Three New Cytochalasins Produced by an Endophytic Fungus in the Genus *Rhinocladiella*

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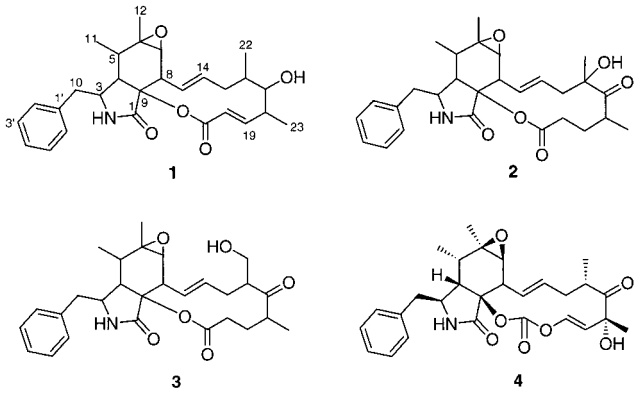
Three new cytotoxic cytochalasins (**1**−**3**) and the previously reported cytochalasin E (**4**) were isolated from a culture of the endophytic fungus *Rhinocladiella* sp. using bioassay-guided fractionation. Extensive NMR and HRCIMS experiments identified these new compounds as 22-oxa-[12]-cytochalasins.

In the course of screening for new bioactive natural products, an extract of the endophytic fungus *Rhinocladiella* sp. '309' was found to exhibit potent activity against several human cancer cell lines. *Rhinocladiella*, commonly found as a saprophyte on dead tree limbs, was isolated living as an endophyte of a viney medicinal plant, *Tripterygium wilfordii* (family Celastraceae). Because of its use in traditional medicine as a treatment for arthritis and other autoimmune diseases, *T. wilfordii* was collected from many locations in Asia and a study of its endophytes and their activity was undertaken. Previous bioassay-guided fractionation of an ethyl acetate extract of this same fungus '309' resulted in the isolation of cytochalasin E.[1]

Cytochalasins are a known class of mold metabolites first discovered in 1966.[2] There are now more than 20 known cytochalasins, isolated from a variety of fungal species, including *Helminthosporium* sp., *Phoma* sp., *Xylaria* sp., *Hypoxylon* sp., and *Chalara* sp.[3,4] Structurally, cytochalasins are composed of a highly substituted isoindolone ring with a benzyl group at the C-3 position and fused to an 11- to 14-membered macrocyclic ring.

The cytochalasins exhibit a broad spectrum of activity, including, but not limited to, antibiotic and antitumor activity,[5] inhibition of HIV-1 protease,[6] and phytotoxic activity.[7] The cytochalasins are widely used as biological probes, but their therapeutic application has been limited by their toxicity. Initial bioassay-guided fractionation of the fermentation extract from *Rhinocladiella* sp. resulted in the isolation of cytochalasin E as the most active component as well as the major metabolite. However, it was observed that the extract also contained a number of other compounds structurally related to cytochalasin E. In an attempt to find other, less toxic, more specific compounds, isolation and structure determination of these additional compounds was pursued and resulted in the discovery of three new 22-oxa-[12]-cytochalasins **1**, **2**, and **3**.

Based on the molecular weight of 465.3 (*m*/*z* 466.2585 $[M + H]^+$) as determined by HRCIMS, compound **1** was given the molecular formula $C_{28}H_{35}NO_5$ (calcd $[M + H]^+$ 466.2593). Analysis of the ^1H and ^{13}C NMR data for **1** indicated two ester and/or amide carbonyls, a monosubstituted benzene ring, four olefinic carbons, two methylene groups, eight methines, two quaternary carbons, and four



methyl groups. The molecule must contain two exchangeable protons, because only 33 protons are accounted for in the DEPT spectrum. The molecular formula requires 12 degrees of unsaturation. The four olefinic carbons, six aromatic carbons, and two other sp^2 carbons account for eight degrees of unsaturation. The remaining four degrees of unsaturation suggest that the molecule contains four rings in addition to the aromatic ring.

