Sequoiatones A and B: Novel Antitumor Metabolites Isolated from a Redwood Endophyte

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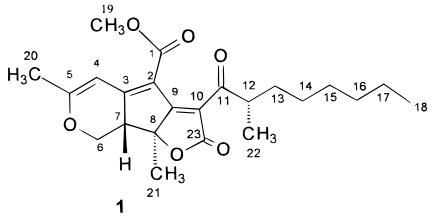
Received February 16, 1999

Sequoiatones A and B were isolated from the fungus *Aspergillus parasiticus*, an endophytic fungus of the coast redwood, *Sequoia sempervirens*. The compounds were isolated from the methanol extract of the mycelial mat of the fungus when grown in liquid culture for 21 days. The compounds were isolated because of their brine shrimp lethality, which served as an excellent guide for chromatographic purification. Full details of the isolation and characterization of sequoiatones A and B are provided herein, along with the test results from the NCI human tumor 60 cell-line screen.

Ever since the use of the fungal metabolite penicillin heralded the age of antibiotics in the 1940s, fungi and bacteria have provided an amazing array of important biologically active compounds. Many anticancer, antifungal, and antibacterial chemotherapeutics are either microbial metabolites or semisynthetic derivatives. The increasing incidence of certain cancers, of drug resistance in pathogenic microbes, and of infectious diseases in immunocompromised individuals necessitate the discovery of new chemotherapeutic agents. Investigating the secondary metabolites of microorganisms isolated from unusual or specialized ecological niches may increase the chance of finding novel compounds. For the past 7 years our laboratory, has been investigating the endosymbiotic microorganisms of conifers, which have received little attention as a source of bioactive metabolites. Plant endosymbionts are generally nonpathogenic in nature, but may produce secondary metabolites that enable them to survive in the competitive world of plant interstitial space. These bioactive compounds might prove suitable for specific medicinal or agrochemical applications.

A sample of *Aspergillus parasiticus* isolated from the inner bark of a coast redwood tree (*Sequoia sempervirens*) has proven a prolific source of novel bioactive metabolites. Two compounds, sequoiatones A and B, which were isolated from the mycelial organic extract, represent a new carbon skeleton. They were isolated on the basis of their brine shrimp lethality, which proved an effective guide for purification.

Sequoiatone A (**1**) was isolated as an optically active yellow crystalline solid. A molecular formula of $C_{23}H_{30}O_6$ and nine degrees of unsaturation were established by HREIMS. The ^{13}C NMR spectrum revealed 23 carbons. Nine carbons were sp^2-hybridized: three were designated carbonyl carbons because of the three C=O stretching vibrations in the IR spectrum at 1740, 1698, and 1680 cm^{-1}. The carbons resonating at δ 200.7 and 177.2 indicated ketone and ester functionalities, respectively. The third carbonyl carbon was not defined without additional information. The DEPT experiment showed that the six remaining sp^2-hybridized carbons constituted one trisubstituted and two tetrasubstituted olefins. The UV spectrum, with λ_{max} 378 (log ϵ 4.58), indicated a conjugated triene system. The three remaining degrees of unsaturation suggested that the compound has three rings. The sp^3-hybridized carbons included one quaternary, two methine, six methylene, and five methyl carbons. Hydrogens were completely accounted for, eliminating the possibility of a hydroxyl moiety.



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An HMQC experiment established one-bond proton and carbon connectivities for sequoiatone A (**1**), and an HMBC experiment established long-range proton and carbon connectivities. ^1H NMR and $^1H-^1H$ COSY provided evidence of an eight-carbon aliphatic side chain. It included five methylene groups: the proximal methylene protons resonated at δ 1.68 and 1.45, while the remainder generated a broad multiplet (δ 1.3−1.2) that integrated to eight protons. The proximal methylene protons were directly attached to a carbon resonating at δ 31.7 (HMQC) and were spin-coupled to two methylene protons at δ 1.26 and to a methine at δ 3.32. The methine was further coupled to a methyl doublet resonating at δ 1.12. The distal methylene group (δ 1.24) was spin-coupled to a terminal methyl group at δ 0.84. The overlapping chemical shifts of the methylene protons made carbon assignments difficult. Comparison to model systems and calculations were used to assign carbon chemical shifts to three of the methylene carbons. This eight-carbon moiety resulted in a facile loss of *m*/*z* 113 in the EIMS, yielding a base peak of *m*/*z* 289. The chemical shift of the methine proton (δ 3.32) suggested that it was connected to the rest of the compound through a carbonyl moiety. The HMBC experiment, which was optimized for 10 Hz coupling, supported this connectivity. The methyl doublet at δ 1.12 showed long-range correlations to the ketone carbon and to carbons at δ 44.2 and 31.7. These latter carbons were directly attached (HMQC) to the methine proton at δ 3.32 and to the methylene

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10.1021/jo990277l CCC: $18.00
Published on Web 07/07/1999

