Sequoiatones C−F, Constituents of the Redwood Endophyte *Aspergillus parasiticus*

Andrea A. Stierle,* Donald B. Stierle, and Timothy Bugni

Department of Chemistry and Geochemistry, Montana Tech of the University of Montana, Butte, Montana 59701

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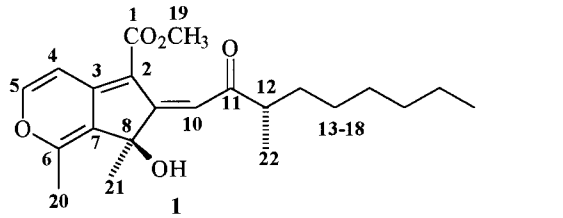
Aspergillus parasiticus, a fungal isolate from a coast redwood tree (*Sequoia sempervirens*), has been shown to produce four new compounds, sequoiatones C−F (**1**−**4**). The structures of these compounds, all of which are cytotoxic to brine shrimp, were deduced by spectral analysis.

Investigating the secondary metabolites of microorganisms isolated from unusual or specialized ecological niches may increase the chance of finding novel, bioactive compounds. For almost 10 years our laboratory has been investigating the endosymbiotic microorganisms of conifers and Native American medicinal plants, which have received little attention as sources of bioactive metabolites.[1] Plant endosymbionts are generally nonpathogenic in nature, but may produce compounds that enable them to survive in the competitive world of plant interstitial space. Some of these compounds could have useful medicinal or agrochemical applications.[2]

An *Aspergillus parasiticus* Speare isolate from the inner bark of a coast redwood tree *Sequoia sempervirens* (D. Don) Endl. (Taxodiaceae) has proven a prolific source of novel bioactive metabolites. Two of these metabolites, sequoiatones A and B, have been previously described. They were characterized by spectroscopic methods, and their absolute stereochemistry was established by X-ray crystallography.[3] The sequoiatones were isolated because of their brine shrimp lethality, which was used as a fractionation guide. They were sent to NCI for evaluation in the primary in vitro human tumor cell line screen and showed moderate, selective activity.[3] We report herein the isolation and characterization of four new sequoiatones, C−F (**1**−**4**).

The $CHCl_3$ partition of the MeOH extract of the mycelia of *A. parasiticus* was found to be active in the brine shrimp lethality test.[4] Fractionation on LH-20 and Si gel yielded four new compounds (**1**−**4**) that showed spectral similarities to sequoiatones A and B.[3] One-bond proton−carbon connectivities were established by HMQC experiments, and long-range proton and carbon connectivities were established by HMBC experiments.

Sequoiatone C (**1**) was isolated as an optically active pale yellow solid. It had a molecular weight of *m*/*z* 374 amu in the EIMS and a molecular formula of $C_{22}H_{30}O_5$, with eight degrees of unsaturation established by HREIMS. The ^{13}C NMR spectrum revealed 22 carbons (Table 1). Ten carbons were sp^2-hybridized: two were designated as carbonyl carbons because of the two $\nu_{C=O}$ stretching vibrations in the IR spectrum at 1680 and 1650 cm^{-1}. The carbons resonating at δ 208.0 and 165.5 indicated a ketone and a probable methyl ester moiety, respectively. The DEPT experiment showed that the eight remaining sp^2-hybridized carbons constituted one disubstituted, one trisubstituted, and two tetrasubstituted olefins. The UV spectrum, with λ_{max} 437 (log ϵ 3.93), indicated a conjugated triene or tetraene system. The two remaining degrees of unsaturation required a bicyclic system, consistent with sequoiatone B. The sp^3-hybridized carbons included one quaternary, one methine, five methylene, and five methyl carbons. These assignments accounted for 29 hydrogens. The last hydrogen was part of a hydroxyl group, as indicated by the broad O−H stretching vibration from 3500 to 3100 cm^{-1} in the IR spectrum.



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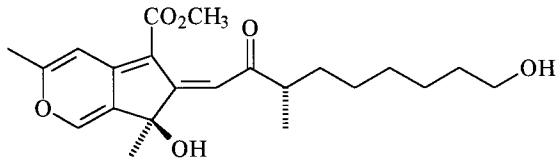

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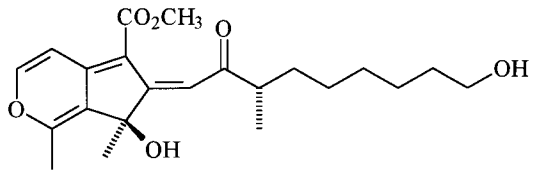

