Sphaeric Acid, a New Succinic Acid Derivative from a *Sphaeropsis* sp.

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The structure of sphaeric acid (**1**), a novel succinic acid derivative isolated from the fermentation broth of a *Sphaeropsis* sp., was determined by spectral data and synthetic transformation to the diol of sphaeric acid and subsequently to a pair of γ-lactones (**2** and **3**).

Endophytic fungi may exist in every well-established plant on our planet. These organisms exist in a range of biological descriptions extending from symbiotic to near pathogenic. Very often such microbes may either mimic or produce some of the same compounds as the host plant that supports them. For instance, all of the known phytohormones are produced by one or more plant-associated microbes. More recently, our search for endophytes of Taxaceae (yews and torreyas) yielded several microbes producing taxol[1−3] and other bioactive compounds.[4−7] Some of these endophytes, while not producing taxol, were of interest to us because of either their taxonomic or chemical novelties. This report deals with the discovery of sphaeric acid, a unique compound from *Sphaeropsis* sp., an endophytic fungus of *Taxus globosa* (Mexican yew).

Sphaeric acid (**1**) is a member of a class of alkylated succinic acid derivatives. Roccellic acid[8] (**4**) and pedicellic acid[9] (**5**) are the members of this class most similar to sphaeric acid, being 2-alkyl-3-methyl succinic acids. Pedicellic acid is the only example of this skeleton found in higher plants, while roccellic acid was isolated from lichens. Sphaeric acid, a novel compound, is now reported from a fungus.

Sphaeropsis sp. was isolated from the inner bark of a surface-sterilized *T. globosa* sample collected in the Sierra Madre Oriental Mountain Range in June 1992. Sphaeric acid was isolated from the methylene chloride extract of the fermentation medium by size-exclusion chromatography and reversed-phase HPLC to give a yellow oil. Crude NMR analysis of the methylene chloride extract of the mycelial mass showed no presence of sphaeric acid. The HRCIMS gave a molecular formula of $C_{19}H_{41}O_4Si_2$ for the protonated TMS derivative. This gave a molecular formula of $C_{13}H_{24}O_4$ for sphaeric acid, which required two sites of unsaturation. The IR signal at 1707 cm^{-1}, the carbon NMR signals at 182.3 and 182.1 ppm, and the two D_2O exchangeable protons at 11.5 ppm in the proton NMR suggested the presence of two carboxylic acid groups.

The carbon NMR data indicated that there were two methyl groups and two methines in the molecule; the remainder of the signals were methylenes. This, as well as the large proton signal at 1.2 ppm, suggested the presence of a long alkyl chain. The triplet at 0.85 ppm, which integrated to three protons, was typical for a terminal methyl of an alkyl chain.

The COSY spectrum of sphaeric acid showed that the two methines at 2.7 and 2.5 ppm coupled to each other (Table 1). Additionally, the methine at 2.5 ppm coupled to the methylene protons at 1.6 ppm, and the methine at 2.7 ppm coupled to the signal at 1.2 ppm. Because the methine at 2.7 ppm is a doublet of quartets, it must be coupled to a methyl group within the 1.2-ppm signal as well as the other methine.

Table 1. Sphaeric Acid COSY Data

^{1}H	COSY
2.7	1.2, 2.5
2.5	1.6, 2.7
1.6	1.2, 2.5
1.2	0.85, 2.7
0.85	1.2

The relative stereochemistry of the two chiral carbons is based on the pair of synthetic lactone derivatives (**2** and **3**). Sphaeric acid was reduced with lithium aluminum hydride to produce the diol, which, upon oxidation with pyridinium chlorochromate, produced the pair of lactones.

The synthesis of the lactones, instead of the dialdehyde, can be explained through the formation of an intermediate similar to the aldehyde hydrate formed in an aqueous chromate oxidation of a primary alcohol to a carboxylic acid. Instead of nucleophilic attack of the aldehyde by a water molecule, which leads to the formation of a carboxylic acid, the neighboring hydroxyl group attacks leading to the formation of the lactone (Scheme 1).

