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Cytonic Acids A and B: Novel Tridepside Inhibitors of hCMV Protease from the Endophytic Fungus *Cytonaema* Species

Bingying Guo,* Jin-Rui Dai, Siewbee Ng, Yicun Huang, Chungyan Leong, Waichung Ong, and Brad K. Carté†

Centre for Natural Product Research, Institute of Molecular and Cell Biology, 59A Science Park Drive, The Fleming, Singapore Science Park, Singapore 118240

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Two novel human cytomegalovirus protease inhibitors, cytonic acids A (**1**) and B (**2**), have been isolated from the solid-state fermentation of the endophytic fungi *Cytonaema* sp. Their structures as *p*-tridepside isomers were elucidated by MS and NMR methods.

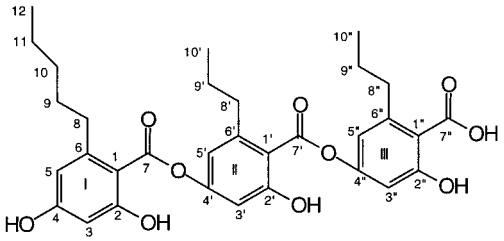
Human cytomegalovirus (hCMV) is a ubiquitous opportunistic pathogen. It causes disease in congenitally infected immune-deficient infants and adults.[1] A major function of the hCMV protease in infected cells is to proteolytically process the 44-kDa hCMV assembly protein precursor to yield the 37-kDa mature assembly protein.[2] This mature protein is essential for assembly of the virus nucleocapsid. In the presence of inhibitors of this protease, cleavage is disrupted, and the formation of infectious virus is thus prevented.

During our search for potential new anti-viral drugs using an hCMV protease inhibition assay, a series of active tridepsides was isolated from solid-state fermentation of the fungus *Cytonaema* sp. Two compounds have been purified and identified as novel *p*-tridepsides, named as cytonic acids A (**1**) and B (**2**). This report describes the isolation and structure elucidation of these two compounds.

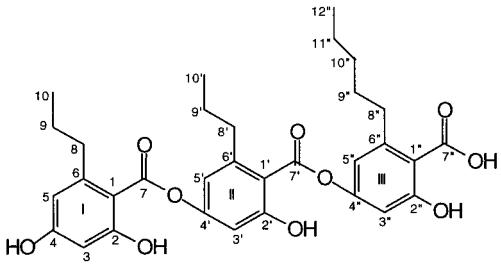
Results and Discussion

A solid-state fermentation extract of the fungus *Cytonaema* sp. showed activity against hCMV protease using high throughput screening (the IC_{50} of crude extract was 31 μg/mL). Following bioassay-guided fractionation, two active compounds, **1** (IC_{50} = 43 μmol) and **2** (IC_{50} = 11 μmol), were obtained.

The ^{1}H NMR spectra of compounds **1** and **2** displayed six aromatic protons (δ 6.23−6.67) and 25 aliphatic protons (δ 0.90−3.12). The ^{13}C NMR spectra of both compounds gave 32 carbon signals. Based on interpretation of their ^{13}C and DEPT spectral data, both compounds contain three methyl groups, eight methylenes, six aromatic methines, 12 aromatic quaternary carbons, and three carbonyl groups. Among the 12 aromatic quaternary carbons, six should be connected to −OR groups (δ 153.3−164.4), three to electron-withdrawing groups (δ 105.5−117.9), and the other three to alkyl groups (δ 146.0−149.4). The ^{1}H−^{1}H COSY data revealed that six aromatic protons are in three pairs, the protons of each pair are meta to each other (J = 2.13−2.43 Hz). Therefore, there should be three aromatic units within both **1** and **2**. Each aromatic unit is 4-subsituted and contains two *m*-protons. The ^{1}H−^{1}H COSY data also indicate that the eight methylenes and three methyl groups belong to three side chains. One *n*-C_5H_{11} side chain and two *n*-C_3H_7 side chains are evident. There are also one carboxylic carbonyl (δ 170.5) and two ester carbonyls (δ 165.8 and 168.9) in each compound. It is proposed that the three aromatic units are connected through ester bonds. All three aromatic units are 6-substituted 2,4-dihydroxylbenzoic acids. They are 1,4-connected, forming two esters, leaving the third carbonyl group free as an aromatic acid.



