Circumdatin G, a New Alkaloid from the Fungus *Aspergillus ochraceus*

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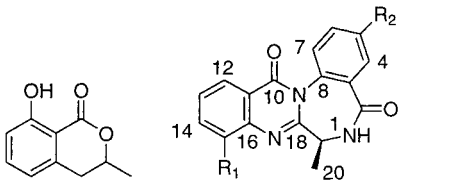
The crude extract of the broth of *Aspergillus ochraceus* was found to inhibit the final stage of polyprotein processing during hepatitis C virus replication. Bioassay-guided fractionation led to the isolation of the known compound mellein as the active component of the extract. Also isolated were circumdatin F and a new alkaloid, circumdatin G. The structure of circumdatin G was determined by spectroscopic analysis.

Hepatitis C virus (HCV) has a positive-strand RNA genome that contains a single large open reading frame encoding a polyprotein. During replication of HCV the final stages of polyprotein processing are performed by the viral protease NS3, a 70 kDa protein that possesses serine protease activity at its amino terminal and a helicase function at its carboxyl terminal.[1] The bifunctional role of NS3 protease makes this protein an attractive target for antiviral therapy.[1] A crude extract of *Aspergillus ochraceus* was found to be active in the HCV screen. Bioassay-guided fractionation led to the isolation of the known compound mellein (**1**)[2] (also known as ochracin) as the active component. Also isolated from the active fraction were circumdatin F (**2**)[3] and a new alkaloid, circumdatin G (**3**). Circumdatins A−F have been previously isolated from *A. ochraceus* and have been suggested as good chemotaxonomic markers for this species.[3,4] The alkaloid ring system present in the circumdatins has also been found in the asperlicins[5] and benzomalvins.[6]

A $CHCl_3$ extract from the lyophilized whole broth of *A. ochraceus* was separated by Sephadex LH-20 (CH_3OH), followed by C18 reverse-phase HPLC, to give mellein (**1**)[2] as the active component of the extract. Also isolated from the active fraction were circumdatin F (**2**)[3] and a new alkaloid, circumdatin G (**3**).



(**1**) mellein

(**2**) R_1=H, R_2=H circumdatin F
(**3**) R_1=OH, R_2=H circumdatin G
(**4**) R_1=H, R_2=OH circumdatin C

Examination of the NMR data of **2** (Table 1) indicated that **2** was identical to the previously reported circumdatin F.[3] Circumdatin F (**2**) was originally isolated in trace quantities and the structure assigned by comparison with circumdatin C (**4**).[4] As only ^{1}H NMR and MS data were previously reported for circumdatin F (**2**), the spectroscopic data are given in Table 1 and the Experimental Section. The stereochemistry at C-1 of **2** was assigned by comparison of the sign of the optical rotation value of **2** (−18.9°) with that of circumdatin C (**4**) (−75°), whose stereochemistry was determined by degradation to L-alanine.

The structure of alkaloid **3** was similar to that of circumdatin F (**2**). A molecular formula of $C_{17}H_{13}N_3O_3$ for **3**, determined by HRESIMS of the $[M + H]^+$ mass ion peak, indicated that **3** contained an extra oxygen atom compared with **2**. Examination of the ^{1}H and COSY NMR spectra of **3** (Table 1) indicated the presence of a 1,2,3-trisubstituted aromatic ring (δ 7.59, overlapped; 7.37, dd, J = 8.0, 8.0 Hz; 7.24, dd, J = 1.5, 8.0 Hz) in place of the 1,2-disubstituted aromatic ring present in circumdatin F (**2**). HMBC correlations from H-14 to C-16 and from H-15 to C-11 allowed the oxygen to be located at C-15 as a phenolic hydroxy group. The stereochemistry at C-1 of **3** was assigned by comparison of the sign of the optical rotation value of **3** (−21.7°) with that of circumdatin C (**4**) (−75°) as for circumdatin F (**2**).