The $^1H-^1H$ connectivities as determined from the COSY spectrum with the aid of pulse-field gradient heteronuclear multiple quantum coherence (PFG−HMQC) led to the recognition of four fragments A, B, C, and D (Figure 1). The geometry of both the 13,14- and the 19,20-double bonds was determined to be E from the large coupling constants (J = 15.5 and 16.0 Hz, respectively). The chemical shifts of δ_C 61.4 (C-7) in partial structure A and of δ_C 78.9 (C-17) in B indicate both carbons are oxygenated.

The PFG heteronuclear multiple bond coherence (PFG−HMBC) spectrum was used to connect the four partial structures to determine the total structure of **1** as illustrated. Partial structure A was connected to fragment B by HMBC correlations of the carbon signal at δ_C 32.6 (C-16) to protons at δ_H 2.12 (H-15) and the carbon signal at δ_C 43.3 (C-15) to δ_H 0.96 (CH_3-22). The aromatic ring D was connected to partial structure C based on the correlations of the carbon signal at δ_C 45.8 (C-10) to the proton signal at δ_H 7.18 (H-2′).

The δ-lactam ring, rather than a lactone, was suggested by the chemical shifts of both the C-3 carbon and its proton (δ_C 54.2 and δ_H 3.68, respectively). The carbonyl carbon at

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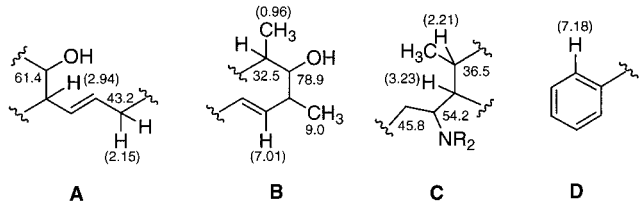

Figure 1. Partial structures of **1** as determined by COSY and HMQC correlations.

C-1 (δ_C 172.8) showed long-range heteronuclear correlations to the protons at δ_H 3.68, 3.13, and 2.94 (H-3, 4 and 8). Because the methine proton signal at δ_H 3.13 (H-4) was coupled to only two other protons at δ_H 3.68 (H-3) and 2.21 (H-5), it must also be attached to a quaternary carbon. This quaternary carbon was identified by the HMBC correlation of δ_H 3.13 (H-4) to δ_C 85.6 (C-9).

The six-membered cyclohexane ring of **1** was assigned by the correlation of the methyl singlet at δ_H 1.15 (CH_3-12) to δ_C 61.4 (C-7), 57.5 (C-6), and 36.5 (C-5) and by the correlation of the proton at δ_H 2.21 (H-5) to δ_C 57.5 (C-6). The δ-lactam, phenyl group, and six-membered ring are responsible for three of the five rings in compound **1**. The two remaining rings were accounted for by an epoxide at C-6/C-7, which was suggested by the chemical shifts of these two carbons (δ_C 57.5 and 61.4, respectively) and by the formation of a macrocyclic ring. Although no correlations can be observed, the macrocyclic ring is closed by the linkage of the quaternary carbon at δ_C 85.6 (C-9), which is typical of an oxygenated quaternary carbon, to the ester carbonyl at δ_C 167.3 (C-21).

The ^{1}H and ^{13}C NMR spectra of **2** showed 28 carbons and 35 protons, with two of the protons capable of exchanging. A molecular formula of $C_{28}H_{35}NO_6$ (MW 481.2) was calculated for **2** by HRCIMS (*m*/*z* 482.2536 $[M + H]^+$). The ^{1}H and ^{13}C NMR spectra of **2** closely resembled those for **1**. The main differences in the ^{13}C NMR spectra are the presence of an aliphatic ketone carbonyl (δ_C 220.4) and of two additional methylene carbons with the loss of two olefinic carbons. From detailed analysis of the COSY, PFG−HMQC, and PFG−HMBC, it was evident that the structural differences between **1** and **2** were restricted to the macrocyclic ring. Compound **1** showed carbon resonances at δ_C 121.7 and 156.8 for the 19,20-double bond conjugated to the ester, but the corresponding signals were not observed in the ^{13}C NMR of **2**. Instead, two methylene carbons appeared at δ_C 28.1 (C-19) and 32.7 (C-20). The protons of these two methylenes (δ_H 1.98 and 1.59 for H-19 and δ_H 2.36 and 2.54 for H-20) showed long-range heteronuclear correlations to the ester carbonyl at δ_C 171.7 (C-21) and to δ_C 39.4 (C-18). The carbon signal at δ_C 39.4 (C-18) was also correlated to a methyl proton signal at δ_H 1.06 (CH_3-23). This methyl resonated as a doublet and thus confirmed its bonding to a methine carbon. The ketone carbon (δ_C 220.4, C-17) was correlated to δ_H 2.53 (H-15a), 2.30 (H-15b), 1.62 (CH_3-22), and 1.06 (CH_3-23). In addition, the HMBC spectrum displayed cross-peaks from the hydroxyl proton (δ_H 3.76) to C-17 (δ_C 220.4), C-16 (δ_C 79.4), and C-22 (δ_C 23.8). These correlations placed the hydroxyl group on C-16 (δ_C 39.4) and established the connectivity as illustrated in **2**.