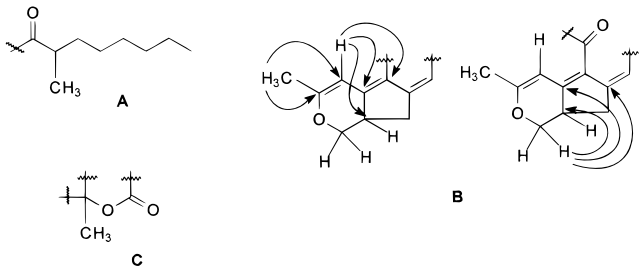


Figure 1. Structural fragments A−C for sequoiatone A (**1**) with selected HMBC correlations.

protons at δ 1.68 and 1.45, respectively. These data generated structural fragment A, which accommodated $C_9H_{17}O$ (Figure 1). The remainder of the molecule was $C_{14}H_{13}O_5$ with eight degrees of unsaturation.

The two remaining fragments of sequoiatone A (**1**) were defined by NMR experiments. The olefinic proton signal at δ 6.44 provided a starting point. An HMQC experiment indicated that it was attached to a carbon resonating at δ 100.0. $^1H-^1H$ COSY showed allylic coupling between this proton and a broadened methyl singlet resonating at δ 2.10, which, in turn, showed HMBC correlations to the carbon at δ 100.0 and to a quaternary carbon at δ 171.4. The δ 6.44 olefinic proton showed long-range correlations to two quaternary carbons resonating at δ 112.8 and 170.0, which generated the dienyl portion of the triene system. The chemical shift pattern of the dienyl carbons (δ 171.4, 100.0, 170.0, 112.8) was typical of attachment to oxygen at the downfield end and attachment to a carbonyl moiety at the upfield end of the conjugated system.[1]

The olefinic proton (δ 6.44) also showed long-range correlations to a methine carbon resonating at δ 43.2, which was attached to a proton at δ 2.72 (HMQC). The $^1H-^1H$ COSY experiment indicated that the proton at δ 2.72 was spin-coupled to two methylene protons at δ 4.71 and 3.95, which were directly attached to a carbon resonating at δ 66.1. The proton at δ 4.71 was spin-coupled to the methine proton (δ 2.72) with a vicinal coupling of 5.7 Hz, typical of axial−equatorial coupling in a six-membered ring.[2] The proton at δ 3.95 showed axial−axial coupling to the methine proton (J = 14.4 Hz).[2] The cis relationship of the two protons at δ 4.71 and 2.72 was supported by correlations in the ^1H NOESY experiment.

The downfield shift of the methylene carbon and protons indicated attachment to oxygen. The proton at δ 4.71 showed long-range correlations (HMBC) to the two quaternary carbons at δ 159.2 and 170.0 (part of the diene) and to the methine carbon at δ 43.2. These last two carbons also showed long-range correlations to the sole olefinic proton at δ 6.44. The HMBC correlations between the methylene proton at δ 4.71 and the olefinic carbon at δ 159.2, in conjunction with the UV data, confirmed that all three olefins were conjugated. ^4J(CH) correlations, though not commonly reported, have been seen in other rigid, highly conjugated systems.[3] The

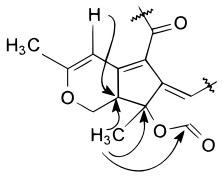


Figure 2. HMBC correlations between structural fragments B and C.

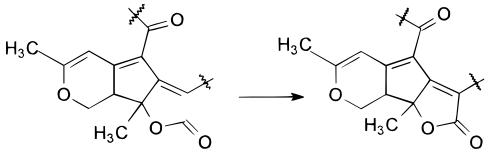


Figure 3. Establishment of tricyclic system for compound **1**.

chemical shifts of both the methylene carbon at δ 66.1 and the terminal olefinic carbon at δ 171.4 indicated attachment to oxygen. If both carbons were attached to the same oxygen, the proposed dihydropyran ring could be generated, as seen in fragment B (C_9H_7O), which accounted for four additional degrees of unsaturation (Figure 1). This left $C_5H_6O_4$ and four degrees of unsaturation for the undefined portions of compound **1**.

Characterization of the third fragment of the molecule relied on the HMBC experiment. The methyl singlet at δ 1.39 exhibited long-range correlations to a quaternary, oxygen-bearing carbon at δ 90.8 and to the ester carbonyl at δ 177.2. The chemical shift of the methyl protons was typical of attachment to an oxygen-bearing carbon. The HMBC experiment also showed long-range correlation between the methyl at δ 1.39 and the methine carbon resonating at δ 43.2, indicating a point of connection to fragment B. These data generated the ester moiety of fragment C (Figure 1). Fragment C contained $C_3H_3O_2$, with $C_2H_3O_2$ remaining for characterization. The remaining fragment consisted of a methyl singlet at δ 3.76, which showed long-range correlation to a quaternary carbon at δ 163.0 and which defined a methyl ester.