Mellein (**1**) inhibited HCV protease with an IC_{50} value of 35 μM. Mellein (**1**) has been reported to have antifungal activity[2,7] but was found to be inactive against HIV-1 reverse transcriptase.[7] Other reports of natural products with activity against HCV protease include Sch 68631 (IC_{50} 7.7 μM)[8] and Sch 351633 (IC_{50} 24.7 μM).[9] Although Sch 351633 was claimed to be novel, the structure is identical to the known compound patulin.[10]

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Jasco DIP-1000 digital poloarimeter. UV spectra were recorded on a Pharmacia Biotech Ultrospec 2000 spectrophotometer. IR spectra were recorded on a Bio-Rad FTS 135 spectrometer. NMR spectra were recorded on a Bruker AMX 500 fitted with a Nalorac 3 mm inverse ^{1}H/BB probe with a z gradient coil using standard pulse sequences. HRMS were recorded on a VG Autospec-Q instrument at a resolving power of 10 000 (10% valley definition) using electrospray ionization in positive ion detection.

Microorganism and Fermentation. *A. ochraceus* Wilhelm was isolated from sediment collected in the Sea of Japan in 1995 by the Pacific Institute of Bioorganic Chemistry, Russian Academy of Science. The strain is deposited in the Centre for Natural Products Research fungal culture collection in liquid nitrogen. The strain was subcultured using Petri dishes with a medium composed of 1% glucose, 0.1% yeast extract, and 1.5% agar in natural seawater for 7 days at 24

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Table 1. NMR Data of Circumdatin F (**2**) and Circumdatin G (**3**) in CD_3OD

	2		**3**	
position	^{13}C (δ)	^{1}H (δ, mult., J in Hz)	^{13}C (δ)	^{1}H (δ, mult., J in Hz)
2	169.4		169.9	
3	131.9		132.2	
4	129.9	7.89 (dd, 1.5, 7.5)	130.2	7.80 (dd, 1.5, 8.0)
5	129.7	7.61 (ddd, 1.5, 7.0, 7.5)	129.9	7.60[a]
6	132.1	7.69 (ddd, 1.5, 7.0, 8.0)	132.3	7.68 (ddd, 1.5, 7.0, 8.0)
7	129.4	7.64 (dd, 1.5, 8.0)	129.8	7.62[a]
8	134.7		135.2	
10	163.2		163.8	
11	122.1		123.0	
12	127.5	8.26 (dd, 1.5, 8.0)	116.7	7.59[a]
13	128.3	7.58 (ddd, 1.0, 7.0, 8.0)	129.4	7.37 (dd, 8.0, 8.0)
14	135.8	7.87 (ddd, 1.5, 7.0, 8.0)	120.9	7.24 (dd, 1.5, 8.0)
15	128.3	7.78 (dd, 1.0, 8.0)	156.5	
16	147.4		136.9	
18	157.0		155.4	
19	51.0	4.43 (q, 6.5)	51.3	4.41 (q, 6.5)
20	14.9	1.66 (d, 6.5)	15.1	1.72 (d, 6.5)

[a] Peaks overlapped.

°C. Colonies on a Petri dish were then used to inoculate 3 × 250 mL Erlenmeyer flasks each containing 50 mL of seed medium composed of 0.4% glucose, a 1% malt extract, and 0.4% yeast extract in natural seawater with the pH being adjusted to 5.5 before sterilization. The seed flasks were incubated for 5 days at 24 °C on a rotary shaker (200 rpm). A 20 mL sample of seed culture was used to inoculate 5 × 2 L Erlenmeyer flasks containing 400 mL of production medium composed of 3.5% glucose, 0.5% peptone, 1% soluble starch, 2% soybean flour, 0.3% beef extract, 0.5% yeast extract, and 0.05% KH_2PO_4 in natural seawater with the pH being adjusted to 5.8 before sterilization. Fermentation was carried out for 9 days at 24 °C on a rotary shaker (200 rpm).

Biological Assay. Inhibition of HCV NS3 protease was measured in a 96-well plate scintillation proximity assay (SPA) using recombinant enzyme and a peptide substrate that is biotinylated at one end and ^{3}H-labeled on the other. Binding of intact peptide substrate to steptavidin-coated SPA bead gives rise to scintillation, while cleavage of the peptide by NS3 protease results in the release of the ^{3}H-labeled arm, generating no signal. Recombinant HCV NS3 protease was refolded in refolding buffer (0.4 M CHAPS, 1 M DTT, 1 M $MgSO_4$, and glycerol in 0.5 M Tris-HCl) for 2 h at 4 °C prior to the assay. Test samples were dissolved in 12.5% (v/v) DMSO. Refolded HCV NS3 protease (40 μL) and test sample (10 μL) were incubated at room temperature for 1 h, and then peptide substrate (10 μL) was added. After overnight incubation 10 μL of stop solution (0.4 mg/mL SPA beads in 100 mM MES, pH 5.0) was added and the plate shaken. The beads were allowed to settle for 30 min before measurement of generated light signals using a Microbeta counter.

