10-Membered Macrolides from the Insect Pathogenic Fungus *Cordyceps militaris* BCC 2816

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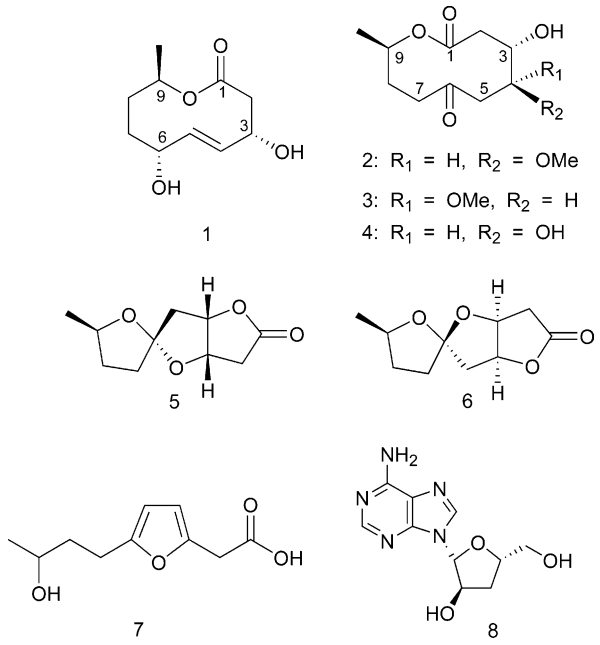
Three new 10-membered macrolides (**1**−**3**) have been isolated from the entomopathogenic fungus *Cordyceps militaris* BCC 2816, together with six known compounds, cepharosporolides C (**4**), E (**5**), and F (**6**), 2-carboxymethyl-4-(3′-hydroxybutyl)furan (**7**), cordycepin (**8**), and pyridine-2,6-dicarboxylic acid. The structures were determined by analysis of NMR data, and an X-ray analysis was performed to confirm the structure of **1**. The antimalarial activity of **1**−**4** and **8** against *Plasmodium falciparum* K1 was evaluated.

The genus *Cordyceps* is a rich source of biologically active secondary metabolites, for example, antimalarial erythrostominones,[1] antimalarial cordypyridones,[2] and antitumor sterols.[3] Although there has been considerable investigation on the genus *Cordyceps*, little has been reported about secondary metabolites from *C. militaris*.[4,5] Cordycepin (3′-deoxyadenosine), possessing antifungal, antivirus, and antitumor activities, is one of a few secondary metabolites previously isolated from *C. militaris*. We describe herein the isolation and structural elucidation of the new 10-membered macrolides (**1**−**3**) from *C. militaris* BCC 2816 together with six known compounds, cepharosporolides C (**4**), E (**5**), and F (**6**),[6] 2-carboxymethyl-4-(3′-hydroxybutyl)-furan (**7**),[7] pyridine-2,6-dicarboxylic acid, and cordycepin (**8**) and the antimalarial activity of **1**−**4** and **8** against *Plasmodium falciparum* K1 (multidrug-resistant strain).