Fragments B and C could be assembled as shown in Figure 2. Logical connecting points generated the tricyclic system in Figure 3. The alternating shielded and deshielded chemical shifts of the triene were consistent with systems with an electron-withdrawing moiety at one end and an electron-donating moiety at the other end.[1] The IR spectrum supported the presence of the $\alpha\beta$-unsaturated γ-lactone. The νC=O of a γ-lactone is 1770 cm^{-1}. Conjugation shifts the carbonyl frequency to 1740 cm^{-1} when the spectrum is run in $CHCl_3$,[4] consistent with the IR spectrum of **1**.

It remained to connect the methyl ester and aliphatic side chain to the tricyclic system. The 2D ^1H NOESY experiment provided evidence for the structure shown. Nuclear Overhauser correlations were seen between the methoxyl protons resonating at δ 3.76 and the olefinic proton at δ 6.44. HyperChem modeling of compound **1** using molecular mechanics geometry optimization[5] indicated that these protons were 3.29 Å apart, adequate for nuclear Overhauser effects.[6] If the points of attach-

(1) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: Weinheim, 1987; pp 462, 278.

(2) *Applications of Nuclear Magnetic Resonance in Organic Chemistry*; Jackman, L. M., Sternhell, S., Eds.; International Series of Monographs in Organic Chemistry; Pergammon Press: Oxford, ***1969***; Vol. 5, p 288.

(3) Muller, N. *Magn. Reson.Chem.* **1985**, *23*, 688−689.

(4) Pavia, L.D.; Lampman, G. M.; Kriz, G.S. *Introduction to Spectroscopy*; Saunders College: Philadelphia, 1979; p 59.

(5) HyperChem Computational Chemistry, Autodesk, Inc.: Sausalito, CA, 1993.

(6) Kemp, W. *Organic Spectroscopy*, 3rd ed.; W. H. Freeman: New York, 1991; p 212.

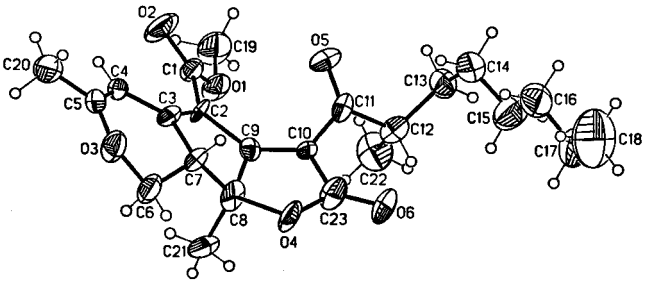
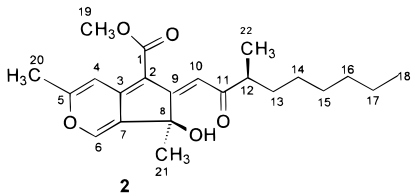


Figure 4. ORTEP drawing of sequoiatone A (**1**).

ment of the methyl ester and fragment A were reversed, these protons would be over 7 Å apart and NOE would not be possible.[6]

Sequoiatone A represents a unique skeletal system. Although the structure proposed could be readily generated from the available data, absolute stereochemistry and absolute structure proof were provided by X-ray crystallography. A suitable crystal that formed in the orthorhombic space group $P2_12_12_1$ was selected for the study, which confirmed the structure proposed and the stereochemistry shown. A computer-generated ORTEP drawing is shown in Figure 4.

Sequoiatone B (**2**) was isolated as an amorphous yellow solid. It displayed a molecular ion at *m*/*z* 374 and a molecular formula of $C_{22}H_{30}O_5$ (HREIMS), which required eight degrees of unsaturation. Compound **2** exhibited several spectral similarities to compound **1**, including the loss of *m*/*z* 113 in the EIMS spectrum. The UV spectrum for compound **2** indicated a chromophore with more extended conjugation than in compound **1**, with a λ_{max} of 428 nm (log ϵ 4.22). The IR spectrum exhibited two carbonyl absorbances at 1680 and 1650 cm^{-1} rather than three as in compound **1**. The absence of the carbonyl stretching frequency at 1740 cm^{-1} indicated the absence of an unsaturated γ-lactone in sequoiatone B. The ^{13}C NMR spectrum showed a ketone carbonyl carbon at δ 208.4 and a possible methyl ester carbonyl at δ 165.8, reminiscent of sequoiatone A. Despite the absence of one carbonyl, however, the ^{13}C NMR spectrum showed an additional sp^2-hybridized carbon, for a total of 10. If two of these were carbonyl carbons, the remaining eight would be olefinic, which satisfied six degrees of unsaturation overall. The two remaining degrees could be accommodated with two rings. Compound **2**, therefore, had one more olefin, one less ring, and one less carbonyl carbon than compound **1**.