Extraction and Isolation. The lyophilized whole broth (40 g) was extracted with $CHCl_3$ (3 × 200 mL) to give 560 mg of extract. A 150 mg aliquot was separated by Sephadex LH-20 (CH_3OH) to give six fractions. The active fractions, 3 (15 mg) and 4 (13 mg), were combined and separated by semipreparative C18 HPLC using a CH_3CN/H_2O gradient to yield mellein (**1**) (0.6 mg), circumdatin F (**2**) (2.0 mg), and circumdatin G (**3**) (1.7 mg).

Circumdatin F (2): white powder; $[\alpha]_D$ −18.9° (*c* 0.11, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 213 (2.79), 227 (2.83), 268 (2.24), 278 (2.17), 310 (1.85) nm; IR (film) 1698, 1675, 1626, 1605, 1471, 1368, 1315, 1165, 960 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD): see Table 1; (+)-APCIMS *m/z* 292 $[M + H]^+$; HRESIMS *m/z* 292.1084 $[M + H]^+$ (calcd for $C_{17}H_{14}N_3O_2$, 292.1086).

Circumdatin G (3): white powder; $[\alpha]_D$ −21.7° (*c* 0.19, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 215 (2.58), 235 (2.64), 276 (1.96), 278 (2.17), 327 (1.98) nm; IR (film) 3476, 1700, 1675, 1626, 1602, 1470, 1370, 1314, 1166, 961 cm^{-1}; ^{1}H and ^{13}C NMR (CD_3OD): see Table 1; (+)-APCIMS *m/z* 308 $[M + H]^+$; (+)-HRESIMS *m/z* 308.1026 $[M + H]^+$ (calcd for $C_{17}H_{14}N_3O_3$, 308.1035), 325.1299 $[M + NH_4]^+$ (calcd for $C_{17}H_{17}N_4O_3$, 325.1300).

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References and Notes

(1) Perni, R. B. *Drug News Perspect.* **2000**, *13*, 69−77.
(2) (a) Krohn, K.; Bahramsari, R.; Flörke, U.; Ludewig, K.; Kliche-Spory, C.; Michel, A.; Aust, H.-J.; Draeger, S.; Schulz, B.; Antus, S. *Phytochemistry* **1997**, *45*, 313−320, and references therein. (b) Cole, R. J.; Cox, R. H. *Handbook of Toxic Fungal Metabolites*; Academic Press: New York, 1981; pp 129−132.
(3) Rahbæk, L.; Breinholt, J. *J. Nat. Prod.* **1999**, *62*, 904−905.
(4) Rahbæk, L.; Breinholt, J.; Frisvad, J. C.; Christophersen, C. *J. Org. Chem.* **1999**, *64*, 1689−1692.
(5) Sun, H. H.; Barrow, C. J.; Sedlock, D. M.; Gillium, A. M.; Cooper, R. *J. Antibiot.* **1994**, *47*, 515−522.
(6) Sun, H. H.; Byard, S. J.; Cooper, R. *J. Antibiot.* **1994**, *47*, 599−601.
(7) Höller, U.; König, G.; Wright, A. D. *J. Nat. Prod.* **1999**, *62*, 114−118.
(8) Chu, M.; Mierzwa, R.; Truumees, I.; King, A.; Patel, M.; Berrie, R.; Hart, A.; Butkiewicz, N.; DasMahapatra, B.; Chan, T.-M.; Puar, M. S. *Tetrahedron Lett.* **1996**, *37*, 7229−7232.
(9) Chu, M.; Mierzwa, R.; He, L.; King, A.; Patel, M.; Pichardo, J.; Hart, A.; Butkiewicz, N.; Puar, M. S. *Bioog. Med. Chem. Lett.* **1999**, *9*, 1949−1952.
(10) (a) Reference 2b, pp 511−515. (b) Boukouvalas, J.; Maltais, F. *Tetrahedron Lett.* **1995**, *36*, 7175−7176, and references therein. (c) Shephard, G. S.; Leggott, N. L. *J. Chromatogr. A* **2000**, *882*, 17−22, and references therein.

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