1



2

Both compounds **1** and **2** showed a $[M - H]^-$ peak at *m/z* 579 on negative ESIMS. Negative mode HRMS gave a peak at *m/z* 579.2214 $[M - H]^-$ for compound **1**, corresponding to the formula $C_{32}H_{36}O_{10}$. The same formula was deduced for compound **2** from the ESIMS and NMR data. MS data also suggested that each aromatic unit contains one −OH group and one −COOR group. This was confirmed by the fragmentation of ion 195, which gave peaks at 151, 177, and 178, indicating loss of CO_2, H_2O, and −OH, respectively. Similarly, MS/MS of ion 223 yielded fragments 179, 205, and 206. The major fragment ions of compounds **1** and **2** are given in the Supporting Information. These spectral data are in accordance with that of tridepsides.[3−13] By comparing the MS and NMR spectral data with those of known tridepsides, gyrophoric acid (see Supporting Information), lasalic acid, and crustic acid,[3,4] the structures of compounds **1** and **2** were established as the derivatives of *p*-tridepside.

The structures of compounds **1** and **2** were confirmed by NOESY, HMQC, and HMBC experiments. Major long-

* To whom correspondence should be addressed. Tel.: (65) 773-7071. Fax: (65) 773-7072. E-mail: bingying@cnpr.nus.edu.sg.

† Current address: Ancile Pharmaceuticals, 10555 Science Center Dr., Suite B, San Diego, CA 92121.

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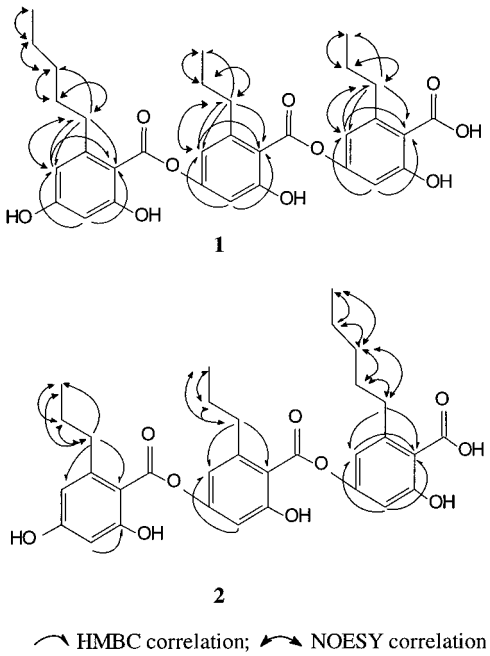

Figure 1. Major HMBC and NOESY correlations for compounds **1** and **2**.

range couplings (^2J and ^3J) observed in HMBC and major NOSEY correlations are shown in Figure 1.

In the HMBC spectra of compound **1**, long-range correlation between H-3 and H-5 to C-1 indicated that the carbonyl-substituted C-1 (δ 105.6) is at the *m*-position relative to both methine carbons. The correlations between H-5 and C-8 (δ 39.6) and between C-5 (δ 112.2), C-6 (δ 148.9), and H-8 indicated that the alkyl side chain is connected to C-6, and that C-5 and C-6 are in *o*-positions. The connectivities from C-8 to C-12 of the side chain $-C_5H_{11}$ were elucidated according to the $^1H-^1H$ COSY spectra. The skeletons of ring II and ring III were deduced in the same way. Since the −COOR-substituted C-1′ (δ 117.9) is para to the −O-acyl-substituted C-4′, this is a *p*-depside. In the HMBC spectrum of compound **2**, the relationships between C-1″, C-5″, and H-8″ indicate that the $-C_5H_{11}$ side chain is attached to ring III and that it is also a *p*-depside. Compounds **1** and **2** were named cytonic acids A and B, respectively.

Both compounds **1** and **2** showed in vitro inhibitory activities to hCMV protease. The IC_{50} for **1** was 43 μmol and for **2**, 11 μmol.

Experimental Section

General Experimental Procedures. HPLC analyses and isolations were carried out on Waters LC systems, including a 600E pump, a 996 photodiode array detector, and a 490 programmable multiwavelength detector. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained on a Bruker AVANCE-400 digital NMR spectrometer, using solvent peaks as reference standard. LC/MS, MS/MS spectra were obtained on a Perkin-Elmer API 300 spectrometer operating with ESI and heated nebulizer ion sources in negative mode (CAD, 2 V; OR, −30 V; RO_3, 35 V; CEM, 3000 V), linked to a Hewlett-Packard 1100 HPLC system using a Novapak C_{18} column (3.9 mm × 50 mm). HRMS spectra were obtained on a PerSeptive Biosystems Mariner TOF spectrometer operating with an ESI ion source in negative mode (spray tip potential, 3300 V; nozzle potential, 100 v; resolution, 5219). IR spectra were obtained with a Bio-Rad FTS-130 Fourier transform infrared spectrometer. UV spectra were measured on an Ultraspec 2000 UV/vis spectrophotometer. The scintillation was counted on a Wallac 1450 Microbeta Trilux counter.