The molecular formula of **3** was determined to be the same as for **2** by HRCIMS: $C_{28}H_{35}NO_6$ (*m*/*z* 482.2530 $[M + H]^+$). A total of 28 carbons and 35 protons could be counted in the ^{13}C NMR and ^{1}H NMR spectra of **3**, which closely paralleled those for **2**. Comparison of the carbon spectra of **2** and **3** showed that **3** contained an oxygenated methylene carbon (δ_C 64.5, C-22) and a methine (δ_C 50.5, C-16) carbon not present in **2**. In addition, the carbon spectrum of **3** was missing an oxygenated methine (δ_C 79.4, C-16 in **2**) and a methyl group (δ_C 23.8, C-22 in **2**). Therefore, it appeared that the C-22 methyl group of **2** has been oxidized to a primary alcohol, while the tertiary alcohol at C-16 was reduced to a methine. The protons of the oxygenated methylene (δ_H 3.67 and 3.78, H-22) in **3** showed heteronuclear correlations to δ_C 216.9 (C-17), δ_C 50.5 (C-16), and δ_C 33.4 (C-15) in the HMBC experiment, and homonuclear correlations to δ_H 2.86 (C-16) in the COSY experiment. These correlations establish the connectivity as illustrated for **3**. A similar oxidation at C-22 has been reported for phomacin A going to phomacin B.[8]

Table 1. COSY and HMBC Correlation Data for **1** in CD_2Cl_2

position	COSY	HMBC
1		3.68, 3.13, 2.94
3	3.13, 2.80	3.13, 2.80, 2.21
4	3.68, 2.21	3.68, 2.80, 2.21, 0.97
5	3.13, 0.97	3.68, 3.13, 1.15, 0.97
6		3.13, 2.21, 1.15
7	2.94	2.94, 1.15
8	6.04, 2.68	2.68
9		3.68, 3.13, 2.94
10	3.68	7.18, 3.13
11	2.21	2.21
12		2.68, 2.21
13	5.12, 2.94, 2.12	2.94, 2.68
14	6.04, 2.12	2.94
15	5.12	6.04, 0.96
	6.04, 5.12	
16	3.78, 0.96	2.12, 2.09, 0.96
17	2.73, 1.63	7.01, 2.12, 2.09, 1.04
18	7.01, 3.78, 1.04	7.01, 5.67, 1.04
19	5.67, 2.73	1.04
20	7.01	7.01
21		7.01, 5.67
22	1.63	
23	2.73	
1′		7.34, 2.80
2′	7.34	7.34, 7.27, 7.18, 2.80
3′	7.27, 7.18	7.34, 7.27, 7.18
4′	7.34	7.18

Compound **4** was determined to have the molecular formula $C_{28}H_{33}NO_7$ by HRCIMS (*m*/*z* 496.2329). By comparison of ^{1}H and ^{13}C NMR, **4** was identified as cytochalasin E.

