New Phomopsolides from a *Penicillium* sp.

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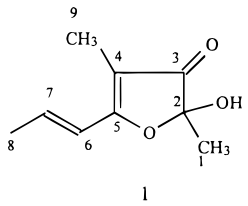
Investigation of the bioactive compounds from a *Penicillium* sp. isolated from the inner bark of the Pacific yew, *Taxus brevifolia*, led to the isolation of the known furanone **1**, and a series of phomopsolides. The phomopsolide fractions contained phomopsolides A and B, which have previously been described, and three new phomopsolides. The structures of the new phomopsolides were deduced by comparison of their NMR spectra to those of the known compounds.

For the past five years we have been investigating the endosymbionts found in the inner bark of the Pacific yew, *Taxus brevifolia*.[1] In the course of these investigations, we have isolated a variety of fungi. The $CHCl_3$ extract of one of these fungal cultures showed strong antibiotic activity against *Staphylococcus aureus* and *Vibrio harveyii* in the disk assay. The fungus, a *Penicillium* sp., was grown in a soytone−glucose broth with added $Mg_3(PO_4)_2 \cdot 12H_2O$[2] for 21 days in still culture. The CH_2Cl_2 extract of the broth showed strong antimicrobial activity in the disk assay. The fractionation by CCCC and HPLC of this extract gave several compounds that exhibited activity aganist *S. aureus*.

The least polar of these compounds was identified as furanone **1**, by comparison of its ^{1}H-NMR spectrum to the known compound.[3] Furanone **1** was previously isolated from the fungus *Stemphylium radicinum*,[4] although the carbon data for this compound have not been reported previously. Two of the other antimicrobial compounds were identified as phomopsolide A (**2**) and phomopsolide B (**4**), which were identified by comparison of their NMR spectra with the known compounds.[4] These two compounds have been isolated previously from *Phomopsis oblonga* and been found to be feeding deterrents for the elm bark beetle.[4,5] In addition to these compounds, three new antimicrobial phomopsolides **3**, **5**, and **6** were identified.



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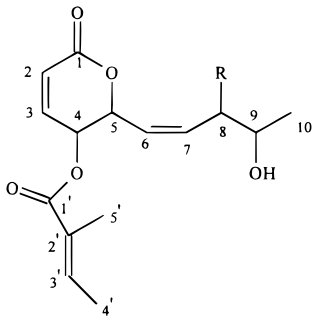
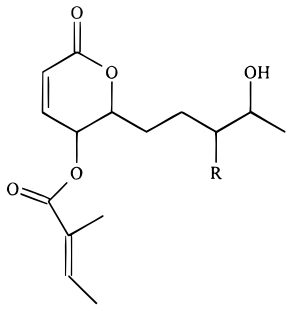


2 R =O Phomopsolide A
3 R =O trans 6,7 double bond
4 R OH trans 6,7 double bond Phomopsolide B



5 R OH
6 R =O

Compound **3** was found to be an isomer of **2** by mass spectrometry. The ^{1}H-NMR spectra of these two compounds were very similar except for the resonances around the C-6/C-7 double bond. These protons, which appeared very close in chemical shift in **2** (δ 6.40 and 6.39) were shifted farther downfield (δ 6.93 and 6.72) and exhibited a large 15.3 Hz coupling constant in **3**, consistent with an *E* double bond in **3**.

Two antimicrobial 6,7-dihydrophomopsolides were also isolated from this fungus. The ^{1}H-NMR spectrum of **5** lacked the two olefinic protons at C-6 and C-7, and these were replaced by two methylene groups at δ 2.05 and δ 1.66. The methylene protons at δ 1.66 were coupled to the methine proton at δ 3.35, which was coupled to the methine proton at δ 3.58, which in turn was coupled to the terminal methyl protons. This established the structure of **5** as a 6,7-dihydrophomopsolide B. The ^{1}H-NMR spectrum of the second dihydro derivative **6** had two coupled methylenes at δ 2.02 and δ 2.72, a quartet methine at δ 4.20, and the terminal methyl doublet. This established a terminal end similar to that found in phomopsolide A and established the structure of **6** to be 6,7-dihydrophomopsolide A.

The furanone and all of the phomopsolides showed strong activity in our antimicrobial disk assay against *S. aureus*. The activities of these compounds are compared to the known antibiotics tetracycline, penicillin G, and streptomycin in Table 3.

