

Pimarane Diterpene and Cytochalasin Derivatives from the Endophytic Fungus *Eutypella scoparia* PSU-D44

Wipapan Pongcharoen,[†] Vatcharin Rukachaisirikul,^{*‡} Souwalak Phongpaichit,[‡] Nattawat Rungjindamai,[‡] and Jariya Sakayaroj[§]

Department of Chemistry, Faculty of Science, Prince of Songkla University, Songkhla, 90112, Thailand, Department of Microbiology, Faculty of Science, Prince of Songkla University, Songkhla, 90112, Thailand, and National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Klong Luang, Pathumthani, 12120, Thailand

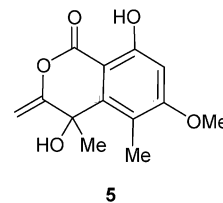
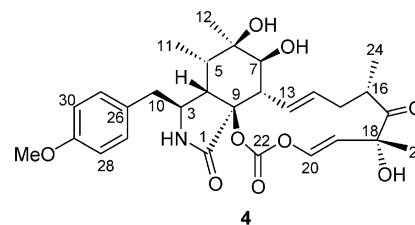
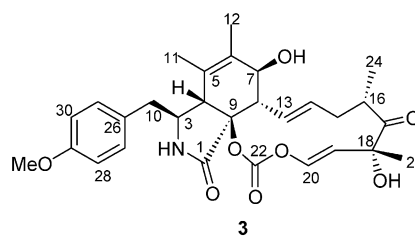
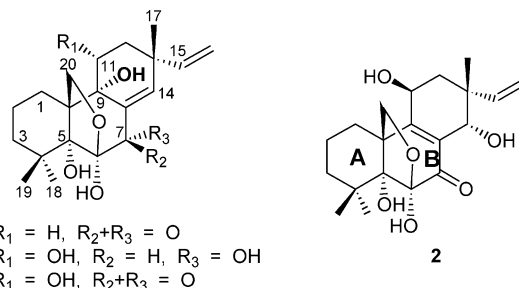
Received February 11, 2006

Two pimarane diterpenes, named scopararanes A (**1**) and B (**2**), and two cytochalasins, named scoparasins A (**3**) and B (**4**), along with 4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methylenoisochroman-1-one (**5**) and diaportheins A (**6**) and B (**7**) were isolated from a culture broth of the endophytic fungus *Eutypella scoparia* PSU-D44. Their antimicrobial activities against *Staphylococcus aureus* ATCC 25923 and *Microsporium gypseum* SH-MU-4 were examined.

In the course of our ongoing search for antimicrobial substances from plants and endophytic fungi, the ethyl acetate extract from the culture broth of the endophytic fungus *Eutypella scoparia* PSU-D44 exhibited interesting antibacterial and antifungal activities against *Staphylococcus aureus* ATCC 25923 and *Microsporium gypseum* SH-MU-4. Investigation of the extract of the culture filtrate led to the isolation and structural elucidation of two new pimarane diterpenes, scopararanes A (**1**) and B (**2**), and two new cytochalasins, scoparasins A (**3**) and B (**4**), together with three known compounds, 4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methylenoisochroman-1-one (**5**)¹ and diaportheins A (**6**) and B (**7**).² All isolated compounds were tested for antimicrobial activities against *S. aureus* ATCC 25923 and *M. gypseum* SH-MU-4.

Scopararane A (**1**) was obtained as a white solid with the molecular formula C₂₀H₂₈O₅, as deduced from the HREIMS. The UV and IR spectra were almost identical to those of diaporthein B (**7**).² The ¹H NMR data were similar to those of **7** except that the oxymethine proton resonance (δ 4.01, dd, J = 11.7 and 4.2 Hz, H-11) in **7** was replaced by the methylene proton signal (δ 1.71, m) in **1**. Comparison of the ¹³C NMR data with those of **7** showed analogy of the chemical shifts except for C-11 and C-12. The methylene protons were then attributed to H₂-11. This was confirmed by their HMBC cross-peaks with C-8 (δ 135.0), C-9 (δ 74.6), C-12 (δ 29.5), and C-13 (δ 39.3). The relative configuration was identical to that in **7** according to NOEDIFF results of H-20a (δ 4.04, d, J = 9.9 Hz)/Me-19 (δ 1.43, s), H-20b (δ 3.73, d, J = 9.9 Hz)/H₂-11, and H₂-11/Me-17 (δ 1.16, s). Thus, scopararane A (**1**) was elucidated as a new pimarane diterpene derivative.