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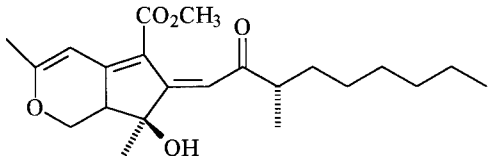

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Both mass spectral and NMR data indicated the presence of an eight-carbon aliphatic side chain analogous to sequoiatones A and B.[3] The facile loss of *m*/*z* 113 amu in the EIMS generated a base peak of *m*/*z* 261. Examination of both HMBC and $^1H-^1H$ COSY correlations indicated that the side chain was a *sec*-octyl moiety, consistent with sequoiatones A and B. The side chain accommodated $C_9H_{17}O$. The remainder of the molecule was $C_{13}H_{13}O_4$ with seven degrees of unsaturation.

The rest of the molecule was characterized by additional NMR experiments. The olefinic proton resonating at δ 7.19

* To whom correspondence should be directed. Tel: 406-496-4717. Fax: 406-496-4135. E-mail: astierle@mtech.edu.

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Table 1. NMR Data for Sequoiatone C (**1**) in $CDCl_3$ [a]

position	^{13}C	^{1}H		COSY	HMBC
1	165.5				
2	106.0				
3	169.0				
4	108.5	7.11	d (5.7)	H-5	C-2, C-5, C-7, C-20
5	152.4	7.34	d (5.7)	H-4	C-4, C-6
6	152.5				C-5, C-7, C-9
7	131.0				
8	78.6				
9	153.5				
10	113.0	7.19	s		C-2, C-3, C-4, C-8, C-11
11	208.0				
12	47.4	2.66	bhex (7.2)	H-22, H-13	
13	34.4	1.68	m	H-12	
		1.45	m		
14	34.3	1.4−1.18	8H m		
15	29.8				
16	32.1				
17	23.0				
18	14.1	0.85	3H t (7.9)	H-17	C-16,C-17
19	50.8	3.80 (s)	3H s		C-1
20	16.2	2.39 (s)	3H s	H-4	C-6, C-7
21	25.2	1.50 (s)	3H s		C-3, C-7, C-8
22	17.1	1.10 (s)	3H d (7.2)		C-11, C-12, C13
OH		7.51 (s)			C-7, C-8

[a] *J* values in Hz are shown in parentheses.

provided a starting point (Table 1). A HMQC experiment showed that it was directly attached to a carbon resonating at δ 113.0. It showed long-range correlations to the ketone carbon at δ 208.0 and to the quaternary olefinic carbons at δ 106.0 and 169.0. These data generated the $\alpha\beta,\gamma\delta$-unsaturated ketone typical of this family of compounds.[3] The olefinic proton at δ 7.19 also showed long-range correlation to the quaternary carbon at δ 78.6, which showed a point of connection to the bicyclic portion of the molecule. Both the hydroxyl proton at δ 7.51 and the methyl protons at δ 1.50 showed long-range correlations to this same oxygen-bearing carbon and to the olefinic carbon at δ 131.0. These connectivities established the B ring common to the sequoiatone skeleton.

The remaining olefinic protons, resonating at δ 7.11 and 7.34 (directly attached to carbons resonating at δ 108.5 and 152.4, respectively), were inconsistent with the normal sequoiatone skeleton, however. These two protons were mutually spin coupled with $J = 5.7$ Hz; the sequoiatone model did not accommodate a disubstituted olefin.[3] The coupling constant was too small for olefinic coupling in a six-membered ring carbocycle, but was consistent with olefinic coupling in a pyran ring.[5] The proton at δ 7.11 showed long-range correlations (HMBC) to the olefinic carbons resonating at δ 152.4 and 131.0. The proton at δ 7.34 showed long-range coupling to the carbons resonating at δ 108.5 and 152.6. These data generated the pyran ring typical of sequoiatones, but with a different pattern of methylation. Both of the quaternary olefinic carbons in the pyran ring showed long-range correlations to methyl protons resonating at δ 2.39. This placed the methyl group at position 6 of the pyran ring. The proton at δ 7.11 also showed long-range correlation to the carbon resonating at δ 106.0 (γ to the ketone carbon), establishing the typical trienyl backbone of the sequoiatones.