The relative stereochemistry was assigned by comparison of the lactones to the known compounds (+)-pilocarpine (**6**) and (+)-isopilocarpine[10] (**7**). The chemical shifts of the methylene protons adjacent to the oxygen in the lactone ring were particularly diagnostic. The COSY data for the mixture of lactones led to the assignments of these methylene protons for each isomer (Table 2). Lactone **2** was assigned the protons at 4.3 and 3.7 ppm, and lactone **3** was assigned the protons at 4.4 and 3.8 ppm. The two isomers, pilocarpine and isopilocarpine, have different chemical shifts for the two protons of the methylene groups (4.05 and 4.15 for pilocarpine and 3.89 and 4.39 for isopilocarpine). The trans stereochemistry was chosen because of the similarity of the methylene protons of the synthesized lactones (4.4/4.3 and 3.8/3.7) to the isopilocarpine signals giving the relative stereochemistry shown. The two lactones had different retention times in the GC/MS, which allowed for the assignment of each isomer. The base peak of 100 for lactone **2** is achieved via the common McLafferty rearrangement. However, lactone **3**, with the carbonyl on the opposite side of the ring, cannot undergo this rearrangement and must fragment in other ways, resulting in the smaller mass fragments observed.

Sphaeric acid was isolated because of its positive result in a brine shrimp toxicity assay.[11] There was some concern

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Scheme 1. Formation of lactone 2 via an aldehyde alkoxylate

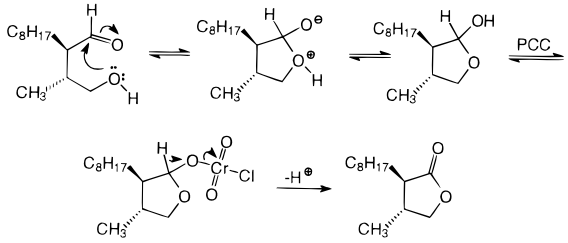
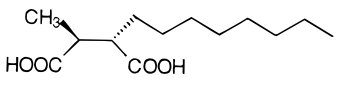


Table 2. Lactone COSY Data

^{1}H	COSY
2.1	1.6, 1.7, 2.2, 2.3, 3.8, 4.4
2.2	1.2, 2.1
2.3	1.1, 2.1, 3.8, 4.4
3.7	2.3, 4.3
3.8	2.1, 4.4
4.3	2.3, 3.7
4.4	2.1, 3.8



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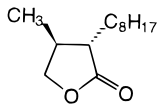

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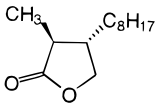

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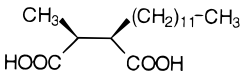

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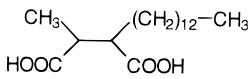

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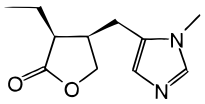

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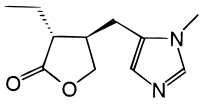

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that the brine shrimp toxicity may have been due to the acidity of sphaeric acid. However, the concentrations tested only lowered the pH only to approximately 5.6, while a pH as low as 4.0 seemed to show no toxicity to the brine shrimp over the 24 hours of testing. Purified sphaeric acid was also tested for other activities and gave a positive result in a mouse thymocyte proliferation assay without being cytotoxic at the concentrations tested. This is a general screening assay for agents that affect interleukin-1 action, which plays a central role in T-cell activation of the immune system.[12] Sphaeric acid was also tested in antibiotic disk diffusion assays showing minimal inhibition against *Staphylococcus aureus* and *Bacillus subtilis* and showing no inhibition against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Geotrichum candidum*, *Aspergillus niger*, and *Fusarium oxysporum*.

Experimental Section

General Experimental Procedures. UV−vis absorptions were recorded on a Beckman DU-50 UV−vis spectrophotometer, and IR spectra were recorded on a Perkin−Elmer model 1600 FTIR. Optical rotations were collected on a Perkin−Elmer model 241 MC polarimeter. NMR data were collected on either a Bruker AC 300, a Bruker DPX 300, or a Bruker DRX 500 spectrometer. ^{13}C multiplicities were determined by DEPT spectra. Mass spectra were recorded on a VG 10E-HF mass spectrometer, and TMS derivatives were prepared by reacting the sample with BSA [*N*,*O*-bis(trimethylsilyl)acetamide]. Solvents used throughout were ACS grade (Fisher) or HPLC grade (Fisher and EM Science), and H_2O was distilled on a Wheaton Autostill 1.5. Agars were prepared from Difco Microbiological agars or corresponding Difco broths and Difco Bacto agar.