Microorganism and Fermentation. The fungal strain F32027 (*Cytonaema* sp.) is an endophyte isolated from *Quercus* sp. in the U.K. by P.J. Fisher, University of Exeter, and deposited in the Centre for Natural Product Research culture collection. The strain was subcultured on malt extract agar (CM057B, Oxoid) for 7 days at 24 °C. It was used to inoculate 250-mL Erlenmeyer flasks each containing 50 mL of seed medium composed of 0.4% glucose, 1% malt extract, and 0.4% yeast extract. The pH of the medium was adjusted to 5.5 before sterilization. The seed flasks were incubated for 5 days at 24 °C on a rotary shaker at 200 rpm. A volume of 20 mL of seed culture was used to inoculate 200 mL of liquid medium. The medium was then added to Vermiculite in a plastic box with a surface area of 484 cm^2. The medium is composed of 3% maltose, 1% glucose, 0.008% yeast extract, and 0.2% peptone. It also contained trace amounts of KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeCl_3$, $ZnSO_4$, and $CaCl_2$. The pH was adjusted to 6.0. An aliquot of 200 mL of the liquid medium was added to 24 g of Vermiculite contained in plastic box and sterilized for further fermentation. The fermentation was carried out for 15 days at 24 °C under static conditions.

Biological Assays. The in vitro hCMV protease activity was measured in a 96-well scintillation proximity assay (SPA),[14] using a ^3H-labeled biotinylated oligopeptide, containing the cleavage site, as the substrate. The binding of the biotin to streptavidin SPA bead brings the ^3H to close proximity with the scintillant and emits photons that can be measured by a scintillation counter. The samples were dissolved in 12.5% aqueous DMSO and added to the protease solution (100 mM HEPES, 30% glycerol, 10 mM DTT). After a 45-min incubation, substrate was added. The enzymatic reaction was incubated at room temperature for 2 h. The reaction was stopped by adding 150 μL of streptavidin SPA beads (0.267 mg/mL in 3 mM $ZnCl_2$ solution). The scintillation was measured after a further 105-min settling period.

Extraction and Isolation. The freeze-dried fermentation (84 g) was extracted with CH_2Cl_2−MeOH (1:1) 3 times (10 mL solvent for each 1 g of dry material) and evaporated to dryness under vacuum. The dry extract (3.4 g) was first partitioned between hexane and 90% MeOH in H_2O (1:1) 3 times. The 90% MeOH portion was adjusted to 70% with H_2O, and then further partitioned 3 times with $CHCl_3$. The active $CHCl_3$ fraction (1.04 g) was then subjected to silica vacuum liquid chromatography, eluted with CH_2Cl_2−MeOH gradient. An enriched active fraction (410 mg) was obtained. It was fractionated by reversed-phase preparative HPLC (NovaPak C_{18} column, 5 μm, 25 mm × 100 mm) using gradient elution (flow rate, 10 mL/min; solvent A, 0.1% HCOOH in H_2O; solvent B, MeCN; solvent B increased from 50% to 80% in 30 min). One major active fraction (21 mg) was further purified by normal-phase HPLC (NovaPak silica column, 5 μm, 25 mm × 100 mm) with hexanes−EtOAc−HOAC (9:1:0.08%) as mobile phase (flow rate of 10 mL/min), to give compounds **1** (7.9 mg) and **2** (3.1 mg).

Cytonic acid A (1): white powder; UV (MeOH) λ_{max} (log ϵ) 211 (4.7), 253 (4.1), 284 (3.9) nm; IR ν_{max} (KBr) 3450, 1766, 1762, 1651, 1575, 1557, 1538, 1135, 1035, 824 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 6.67 (1H, d, J = 2.1 Hz, H-3″), 6.65 (1H, d, J = 2.2 Hz, H-5″), 6.53 (1H, d, J = 2.4 Hz, H-3′), 6.47 (1H, d, J = 2.4 Hz, H-5′), 6.29 (1H, d, J = 2.4 Hz, H-5), 6.23 (1H, d, J = 2.4 Hz, H-3), 3.10 (2H, t, J = 7.6 Hz, H-8′), 2.88 (2H, t, J = 7.7 Hz, H-8), 2.84 (2H, t, J = 7.7 Hz, H-8″), 1.55−1.77 (6H, m, H-9, H-9′ and H-9″), 1.30−1.42 (4H, m, H-10 & H-11), 0.97 (3H, t, J = 7.1 Hz, H-10″), 0.96 (3H, t, J = 7.3 Hz, H-10′), 0.90 (3H, t, J = 7.1 Hz, H-12); ^{13}C NMR (CD_3OD, 100 MHz) δ 170.5 (s, C-7″), [a]168.9 (s, C-7), [a]165.8 (s, C-7′), 164.4 (s, C-4), 164.3 (s, C-2), 162.7 (s, C-2″), 160.3 (s, C-2′), 154.3 (s, C-4″), 153.3 (s, C-4′), δ 149.1 (s, C-6′), 148.9 (s, C-6), 146.3 (s, C-6″), [b]117.9 (s, C-1′), [b]117.1 (s, C-1″), 115.6 (d, C-5″), 114.9 (d, C-5′), 112.2 (d, C-5), 108.6 (d, C-3″), 108.1 (d, C-3′), 105.6 (s, C-1), 102.0 (d, C-3), 39.6 (t, C-8), 38.5 (t, C-8′), 35.8 (t, C-8″), 33.0 (t, C-10), 32.6 (t, C-9″), 26.4 (t, C-9), 26.0 (t, C-9′), 23.6 (t, C-11), 14.6 (q, C-10′ and C-10″), 14.4 (q, C-12); ESIMS (negative) *m/z*