The remaining fragment consisted of a methyl singlet at δ 3.80, which showed long-range correlation to a quaternary carbon at δ 165.5 and which defined a methyl ester. There was only one point of attachment available to this moiety, at C-2, which generated **1**. Both the IR and UV spectra supported this structure. Molecular mechanics modeling of **1** using Hyperchem conjugate gradient geometry optimization[6] showed that compound **1** is a planar molecule: the bond torsion angles throughout the conjugated system are less than 5°. Conjugate resonance effects are optimized in planar systems.[7,8] The ketone $\nu_{C=O}$ at 1650 cm^{-1} requires an $\alpha\beta,\gamma\delta$-unsaturated ketone with maximum orbital overlap. The methyl ester $\nu_{C=O}$ at 1680 cm^{-1} also requires $\alpha\beta,\gamma\delta$-unsaturation with a δ-OR moiety.[9]

Sequoiatone D (**2**) was similar to sequoiatone B.[3] Isolated as an optically active yellow solid, **2** gave a molecular ion of *m*/*z* 390 and a molecular formula of $C_{22}H_{30}O_6$ by HREIMS. This was 16 amu larger than sequoiatone B, with a difference of one oxygen in the molecular formula. The loss of 113 amu typical of this family of compounds was not seen in **2**. Rather, there was a facile loss of 129 amu. These data suggested that sequoiatone D (**2**) is a hydroxylated derivative of sequoiatone B. The UV spectrum showed λ_{max} 428 (log ϵ 3.95), and the IR spectrum showed a broad O−H stretch at 3300 cm^{-1} and carbonyl absorptions at 1680 and 1650 cm^{-1}, both identical to sequoiatone B. The downfield regions of the ^{1}H NMR and ^{13}C NMR (Table 2) spectra were also virtually identical to that of sequoiatone B. These data indicated that the bicyclic ring system of sequoiatone B was intact. There were two discrepancies in both the mid- and high-field regions of the ^{1}H NMR spectrum; however, the terminal methyl triplet at δ 0.85 was absent and a two-proton triplet was present at δ 3.62. The $^{1}H-^{1}H$ COSY experiment showed that this distal triplet methylene at δ 3.62 was spin coupled to a methylene group at δ 1.52 that was further spin coupled into the multiplet at δ 1.2−1.4. As in sequoiatones A and B, the methine proton at δ 2.67 was spin coupled to a methyl at δ 1.10 and to two methylene protons resonating at δ 1.66 and 1.43. The chemical shift of the terminal methylene triplet was ideal for attachment to the additional hydroxyl moiety, generating the proposed structure for **2**.

Sequoiatone E (**3**) combined the ring system of sequoiatone C (**1**) and the alcoholic side chain of sequoiatone D (**2**). Compound **3** gave a molecular ion of *m*/*z* 390 and a molecular formula of $C_{22}H_{30}O_6$ by HREIMS, with eight sites of unsaturation. The ^{1}H NMR (Table 2) absorption of the olefinic protons of **1** and **3** were superimposable, and $^{1}H-^{1}H$ COSY and HMBC experiments confirmed that the bicyclic systems were identical, as were the UV and IR spectra. The side chain, however, clearly terminated in a primary alcohol. Comparison of the ^{1}H NMR, $^{1}H-^{1}H$ COSY, ^{13}C NMR (Table 2), and HMBC experiments of compounds **2** and **3** demonstrated that **3** is the C-18 alcohol of sequoiatone C (**1**).

Sequoiatone F (**4**) gave a molecular ion of *m*/*z* 376 in the EIMS and a molecular formula of $C_{22}H_{32}O_5$ in the HREIMS, with seven degrees of unsaturation. The eight-carbon aliphatic side chain was indicated by the facile loss of *m*/*z* 113 amu in the EIMS. ^{1}H NMR (Table 2), $^{1}H-^{1}H$ COSY, ^{13}C NMR (Table 2), and HMBC experiments confirmed the presence of this moiety, which has been fully described previously.[3]

The olefinic proton at δ 6.33 in **4** was attached to a carbon resonating at δ 100.3 (HMQC) and showed a small, allylic coupling to a methyl resonating at δ 2.05. The olefinic proton showed long-range coupling to a methylene carbon at δ 67.5 and to a methine carbon at δ 47.9. The methylene carbon was directly attached to protons resonating at δ 4.67 and 3.90, which showed mutual geminal coupling ($J = 10.5$ Hz). These protons were both spin

