 (4.59), 271 (2.99) nm; IR (KBr) ν_{max} 3436, 2927, 1714, 1632, 1473, 1263, 1223, 1012, 797, 602 cm^{-1}. ^1H NMR, COSY, NOESY (400 MHz, $CDCl_3$) δ 6.86 (1H, d, J = 7.5 Hz, H-14), 6.62 (1H, d, J = 7.5 Hz, H-13), 4.50 (1H, s, OH-16), 3.73 (1H, br d, J = 10.9 Hz, H-20a), 3.42 (1H, d, J = 10.9 Hz, H-20b), 2.99 (1H, dd, J = 16.5, 7.8 Hz, H-11a), 2.79 (1H, m, H-7a), 2.63 (1H, d, J = 16.5 Hz, H-11b), 2.20 (3H, s, H_3-22), 1.83 (1H, ddd, J = 13.7, 4.7, 3.1 Hz, H-3a), 1.76−1.55 (4H, m, H-1a, H-6a, H-7b, H-9), 1.48 (1H, m, H-2a), 1.41 (1H, m, H-2b), 1.34 (1H, m, H-6b), 1.23 (3H, s, H_3-18), 1.15 (1H, dd, J = 11.6, 3.7 Hz, H-5), 1.04 (1H, br s, OH-20), 0.99 (3H, s, H_3-19), 0.95 (1H, m, H-3b), 0.93 (1H, m, H-1b), 0.45 (3H, s, H_3-21); ^{13}C NMR, HSQC, HMBC (100.6 MHz, $CDCl_3$) δ 150.3 (C, C-16), 144.1 (C, C-12), 135.6 (C, C-17), 129.1 (CH, C-14), 120.8 (C, C-15), 116.5 (CH, C-13), 65.3 (CH_2, C-20), 62.5 (CH, C-9), 53.4 (CH, C-5), 47.7 (C, C-8), 41.4 (CH_2, C-1), 38.6 (C, C-4), 37.3 (C, C-10), 35.7 (CH_2, C-3), 34.8 (CH_2, C-7), 32.5 (CH_2, C-11), 31.0 (CH_3, C-18), 26.7 (CH_3, C-19), 19.7 (CH_2, C-6), 18.3 (CH_2, C-2), 16.7 (CH_3, C-21), 15.5 (CH_3, C-22); APCIMS *m/z* pos. 311.2 $[M - OH]^+$ (100), neg. 309.2 $[M - H_3O]^-$ (6), 327.2 $[M - H]^-$ (100); HREIMS *m/z* 328.2413 (calcd for $C_{22}H_{32}O_2$, 328.2402).

3,16-Bis(α-methoxy-α-(trifluoromethyl)phenylacetyl)dasyscyphin D (3). Dasyscyphin D **1** (1.0 mg, 3.0 μmol) was dissolved in dry CH_2Cl_2 (1 mL) under argon atmosphere. MTPA chloride (5.0 mg, 19.8 μmol), DMAP (1.0 mg, 8.2 μmol), and dry triethylamine (10 μL, 7.2 mg, 6.9 μmol) were added. The solution was stirred at room temperature for 1 h. Ether (5 mL) was added, and the resulting solution was washed with aqueous 1 M HCl (5 mL) and saturated aqueous Na_2CO_3 (5 mL). The organic phase was dried over Na_2SO_4 and the solvent was removed in vacuo to furnish the diesters as yellow oils.

Compound 3a. Prepared from (+)-MTPA chloride. Yield: 1.0 mg (44%). Characteristic ^1H NMR shifts: COSY, NOESY (500 MHz, $CDCl_3$) δ 4.72 (H-3), 1.73 (H_α-2), 1.65 (H_β-2), 1.11 (H-1), 0.94 (H-5), 0.78 (H_3-19), 0.73 (H_3-20), 0.43 (H_3-21).

Compound 3b. Prepared from (−)-MTPA chloride. Yield: 1.0 mg (44%). ESIMS: *m/z* 761 $[M + H]^+$ (46), 783 $[M + Na]^+$ (86), 799 $[M + K]^+$ (88). Characteristic ^1H NMR shifts: COSY, NOESY (500 MHz, $CDCl_3$): δ 4.69 (H-3), 1.69 (H_α-2), 1.55 (H_β-2), 1.20 (H-5), 1.10 (H-1), 0.88 (H_3-19), 0.75 (H_3-20), 0.42 (H_3-21).

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Supporting Information Available: ^1H, ^{13}C, and 2D NMR spectra of compounds **1** and **2** and CIF of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Saccardo, P. A. *Syll. Fung.* **1889**, *8*, 437.
(2) Rojas de la Parra, V.; Mierau, V.; Anke, T.; Sterner, O. *Tetrahedron* **2006**, *62*, 1828–1832.
(3) Mierau, V.; Rojas de la Parra, V.; Sterner, O.; Anke, T. *J. Antibiot.* **2006**, *59*, 53–56.
(4) Mukku, V. J. R. V.; Edrada, R. A.; Schmitz, F. J.; Shanks, M. K.; Chaudhuri, B.; Fabbro, D. *J. Nat. Prod.* **2003**, *66*, 686–689.
(5) Kwak, J. H.; Schmitz, F. J.; Kelly, M. *J. Nat. Prod.* **2000**, *63*, 1153–1156.
(6) Goclik, E.; Koenig, G. M.; Wright, A. D.; Kaminsky, R. *J. Nat. Prod.* **2000**, *63*, 1150–1152.
(7) Andersen, R.; Nodwell, M.; Mui, A. (University of British Columbia, Can.) PCT Int. Appl. 2007147251, 2007.
(8) Andersen, R.; Nodwell, M.; Mui, A. (University of British Columbia, Can.) PCT Int. Appl. 2007147252, 2007.
(9) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543–2549.
(10) Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512–519.
(11) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.
(12) Ono, M.; Sawamura, H.; Ito, Y.; Mizuki, K.; Nohara, T. *Phytochemistry* **2000**, *55*, 873–877.
(13) Oku, N.; Matsunaga, S.; Wada, S.; Watabe, S.; Fusetani, N. *J. Nat. Prod.* **2000**, *63*, 205–209.
(14) Orihara, Y.; Yang, J. W.; Komiya, N.; Koge, K.; Yoshikawa, T. *Phytochemistry* **2002**, *59*, 385–389.
(15) Sunazuka, T.; Handa, M.; Nagai, K.; Shirahata, T.; Harigaya, Y.; Otoguro, K.; Kuwajima, I.; Omura, S. *Tetrahedron* **2004**, *60*, 7845–7859.
(16) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. *J. Org. Chem.* **1997**, *62*, 7512–7515.
(17) Smith, P. M.; Thomas, E. J. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3541–3556.
(18) Breitenbach, J.; Kränzlin, F. *Pilze der Schweiz*; Mykologia: Luzern, 1984; Vol. 1, Ascomyceten.

NP800355A