All three new cytochalasins are members of the 22-oxa-[12]-cytochalasin group. To date, only four other 22-oxa-[12]-cytochalasins have been reported: rosellichalasin;[9] 22-oxa-[12]-cytochalasa-6(12),13,19-triene-1,21-dione-7,18-dihydroxy-16,18-dimethyl-10-phenyl-(7*S**,13*E*,16*S**,18*S**,19*E*);[10] and phomacins A and B.[8] Evidence suggests that these 22-oxa-[12]-cytochalasins are derived from a Baeyer−Villiger type oxidation of a carbocyclic precursor of the [11]-cytochalasin group. A similar situation is known to occur with the [13]-cytochalasins to yield 24-oxa-[14]-cytochalasins, as has been demonstrated by the incorporation of deoxaphomin into cytochalasin B.[11]

The cytochalasins were tested against three different cell lines derived from human cancers. Based on the data presented in Table 2, cytochalasin E (**4**) is significantly more potent (15−100-fold) against all cell lines tested. Compounds **1**, **2**, and **3** show no significant selectivity in the cell lines tested.

Experimental Section

General Experimental Procedures. ^{1}H and 2D NMR experiments were performed on a Varian Unity 500 MHz

Table 2. Cytotoxicity Analysis in Human Tumor Cells

	(IC_{100} μg/mL)		
compound	2780S	HCT-116	SW-620
cytochalasin E (**4**)	< 0.0153	0.977	0.244
1	3.91	15.6	3.91
2	15.6	62.5	15.6
3	3.91		15.6

spectrometer, while ^{13}C NMR experiments (100 MHz) were performed on a Varian Unity 400 MHz spectrometer. NMR spectra were recorded using CD_2Cl_2 solutions, and the chemical shifts were referenced relative to the corresponding solvent signals (δ_H 5.32 and δ_C 54.0). The IR spectra were recorded on a Perkin-Elmer 16PC FTIR spectrometer. Mass spectral data were acquired by the University of Illinois, Urbana, Mass Spectrometry Facility.

Fungal Material. The endophytic fungus *Rhinocladiella* sp. was isolated from the perennial twining vine *Tripterygium wilfordii* and identified by Gary Strobel. It was obtained using the standard protocol for the isolation of endophytic microbes from plant materials.[12] It was identified on the basis of apical conidia appearing on the new growing points of conidiophores. The conidiophores became elongated by sympodial growth. The conidia themselves were subhyaline to dark and mostly one-celled ovoid to oblong-ellipsoid. The organism perfectly matched the description of *Rhinocladiella*.[13] A voucher of the fungus has been deposited in the Cornell University fungal herbarium, CUP. A subculture of the *Rhinocladiella* sp. was used to inoculate three 2-L Erlenmeyer flasks, each containing 500 mL of autoclaved modified M-1-D medium.[14] The cultures were grown at room temperature under still conditions for 21 days.

Extraction and Isolation. The fungal cultures were filtered through cheesecloth, and the filtrate (1.5 L) was extracted with EtOAc (2 × 1.5 L) and once with CH_2Cl_2 (1.5 L). The organic extracts were combined and concentrated in vacuo to yield 180 mg of a brown oil. This extract was chromatographed on a C_{18} column (1.5 × 7.0 cm) using a stepwise gradient of CH_3CN-H_2O (50 mL; 40, 50, 60, 70, 80, 90, and 100% CH_3CN in H_2O); collecting 10-mL fractions. Similar fractions were combined based on TLC. Combined fractions 7 and 8 were subjected to semipreparative reversed-phase HPLC (Supelcosil SPLC-18; 25 cm × 10 mm; 5 μm; 60% CH_3CN in H_2O) to yield **4** (15.2 mg; t_R 8.6 min) and **1** (2.4 mg; t_R 10.9 min). Fraction 6 was chromatographed on reversed-phase HPLC (Supelcosil SPLC-18; 25 cm × 10 mm, 5 μm) with a solvent gradient of 50% CH_3CN in H_2O to 100% CH_3CN to give **4** (7.2 mg; t_R 16.1 min) and **2** (3.6 mg; t_R 15.2 min). Fractions 4 and 5 were combined and chromatographed using the same method as described for fraction 6; this fractionation yielded 2.3 mg **3** (t_R 11.8 min).