Scopararane B (**2**) was obtained as a colorless gum with the molecular formula C₂₀H₂₈O₆, as deduced from HREIMS. The UV spectrum showed a maximum absorption band at λ_{\max} 246 nm, while IR hydroxyl and the carbonyl stretching frequencies were found in the region of 3414 and 1684 cm⁻¹, respectively. The ¹H NMR data were similar to those of **7**² except that the signal of the β -olefinic proton (δ 6.80, d, J = 1.8 Hz) of the α,β -unsaturated ketone in **7** was replaced, in **2**, by an oxymethine proton (δ 4.43, s). The oxymethine proton was attributed to H-14 on the basis of the ³J HMBC cross-peaks of H-14 with C-7 (δ 194.6), C-9 (δ 164.6), C-15 (δ 143.8), and C-17 (δ 24.3). The ¹³C chemical shifts of C-8 (δ 132.3) and C-9 suggested the presence of a C₈–C₉ double bond. The relative configuration of rings A and B was identical to that in **7** according to NOEDIFF results (see Supporting Information).



Irradiation of H-20b (δ 3.29, d, J = 9.3 Hz) affected Me-17 (δ 1.03, s), but not H-11 (δ 4.42, dd, J = 6.3 and 4.8 Hz), indicating α -equatorial and β -axial location of H-11 and Me-17, respectively. The coupling constants derived from coupling between H-11 and H-12a (J = 4.8 Hz) and H-12b (J = 6.3 Hz) confirmed the α -equatorial position of H-11. Signal enhancement of H-14, upon irradiation of Me-17 in the NOEDIFF experiment, established the β -equatorial orientation of H-14. The relative configuration of C-11 and C-14 was identical to that in sphaeropsidin E.³ Therefore, scopararane B (**2**) was assigned as a new pimarane diterpene derivative.

* To whom correspondence should be addressed. Tel: +66-74-288-435. Fax: +66-74-212-918. E-mail: vatcharin.r@psu.ac.th.

[†] Department of Chemistry, Prince of Songkla University.

[‡] Department of Microbiology, Prince of Songkla University.

[§] National Center for Genetic Engineering and Biotechnology (BIOTEC).

Table 1. ^1H and ^{13}C NMR Data of Scoparasin A (**3**) and B (**4**)

position	3		4	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1		169.9, qC		170.0, qC
2-NH	5.81, br, s		6.11, br, s	
3	3.35, t (6.6)	59.2, CH	3.68, m	53.8, CH
4	3.81, br, s	48.2, CH	3.00, dd (5.2, 2.7)	48.1, CH
5		125.4, qC	2.29, dd (7.5, 4.8)	35.8, CH
6		131.7, qC		57.3, qC
7	4.15, dd (5.7, 3.6)	70.0, CH	2.65, m	60.6, CH
8	2.81, m	50.0, CH	2.64, m	45.9, CH
9		86.2, qC		87.0, qC
10	2.78, m	43.2, CH ₂	a: 2.85, td (13.5, 4.5) b: 2.62, m	44.2, CH ₂
11	1.46, br, s	17.7, CH ₃	1.11, d (7.2)	13.2, CH ₃
12	1.61, br, s	13.9, CH ₃	1.25, s	19.7, CH ₃
13	6.13, br, dd (15.0, 10.5)	129.4, CH	5.89, ddm (15.0, 9.9)	128.5, CH
14	5.31, ddd (15.0, 11.1, 3.9)	133.6, CH	5.23, ddd (15.0, 10.0, 3.6)	131.5, CH
15	a: 2.73, m b: 2.07, m	39.0, CH ₂	a: 2.67, m b: 2.15, dm (13.8)	39.1, CH ₂
16	2.87, m	40.9, CH	2.93, ddd (11.4, 6.9, 2.4)	40.8, CH
17		211.5, qC		211.7, qC
18-OH	4.42, br, s	76.6, qC	4.43, br, s	77.0, qC
19	5.59, d (11.7)	120.5, CH	5.61, d (11.7)	120.4, CH
20	6.60, d (11.7)	142.5, CH	6.51, d (11.7)	142.2, CH
22		149.0, qC		149.4, qC
24	1.11, d (6.6)	20.2, CH ₃	1.16, d (6.6)	20.1, CH ₃
25	1.44, s	24.6, CH ₃	1.50, s	24.4, CH ₃
26		128.6, qC		127.9, qC
27, 31	7.00, d (8.7)	130.3, CH	7.06, d (8.7)	130.5, CH
28, 30	6.80, d (8.7)	114.3, CH	6.87, d (8.7)	114.3, CH
29		158.7, qC		158.9, qC
29-OCH ₃	3.72, s	55.3, CH ₃	3.79, s	55.3, CH ₃