To look at the compound production as a function of growth media we also grew this organism in unamended soytone−glucose broth. The comparison of metabolite production in these media is tabulated in Table 4. It is well-known that antibiotic production can be altered by the addition of certain nutrients,[2] and we are currently

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Table 1. ^{1}H NMR (300 MHz) Data for Compounds **1-6** ($CDCl_3$, δ in ppm, J in Hz)

C#	1	2	3	4	5	6
1	1.51					
2		6.23 (d, 9.7)	6.24 (d, 9.6)	6.23 (d, 9.7)	6.18 (d, 9.8)	6.16 (d, 9.7)
3		7.10 (dd, 9.7, 5.4)	7.02 (d, 9.6, 5.4)	6.96 (dd, 9.6, 5.4)	6.98 (dd, 9.8, 5.7)	6.97 (dd, 9.7, 5.7)
4		5.63 (m)	5.47 (dd, 5.4, 2.8)	5.33 (dd, 5.4, 3.0)	5.26 (dd, 5.7, 2.7)	5.25 (m)
5		5.96 (m)	5.29 (m)	5.07 (dd, 5.4, 2.8)	4.57 (m)	4.55 (m)
6	6.33 (dd, 15.6,1.5)	6.40 (m)	6.93 (dd, 15.3, 3.6)	5.84 (dd, 15.7, 5.4)	2.05 (m, 6.5)	2.02 (m, 2H)
7	6.83 (dq, 15.6, 6.9)	6.39 (m)	6.72 (d, 15.3)	5.94 (dd, 15.7, 6.0)	1.66 (q, 6.3)	2.72 (m, 2H)
8	1.95 (dd, 6.9, 1.5)			3.86 (t, 6.0)	3.35 (q, 6.3)	
9	1.68	4.34 (q, 6.3)	4.40 (q, 6.4)	3.55 (p, 6.3)	3.58 (p, 6.2)	4.20 (q, 6.3)
10		1.37 (d, 6.3)	1.37 (d, 6.4)	1.10 (d, 6.3)	1.16 (d, 6.2)	1.32 (d, 6.3)
1′						
2′						
3′		6.84 (m)	6.79 (m)	6.85 (m)	6.90 (br q, 6.6)	6.85 (br q, 6.6)
4′		1.75 (d, 6.3)	1.76 (d, 6.4)	1.75 (d, 6.3)	1.78 (d, 6.6)	1.76 (d, 6.6)
5′		1.76 (br s)	1.75 (br s)	1.76 (br s)	1.80 (br s)	1.77 (br s)

Table 2. ^{13}C-NMR (75 MHz) Data for Compounds **1**−**6** ($CDCl_3$, δ in ppm)

carbon #	1	2	3	4	5	6
1	22.1	162.5	161.9	162.5	163.0	162.8
2	102.3	124.6	125.0	124.7	124.8	124.7
3	203.1	141.5	141.3	141.1	140.8	141.0
4	106.7	63.6	63.0	63.4	62.9	63.1
5	176.9	77.3	77.6	78.6	78.8	77.9
6	139.5	143.1	140.6	135.0	28.4	24.1
7	118.9	124.9	126.3	124.5	26.3	32.5
8	18.8	202.5	200.6	70.5	70.7	211.8
9	5.2	73.5	72.5	76.1	75.1	72.8
10		19.9	20.2	18.7	19.6	19.8
1′		166.9	166.9	166.7	166.9	166.7
2′		127.8	127.6	127.5	127.5	127.5
3′		139.4	139.4	139.8	139.7	139.9
4′		14.9	15.0	14.5	14.5	14.6
5′		12.4	12.3	11.9	12.0	12.0

Table 3. Comparison of Antibiotic Activity of Compounds **1**−**6** to Several Known Antibiotics Against *S. aureus*

compound	concentration	zone of inhibition (mm)
tetracycline	30 μg	38
penicillin G	10 units	22
streptomycin	10 μg	13
1	100 μg	9
2	25 μg	22
3	25 μg	22
4	25 μg	20
5	100 μg	10
6	50 μg	9

Table 4. The Production of Compounds **1**−**6** as a Function of Growth Media (Isolated Yields in mg/L Broth)

compound	glucose−soytone broth	glucose−soytone−$Mg_3(PO_4)_2$ broth
1	4	22
2	11	1
3	18	5
4	9	42
5	9	18
6	11	16

investigating the effect of this media alteration on antibiotic production by our fungi.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker DPX-300 spectrometer. ^{1}H-NMR spectra were recorded at 300 MHz and the ^{13}C-NMR spectra were recorded at 75 MHz. All of the chemical shifts were recorded with respect to the deuterated solvent shift. The IR spectra were recorded on a Perkin-Elmer 1310 spectrometer. The optical rotation was recorded on a Perkin-Elmer 241 MC polarimeter using a 1-mL cell. The CCCCs were performed on a P.C. Inc. MLPC instrument. All of the mass spectra were provided by the Montana State Mass Spectrometer facility at Montana State University. All solvents used were spectral grade.