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The DEPT experiment indicated that seven of the sp^2-hybridized carbons were quaternary and that three were methines. The HMQC experiment indicated that each of the methine carbons were attached to a singlet proton (δ 7.38, 7.18, and 6.92); three olefins, therefore, were trisubstituted, and the fourth was tetrasubstituted. Once again, the λ_{max} 428 (log ϵ 4.22) in the UV spectrum indicated a conjugated trienyl or tetraenyl system. The sp^3-hybridized carbons included one quaternary carbon,

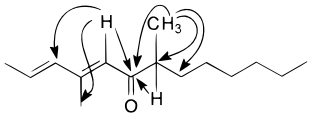


Figure 5. HMBC correlations between the aliphatic side chain and adjacent fragments of compound **2**.

one methine, five methylenes, and five methyl groups, including one methoxyl moiety, likely a methyl ester. These assignments accommodated all but one hydrogen, indicating the presence of a hydroxyl moiety, which was confirmed by the broad O−H stretching vibration from 3500 to 3100 cm^{-1} in the IR spectrum.

NMR experiments suggested that the eight-carbon aliphatic side chain (fragment A) of compound **1** was also present in compound **2**. The ^1H NMR spectrum of compound **2** showed 10 contiguous methylene protons. The proximal methylene protons absorbed at δ 1.66 and 1.45 and were directly attached to a carbon resonating at δ 34.4. The remainder of the methylene protons generated a broad, eight- proton multiplet that resonated between δ 1.4 and 1.2. The proximal methylene protons were spin-coupled to a deshielded methine at δ 2.67, which was further coupled to a methyl doublet at δ 1.10 and to another methylene group at δ 1.32. The distal methylene protons (δ 1.24) were directly attached to a carbon resonating at δ 23.0 (HMQC) and were spin-coupled to a methylene group at δ 1.37 and to a terminal methyl triplet at δ 0.85. Once again, the overlapping proton signals made it difficult to accurately determine ^{13}C NMR assignments for three of the methylene carbons. Calculations and comparison to model systems generated the assignments shown.

An HMBC experiment provided connectivity information between the aliphatic side chain and the rest of the molecule. Both the methine proton at δ 2.67 and the methyl doublet at δ 1.10 showed long-range correlations to the ketone carbonyl carbon at δ 208.4 (Figure 5). An olefinic proton at δ 7.18 that was directly attached to a carbon at δ 113.8 (HMQC) also showed long-range correlation to the ketone carbonyl. The proton at δ 7.18 showed additional long-range correlations to carbons resonating at δ 169.0, 113.3, 107.0, and 78.0. Once again, ^4J(CH) coupling was seen in this rigid, conjugated system.[3] It also appears that we are seeing ^5J(CH) coupling between the proton at δ 7.18 and the carbon at δ 107.0. An HMQC experiment indicated that the carbon resonating at δ 107.0 was attached to an olefinic proton at δ 6.92, which showed long-range correlations to quaternary carbons resonating at δ 164.3 and 135.8, and a methyl carbon at δ 20.7. The methyl carbon was attached to methyl protons resonating at δ 2.25 (HMQC). $^1H-^1H$ COSY also showed allylic coupling between this methyl group and the olefinic proton at δ 6.92.

These data generated a trienyl backbone analogous to that of compound **1**. The chemical shifts of the olefinic carbons were also similar, with the chemical shift pattern indicating attachment to oxygen at the lowfield terminus and conjugation with a carbonyl at the highfield terminus of the triene. The ^1H NMR data did not suggest a conjugated tetraene. The two remaining olefinic carbons, with chemical shifts at δ 142.3 and 135.8, were not consistent with the alternating resonance patterns of the other carbons. Rather, the fourth olefin was connected through the ethereal oxygen. The fragment depicted in

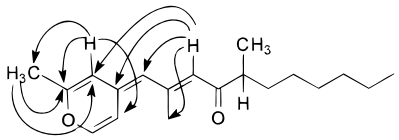


Figure 6. Trienyl backbone and side chain of compound **2**.

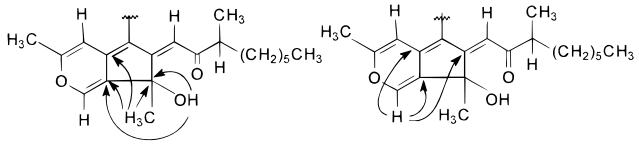


Figure 7. HMBC correlations from the southern half of compound **2** to triene backbone.

Figure 6 constituted six sites of unsaturation, with two as yet undefined. It used $C_{19}H_{23}O_2$, with $C_3H_7O_3$ unassigned. These three carbons included a methyl ester moiety and a methyl group. Two of the oxygens could be assigned to the methyl ester and the third to the hydroxyl moiety.

The final connectivities could be tackled from two directions using correlations from an HMBC experiment. The ^{1}H NMR indicated one exchangeable proton at δ 7.51. This hydroxyl proton could be correlated to quaternary carbons resonating at δ 135.8 and 78.0. A methyl singlet at δ 1.50 showed these same long-range correlations. The chemical shift of the methyl group is typical of attachment to an alcoholic carbon. The methyl protons were also correlated to the quaternary carbon at δ 169.0, which is part of the triene backbone. The olefinic proton resonating at δ 7.38 showed long-range correlations to three quaternary carbons at δ 169.0, 135.8, and 153.1, which provided connectivity to the triene backbone and to the final olefinic carbon (Figure 7). The methyl ester could be attached at only one site on the bicyclic ring system, which generated the final structure for sequoiatone B (**2**).