Compound **1** was isolated as a white solid, from which the molecular formula was determined as $C_{10}H_{16}O_4$ by HREIMS. The IR spectrum showed absorption bands at 3448 (O−H) and 1720 cm^{-1} (lactone carbonyl). The ^{13}C NMR spectrum showed 10 carbon signals; one lactone carbonyl carbon (δ 170.3), two olefinic methine carbons (δ 130.3 and 133.0), three oxymethine carbons (δ 66.8, 74.3, and 72.9), three methylene carbons (δ 31.3, 37.0, and 44.0), and one methyl carbon (δ 20.6). These suggested that **1** might be a 10-membered macrolactone with one double bond, two hydroxyl groups, and one methyl group. The ^1H NMR spectrum showed signals of the secondary methyl group [δ 1.14 (d, J = 6.0 Hz)], three methylene groups [δ 2.48 (dd, J = 3.6 and 12.0 Hz), 2.53 (dd, J = 3.9 and 12.0 Hz), 1.66 (m), 1.96 (m), 1.57 (m), and 1.77 (m)], three oxymethine protons [δ 4.63 (m), 4.12 (m) and 4.77 (m)], and two olefinic protons [δ 5.76 (dd, J = 3.0 and 16.0 Hz) and 5.63 (ddd, J = 1.2, 8.1 and 16.0 Hz)]. In the COSY spectrum, the doublet methyl protons were vicinally coupled with the oxymethine proton at δ 4.77 (m, H-9). The methylene protons, H-8, at δ 1.57 and 1.77 were vicinally coupled with H-9 and methylene protons, H-7, at δ 1.66 and 1.96. The configuration of the double bond was *trans* from the coupling constant (J = 16.0 Hz). Each olefinic proton was further coupled with an oxymethine proton (δ 4.63 or 4.12). The oxymethine protons at δ 4.63 and 4.12 were assigned to allylic protons, H-3 and H-6, respectively, on the basis of ^3J HMBC correlations (see Supporting Information). Furthermore, two nonequivlent methylene protons, H-2 [δ 2.48 (dd, J = 3.6 and 12.0 Hz), 2.53 (dd, J = 3.9 and 12.0 Hz)], gave HMBC cross-peaks with the lactone carbonyl carbon (δ 170.3, C-1), the oxymethine carbon (δ 66.8, C-3), and the olefinic oxymethine carbon (δ 133.0, C-4). The connection of the structural units to form a 10-membered lactone was achieved on the basis of HMBC correlations between H-7/C-5 and H-9/C-1. The structure of **1** was then confirmed by X-ray crystallographic analysis, which also established the relative stereochemistry (Figure 1). Compound **1** is closely related to decarestrictine C_1, which was isolated from *Penicillium simplicissimum* and *P. corylophilum*,[8,9] differring only in the configuration at C-6.



Compound **2** was isolated as a colorless gum. The EIMS exhibited a fragment ion at m/z 229 $(M - CH_3OH)^+$, which corresponded to the formula $C_{10}H_{14}O_4$. The IR spectrum showed absorption bands at 3466 (O−H), 1735 (lactone

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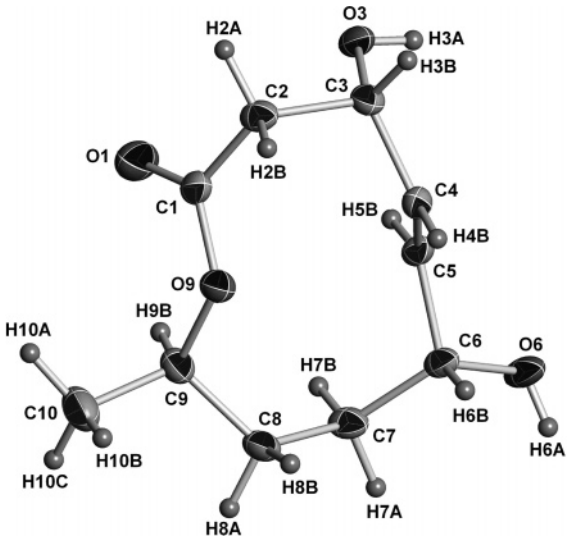

Figure 1. Crystal structure of **1**.

carbonyl), and 1728 cm^{-1} (ketone carbonyl). The ^1H NMR signals were similar to those of the co-metabolite, cepharosporolide C (**4**),[6] except for the presence of an additional singlet of a methoxyl group at δ 3.43. A carbon signal at δ 57.4 in the ^{13}C NMR spectrum supported the ^1H NMR data, suggesting that **2** was a methyl ether derivative of **4**. The methoxyl group was assigned to be at C-4 (δ 81.9) by a HMBC correlation from the methyl protons to C-4 (see Supporting Information). The relative stereochemistry of oxymethine protons at δ 4.11 (ddd, 3.0, 9.0, and 12.0 Hz, H-3) and 3.35 (ddd, 3.0, 7.5, and 9.0 Hz, H-4) was assigned as *trans* by the coupling constant value of 9.0 Hz[6] and the following NOEDIFF results. Irradiation of H-4 did not show signal enhancement of H-3 and vice versa. Therefore, **2** was assigned to be a methyl ether derivative of **4**. Cross-peaks in the COSY and HMBC spectra supported the assigned structure. Since compound **2** gave the specific rotation value $[\alpha]^{29}_D$ +59°, similar to **4** ($[\alpha]^{29}_D$ +68°, compared with +75°, the value cited in the previous literature[6]), the stereochemistry of **2** should be identical to that of **4**.