Biological Testing. The antibiotic activity of the crude column fractions and pure compounds was made by dissolving the material in $CHCl_3$, applying a small amount of material to a DIFCO concentration disk and placing on a lawn of *S. aureus* on an agar Petri dish. The zones of inhibition were recorded by measuring the zone where no growth of the organism occurred.

Extraction and Isolation. The isolation of this fungus from *T. brevifolia* has been previously described.[1] This fungus was identified as a *Penicillium* sp. and is deposited in the Agricultural Research Culture Collection (NRRL# 21208). *Penicillium* sp. was grown in media A broth (10 g soytone + 40 g glucose + 10 g $Mg_3(PO_4)_2 \cdot 12\ H_2O$/L of broth) (14 × 500 mL) in still culture. After 21 days the mycelia was filtered off and the broth extracted with CH_2Cl_2 (3 times with 1 L of CH_2Cl_2) to give 1.37 g (0.20 g/L) of a brown oil. This crude extract was fractionated on CCCC using hexane−EtOAc−MeOH−H_2O 2:2:2:1, under normal phase conditions to give several major bioactive fractions. The least polar fraction contained pure **1**. Subsequent fractions were found to be mixtures of phomopsolides by ^{1}H-NMR spectroscopy and were further purified by HPLC on Si gel using hexane−isopropyl alcohol as eluents. This fungus was also grown in mycological broth (10 g soytone + 40 g glucose/L of broth), (6 × 2 L) in still culture for 21 days and worked up as above. The CH_2Cl_2 extract gave 1.6007 g (0.13 g/L) of a brown oil. The relative amounts of the metabolites are tabulated in Table 4.

Furanone 1: isolated as a brown solid (158 mg, 11.5% of the crude extract); mp 97−99 °C; $[\alpha]^{20}_D$ +20.3° (*c* 0.022 MeOH), which exhibited comparable spectral (^{1}H NMR) data to published values;[3] ^{1}H- and ^{13}C-NMR data appear in Tables 1 and 2 for comparison.

Phomopsolide A (2): isolated as a colorless oil (8 mg, 0.6% of the crude extract); $[\alpha]^{20}_D$ +310° (*c* 0.021 MeOH), which exhibited comparable spectral (^{1}H NMR and ^{13}C NMR) data to published values;[4] ^{1}H- and ^{13}C-NMR data appear in Tables 1 and 2 for comparison.

Phomopsolide B (4): isolated as a colorless oil (35 mg, 2.5% of the crude extract); $[\alpha]^{20}_D$ +250° (*c* 0.030 MeOH), which exhibited comparable spectral (^{1}H NMR

and ^{13}C NMR) data to published values;[4] ^{1}H- and ^{13}C-NMR data appear in Tables 1 and 2 for comparison.

6,7-(*E*) Phomopsolide A (3): isolated as a colorless oil (294 mg, 21.5% of the crude extract); $[\alpha]^{20}_{D}$ +186° (*c* 0.0257 MeOH); IR (neat) ν_{max} 3500, 2980, 2925, 1709, 1637, 1244, 1100, 824, 730; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; EIMS *m*/*z* 252 (1), 224 (14), 167 (29), 150 (55), 83 (81), 55 (100); HRCIMS *m*/*z* 295.1172 (calcd for $C_{15}H_{18}O_6$ + H, 295.1187).

6,7-Dihydrophomopsolide B (5): isolated as a colorless oil (126 mg, 9.2% of the crude extract); $[\alpha]^{20}_{D}$ +110° (*c* 0.0045 MeOH)); IR (neat) ν_{max} 3500, 2965, 2915, 1708, 1645, 1250, 1125, 820, 727; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; EIMS *m*/*z* 298 (4), 253 (12), 181 (35), 153 (68), 97 (46), 83 (96), 55 (100); HREIMS 298.1408 (calcd for $C_{15}H_{22}O_6$, 298.1416).

6,7-Dihydrophomopsolide A (6): isolated as a colorless oil (112 mg, 8.2% of the crude extract); $[\alpha]^{20}_{D}$ +167° (*c* 0.0297 MeOH)); IR (neat) ν_{max} 3500, 2980, 2920, 1705, 1245, 1120, 820, 775; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; EIMS *m*/*z* 296 (0.4), 224 (20), 153 (8), 83 (100), 55 (52); HRCIMS 297.1328 (calcd for $C_{15}H_{20}O_6$ + H, 297.1338).

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