Table 2. ^{1}H and ^{13}C NMR Data for Sequoiatones D−F (**2**−**4**) in $CDCl_3$ [a]

position	**2**		**3**		**4**	
1		165.9		165.5		164.9
2		113.6		106.0		115.0
3		168.8		169.0		169.4
4	6.92 s	107.0	7.11 d (5.7)	108.5	6.33 bs	100.3
5		164.1	7.34 d (5.7)	152.4		169.7
6α	7.38 s	142.3		152.5	3.90 dd (14.5, 10.5)	67.5
6β					4.67 dd (10.5, 5.5)	
7		135.8		131.0	3.08 dd (14.5, 5.5)	47.9
8		76.2		78.6		79.8
9		152.1		153.5		158.4
10	7.18 s	113.8	7.19 s	113.0	7.14 s	115.3
11		206.4		208.0		208.1
12	2.67 hex (7.2)	47.8	2.66 hex (7.2)	47.4	2.67 hex (7.2)	47.8
13	1.67 m	34.4	1.68 m	34.4	1.67 m	33.7
13	1.43 m		1.45 m		1.37 m	
14	1.4−1.2	29.8	1.4−1.2	34.3	1.4−1.2	27.3
15	1.4−1.2	32.1	1.4−1.2	29.8	1.4−1.2	29.4
16	1.4−1.2	34.3	1.4−1.2	32.1	1.4−1.2	31.7
17	1.52	32.2	1.51	23.0	1.2	22.6
18	3.62 t (7.9)	62.8	3.65 t (7.9)	62.1	0.85 t	14.1
19	3.80 s	51.3	3.80 s	50.8	3.83 s	51.3
20	2.25 bs	20.7	2.39 bs	16.2	2.05 bs	21.6
21	1.50 s	27.7	1.50 s	25.2	1.22 s	22.5
22	1.10 d (7.2)	17.8	1.10 d (7.2)	17.1	1.10 d (7.2)	17.0
OH	7.51 s		7.65 s		7.17 s	

[a] *J* values in Hz are shown in parentheses.

coupled to the methine proton at δ 3.08, which was directly attached to the carbon at δ 47.9. The protons at δ 4.67 and 3.08 were coupled by $J = 5.5$ Hz, typical of axial−equatorial coupling in a six-membered ring. The proton at δ 3.08 also showed diaxial coupling to the proton at δ 3.90 ($J = 14.5$ Hz).[3] These data generated a saturated A ring reminiscent of sequoiatone A.[3] Both methylene protons showed long-range correlations to the carbon resonating at δ 79.8, which allowed closure of the B ring and generated the structure of **4** as shown. The similarity of spectral data, particularly the magnitude of $J_{6,7}$, indicated that the stereochemistry of **4** was consistent with that of sequoiatone A. The use of 1D-difference NOE experiments to confirm the relative stereochemistry of the ring system was inconclusive. When H-7 was irradiated, there was a small NOE enhancement of H-6β, which is consistent with the axial−equatorial relationship of the two protons. There was no enhancement of methyl protons on C-21. Unfortunately, although the absence of an NOE enhancement supports the proposed stereochemistry, it is not appropriate to use the lack of an NOE effect as a supporting argument.

The UV and IR spectral data of **4** were consistent with that of sequoiatone A, the only other dihydrosequoiatone reported to date.[3] These data suggest that bicyclic **4** exhibits the same degree of nonplanarity as tricyclic sequoiatone A. Hyperchem molecular modeling using conjugate gradient geometry optimization to drive the minimization provided structural insights.[6] The minimized model of **4** indicated that the dihydropyran ring is not planar, unlike the fully unsaturated systems of sequoiatone B and compounds **1**−**3**. The bond torsion angle C3−C2−C9−C10 is 24°, and C10−C9−C11−O is 85°. Nonplanar π bonds are not conjugated.[3]

Sequoiatones C−F (**1**−**4**) were also active in the brine shrimp lethality assay. The LD_{50} of **1** was 260 μM, **2** 1300 μM, **3** 640 μM, and **4** 260 μM.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 MC polarimeter using a 1 mL cell. IR spectra were recorded on a Perkin-Elmer 1310 spectrometer. ^{1}H and ^{13}C NMR spectra were run in $CDCl_3$ on Bruker DPX-300 or DRX-500 spectrometers. ^{1}H NMR spectra were recorded at 500 MHz, and the ^{13}C NMR spectra were recorded at 125 MHz unless otherwise noted. All of the chemical shifts were recorded with respect to the deuterated solvent shift ($CDCl_3$, δ 7.24 for the proton resonance and δ 77.0 for the carbon). Mass spectral data were provided by the Montana State Mass Spectrometry facility at Montana State University. All solvents used were spectral grade.