Scoparasin A (**3**) was obtained as a white solid with the molecular formula $\text{C}_{29}\text{H}_{35}\text{NO}_8$ on the basis of the HREIMS. The UV spectrum with maximum absorption bands at λ_{max} 224, 275, and 284 nm indicated that **3** had a conjugated aromatic chromophore. A strong absorption band at 1761 cm^{-1} in the IR spectrum indicated the presence of a vinyl carbonate moiety,^{4,5} while an absorption band at 1716 cm^{-1} was assigned to ketone and lactam carbonyl functional groups.^{4,5} The carbonyl carbon signals at δ 149.0, 169.9, and 211.5 were in agreement with the IR data. The ^1H and ^{13}C NMR data (Table 1) suggested that **3** was structurally related to cytochalasin K.⁴ In the ^1H NMR spectrum, they differed only in the signal pattern of the phenyl ring attached at C-10. Compound **3** possessed a *para*-methoxybenzene ring according to the presence of the characteristic proton resonances, δ 7.00 (d, $J = 8.7$ Hz, 2H), 6.80 (d, $J = 8.7$ Hz, 2H), and 3.72 (s, 3H). The relative configuration was identical to that of cytochalasin K on the basis of the following NOEDIFF results. Irradiation of H-3 α (δ 3.35) enhanced the signal intensity of only H-27 and H-31 (δ 7.00), but not H-4 (δ 3.81), indicating the *trans* relationship of H-3/H-4. Irradiation of H-8 (δ 2.81) affected the signal intensity of H-4, but not H-7 (δ 4.15), suggesting *cis* and *trans* relationships of H-4/H-8 and H-7/H-8, respectively. Upon irradiation of H-19 (δ 5.59), the signal intensity of H-16 (δ 2.87, m) and Me-25 (δ 1.44, s) was enhanced, indicating the *cis* relationship of H-19/H-16 and H-19/Me-25. Therefore, scoparasin A (**3**) was determined as a methyl ether derivative of cytochalasin K.

Scoparasin B (**4**) was obtained as a white solid with the molecular formula $\text{C}_{29}\text{H}_{37}\text{NO}_9$ on the basis of the HREIMS. The UV and the IR data were similar to those of **3**. The ^1H NMR spectrum (Table 1) was also similar to that of **3** except for an additional signal of

methine proton (δ 2.29, dd, $J = 7.5$ and 4.8 Hz) in **4**. In addition, signals of two methyl groups in the perhydroisoindolyl residue (Me-11, δ 1.11, d, $J = 7.2$ Hz and Me-12, δ 1.25, s) in **4** resonated at higher field than those in **3**. In the COSY spectrum, the additional methine proton was coupled with Me-11. The HMBC cross-peaks of Me-11 with C-4 (δ 48.1), C-5 (δ 35.8), and C-6 (δ 57.3) and those of Me-12 with C-5, C-6, and C-7 (δ 60.6) indicated that they were located at C-5 and C-6, respectively. Consequently, the methine proton was attributed to H-5. The chemical shift of C-6 suggested the presence of a hydroxyl substituent at C-6. Signal enhancement of H-3 α (δ 3.68, m) and Me-12 upon irradiation of Me-11 established their *cis* α -relationship. The relative configuration of the macrocyclic ring was identical to that of **3** on the basis of NOEDIFF results (see Supporting Information). Thus, scoparasin B (**4**) was identified as a new cytochalasin derivative.