The stereochemistry of the trisubstituted olefin (9,10) and the conformation of the $\alpha\beta$-unsaturated ketone could be established by 1D difference NOE spectroscopy, infrared spectroscopy, and molecular modeling. There are four possible conformational modes available for the 9,-10-olefin of sequoiatone B: *Z-s-cis*; *Z-s-trans*; *E-s-cis*; and *E-s-trans*. All four of these conformers were modeled using both molecular mechanics geometry optimization and HyperChem PM3 semiempirical geometry optimization. Conjugate gradient methods (Fletcher−Reeves and Polak−Ribiere) were chosen to drive the minimization.[5] The *Z-s-cis* conformer was the lowest in energy (36 kcal/mol), followed by the *Z-s-trans* (42 kcal/mol), *E-s-cis* (46 kcal/mol), and *E-s-trans* (51 kcal/mol). In a 1D-difference NOE experiment, irradiation of olefinic H-10 showed a 3% enhancement of methoxyl H-19. NOE's are possible only when the internuclear distance (*r*) is not more than 4 Å. In the energy-minimized structures, only the *Z* isomers allow this proximity between H-10 and H-19. Both *Z* conformers have *r*[H-10−H-19] of 2.95 Å. For both of the *E* isomers, *r*[H-10 and H-19] is greater than 5 Å.

A second 1D-difference NOE experiment showed a 10.5% enhancement of methine H-12 and a 3% enhancement of methyl H-22 when olefin H-10 was irradiated. The close proximity of H-10 and H-12 is possible in the *E-s-cis* and *Z-s-cis* conformers, where *r*[H10−H12] are 2.19 and 2.20 Å, respectively. Both s-trans conformers have internuclear distances approaching 4 Å.

It is difficult to launch a rigorous discussion of infrared spectroscopy unless the spectra were run in dilute, nonpolar solvents. All of our spectra were run in chloroform stabilized with 1% ethanol, which shifts carbonyl stretching vibrations to lower frequencies, by about 15 cm^{-1}.[7,8] Yet certain trends in the infrared spectra clearly support the conformational assignment shown. In sequoiatone B (**2**), the ester carbonyl stretching vibration was 1680 cm^{-1}, which required $\alpha\beta,\gamma\delta$-unsaturation in conjugation with an electron-donating group in the δ position (ether oxygen).[6] Conjugation effects of νC═O are maximized in planar systems (bond torsion angle of 0°) and minimized if planarity is compromised by steric constraints.[7,8] Neither of the energy-minimized *E* conformers were planar: bond torsion angles of the $\alpha\beta$-unsaturated esters of both *E* conformers were 6°. The *Z-s-cis* conformer was clearly planar, however, with a bond torsion angle of 0°.

The νC═O of the unsaturated ketone (1650 cm^{-1}) also required $\alpha\beta,\gamma\delta$-unsaturation. Conjugation effects are maximized in a planar system, but in both *E* conformers, the bond torsion angle for the $\alpha\beta$-unsaturated ketone was 10°. For the energy-minimized *Z* conformers, the bond torsion angle was 3.5°. These data supported the *Z-s-cis* conformation, as shown.

A molecular formula search yielded a *Penicillium funiculosum* metabolite isolated by Katayama in 1989 with spectral data that were virtually identical to that of sequoiatone B (**2**).[9] The structure of the compound was not characterized in the publication. Communication with the author confirmed that he and his colleagues have not resolved the structure to date.[10]

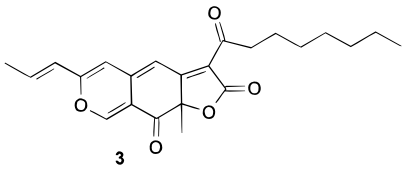
The spectral data for sequoiatones A and B showed many similarities, but there were a few key discrepancies that must be addressed. Although both compounds possess the same aliphatic side chain, the chemical shifts of the two methine protons (H-12) differed by 0.65 ppm. HyperChem modeling of both compounds using PM3 semiempirical geometry optimization provided a reasonable explanation for the discrepancy.[4] In the lowest energy conformer of sequoiatone A (**1**), H-12 is 2.37 Å from the ester carbonyl oxygen and resides in the anisotropic deshielding zone of that carbonyl. In sequoiatone B (**2**), the bicyclic system allows more degrees of freedom than the tricyclic system. In its lowest energy conformation, H-12 is not within such a zone and resonates at δ 2.67, which is more typical of a proton α to a carbonyl.