Yew Broth. Yew needles and small stems (*T. brevifolia* collected in the Flathead National Forest) (5 g) were placed in a beaker with 500 mL of H_2O. The H_2O was boiled for 5 min and then allowed to simmer for 1 h without heating. The broth was passed through cheesecloth to remove the yew debris, and the broth was frozen in 10-mL portions.

M-1-D Agar. Major salts: $Ca(NO_3)_2$ − (0.28 g/L), KNO_3 − (0.08 g/L), KCl − (0.06 g/L); minor salts: $FeCl_3 \cdot 6H_2O$ − (2.0 mg/L), $MnSO_4$ − (5.0 mg/L), $ZnSO_4 \cdot 7H_2O$ − (2.5 mg/L), H_3BO_3 − (1.4 mg/L), KI − (0.7 mg/L); $MgSO_4$ anhydrous − (0.36 g/L), $NaH_2PO_4 \cdot H_2O$ − (0.02 g/L), ammonium tartrate − (5.0 g/L), sucrose − (30 g/L), yeast extract (Difco) (0.25 g/L), agar (15 g/L).

Fungal Isolation. Twig samples of *T. globosa*, mexican yew, were surface sterilized with 95% EtOH. After evaporation of the EtOH in a sterile laminar flow hood, the outer bark of the twigs were removed. Small pieces (ca. 1 cm) of inner bark (phloem-cambium and xylem tissues) were removed and placed on water agar plates. After a period of initial growth, hyphal tip transfers of the developing *Sphaeropsis* sp. were grown on potato dextrose agar and visually checked for purity. This fungus is deposited in the Montana State University mycological collection as no. 2083.

Culture Conditions. The *Sphaeropsis* sp. was maintained on M-1-D agar containing 1% yew broth. It was grown in still culture in 2-L Erlenmeyer flasks and 1000-mL Roux flasks in R-1 media [Bacto soytone (Difco) − 1.0 g/L, sucrose − 10 g/L, 1.0 M $KHPO_4$ (1.0 mL/L)] containing 1% yew broth for 21 days. The amount of liquid used in each flask was chosen to give the largest surface area for growth.

Extraction and Isolation. The fungal mycelia were removed by filtration through eight layers of cheesecloth, and the filtrate was extracted once with 400 mL of CH_2Cl_2. The filtrate was acidified to pH = 1 and extracted with two additional 400-mL portions of CH_2Cl_2. The extracts were combined and evaporated to dryness on a rotoevaporator (23.5 mg/L). The extracts were chromatographed on a Sephadex LH-20 column (2.8 cm × 110 cm) and eluted with 1:1 $CHCl_3$−MeOH. Nine total fractions were collected, and isolation of the active compound was carried out by activity-guided fractionation using a brine shrimp toxicity bioassay. Fractions 4 and 5 (19.5 mg) were then chromatographed by reversed-phase HPLC on a preparative Rainin Dynamax 60-Å C18 column by gradient elution using a linear gradient from 70% MeOH in H_2O to 100% MeOH, followed by a CH_2Cl_2 wash. Four fractions were collected, with fraction 3 (11.6 mg) exhibiting activity. This fraction was submitted to a final separation on a Toyopearl TSK gel HW-40F column (2.8 cm × 40 cm) and eluted with 2:1 MeOH−H_2O. Sixteen fractions were collected, with fraction 2 giving pure sphaeric acid (7.1 mg).

Brine Shrimp Toxicity Bioassay. The sample was dissolved in 30 μL of MeOH and added to a test vial of artificial seawater (3.0 mL). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial. The brine shrimp were observed periodically over a 24-h period. A positive assay was the death of all or most of the brine shrimp. Pure sphaeric acid caused the death of 57% of the brine shrimp over 24 h at a concentration of <0.33 mg/mL. At a concentration of 0.5 mg/mL, sphaeric acid produced a pH of 5.6, while a pH as low as 5.0 resulted in no brine shrimp deaths.