Diaporthin A (**6**) exhibited ^1H and ^{13}C NMR, HMQC, and HMBC data identical to those of diaporthin A with a reported β -hydroxyl group at C-7.¹ The β -disposition of 7-OH has been assigned by comparison of its specific rotation with those of sphaeropsidins.¹ However, in this investigation it was observed that the H-7 resonance (δ 4.59, d, $J = 1.8$ Hz) in the NOEDIFF experiment was strongly enhanced by irradiation of H-14 (δ 5.98, t, $J = 1.8$ Hz), indicating that H-7 rather than 7-OH was located at the β -equatorial position.

All isolated compounds were tested for antimicrobial activities against *S. aureus* ATCC 25923 and *M. gypseum* SH-MU-4. Compound **7** gave the lowest minimum inhibitory concentration (MIC) value of $87.8\ \mu\text{M}$ against *S. aureus*, while compound **4** exhibited the best activity against *M. gypseum* SH-MU-4 with a MIC value of $30.3\ \mu\text{M}$.

Experimental Section

General Experimental Procedures. Melting points were measured on an electrothermal melting point apparatus (Electrothermo 9100). Optical rotations were measured on a JASCO P-1020 polarimeter. Infrared spectra (IR) were determined on a Perkin-Elmer 783 FTS165 FT-IR spectrometer. Ultraviolet (UV) absorption spectra were determined in MeOH on a Shimadzu UV-160A spectrophotometer. ^1H and ^{13}C NMR spectra were recorded in CDCl_3 on a 300 MHz Bruker FTNMR Ultra Shield spectrometer. Mass spectra were obtained on a MAT 95 XL mass spectrometer (ThermoFinnigan). Thin-layer chromatography (TLC) and pre-coated TLC were performed on silica gel GF₂₅₄ (Merck). Column chromatography was carried out on silica gel (Merck) type 100 (70–230 mesh ASTM).

Fungal Material. The endophytic fungus *E. scoparia* PSU-D44 was isolated from the leaves of *Garcinia dulcis*, collected in Songkhla Province, Thailand, in 2005. This fungus was deposited as PSU-D44 at the Department of Microbiology, Faculty of Science, Prince of Songkla University.

Extraction and Isolation. The endophytic fungus *E. scoparia* PSU-D44 was grown on potato dextrose agar (PDA) at $25\ ^\circ\text{C}$ for 5 days. Three pieces ($0.5 \times 0.5\ \text{cm}^2$) of mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300 mL of potato dextrose broth (PDB) at room temperature for 4 weeks. The culture (5 L) was filtered to give the filtrate and mycelia. The filtrate was extracted three times with EtOAc to afford a broth extract (212.1 mg) as a brown gum. The crude EtOAc extract was separated by column chromatography with a gradient system of increasing polarity (EtOAc–light petroleum and EtOAc–MeOH) to afford eight fractions (A–H). Fraction A was further purified by pre-coated TLC using 100% CH_2Cl_2 as a mobile phase (2 runs) to give **1** (4.4 mg) and **5** (3.7 mg). Fraction B gave **7** (20.5 mg). Fraction E was subjected to column chromatography using a gradient of acetone–light petroleum followed by a gradient of acetone–MeOH to give four subfractions (E1–E4). Fraction E2 contained **6** (11.3 mg). Fraction E3 was subjected to pre-coated TLC using 1% MeOH in CH_2Cl_2 as a mobile phase (5 runs) to afford **2** (1.3 mg). Fraction G was further separated by column chromatography with a gradient system of CH_2Cl_2 –MeOH to give three subfractions (G1–G3). Subfraction G2 was then subjected to column chromatography with a gradient system of CH_2Cl_2 –MeOH to afford **3** (3.2 mg) and **4** (14.9 mg).