Compound **3**, isolated as a colorless gum, had the same molecular formula as **2** by HRMS and ^{13}C NMR data. The IR, ^1H NMR, and ^{13}C NMR spectra were similar to those of **2**. The location of the methoxyl group was assigned according to the HMBC correlation from the methyl protons (δ 3.41) to C-4 (δ 78.8). The NOE enhancement of the oxymethine proton at δ 4.28 (ddd, 3.0, 3.0, and 11.0 Hz, H-3) was observed after irradiation of the other oxymethine proton at δ 3.98 (ddd, 3.0, 4.5, and 11.0 Hz, H-4), suggesting that they were in a *cis* relationship. The coupling constant value of 3.0 Hz supported the assigned stereochemistry. From these results together with COSY and HMBC data, compound **3** was the second new methyl ether derivative of **4**. Since the other 10-membered lactones (**1** and **2**) isolated from the same extract had the same configurations at C-3 and C-9, it was concluded that compound **3** is the C-4 epimer of **2**.

The known compounds (**4**−**8**) and pyridine-2,6-dicarboxylic acid were identified by comparison of NMR spectral data with those previously reported. Compounds **1**−**4** and cordycepin (**8**) were subjected to in vitro antimalarial activity assay against *Plasmodium falciparum* K1. Cordycepin inhibited the growth of the parasite with an IC value of 4.5 μg/mL, while macrolides **1**−**4** were inactive up to a concentration of 20 μg/mL.

Experimental Section

General Experimental Procedures. Melting points were determined on an Electrothermal 9100 melting point apparatus and are uncorrected. Optical rotations were measured in MeOH at the sodium D line (590 nm) on an AUTOPOL II automatic polarimeter. Ultraviolet spectra (UV) were measured with a Specord S100 spectrophotometer (Analytik Jena Ag). Infrared spectra (IR) were obtained on a FTS165 FT-IR spectometer or a Perkin-Elmer Spectrum GX FT-IR system. ^1H and ^{13}C NMR spectra were recorded on a Varian UNITY INOVA 500 MHz or Bruker AMX 400 MHz spectrometer using deuteriochloroform solutions unless ortherwise stated with tetramethylsilane (TMS) as internal standard. EI and HREI mass spectra were measured on a ThermoFinnigan MAT95XL spectrometer. Thin-layer chromatography (TLC) and precoated TLC were performed on silica gel 60 GF_{254} (Merck). Column chromatography (CC) was performed on silica gel (Merck) type 100 (70−230 Mesh ASTM) eluted with either gradient system A ($CHCl_3$−15% MeOH−$CHCl_3$), B ($CHCl_3$−55% MeOH−$CHCl_3$), or C (light petroleum−80% EtOAc−light petroleum), unless otherwise stated. Light petroleum had a bp of 40−60 °C

Fungal Material. *Cordyceps militaris* was collected on Lepidoptera pupa, identified and isolated by Dr. Nigel L. Hywel-Jones. This fungus was deposited at the Thailand BIOTEC Culture Collection as BCC 2816 on September 15, 2000.

Fermentation and Isolation. BCC 2816 was maintained on potato dextrose agar at 25 °C for 15 days, which was cut into pieces (1 × 1 cm), and inoculated into 2 × 250 mL Erlenmeyer flasks containing 25 mL of Difco potato dextrose broth (PDB; composition, potato starch 4.0 g, dextrose 20.0 g, per liter) (10 pieces for each flask). After incubation at 25 °C for 6 days on a rotary shaker (200 rpm), each primary culture was transferred into a 1000 mL Erlenmeyer flask containing 250 mL of the same liquid medium (PDB) and incubated at 25 °C for 6 days on a rotary shaker (200 rpm). Each 25 mL portion of the secondary cultures (in 2 flasks) was transferred into 20 × 1000 mL Erlenmeyer flasks each containing 250 mL of PDB, and static fermentation was carried out at 25 °C for 27 days. The flask cultures were filtered to separate into filtrate and wet mycelia. The filtrate was extracted twice with an equal amount of EtOAc. The combined EtOAc solution was dried over anhydrous $MgSO_4$ and evaporated in vacuo to obtain a mixture of light brown oil and solid (2.31 g). The mycelial cakes were extracted with MeOH (500 mL). To the extract was added H_2O (50 mL), and the mixture was washed with hexane (500 mL). The aqueous MeOH layer was concentrated under reduced pressure. A mixture of EtOAc (600 mL) and H_2O (100 mL) was added to the concentrated solution. The EtOAc layer was evaporated to dryness to obtain a mixture of brown oil and solid (579 mg). The extracts from culture filtrate and mycelia were separately subjected to chromatographic fractionation. The extract from filtrate was fractionated by CC over Sephadex LH20 using MeOH as eluent to afford five fractions (A1−A5). Fraction A2 (478 mg) was further purified by CC with gradient system A to yield five fractions (B1−B5). Fraction B2 (85 mg, eluted with $CHCl_3$) was subjected to CC eluted with gradient system C to afford **5** (25 mg) and **6** (12 mg). Compound **4** (45 mg) was obtained from fraction B4 (78 mg, eluted with 5% MeOH−$CHCl_3$) after CC using gradient system C. Fraction A4 (236 mg) was fractionated by CC with gradient system B to yield five fractions. The fourth fraction (96 mg, eluted with 15% MeOH−$CHCl_3$) gave **8** (36 mg) after purification on CC with gradient system B. The crude extract from mycelia was fractionated by CC over Sephadex LH20 using MeOH as eluent to afford seven fractions (C1−C7). Fraction C2 (126 mg), upon CC with gradient system B, yielded **1** (16 mg), **4** (17 mg), **7** (9 mg), and a mixture of **5** and **6** (33 mg). Compound **2** (3 mg) was obtained from fraction C3 (245 mg) after CC with gradient system A followed by PTLC with 30% EtOAc−light petroleum. Fraction C4 (93 mg) was purified by CC with gradient system B to afford three fractions. Further separation of the second fraction (15 mg, eluted with 10%