579 [M−H]$^-$ (2.2%), 195 (100%), 177 (54.1%), 401 (42.2%), 151 (32.4%), 205 (26.8%), 133 (14.6%), 223 (15.5%), 373 (9.2%); ESIMS/MS (negative) for 579: 579, 401, 373, 223, 195; ESIMS/MS (negative) for 401: 195, 223, 401; ESIMS/MS (negative) for 373: 195, 373; ESIMS/MS (negative) for 223: 223, 179; ESIMS/MS (negative) for 195: 151, 195; heated nebulizer APCI MS (negative) *m*/*z* 151 (100%), 195 (91.5%), 179 (48.3%), 137 (31.3%), 177 (22.5%), 223 (16.2%), 205 (18.6%). HRMS (negative) *m*/*z* 579.2214 (calcd for $C_{32}H_{35}O_{10}$, 579.2227, [M − H]$^-$). ([a,b] Assignments with same superscript may be interchanged.)

Cytonic acid B (2): white powder; UV (MeOH) λ_{max} (log ϵ) 212 (4.7), 250 (4.1), 286 (3.9) nm; IR ν_{max} (KBr) 3450, 1765, 1760, 1651, 1574, 1557, 1538, 1137, 1034, 828 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 6.67 (1H, d, J = 2.2 Hz, H-3″), 6.65 (1H, d, J = 2.2 Hz, H-5″), 6.52 (1H, d, J = 2.4 Hz, H-3′), 6.46 (1H, d, J = 2.4 Hz, H-5′), 6.29 (1H, d, J = 2.4 Hz, H-5), 6.23 (1H, d, J = 2.4 Hz, H-3), 3.12 (2H, t, J = 7.6 Hz, H-8″), 2.88 (2H, t, J = 7.7 Hz, H-8′), 2.82 (2H, t, J = 7.7 Hz, H-8), 1.65−1.77 (4H, m, H-9 & H-9′), 1.56−1.65 (2H, m, H-9″), 1.31−1.41 (4H, m, H-10″ and H-11″), 1.00 (3H, t, J = 7.4 Hz, H-10), 0.97 (3H, t, J = 7.4 Hz, H-10′), 0.90 (3H, t, J = 7.0 Hz, H-12″); ^{13}C NMR (CD_3OD, 100 MHz) δ 170.5 (s, C-7″), [c]168.9 (s, C-7), [c]165.8 (s, C-7′), [d]164.4 (s, C-4), [d]164.3 (s, C-2, C-2″), 160.2 (s, C-2′), 154.3 (s, C-4″), 153.3 (s, C-4′), 149.4 (s, C-6′), 148.9 (s, C-6), 146.0 (s, C-6″), [e]117.9 (s, C-1″), [e]117.2 (s, C-1′), 115.6 (d, C-5″), 114.8 (d, C-5′), 112.2 (d, C-5), 108.6 (d, C-3″), 108.0 (d, C-3′), 105.5 (s, C-1), 102.0 (d, C-3), 39.6 (t, C-8′), 37.7 (t, C-8), 36.4 (t, C-8″), 33.2 (t, C-10″), 32.8 (t, C-9″), 26.4 (t, C-9′), 25.9 (t, C-9), 23.7 (t, C-11″), 14.5 (q, C-10 and C-10′), 14.4 (q, C-12″); ESIMS (negative) *m*/*z* 579 [M − H]$^-$ (2.0%), 223 (100%), 401 (70.3%), 177 (52.7%), 133 (8.1%); ESIMS/MS (negative) for 579: 579, 401, 223, 195, 373, 151; ESIMS/MS (negative) for 401: 401, 223, 195, 177; ESIMS/MS (negative) for 373: 195, 177, 151; ESIMS/MS (negative) for 223: 223, 179; MS/MS (negative) for 195: 151, 195; heated nebulizer APCI MS (negative) *m*/*z* 223 (100%), 177 (100%), 179 (75.5%), 133 (70.6%), 401 (2.7%). ([c,d,e] Assignments with same superscript may be interchanged.)

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Supporting Information Available: Structure of gyrophoric acid and LC−MS/MS fragmentation routes of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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