Scoparane A (1): white solid; $[\alpha]_D^{29} +57.4$ (*c* 1.00, MeOH); UV-(MeOH) λ_{\max} nm (log ϵ) 231 (3.31); FT-IR (neat) $\nu_{\text{cm}^{-1}}$ 3394, 1701; ^1H NMR (300 MHz) 6.79 (1H, d, *J* = 1.5 Hz, H-14), 5.87 (1H, dd, *J* = 17.4, 10.5 Hz, H-15), 5.12 (1H, d, *J* = 17.7 Hz, H-16a), 5.07 (1H, d, *J* = 10.5 Hz, H-16b), 5.06 (1H, brs, 5-OH), 4.04 (1H, d, *J* = 9.9 Hz, H-20a), 3.73 (1H, d, *J* = 9.9 Hz, H-20b), 2.20 (1H, td, *J* = 13.5, 4.8 Hz, H-12a), 2.08 (2H, m, H-1), 1.71 (2H, m, H-11), 1.60 (1H, m, H-3a), 1.55 (1H, m, H-12b), 1.48 (1H, m, H-2), 1.43 (3H, s, H-19), 1.24 (1H, m, H-3b), 1.19 (3H, s, H-18), 1.16 (3H, s, H-17); ^{13}C NMR (75 MHz) 196.0 (C, C-7), 150.5 (CH, C-14), 144.9 (CH, C-15), 135.0 (C, C-8), 112.9 (CH₂, C-16), 104.3 (C, C-6), 81.7 (C, C-5), 74.6 (C, C-9), 68.5 (CH₂, C-20), 49.3 (C, C-10), 39.3 (C, C-13), 37.4 (CH₂, C-3), 37.1 (C, C-4), 29.5 (CH₂, C-12), 27.2 (CH₂, C-11), 27.0 (CH₃, C-18), 24.7 (CH₃, C-17), 23.5 (CH₃, C-19), 22.1 (CH₂, C-1), 17.5 (CH₂, C-2); HREIMS *m/z* 330.1823 [*M* - H₂O]⁺ (calcd for C₂₀H₂₆O₄ 330.1831); EIMS *m/z* (% relative intensity) 330 (43), 257 (36), 228 (30), 215 (61), 187 (41), 167 (100), 149 (32), 91 (31).

Scoparane B (2): colorless gum; $[\alpha]_D^{29} +232.5$ (*c* 0.04, MeOH); UV-(MeOH) λ_{\max} nm (log ϵ) 246 (3.91); FT-IR (neat) $\nu_{\text{cm}^{-1}}$ 3414, 1684; ^1H NMR (300 MHz) 6.13 (1H, dd, *J* = 17.7, 10.2 Hz, H-15), 5.34 (1H, dd, *J* = 10.2, 0.9 Hz, H-16a), 5.27 (1H, dd, *J* = 17.7, 0.9 Hz, H-16b), 5.06 (1H, brs, 5-OH), 4.45 (1H, d, *J* = 9.3 Hz, H-20a), 4.43 (1H, s, H-14), 4.42 (1H, dd, *J* = 6.3, 4.8 Hz, H-11), 3.29 (1H, d, *J* = 9.3 Hz, H-20b), 2.24 (1H, dd, *J* = 14.4, 4.8 Hz, H-12a), 2.01 (1H, dd, *J* = 14.4, 6.3 Hz, H-12b), 1.83 (1H, m, H-1), 1.70 (1H, m, H-1), 1.65 (2H, m, H-2), 1.60 (1H, m, H-3a), 1.56 (3H, s, H-19), 1.32 (1H, m, H-3b), 1.27 (3H, s, H-18), 1.03 (3H, s, H-17); ^{13}C NMR (75 MHz) 194.6 (C, C-7), 164.6 (C, C-9), 143.8 (CH, C-15), 132.3 (C, C-8), 115.1 (CH₂, C-16), 104.9 (C, C-6), 81.1 (C, C-5), 70.4 (CH₂, C-20), 70.0 (CH, C-11), 64.5 (CH, C-14), 54.2 (C, C-10), 40.1 (CH₂, C-12), 39.8 (C, C-13), 37.6 (CH₂, C-3), 36.8 (C, C-4), 28.0 (CH₃, C-18), 24.3 (CH₃, C-17), 24.1 (CH₃, C-19), 23.8 (CH₂, C-1), 17.8 (CH₂, C-2); HREIMS *m/z* 346.1748 [*M* - H₂O]⁺ (calcd for C₂₀H₂₆O₅ 346.1780); EIMS *m/z* (% relative intensity) 346 (6), 328 (56), 255 (46), 229 (42), 213 (49), 187 (70), 171 (42), 145 (46), 105 (40), 95 (51), 91 (60), 69 (100).

Scoparasin A (3): white solid; $[\alpha]_D^{29} +114.8$ (*c* 0.53, MeOH); UV-(MeOH) λ_{\max} nm (log ϵ) 224 (3.85), 275 (3.16), 284 (3.12); FT-IR (neat) $\nu_{\text{cm}^{-1}}$ 3386, 1761, 1716; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 525.2352 [*M*]⁺ (calcd for C₂₉H₃₅NO₈ 525.2363); EIMS *m/z* (% relative intensity) 525 (5), 367 (15), 246 (28), 228 (17), 218 (10), 121 (100).