Compound 1: white powder; ^1H NMR (CD_2Cl_2, 500 MHz) δ 7.34 (2H, dd, J = 7.0, 7.5 Hz, H-3′), 7.27 (1H, t, J = 7.5 Hz, H-4′), 7.18 (2H, d, J = 7.0, H-2′), 7.01 (1H, dd, J = 5.0, 16.0 Hz, H-19), 6.04 (1H, ddd, J = 2.0, 10.0, 15.5 Hz, H-13), 5.67 (1H, dd, J = 2.0. 16.0 Hz, H-20), 5.12 (1H, ddd, J = 4.0, 11.0, 15.5 Hz, H-14), 3.78 (1H, br s, H-17), 3.68 (1H, dd, J = 6.0, 7.5 Hz, H-3), 3.13 (1H, d, J = 6.0 Hz, H-4), 2.94 (1H, dd, J = 5.5, 10.0 Hz, H-8), 2.80 (2H, d, J = 7.5 Hz, H-10), 2.73 (1H, m, H-18), 2.68 (1H, d, J = 5.5 Hz, H-7), 2.21 (1H, quintet, J = 7.5 Hz, H-5), 2.12 (1H, quintet, J = 2.0 Hz, H-15a), 2.09 (1H, quintet, J = 2.0 Hz, H-15b), 1.63 (1H, m, H-16), 1.15 (3H, s, CH_3-12), 1.04 (3H, d, J = 7.0, CH_3-23), 0.97 (3H, d, J = 7.5 Hz, CH_3-11), 0.96 (3H, d, J = 7.5 Hz, CH_3-22); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 172.8 (C-1), 167.3 (C-21), 156.8 (C-19), 138.8 (C-14), 137.8 (C-1′), 129.9 (C-2′), 129.4 (C-3′), 127.5 (C-4′), 125.0 (C-13), 121.7 (C-20), 85.6 (C-9), 78.9 (C-17), 61.4 (C-7), 57.5 (C-6), 54.2 (C-3), 49.6 (C-4), 47.3 (C-8), 45.8 (C-10), 43.6 (C-18), 43.3 (C-15), 36.5 (C-5), 32.6 (C-16), 19.7 (C-12), 18.7 (C-22), 13.0 (C-11), 9.0 (C-23); HRCIMS calcd for $C_{28}H_{36}NO_5$ $[M + H]^+$ 466.2593; found 466.2585.

Compound 2: white powder; IR ($CHCl_3$) ν_{max} 1716, 1602, 1456, 1378, 1316, 1276 cm^{-1}; ^1H NMR (CD_2Cl_2, 500 MHz) δ 7.35 (2H, dd, J = 7.0, 7.5 Hz, H-3′), 7.27 (1H, t, J = 7.0 Hz, H-4′), 7.23 (2H, dd, J = 1.5, 7.5 Hz, H-2′), 5.87 (1H, dd, J = 9.0, 15.5 Hz, H-13), 5.49 (1H, ddd, J = 6.0, 9.0, 15.5 Hz, H-14), 3.66 (1H, dt, J = 4.5, 9.0 Hz, H-3), 3.36 (1H, ddd, J = 3.5, 6.5, 10.0 Hz, H-18), 3.05 (1H, dd, J = 9.0, 13.5 Hz, H-10a), 2.88 (1H, dd, J = 4.5, 13.5 Hz, H-10b), 2.75 (1H, d, J = 5.0 Hz, H-7), 2.54−2.48 (4H, m, H-4, H-8, H-15a, H-20a), 2.36 (1H, ddd, J = 3.5, 12.5, 15.5 Hz, H-20b), 2.30 (1H, dd, J = 5.5, 13.5 Hz, H-15b), 2.02 (1H, dq, J = 4.5, 7.0 Hz, H-5), 1.98 (1H, m, H-19a), 1.62 (3H, s, CH_3-22), 1.59 (1H, m, H-19b), 1.31 (3H, s, CH_3-12), 1.11 (3H, d, J = 7.5 Hz, CH_3-11), 1.06 (3H, d, J = 7.0 Hz, CH_3-23); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 220.4 (C-17), 171.8 (C-1), 171.7 (C-21), 138.4 (C-1′), 131.3 (C-13), 129.8 (C-2′), 129.5 (C-3′), 129.4 (C-14), 127.5 (C-4′), 84.2 (C-9), 79.4 (C-16), 60.1 (C-7), 57.5 (C-6), 55.0 (C-3), 51.5 (C-4), 49.2 (C-8), 46.2 (C-15), 44.0 (C-10), 39.4 (C-18), 36.6 (C-5), 32.7 (C-20), 28.1 (C-19), 23.8 (C-22), 20.6 (C-12), 17.4 (C-23), 14.1 (C-11); HRCIMS calcd for $C_{28}H_{36}NO_6$ $[M + H]^+$ 482.2543; found 482.2536.