The IR spectra of the two compounds also showed discrepancies, primarily in νC═O. In both compounds, the νC═O of the methyl ester carbonyl was 1680 cm^{-1}. According to energy-minimized models, the resonance effects are the same for the methyl ester carbonyls for both **1** and **2**. The ketone νC═O, however, differed significantly in the two compounds. In sequoiatone A (**1**) the ketone νC═O was 1698 cm^{-1}, typical of a saturated

(7) Nakanishi, K.; Solomon, P. H. *Infrared Absorption Spectroscopy*, 2nd ed.; Holden-Day: San Francisco, 1977; p 44.
(8) Parikh, V. M. *Absorption Spectroscopy of Organic Molecules*; Addison-Wesley: Reading, 1974; p 257.
(9) Katayama, M.; Yanagi, M.; Marumo, S. *Agric. Biol. Chem.* **1989**, *53*(12), 3379−3380.
(10) Katayama, M. Personal communication, Government Industrial Research Institute, Nagoya, Japan, 1998.

ketone analyzed in $CHCl_3$. This is not surprising because in the energy-minimized model of **1** the bond torsion angle of the α*β*-unsaturated ketone is 23.8°. Little conjugation effect can be seen in such a nonplanar system, which is reflected in the magnitude of the *ν*C=O. In the energy-minimized *Z-s-cis* model of **2**, however, the bond torsion angle of the α*β*-unsaturated ketone is 3.5° and allows conjugate resonance, which lowered the *ν*C=O frequency to 1650 cm^{-1}.[7,8]

Sequoiatones A and B represent a new carbon skeleton that bears a superficial resemblance to the *Monascus* sp. metabolites monascorubrin **3**[11] and rubropunctatin **4**.[12] The sequoiatones were isolated because of their brine shrimp lethality, which was used as a fractionation guide. Sequoiatone A has an LC_{50} of 4.35×10^{-4} M, and sequoiatone B has an LC_{50} of 1.5×10^{-5} M. The two compounds were also tested against several different bacteria and fungi at several different concentrations in standard disk assays and exhibited no antimicrobial activity. The compounds were sent to the National Cancer Institute for primary in vitro testing against a panel of 60 different human tumor cell lines.[13,14] The compounds showed moderate and somewhat selective inhibition of human tumor cells, with greatest efficacy against breast cancer cell lines. Most of the GI_{50}'s were between 4 and 10 μM with LC_{50}'s > 100 μM.



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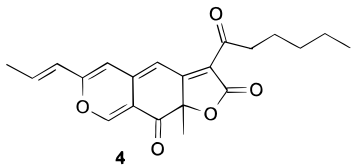

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Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were run in $CDCl_3$ on a Bruker DPX-300 or DRX-500 spectrometer. ^1H 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 chloroform solvent shift (*δ* 7.24 for the proton resonance and *δ* 77.0 for the carbon resonance). IR spectra were recorded on a Perkin-Elmer 1310 spectrometer in $CHCl_3$. Optical rotations were recorded on a Perkin-Elmer 241 MC polarimeter using a 1 mL cell. Mass spectral data were provided by the Montana State Mass Spectrometer facility at Montana State University. All solvents used were spectral grade.

Collection, Extraction, Isolation, and Bioassay Procedures. A piece of outer bark (2 cm^2) was removed from a coast redwood in Santa Cruz, CA. 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 time proliferating hyphae and bacterial colonies were transferred to fresh DIFCO mycological or tryptic soy agar**.** Microorganisms were established as pure cultures using standard procedures. Each microbe was then grown in 100 mL mycological broth cultures (still, 21 days or shaken, 200 rpm, 6 days). At time of harvest, organisms were killed by the addition of 20 mL of MeOH and then homogenized with an Omnimixer. The cultures were extracted with 100 mL of $CHCl_3$ (3×), and the organic layer was reduced in vacuo to an oil. The aqueous layer was lyophilized and then thoroughly extracted with $CHCl_3$/MeOH (1:1, v/v). The freeze-dried extract (FDX) and the $CHCl_3$ extract were tested in the standard disk assay against the following bacteria and fungi: (bacteria) *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, *Vibrio harveyii*, and *Escherichia coli*; (fungi) *Candida albicans*, *Sclerotinia sclerotiorum*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, and *Hansenula anomala*. The extracts were also tested for brine shrimp lethality.[15,16] To determine brine shrimp lethality, specific quantities of an extract, column fraction, or pure compound were transferred to a vial and dissolved in 10 μL of methanol (if necessary, 5 μL of DMSO was added to facilitate dissolution.) Four mL of artificial seawater and approximately 20 brine shrimp (24 h old) were then added to each vial, and the brine shrimp were counted (time 0). The number of surviving brine shrimp was determined at 1, 4, 8, and 24 h. Brine shrimp lethality is reported as % kill in 24 h. Microbes that produced active extracts were marked for further study.

