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

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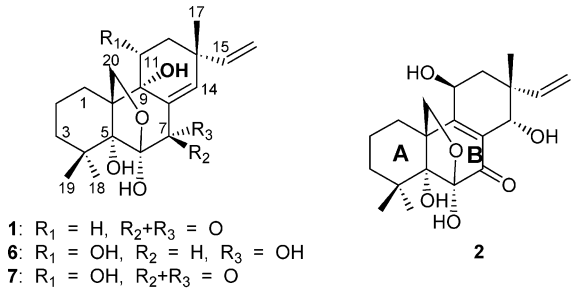
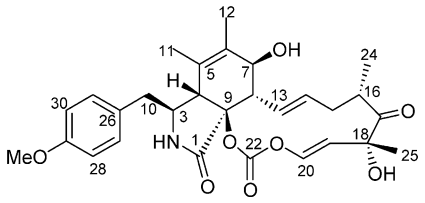
Two pimarane diterpenes, named scopararanes A (**1**) and B (**2**), and two cytochalasins, named scoparasins A (**3**) and B (**4**), along with 4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**5**) and diaportheins A (**6**) and B (**7**) were isolated from a culture broth of the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

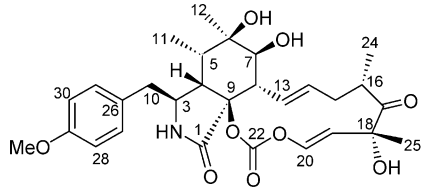
In the course of our ongoing search for antimicrobial substances from plants and endophytic fungi, the ethyl acetate extract from the culture broth of the endophytic fungus *Eutypella scoparia* PSU-D44 exhibited interesting antibacterial and antifungal activities against *Staphylococcus aureus* ATCC 25923 and *Microsporum 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-methyleneisochroman-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.

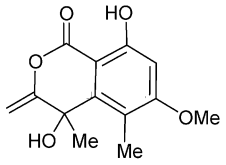
Scopararane A (**1**) was obtained as a white solid with the molecular formula $C_{20}H_{28}O_5$, as deduced from the HREIMS. The UV and IR spectra were almost identical to those of diaporthein B (**7**).[2] The $^1$H NMR data were similar to those of **7** except that the oxymethine proton resonance ($\delta$ 4.01, dd, $J$ = 11.7 and 4.2 Hz, H-11) in **7** was replaced by the methylene proton signal ($\delta$ 1.71, m) in **1**. Comparison of the $^{13}$C 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 $H_2$-11. This was confirmed by their HMBC cross-peaks with C-8 ($\delta$ 135.0), C-9 ($\delta$ 74.6), C-12 ($\delta$ 29.5), and C-13 ($\delta$ 39.3). The relative configuration was identical to that in **7** according to NOEDIFF results of H-20a ($\delta$ 4.04, d, $J$ = 9.9 Hz)/Me-19 ($\delta$ 1.43, s), H-20b ($\delta$ 3.73, d, $J$ = 9.9 Hz)/$H_2$-11, and $H_2$-11/Me-17 ($\delta$ 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 $C_{20}H_{28}O_6$, as deduced from HREIMS. The UV spectrum showed a maximum absorption band at $\lambda_{max}$ 246 nm, while IR hydroxyl and the carbonyl stretching frequencies were found in the region of 3414 and 1684 cm$^{-1}$, respectively. The $^1$H NMR data were similar to those of **7**[2] except that the signal of the $\beta$-olefinic proton ($\delta$ 6.80, d, $J$ = 1.8 Hz) of the $\alpha,\beta$-unsaturated ketone in **7** was replaced, in **2**, by an oxymethine proton ($\delta$ 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 ($\delta$ 194.6), C-9 ($\delta$ 164.6), C-15 ($\delta$ 143.8), and C-17 ($\delta$ 24.3). The $^{13}$C chemical shifts of C-8 ($\delta$ 132.3) and C-9 suggested the presence of a $C_8-C_9$ 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 ($\delta$ 3.29, d, $J$ = 9.3 Hz) affected Me-17 ($\delta$ 1.03, s), but not H-11 ($\delta$ 4.42, dd, $J$ = 6.3 and 4.8 Hz), indicating $\alpha$-equatorial and $\beta$-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 $\alpha$-equatorial position of H-11. Signal enhancement of H-14, upon irradiation of Me-17 in the NOEDIFF experiment, established the $\beta$-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.