Scoparasin B (4): white solid; $[\alpha]_D^{29} -52.7$ (*c* 0.17, MeOH); UV-(MeOH) λ_{\max} nm (log ϵ) 224 (3.63), 276 (2.82), 283 (2.76); FT-IR (neat) $\nu_{\text{cm}^{-1}}$ 3272, 1764, 1716; ^1H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 525.2350 [*M* - H₂O]⁺ (calcd for C₂₉H₃₅NO₈ 525.2363); EIMS *m/z* (% relative intensity) 525 (10), 367 (86), 246 (100), 218 (25), 121 (53).

Antibacterial Activity Testing. MICs were determined by the agar microdilution method.⁶ The test substances were dissolved in DMSO (Merck, Germany). Serial 2-fold dilutions of the test substances were mixed with melted Mueller-Hinton agar (Difco) in the ratio of 1:100 in microtiter plates with flat-bottomed wells (Nunc, Germany). Final

concentration of the test substances in agar ranged from 128 to 0.03 $\mu\text{g/mL}$. *S. aureus* ATCC 25923 was used as a test strain. Inoculum suspensions (10 μL) were spotted on agar-filled wells. The inoculated plates were incubated at 35 °C for 18 h. MICs were recorded by reading the lowest substance concentration that inhibited visible growth. Vancomycin, a positive control drug, exhibited an MIC value of 0.6 μM . Growth controls were performed on agar containing DMSO.

Antifungal Activity Testing. The hyphal extension-inhibition assay⁷ was used. A modification of the NCCLS M38-A broth microdilution test⁸ was performed against *M. gypseum* SH-MU-4. Equal volumes of a suspension of conidia (approximately 4×10^3 conidia/mL) were added to each test dilution to make final concentrations of 1–128 $\mu\text{g/mL}$ in triplicate. Plates were incubated at 25 °C for 72 h. Miconazole, a standard antifungal agent, gave an MIC value of 9.6 μM . The MICs were recorded for the lowest concentration that resulted in a reduction of approximately 50% of the fungal growth.

Acknowledgment. W.P. thanks the Royal Golden Jubilee Ph.D. Program of the Thailand Research Fund for a scholarship. The Higher Education Development Project: Postgraduate Education and Research Program in Chemistry, funded by the Royal Thai Government (PERCH) and the Graduate School, Prince of Songkla University, are gratefully acknowledged for material support. Finally, S.P. thanks the Bioresources Utilization Program (BUP) for a research grant.

Supporting Information Available: Tables of selected HMBC correlations and NOEDIFF data of **1–4**. These data are available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- Chinworrungsee, M.; Kittakoop, P.; Isaka, M.; Chanphen, R.; Tanticharoen, M.; Thebtaranonth, Y. *J. Chem. Soc., Perkin Trans. 1* **2002**, 22, 2473–2476.
- Dettrakul, S.; Kittakoop, P.; Isaka, M.; Nopichai, S.; Suyarnsestakorn, C.; Tanticharoen, M.; Thebtaranonth, Y. *Bioorg. Med. Chem. Lett.* **2003**, 13, 1253–1255.
- Evidente, A.; Sparapano, L.; Bruno, G.; Motta, A. *Phytochemistry* **2002**, 59, 817–823.
- Steyn, P. S.; Heerden, F. R.; Rabie, C. J. *J. Chem. Soc., Perkin Trans. 1* **1982**, 541–544.
- Buchi, G.; Kitaura, Y.; Yuan, S.-S.; Wright, H. E.; Clardy, J.; Demain, A. L.; Glinesukon, T.; Hunt, N.; Wogan, G. N. *J. Am. Chem. Soc.* **1973**, 95, 5423–5425.
- Lorian, V. *Antibiotics in Laboratory Medicine*, 4th ed.; William and Wilkins: Baltimore, 1996; pp 28–32.
- Huang X.; Xie W.; Gong Z. *FEBS Lett.* **2000**, 478, 123–126.
- National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. NCCLS documents M38-A; National Committee for Clinical Laboratory Standards: Wayne, PA, 2002.

NP0600649