Fungus RDWD1-2 was identified as *Aspergillus parasiticus* by Karen Dohrman, Microbial Identification, Inc., Newark, DE. The $CHCl_3$ extract of the RDWD1-2 pilot culture showed very little antimicrobial activity but good brine shrimp lethality. The fungus was grown in 16 × 600 mL DIFCO mycological broth cultures in 2 L Erlenmeyer flasks for 21 days, still. At harvest time, the fungus was again killed with the addition

Table 1. NMR Data for Sequoiatone A, 1 ($CDCl_3$)

	^{13}C-DEPT	^1H		^1H COSY	HMBC
1	163.0s				
2	112.8s				
3	170.0s				
4	100.0d	6.44	bs	H-20	C-2, C-3, C-7
5	171.4s				
6α	66.1t	3.95	dd, *J* = 10.8, 14.4	H-6*β*, H-7	
6*β*		4.71	dd, *J* = 5.7, 10.8	H-6α, H-7	C-3, C-7, C-9
7	43.2d	2.72	dd, *J* = 5.7, 14.4	H-6α, H-6*β*	
8	90.8s				
9	159.2s				
10	115.5s				
11	200.7s				
12	44.2d	3.32	bh, *J* = 7.2	H-13, H-22	
13	31.7t	1.68	m	H-12	
		1.45	m	H-12	
14[a]	27.1t	1.4−1.18	8H, m		
15[a]	29.4t				
16[a]	31.6t				
17	22.7t				
18	14.1q	0.84	3H, t, *J* = 6.9	H-17	
19	51.6q	3.76	3H, s		C-1
20	21.6q	2.10	3H, bs	H-4	C-2, C-4, C-5
21	22.6q	1.39	3H, s		C-7, C-8, C-23
22	16.6q	1.12	3H, d, *J* = 7.2	H-12	C-11, C-12, C-13
23	177.2s				

[a] The carbon chemical shifts C-13 through C-16 were assigned by calculation.[1] *J* values are given in Hz.

(11) Fielding, B. C.; Haws, E. J.; Holker, J. S. E.; Powell, A. D. G.; Robertson, A.; Stanway, D. N.; Whalley, W. B. *Tetrahedron Lett.* **1960**, *5*: 24.

(12) Haws, E. J.; Holker, J. S. E.; Kelly, A.; Powell, A. D. G.; Robertson, A. *J. Chem. Soc.* **1959**, 3598.

(13) Boyd, M.; Paull, K. D. *Drug Dev. Res.* **1995**, *34*, 91−109.

(14) Varma, R. Personal communication, National Cancer Institute Drug Synthesis and Discovery Branch, Bethesda, MD, 1999.

(15) Ferrigni, N. R.; McLaughlin, J. L.; Powell, R, G.; Smith, C. R., Jr. *J. Nat. Prod.* **1984**, *47*: 347.

(16) McLaughlin, J. L. *Abstracts of the International Symposium on Organic Chemistry of Medicinal Natural Products,* IUPAC, Shanghai, Nov 10−14, 1984.

Table 2. NMR Data for Sequoiatone B, 2 ($CDCl_3$)[a]

C no.	^{13}C DEPT	^1H		COSY	HMBC
1	165.8s				
2	113.3s				
3	169.0s				
4	107.0d	6.92	1H, bs	H-20	C-5, C-7, C-20
5	164.3s				
6	142.3d	7.38	1H, s		C-3, C-7, C-9
7	135.8s				
8	78.0s				
9	153.1s				
10	113.8d	7.18	1H, s		C-2, C-3, C-4, C-8, C-11
11	208.4s				
12	47.8d	2.67	1H, bh, $J = 7.2$	H-22, H-13	C-11
13	34.4t	1.66 1.45		H-12	
14	29.4[a]t	1.4−1.2			
15	29.8[a]t				
16	32.1[a]t				
17	23.0t				
18	14.5q	0.85	3H, t, $J = 7.9$	H-17	C-16, C-17
19	51.3q	3.80 (s)	3H, s		C-1
20	20.7q	2.25 (s)	3H, bs	H-4	C-4, C-5
21	27.7q	1.50 (s)	3H, s		C-3, C-7, C-8
22	17.8q	1.10 (s)	3H, d, $J = 7.2$		C-11, C-12, C13
OH		7.51 (s)			C-7, C-8

[a] *J* values are given in Hz.

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 (0.647 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, 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. Mycelial MeOH extract: 11.64 g; mycelial $CHCl_3$−MeOH extract: 3.74 g; mycelial mat: 31.05 g. All three organic extracts were tested for brine shrimp lethality. Most of the activity was concentrated in the mycelial MeOH extract. All subsequent isolation procedures were guided by this bioassay.

The mycelial MeOH extract (5.68 g) was fractionated using centrifugal countercurrent chromatography (CCCC) in normal-phase mode (50:70:80:65 hexane/EtOAc/MeOH/H_2O, upper phase mobile, 800 rpm, 2 mL/min.). The large and intermediate coil were used in tandem, for a combined volume of 325 mL. Brine shrimp lethality was concentrated in the first three fractions, which were applied to a Sephadex LH-20 column ($CHCl_3$/MeOH 1:1). Fraction 6 (LH-20) showed the most brine shrimp lethality and was further resolved by CCCC as described. Fraction 5 from the CCCC separation was applied to a Rainin semipreparative silica gel HPLC column (isocratic: EtOAc/hexane 5:95). Fraction 11 from this separation eluted as a pure amorphous solid, compound **2** (5.5 mg, 0.09% of mycelial extract). Fraction 12 was resolved by a Rainin analytical silica gel HPLC column (isocratic: IPA/hexane 5:95) to yield compound **1** as a pure crystalline solid (1.3 mg, 0.02% of mycelial extract).

