Torreyanic Acid: A Selectively Cytotoxic Quinone Dimer from the Endophytic Fungus *Pestalotiopsis microspora*

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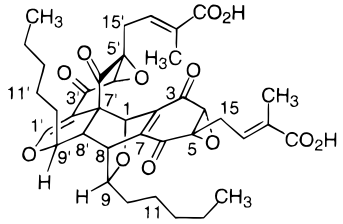
Endophytic fungi reside in the intercellular spaces of higher plants, and a single plant species typically serves as the host for several fungal species. The secondary metabolism of endophytic fungi is largely unexplored, but these fungi should be a fruitful source of biologically active secondary metabolites because of the magnitude of the resource—conservatively 1.5×10^6 species—and their intimate association and coevolution with other organisms.[1,2] As part of a program to explore the biosynthetic potential of endophytic fungi, we investigated the Florida torreya (*Torreya taxifolia*), an endangered species closely related to the taxol-producing Pacific yew (*Taxus brevifolia*).[3,4] We identified the endophyte *Pestalotiopsis microspora* as the likely cause of the Florida torreya's decline and characterized several phytotoxic and antifungal secondary metabolites produced by *P. microspora*.[5] We now wish to report the isolation and structure determination of torreyanic acid (**1**), another metabolite from *P. microspora*. Torreyanic acid (**1**) is an unusual dimeric quinone with selective cytotoxicity against human cancer cell lines.



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P. microspora was cultured in potato dextrose broth, and its organic extract (375 mg/L) was obtained by sequential whole culture extractions with ethyl acetate. The organic extract exhibited a zone of inhibition against *Bacillus subtilis* in an agar diffusion assay. Bioassay-guided fractionation using silica gel (95:5, 90:10 CH_2Cl_2−MeOH) and flash reverse-phase chromatography (C-18, 50% MeOH−H_2O to 100% MeOH) afforded active crystalline torreyanic acid (50 mg/L), which could be separated from contaminating oils by careful MeOH trituration.

Torreyanic acid, $[\alpha]_D$ +92.3° (*c* 0.11, MeOH) and mp 160 °C dec, was structurally analyzed by one- and two-dimensional NMR spectroscopy. The ^{1}H NMR spectrum displays a large methylene envelope at δ 1.20 and two

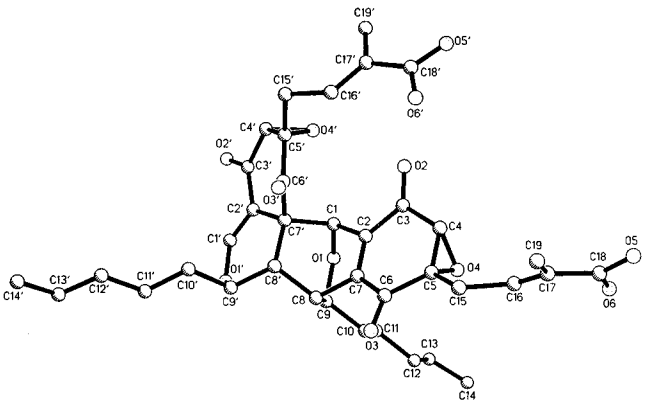
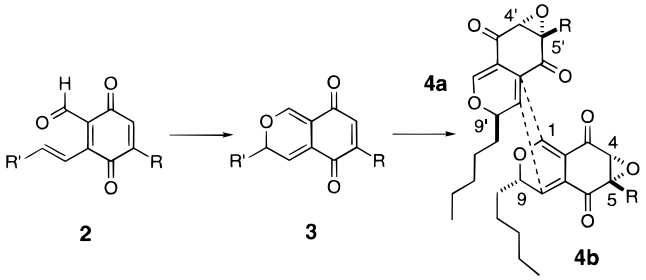


Figure 1. Computer-generated perspective drawing of torreyanic acid (**1**). Hydrogens are omitted for clarity, and the absolute configuration shown is arbitrary.