MeOH−$CHCl_3$) by PTLC using 20% EtOAc−light petroleum yielded **3** (5.5 mg). Pyridine-2,6-dicarboxylic acid (8.6 mg) was obtained from fraction C6.

Compound 1: colorless crystals (MeOH/$CHCl_3$), mp 203−204 °C; $[\alpha]^{29}_D$ −55° (*c* 0.036, MeOH); IR (neat) ν_{max} 3448, 1720 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 5.76 (1H, dd, J = 3.0 and 16.0 Hz, H-4), 5.63 (1H, ddd, J = 1.2, 8.1, and 16.0 Hz, H-5), 4.77 (1H, m, H-9), 4.63 (1H, m, H-3), 4.12 (1H, m, H-6), 2.53 (1H, dd, J = 3.9 and 12.0 Hz, H-2), 2.48 (1H, dd, J = 3.6 and 12.0 Hz, H-2), 1.96 (1H, m, H-7), 1.77 (1H, m, H-8), 1.66 (1H, m, H-7), 1.57 (1H, m, H-8), 1.14 (3H, d, J = 6.0 Hz, H-10); ^{13}C NMR (CD_3OD, 125 MHz) δ 170.3 (C, C-1), 133.0 (CH, C-4), 130.3 (CH, C-5), 74.3 (CH, C-6), 72.9 (CH, C-9), 66.8 (CH, C-3), 44.0 (CH_2, C-2), 37.0 (CH_2, C-7), 31.3 (CH_2, C-8), 20.6 (CH_3, C-10); TOF MS *m/z* 201 $[M + H]^+$ (4), 183 (40), 182 (100), 167 (13), 165 (23), 164 (17), 158 (100); HREIMS *m/z* 200.1046 $[M]^+$ (calcd for $C_{10}H_{16}O_4$, 200.1049).

X-ray Structure Determination of 1. Crystal data of **1**: $C_9H_{16}O_4$, MW 200.23, monoclinic, $P2_1$(No. 4), a = 5.0240(6) Å, b = 7.6550(9) Å, c = 13.2534(16) Å, β = 92.823(2)°, V = 509.09(11) $Å^3$, D_x = 1.306 g/cm^3, Z = 2. A total of 4477 reflections, of which 2342 were unique (2133 observed, $I > 2\sigma(I)$), were measured at room temperature from a 0.254 × 0.246 × 0.078 mm^3 colorless crystal using graphite-monochromated Mo Kα radiation(λ = 0.71073 Å) on a Bruker APEX CCD diffractometer. The crystal structure was solved by direct methods, and all atoms except hydrogen atoms were refined anisotropically by full-matrix least-squares method on F^2 using SHELXTL-NT to give a final R-factor of 0.0430 (R_w = 0.0847).[11]