Sphaeric acid (1): light yellow oil; $[\alpha]^{25}_D$ +7° (*c* 23 mg/mL, CH_3OH); UV (CH_3CN) λ_{max} (ϵ) 205 (159); IR (neat) ν_{max} 2919 (OH), 1707 (C=O, acid), 1460, 1413, 1278, 1225, 931, 720

cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 11.5 (2H, br, D_2O exchange), 2.7 (1H, dq, $J = 9.0$, 7.1 Hz), 2.5 (1H, ddd, $J = 9.0$, 9.0, 4.7 Hz), 1.6 (2H, m), 1.2 (15 H, br s), 0.85 (3H, t, $J = 6.5$ Hz); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 182.3 (s), 182.1 (s), 47.8 (d), 40.9 (d), 31.8 (t), 29.5 (t), 29.3 (t), 29.2 (t), 29.1 (t), 26.6 (t), 22.6 (t), 15.1 (q), 14.1 (q); HRCIMS *m/z* 389.2494 [TMS derivative + H]$^+$ (calcd for $C_{19}H_{41}O_4Si_2$, 389.2543); EIMS *m/z* 181 (6), 154 (32), 142 (18), 111 (20), 97 (39), 83 (21), 69 (47), 55 (100).

Diol of sphaeric acid: colorless oil; UV (MeOH) λ_{max} (ϵ) 210 (290); IR (neat) ν_{max} 3328 (OH), 2922, 2854, 1715 m, 1456, 1378, 1035, 757, 721 cm^{-1}; HRCIMS *m/z* [M + H]$^+$ 217.2171 calcd 217.2168 for $C_{13}H_{29}O_2$; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.7 (2H, m), 3.5 (2H, m), 2.5 (2H, br, D_2O exchange), 1.7 (1H, m), 1.4 (1H, m), 1.2 (14H, br), 0.9 (3H, d, $J = 7.2$ Hz), 0.85 (3H, t, $J = 6.6$ Hz); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 65.2 (t), 62.6 (t), 44.1 (d), 37.5 (d), 32.3 (t), 30.4 (t), 30.0 (t), 29.7 (t), 29.5 (t), 28.1 (t), 23.1 (t), 15.5 (q), 14.5 (q); HRCIMS *m/z* 217.2171 [M + H]$^+$ (calcd for $C_{13}H_{29}O_2$, 217.2168); EIM*S* *m/z* 168 (20), 154 (02), 140 (05), 125 (08), 111 (19), 97 (37), 83 (47), 69 (88), 55 (100).

Lactones 2 and 3: (unseparated due to bioassay requirements) light yellow oil; UV (MeOH) λ_{max} (ϵ) 212 (430); IR (neat) ν_{max} 2923, 2854, 1777 (C═O, lactone), 1456, 1385, 1337, 1167, 1132, 1017 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 4.4 (0.45H, dd, $J = 8.7$, 7.7 Hz), 4.3 (0.55H, dd, $J = 8.5$, 8.0 Hz), 3.8 (0.45H, dd, $J = 9.2$, 8.7 Hz), 3.7 (0.55H, dd, $J = 8.9$, 8.5 Hz), 2.3 (0.55H, m), 2.2 (0.45H, m), 2.1 (1H, m), 1.7 (1H, m), 1.6 (2H, m), 1.5 (1H, m), 1.3 (10H, br), 1.2 (1.5H, d, $J = 6.5$ Hz), 1.1 (1.5H, d, $J = 6.6$ Hz), 0.9 (3H, t, $J = 6.3$ Hz);^{13}C NMR ($CDCl_3$, 125 MHz) δ 180.2 (s), 179.8 (s), 73.0 (t), 72.1 (t), 47.1 (d), 44.2 (d), 41.0 (d), 36.5 (d), 32.6 (t), 32.2 (t), 30.1 (t), 30.0 (t), 29.8 (t), 29.6 (t), 29.5 (t), 29.4 (t), 27.6 (t), 27.2 (t), 23.0 (t), 17.3 (q), 14.4 (q); HREIMS *m/z* **2** 212.1775 M$^+$ (calcd for $C_{13}H_{24}O_2$, 212.1776); **3** 212.1770 M$^+$ (calcd for $C_{13}H_{24}O_2$, 212.1776); EIMS *m/z* **2** 212 (02), 197 (06), 113 (53), 100 (100), 85 (82), 69 (16), 55 (33); **3** 212 (01), 181 (01), 99 (54), 83 (32), 70 (89), 55 (100).

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