Compound 3: white powder; IR ($CHCl_3$) ν_{max} 1718, 1602, 1428, 1276 cm^{-1}; ^1H NMR (CD_2Cl_2, 500 MHz) δ 7.35 (2H, dd, J = 7.0, 7.5 Hz, H-3′), 7.27 (1H, t, J = 7.5 Hz, H-4′), 7.22 (2H, d, J = 7.0 Hz, H-2′), 6.02 (1H, m, H-13), 5.71 (1H, m, H-14), 3.78 (1H, dd, J = 7.5, 11.0 Hz, H-22a), 3.67 (2H, m, H-3, H-22b), 3.22 (1H, m, H-18), 3.02 (1H, dd, J = 9.0, 13.0 Hz, H-10a), 2.86 (2H, m, H-10b, H-16), 2.73 (2H, m, H-7, H-8), 2.50 (2H, m, H-4, H-20a), 2.40 (1H, m, H-15a), 2.34 (1H, m, H-19b), 2.30 (1H, m, H-20b), 2.20 (1H, dt, J = 11.0,14.5 Hz, H-15b), 2.10 (1H, dq, J = 5.0, 7.0 Hz, H-5), 1.34 (1H, m, H-19b), 1.26 (3H, s, CH_3-12), 1.06 (3H, d, J = 7.0 Hz, CH_3-11), 0.99 (3H, d, J = 6.5 Hz, CH_3-23); ^{13}C NMR (CD_2Cl_2, 100 MHz) δ 216.9 (C-17), 171.9 (C-21), 171.8 (C-1), 138.2 (C-1′), 134.8 (C-14), 129.9 (C-2′), 129.5 (C-3′), 128.8 (C-13), 127.5 (C-4′), 84.2 (C-9), 64.5 (C-22), 59.9 (C-7), 57.7 (C-6), 54.9 (C-3), 50.9 (C-4), 50.5 (C-16), 49.5 (C-8), 44.0 (C-10), 43.1 (C-18), 36.9 (C-5), 33.4 (C-15), 33.2 (C-20), 29.0 (C-19), 20.3 (C-12), 13.7 (C-11), 13.3 (C-23); HRCIMS calcd for $C_{28}H_{36}NO_6$ $[M + H]^+$ 482.2543; found 482.2530.

Cytochalasin E (4): white powder; ^1H NMR and ^{13}C NMR, comparable to that in the literature;[1,15] HRCIMS calcd for $C_{28}H_{34}NO_7$ $[M + H]^+$ 496.2335; found 496.2329.

Bioassay for Cytotoxicity. The following human tumor cell lines were used to determine the cytotoxicity: A2780S (ovarian tumor cell line), SW-620 (colon tumor cell line), and HCT-116 (colon tumor cell line). Solutions of pure compounds were made at a concentration of 1.0 mg/mL in MeOH. Cell suspensions were diluted to 2.5 × 10^4 cells/mL and added by pipet (150 μL) into 96-well microtiter plates. Cells were then incubated for approximately 24 h in 5% CO_2 at 37 °C. Aliquots of test solutions (50 μL each) were added to the microtiter plates and then diluted 4-fold up the plate for a total of eight dilutions. After an additional 72-h incubation period, the cells were fixed with a solution of 10X Hanks' Balanced Salt Solution−37% (w/w) formaldehyde solution−water (1:1:8) for 10 min. Next the cells were stained with 0.0075% crystal violet solution for 15 min, and the concentration resulting in total cell kill (IC_{100}) was read.

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Supporting Information Available: ^1H and ^{13}C NMR spectra for **1**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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