Compound 2: colorless gum; $[\alpha]^{29}_D$ +59° (*c* 0.017, MeOH); IR (neat) ν_{max} 3466, 1735, 1728 cm^{-1}; ^1H NMR (500 MHz) δ 5.09 (1H, m, H-9), 4.11 (1H, ddd, J = 3.0, 9.0 and 12.0 Hz, H-3), 3.43 (3H, s, OCH_3), 3.35 (1H, ddd, J = 3.0, 7.5, and 9.0 Hz, H-4), 2.93 (1H, dd, J = 7.5 and 18.0 Hz, H-5), 2.86 (1H, dd, J = 3.0 and 18.0 Hz, H-2), 2.63 (1H, dd, J = 3.0 and 18.0 Hz, H-5), 2.44 (1H, dd, J = 12.0 and 18.0 Hz, H-2), 2.42 (1H, ddd, J = 3.5, 6.5, and 13.5 Hz, H-7), 2.33 (1H, ddd, J = 3.5, 11.0, and 13.5 Hz, H-7), 2.12 (1H, m, H-8), 2.02 (1H, m, H-8), 1.25 (3H, d, J = 6.0 Hz, H-10); ^{13}C NMR (125 MHz) δ 208.6 (C, C-6), 169.2 (C, C-1), 81.9 (CH, C-4), 71.7 (CH, C-9), 68.3 (CH, C-3), 57.4 (CH_3, OCH_3), 41.7 (CH_2, C-5), 40.4 (CH_2, C-7), 39.7 (CH_2, C-2), 33.2 (CH_2, C-8), 19.6 (CH_3, C-10); EIMS *m/z* 198 $[M - CH_3OH]^+$ (8), 170 (10), 142 (27), 127 (15), 111 (25), 101 (100), 83 (35), 71 (13), 55 (33); HREIMS *m/z* 198.0864 $[M - CH_3OH]^+$ (calcd for $C_{10}H_{14}O_4$, 198.0892).

Compound 3: colorless gum; $[\alpha]^{29}_D$ −67° (*c* 0.015, MeOH); IR (neat) ν_{max} 3464, 1740, 1732 cm^{-1}; ^1H NMR (500 MHz) δ 5.04 (1H, m, H-9), 4.28 (1H, ddd, J = 3.0, 3.0, and 11.0 Hz, H-3), 3.98 (1H, ddd, J = 3.0, 4.5, and 11.0 Hz, H-4), 3.41 (3H, s, OCH_3), 2.81 (1H, dd, J = 4.5 and 18.0 Hz, H-5), 2.70 (1H, dd, J = 11.0 and 18.0 Hz, H-2), 2.57 (1H, dd, J = 3.0 and 18.0 Hz, H-2), 2.51 (1H, dd, J = 11.0 and 18.0 Hz, H-5), 2.41 (1H, ddd, J = 3.0, 11.0, and 13.0 Hz, H-7), 2.30 (1H, ddd, J = 3.0, 7.5, and 13.0 Hz, H-7), 2.09 (1H, m, H-8), 1.99 (1H, m, H-8), 1.23 (3H, d, J = 6.0 Hz, H-10); ^{13}C NMR (125 MHz) δ 209.7 (C, C-6), 169.9 (C, C-1), 78.8 (CH, C-4), 72.0 (CH, C-9), 66.7 (CH, C-3), 57.4 (CH_3, OCH_3), 42.9 (CH_2, C-5), 40.5 (CH_2, C-7), 37.4 (CH_2, C-2), 34.0 (CH_2, C-8), 19.5 (CH_3, C-10); EIMS *m/z* 198 $[M - CH_3OH]^+$ (10), 180 (6), 170 (5), 154 (14), 142 (34), 127 (20), 111 (40), 101 (100), 83 (35), 71 (13), 55 (33); HREIMS *m/z* 198.0879 $[M - CH_3OH]^+$ (calcd for $C_{10}H_{14}O_4$, 198.0892).

Biological Assay. The assay for activity against *P. falciparum* K1 was performed using the microculture radioisotope technique as described by Desjardins.[10] The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of $[^3H]$-hypoxanthine by *P. falciparum*. An IC_{50} value of 1.2 ng/mL was observed for the standard compound, dihydroartemisinin, in the same test system.

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Supporting Information Available: Table of HMBC correlations for compounds **1**−**3** is available free of charge via the Internet at http://pubs.acs.org.

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(11) Crystallographic data of compound **1** have been deposited at the Cambridge Crystallographic Data Centre under the reference number CCDC 241928. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (e-mail: deposit@ccdc.cam.ac.uk).

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