Collection, Extraction, and Isolation Procedures. A piece of outer bark (2 cm^2) was removed from a coast redwood in Santa Cruz, California, July 1996. A sterile scalpel was used to remove a tiny piece of phloem (8 mm^2), which was transferred to sterile water agar. The plate was incubated at room temperature for 3−10 days, during which proliferating hyphae and bacterial colonies were transferred to fresh DIFCO mycological or tryptic soy agar. Microorganisms were established as pure cultures using standard procedures. Isolation of individual microbes and determination of biological activity of fungal extracts have been described previously.[3]

One of the fungi isolated in this study was identified as *Aspergillus parasiticus* Speare by Microbial Identification, Inc, Newark, DE. The $CHCl_3$ extract of the pilot culture showed very little antimicrobial activity, but good brine shrimp lethality. The fungus was grown in 54 × 300 mL of DIFCO mycological broth cultures in 1 L Erlenmeyer flasks for 19 days, still. At harvest time, the fungus was again killed with the addition of 50 mL of MeOH/flask. The culture was filtered through cheesecloth, and the mycelial mat and the filtrate were handled separately.

The filtrate was extracted three times with 1 L of $CHCl_3$, and the extract was reduced in vacuo to an oil (2.36 g). The mycelial mat was pulverized in a Waring blender and soaked in 1 L of MeOH overnight. It was filtered through Whatman filter paper, then extracted (1 L of MeOH) and filtered two more times. The mycelial mat was then extracted twice with 1 L of $CHCl_3$−MeOH (1:1 v/v). The mat was air-dried, and the two organic extracts were reduced in vacuo and partitioned between $CHCl_3$ (1 L) and water (1 L), with all of the brine shrimp activity concentrated in these organic extracts. Mycelial MeOH, $CHCl_3$ soluble extract: 9.80 g; mycelial $CHCl_3$−MeOH, $CHCl_3$ soluble extract: 16.74 g; mycelial mat: 98.54 g. All of the extracts were tested for brine shrimp lethality. Most of the activity was concentrated in the mycelial MeOH,

$CHCl_3$ soluble extract. All subsequent isolation procedures were guided by this bioassay.

The mycelial MeOH, $CHCl_3$ extract (9.80 g) was fractionated using a large Sephadex LH-20 column ($CHCl_3$/MeOH, 1:1). The three large LH-20 fractions were further fractionated by preparative flash Si gel and finally purified by preparative HPLC on a Rainin column with a hexane/*i*-PrOH gradient to give sequoiatone C (**1**), 4.6 mg, sequoiatone D (**2**), 3.9 mg, sequoiatone E (**3**), 15.5 mg, and sequoiatone F (**4**), 21.5 mg.

Sequoiatone C (1): yellow solid, $[\alpha]^{25}_D$ +12.7° (*c* 0.63, MeOH); UV (MeOH) λ_{max} (log ϵ) 251 (3.56), 318 (3.51), 437 (3.93); IR ($CHCl_3$) ν_{max} 3400, 2920, 1680, 1650, 1495, 1448, 822 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m*/*z* 374 (20), 290 (6), 262 (19), 261 (100), 187 (20), 57 (10); HREIMS *m*/*z* 374.2084 (calcd for $C_{22}H_{30}O_5$, 374.2093).

Sequoiatone D (2): yellow oil, $[\alpha]^{25}_D$ +11.9° (*c* 0.46, MeOH); UV (MeOH) λ_{max} (log ϵ) 251 (3.55), 314 (3.45), 428 (3.95); IR ($CHCl_3$) ν_{max} 3300, 2910, 1680, 1650, 1490, 1440, 1160, 835 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; EIMS *m*/*z* 390 (8), 342 (9), 261 (100), 219 (25), 69 (10); HREIMS *m*/*z* 390.2044 (M+ calcd for $C_{22}H_{30}O_6$, 390.2042).

Sequoiatone E (3): yellow oil, $[\alpha]^{25}_D$ +37° (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (3.74), 319 (3.40), 433 (3.50); IR ($CHCl_3$) ν_{max} 3300, 2925, 2855, 1680, 1650, 1452, 1238 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; EIMS *m*/*z* 390 (10), 261 (100), 219 (32), 69 (15); HREIMS *m*/*z* 390.2050 (calcd for $C_{22}H_{30}O_6$, 390.2042).

Sequoiatone F (4): yellow oil, $[\alpha]^{25}_D$ +8.5° (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (3.59), 312 (3.63), 372 (3.66); IR ($CHCl_3$) ν_{max} 3300, 1698, 1680, 1560, 1210, 1060 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; EIMS *m*/*z* 376 (10), 263 (100), 231 (30), 57 (7); HREIMS *m*/*z* 376.2252 (M+ calcd for $C_{22}H_{32}O_5$, 376.2250).

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(9) IR spectra run in $CHCl_3$ give carbonyl frequencies 15 cm^{-1} less than those run in nonpolar solvents such as CCl_4. This is primarily due to the presence of ethanol, which is added as a stabilizer.[7,8]

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