Scheme 1. A Plausible Biosynthetic Scheme for the Production of 1



2 **3** **4a** **4b**

methyl triplets at δ 0.86, suggesting two long aliphatic chains. The molecular formula $C_{38}H_{44}O_{12}$ derived from HRFABMS is consistent with the 38 carbon resonances observed in the ^{13}C NMR spectrum. With six carbonyls and eight olefinic carbons (^{13}C NMR), compound **1** must contain seven rings. The intense absorption at 1690 cm^{-1} and the shoulder at 1700 cm^{-1} in the IR spectrum indicated both unsaturated carbonyl(s) and a ketone. In addition, the molecular formula and DEPT spectrum indicated two free hydroxyls.

Several fragments of torreyanic acid (**1**) could be obtained from a combination of COSY, HMQC, and HMBC spectra (Table 1). The two flanking 2-methyl-2-butenoic acid residues and two pentyl side chains were easily identified and suggested that the molecule consists of two similar but not identical parts. The partial structure of the right-hand portion of **1** was based on the large number of heteronuclear correlations for H-1 (δ 5.60) and of methylene H-15 (δ 2.94) to C-6 (δ 189.9). Important features for the construction of the left-hand portion are the observed cross peaks of H-1′ (δ 7.82) to the four carbons C-3′, C-2′, C-9′, and C-7′ (δ 189.8, 114.2, 83.1, and 51.3). The two fragments were connected through the correlations of C-8′ (δ 37.9) to H-1 (δ 5.60) and C-9′ (δ 83.1) to H-8 (δ 3.45). In the 1,4-diketo ring, C-3′ (δ 189.8) correlates to H-4′ (δ 3.72), while C-6′ (δ 201.5), the only ketone carbonyl in the molecule, showed cross peaks to H_b-15′ (δ 2.60) as well as to H-8′ (δ 2.73). A bicyclic ether ring was deduced from the correlation between C-9 (δ 72.9) and H-1 (δ 5.60), and this bicyclic ring rigidifies the central core of torreyanic acid. The presence of only two exchangeable protons required that the pairs of oxygenated carbons C-4 (δ 59.8), C-5 (δ 63.5) and C-4′ (δ 66.2), C-5′ (δ 64.9) must be epoxides, further limiting conformational flexibility. Finally, **1** had to be a diacid in order to account for two remaining hydroxyl groups. Some stereochemical features could be antici-

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Table 1. NMR Data for Torreyanic acid (1) in MeOD

^{13}C (100 MHz)[a]	^{1}H (*J* (Hz), 500 MHz)[b,c]	COSY	HMBC	ROESY
1 71.0 d	5.60 (s)		3.76	
2 143.9 s			5.60, 3.76, 3.45	
3 188.8 s			5.60, 3.76	
4 59.8 d	3.76 (s)		2.94	2.94
5 63.5 s			6.66, 3.76, 2.94	
6 *189.9 s[d]			3.45, 2.94	
7 146.7 s			5.60, 4.16, 3.45, 2.73	
8 40.1 d	3.45 (dd, 2.5, 1.5)	4.16, 2.73	4.51, 4.16, 2.73	4.51, 4.16, 2.73
9 72.9 d	4.16 (td, 6.0, 1.5)	3.45, 1.13, 1.05	5.60, 2.73, 1.13, 1.05	4.51, 3.45
10 35.8 t	1.45			
11 25.7 t	1.25		4.16	
	1.13			
12 32.6 t	1.13		1.45, 1.27, 1.24	
	1.05		0.85	
13 ^23.4 t[e]	1.22		0.85	
14 ^14.2 q	0.85 (t, 7.0)		1.25	
15 28.1 t	2.94 (t, 8.0)	6.66	6.66, 3.76	3.76
16 134.3 d	6.66 (td, 7.5, 1.5)	2.94, 1.88	2.94	5.60, 3.76
17 133.4 s			6.66, 2.94, 1.88	
18 ^171.30 s			6.66, 1.88	
19 12.9 q	1.88 (d, 1.0)		6.66	
1′ 159.8 d	7.82 (s)		4.51, 3.72	
2′ 114.2 s			7.82, 5.60, 3.72	
			2.73	
3′ *189.8 s			7.82, 3.72	
4′ 66.2 d	3.72 (s)		2.85, 2.60	2.60
5′ 64.9 s			6.55, 3.72	
6′ 201.5 s			2.73, 2.60	
7′ 51.3 s			7.82, 5.60, 4.51, 3.45, 2.73	
8′ 37.9 d	2.73 (d, 2.5)	3.45	5.60, 4.16, 3.45	4.51, 3.45, 1.25
9′ 83.1 d	4.51 (dd, 10.0, 5.0)	1.45, 1.23	7.82, 3.45, 1.45, 1.24	4.16, 3.45, 2.73
10′ 34.0 t	1.45		4.51, 2.73	
11′ 26.5 t	1.25		4.51	
12′ 32.3 t	1.23		1.45, 1.25, 0.86	
13′ ^23.5 t	1.23		0.86	
14′ ^14.2 q	0.86 (t, 7.0)		1.23	
15′ 29.0 t	2.85 (dd, 15.0, 7.5)	6.55, 2.60	6.55, 3.72, 2.73	
	2.60 (dd, 15.0, 7.5)	6.55, 2.85		3.72
16′ 135.0 d	6.55 (td, 7.5, 1.5)	2.85, 2.60, 1.85	2.85, 2.60	5.60, 3.72
17′ 132.9 s			6.55, 2.85, 2.60	
			1.85	
18′ ^171.28 s			6.55, 1.85	
19′ 12.9 q	1.85 (d, 1.0)		6.55	