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

| | **3** | | **4** | |
|---|---|---|---|---|
| position | $\delta_H$ ($J$ in Hz) | $\delta_C$ | $\delta_H$ ($J$ in Hz) | $\delta_C$ |
| 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)<br>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<br>b: 2.07, m | 39.0, $CH_2$ | a: 2.67, m<br>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 $C_{29}H_{35}NO_8$ on the basis of the HREIMS. The UV spectrum with maximum absorption bands at $\lambda_{max}$ 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 $\delta$ 149.0, 169.9, and 211.5 were in agreement with the IR data. The $^1$H and $^{13}$C NMR data (Table 1) suggested that **3** was structurally related to cytochalasin K.[4] In the $^1$H 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, $\delta$ 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$\alpha$ ($\delta$ 3.35) enhanced the signal intensity of only H-27 and H-31 ($\delta$ 7.00), but not H-4 ($\delta$ 3.81), indicating the *trans* relationship of H-3/H-4. Irradiation of H-8 ($\delta$ 2.81) affected the signal intensity of H-4, but not H-7 ($\delta$ 4.15), suggesting *cis* and *trans* relationships of H-4/H-8 and H-7/H-8, respectively. Upon irradiation of H-19 ($\delta$ 5.59), the signal intensity of H-16 ($\delta$ 2.87, m) and Me-25 ($\delta$ 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 $C_{29}H_{37}NO_9$ on the basis of the HREIMS. The UV and the IR data were similar to those of **3**. The $^1$H NMR spectrum (Table 1) was also similar to that of **3** except for an additional signal of a methine proton ($\delta$ 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, $\delta$ 1.11, d, $J$ = 7.2 Hz and Me-12, $\delta$ 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 ($\delta$ 48.1), C-5 ($\delta$ 35.8), and C-6 ($\delta$ 57.3) and those of Me-12 with C-5, C-6, and C-7 ($\delta$ 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$\alpha$ ($\delta$ 3.68, m) and Me-12 upon irradiation of Me-11 established their *cis* $\alpha$-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.

Diaporthein A (**6**) exhibited $^1$H and $^{13}$C NMR, HMQC, and HMBC data identical to those of diaporthein A with a reported $\beta$-hydroxyl group at C-7.[1] The $\beta$-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 ($\delta$ 4.59, d, $J$ = 1.8 Hz) in the NOEDIFF experiment was strongly enhanced by irradiation of H-14 ($\delta$ 5.98, t, $J$ = 1.8 Hz), indicating that H-7 rather than 7-OH was located at the $\beta$-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 $\mu$M against *S. aureus*, while compound **4** exhibited the best activity against *M. gypseum* SH-MU-4 with a MIC value of 30.3 $\mu$M.

## Experimental Section

**General Experimental Procedures.** Melting points were measured on an electrothermal melting point apparatus (Electrothermal 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. $^1$H 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 precoated TLC were performed on silica gel $GF_{254}$ (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 °C for 5 days. Three pieces (0.5 × 0.5 $cm^2$) 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 precoated 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 precoated 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).