Sequoiatone A, compound 1: yellow crystalline solid; mp 93.4−95.3 °C; $[\alpha]^{25}_D$ −750° (*c* 0.26, MeOH); UV (MeOH) λ_{max} 240 (log ϵ 3.88), 270 (log ϵ 3.97), 378 (log ϵ 4.58); IR ($CHCl_3$) ν_{max} 2930, 2912, 2832, 1740, 1698, 1680, 1555, 1540, 1227, 1200 (br), 1138, 1038, 898 cm^{-1}; ^1H and ^{13}C NMR data shown in Table 1; 2D NOESY($CDCl_3$) δ 6.44−3.76 and 2.10, 4.71−3.95 and 2.72, 3.95−2.72, 3.32−1.68; EIMS 402 (M^+, 1), 290 (15), 289 (100), 215(29), 187(8); HREIMS *m/z* 402.2034 (M^+, calcd for $C_{23}H_{30}O_6$, *m/z* 402.2042).

X-ray Crystallographic Data. Crystal data: pale yellow, 0.012 × 0.16 × 0.78 mm, $C_{23}H_{30}O_6$, FW = 402.2, orthorhombic, space group $P2_12_12_1$, a = 6.329(1) Å, b = 16.644(2) Å, c = 22.387(3) Å, $\alpha = \beta = \gamma = 90°$, V = 2216.6(6) Å^3, Z = 4, D_{calc} = 1.21 g/cm^3, T = 26 °C, radiation Cu Kα (λ = 1.54178 Å, μ = 70.6 cm^{-1}, $F(000)$ = 864, R = 0.081, R_w = 0.082, S = 1.05, 262 parameters. Intensity data were taken as $\theta/2\theta$ scans on a Nicolet R3m diffractometer (Siemens P4 upgraded) for 2788 unique reflections in the range $4° < 2\theta < 110°$, of which 1514 with $I > 2\sigma(I)$ were used for structure solution and refinement. The data were corrected for Lorentz and polarization effects. Absorption corrections were calculated by Gaussian integration (transmission factor range 0.87−0.99); extinction corrections were not needed. The structure was solved by direct methods and refined by full-matrix least-squares with anisotropic thermal parameters, using statistical weighting. Crystallographic calculations were done with **SHELTXTL** program package. Most of the hydrogen positions were located on difference maps; four of the *n*-hexyl protons were not found. Calculated hydrogen positions were used for structure refinement with fixed isotopic thermal parameters, assigned as 1.2 times the U_{iso} values for the carbon atoms. To establish absolute configuration, repeated low-speed scans were measured for the Friedel pairs and symmetry equivalents of the 25 reflections [with $I > 10\sigma(I)$] most sensitive to choice of enantiomer. After data reduction and absorption corrections, observed and calculated values for $\Delta F = (F_{hkl} - F_{h-k-l-})$ were compared for reflections for which $|\Delta F°|/F° > 0.5\%$. Sign agreement for 18 of 20 reflections established absolute configuration.

Sequoiatone B, compound 2: $[\alpha]^{25}_D$ +72.7° (*c* 0.11, MeOH); UV (MeOH) λ_{max} 250 (log ϵ 3.79), 314 (log ϵ 3.69), 358 (log ϵ 3.52), 428 (log ϵ 4.22); IR ($CHCl_3$) ν_{max} 3300 (broad), 2932, 2910, 2832, 1680, 1650, 1580, 1532, 1486, 1438, 1234, 1198, 947, 924, 898, 830 cm^{-1}; ^1H NMR and ^{13}C NMR shown in Table 2; 1D-difference NOE ($CDCl_3$) δ irrad 7.18, enhanced−2.67 (10.5), 1.10 (3); irrad 7.51, enhanced−1.50 (4.7), 1.3 (8.4), irrad 1.50, enhanced−7.51 (3), 7.38 (3); EIMS *m/z* 374 (9), 261 (100), 243 (13), 234 (15), 229 (21), 218 (9), 211 (13), 201 (16), 187 (10): HREIMS *m/z* 374.2084 (M^+, calcd for C_{22} $H_{30}O_5$, 374.2093).

Acknowledgment. We thank our colleagues from the Department of Chemistry, Montana State University: Dr. Scott Busse for assistance with NMR spectroscopy, Dr. L. Joseph Sears for mass spectral data, and Dr. Ray Larsen for X-ray crystallography data. We thank the National Science Foundation Grant No. 9506620 for providing funding for NMR upgrades at the MSU facility.

Supporting Information Available: X-ray crystallography data and ^1H NMR and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

JO990277L