[a] Multiplicity was determined from DEPT spectra. [b] Assignments to carbons were based on HMQC spectra. [c] Those signals without designated splitting patterns were either multiplets or buried by other signals. [d] An asterisk indicates that carbon assignments may be exchanged. [e] A caret indicates that carbon assignments may be exchanged with their primed partner.

pated from NOE data, including the *trans* configuration about the two olefins on the tiglic acid side chain, but our ability to finally prepare crystals (CH_2Cl_2:MeOH) suitable for X-ray analysis obviated further attempts to deduce the structure from spectral data. A single-crystal X-ray analysis[6] of torreyanic acid (**1**) revealed the relative stereochemistry shown in Figure 1.

While the overall structure of torreyanic acid (**1**) could be generated by a Diels−Alder dimerization of two identical monomers, the opposite relative configurations for C-9 and C-9′ require two diastereomeric monomers. Several pathways can be envisioned to produce torreyanic acid (**1**). One plausible path would involve the electrocyclic—possibly acid-catalyzed—closure of achiral **2** to racemic **3**, the enzymatic oxidation of racemic **3** to diastereomers **4a** and **4b**, and the [4 + 2] addition of **4a** and **4b** to give **1** (Scheme 1). The stereochemistry of the final Diels−Alder reaction might be a consequence of keeping the two pentyl side chains opposite one another. Such [4 + 2]-cyclized quinone dimers are rare, but a recent example is the weakly cytotoxic longithorone A isolated from a marine tunicate.[7]

Torreyanic acid was tested in several cell lines derived from human cancers. In general, torreyanic acid is five−ten times more potent in cell lines that are sensitive to protein kinase C (PKC) agonists and causes cell death by apoptosis. IC_{50} values range from 3.5 (NEC) to 45 (A549) μg/mL with a mean value of 9.4 μg/mL for 25 different cell lines. Torreyanic acid also shows G1 arrest of G0 synchronized cells at the 1−5 μg/mL level depending on the cell line. These results suggest that torreyanic acid should be subjected to *in vivo* tests against cell lines that express low amounts of PKC, and such tests are in progress. The importance of examining vanishing species such as the Florida torreya for potentially useful compounds is familiar, and as these results suggest, their endosymbionts should be examined as well.

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Supporting Information Available: Tables of X-ray and spectral data for torreyanic acid (**1**) (14 pages).

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(6) Crystals of **1** form in the monoclinic space group *C*2 with a = 25.273(6), b = 11.634(3), and c = 13.073 Å and β = 100.41(2)°. A total of 2606 symmetry independent 2σ data were collected using graphite-monochromated Cu Kα radiation. The structure was solved by direct methods and refined by full matrix least-squares refinements (SHELX93). The final crystallographic residual is 5.7% for a model with anisotropic non-hydrogen atoms and isotropic riding hydrogens.

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