**Scopararane A (1):** white solid; $[\alpha]^{29}_{D}$ +57.4 (*c* 1.00, MeOH); UV-(MeOH) $\lambda_{max}$ nm (log $\epsilon$) 231 (3.31); FT-IR (neat) $\nu_{cm^{-1}}$ 3394, 1701; $^1$H 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 ($CH_2$, C-16), 104.3 (C, C-6), 81.7 (C, C-5), 74.6 (C, C-9), 68.5 ($CH_2$, C-20), 49.3 (C, C-10), 39.3 (C, C-13), 37.4 ($CH_2$, C-3), 37.1 (C, C-4), 29.5 ($CH_2$, C-12), 27.2 ($CH_2$, C-11), 27.0 ($CH_3$, C-18), 24.7 ($CH_3$, C-17), 23.5 ($CH_3$, C-19), 22.1 ($CH_2$, C-1), 17.5 ($CH_2$, C-2); HREIMS $m/z$ 330.1823 $[M - H_2O]^+$ (calcd for $C_{20}H_{26}O_4$ 330.1831); EIMS $m/z$ (% relative intensity) 330 (43), 257 (36), 228 (30), 215 (61), 187 (41), 167 (100), 149 (32), 91 (31).

**Scopararane B (2):** colorless gum; $[\alpha]^{29}_{D}$ +232.5 (*c* 0.04, MeOH); UV(MeOH) $\lambda_{max}$ nm (log $\epsilon$) 246 (3.91); FT-IR (neat) $\nu_{cm^{-1}}$ 3414, 1684; $^1$H 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 ($CH_2$, C-16), 104.9 (C, C-6), 81.1 (C, C-5), 70.4 ($CH_2$, C-20), 70.0 (CH, C-11), 64.5 (CH, C-14), 54.2 (C, C-10), 40.1 ($CH_2$, C-12), 39.8 (C, C-13), 37.6 ($CH_2$, C-3), 36.8 (C, C-4), 28.0 ($CH_3$, C-18), 24.3 ($CH_3$, C-17), 24.1 ($CH_3$, C-19), 23.8 ($CH_2$, C-1), 17.8 ($CH_2$, C-2); HREIMS $m/z$ 346.1748 $[M - H_2O]^+$ (calcd for $C_{20}H_{26}O_5$ 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; $[\alpha]^{29}_{D}$ +114.8 (*c* 0.53, MeOH); UV-(MeOH) $\lambda_{max}$ nm (log $\epsilon$) 224 (3.85), 275 (3.16), 284 (3.12); FT-IR (neat) $\nu_{cm^{-1}}$ 3386, 1761, 1716; $^1$H and $^{13}$C NMR data, see Table 1; HREIMS $m/z$ 525.2352 $[M]^+$ (calcd for $C_{29}H_{35}NO_8$ 525.2363); EIMS $m/z$ (% relative intensity) 525 (5), 367 (15), 246 (28), 228 (17), 218 (10), 121 (100).

**Scoparasin B (4):** white solid; $[\alpha]^{29}_{D}$ −52.7 (*c* 0.17, MeOH); UV-(MeOH) $\lambda_{max}$ nm (log $\epsilon$) 224 (3.63), 276 (2.82), 283 (2.76); FT-IR (neat) $\nu_{cm^{-1}}$ 3272, 1764, 1716; $^1$H and $^{13}$C NMR data, see Table 1; HREIMS $m/z$ 525.2350 $[M - H_2O]^+$ (calcd for $C_{29}H_{35}NO_8$ 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 $\mu$g/mL. *S. aureus* ATCC 25923 was used as a test strain. Inoculum suspensions (10 $\mu$L) 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 $\mu$M. Growth controls were performed on agar containing DMSO.

**Antifungal Activity Testing.** The hyphal extension−inhibition assay[7] was used. A modification of the NCCLS M38-A broth microdilution test[8] was performed against *M. gypseum* SH-MU-4. Equal volumes of a suspension of conidia (approximately $4 \times 10^3$ conidia/mL) were added to each test dilution to make final concentrations of 1−128 $\mu$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 $\mu$M. The MICs were recorded for the lowest concentration that resulted in a reduction of approximately 50% of the fungal growth.

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**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.

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