Phomacins: Three Novel Antitumor Cytochalasan Constituents Produced by a *Phoma* sp.

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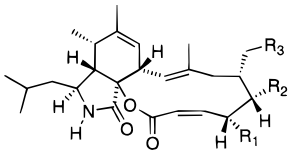
Three novel cytochalasans, phomacins A, B and C, were isolated from a fermentation broth of the fungus *Phoma* sp. and purified by HSCCC (high speed countercurrent chromatography) followed by HPLC. The structures were determined by 1D and 2D NMR techniques. All three compounds have shown potent inhibitory activity against the HT29 colonic adenocarcinoma cell line.

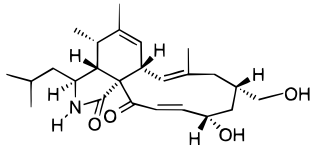
Introduction

The cytochalasans[1] are a group of secondary fungal metabolites that have been isolated from *Phomopsis*, *Chaetomium*, *Zygosporium*, *Hypoxylon*, and *Aspergillus* species.[2,3] The common structural feature of the group is expressed by a perhydroisoindol-1-one moiety bearing a benzyl group (cytochalasins), a *p*-methoxybenzyl group (pyrichalasins), a (indol-3-yl)methyl group (chaetoglobosins) or a 2-methylpropyl group (aspochalasins) at the C-3 position and an 11-, 13- or 14-membered carbocyclic (or oxygen containing) ring connecting the C-8 and C-9 positions.

In the course of screening for new antitumor compounds, a fungal extract was discovered which exhibited potent activity against the HT29 tumor cell line. Bioassay-directed fractionation of the fermentation extract provided three novel, structurally related antitumor agents: phomacins A(**1**), B (**4**), and C (**5**). Phomacins A−C are structurally related to aspochalasin. The substitution at the C-16 position in aspochalasin is unique since the known aspochalasins do not contain any substituents at this position.



1: R_1 = OH, R_2 = OH, R_3 = H
4: R_1 = OH, R_2 = H, R_3 = OH



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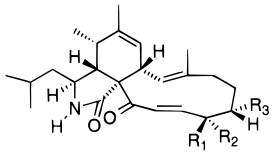

2: R_1 = H, R_2 = OH, R_3 = OH
3: R_1 = OH, R_2 = H, R_3 = OH

Results and Discussion

Phomacins A−C were isolated as white powders. The molecular formulas were determined by high resolution fast atom bombardment mass spectra (HRFABMS) and ion spray mass spectrometry. The IR spectra suggested the presence of NH and OH (broad bands at 3389, 3400, or 3409 cm^{-1} and an amide residue (bands at 1680 or 1660 cm^{-1}) in all three metabolites.

Table 1. ^{13}C NMR Spectral Data for the Phomacins (δ in ppm)

carbon no.	phomacin A (**1**)	phomacin B (**4**)	phomacin C (**5**)
1	174.2	174.2	175.3
3	51.4	52.3	49.1
4	52.3	51.6	51.2
5	34.2	34.2	34.9
6	140.2	140.2	140.4
7	124.1	124.2	125.5
8	39.4	39.5	43.6
9	88.6	88.7	68.1
10	48.4	48.5	48.3
11	13.9	13.9	13.4
12	19.7	19.8	19.9
13	122.0	122.4	124.5
14	138.9	138.0	135.1
15	50.9	45.7	44.1
16	28.3	31.8	33.6
17	79.9	41.2	43.4
18	74.5	68.4	69.7
19	157.5	157.5	148.1
20	118.5	118.5	129.0
21	167.7	167.7	197.5
22	24.9	24.8	24.8
23	21.3[a]	21.3[a]	21.2[a]
24	23.7[b]	23.7[b]	23.6[b]
25	15.4	15.7	16.1
26	20.6	68.4	68.2

[a,b] Assignments may be interchanged.

Structure of Phomacin A (1). A molecular weight of 431 (*m*/*z* 432, $M^+ + H$) was determined giving rise to the molecular formula of $C_{25}H_{38}NO_5$ ($M^+ + H$: 432.2753). The IR spectrum showed an ester carbonyl band at 1702 cm^{-1} and an amide carbonyl at 1680 cm^{-1}. The ^{13}C NMR spectrum of **1** displayed 25 carbons signals (Table 1) and suggested the presence of one α,β-unsaturated ester carbonyl (δ 167.7), one amide carbonyl (δ 174.2), and three carbon−carbon double bonds (δ 118.5−167.7). Three degrees of unsaturation remained which were assigned to three rings. The molecule must contain three exchangeable protons since only 34 protons were identified from the ^{13}C NMR data. The ^{1}H NMR spectrum showed four methyl doublets between δ 0.89 and 1.17 and two methyl singlets at δ 1.69 and 1.36 as well as olefinic protons at δ 5.26, 6.10, 7.13, and 5.96 (Table 2). The ^{1}H−^{1}H connectivities determined from the COSY spectrum suggested the presence of partial structure A (Figure 1). The carbon and proton connectivities were established through a HMQC experiment. Chemical

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(1) In the chemical literature, all compounds containing the perhydroisoindole skeleton, including the macrocycle and the C-substituents of the isoindole system, are named cytochalasans. However, cytochalasins are accepted as the trivial names of the 10-phenyl compounds in the group.[6]

(2) Cole, R.; Cox, R. H. In *Handbook of Toxic Fungal Metabolites*; Academic Press: New York, 1981, p 289.

(3) Turner, W. B.; Aldridge, D. C. In *Fungal Metabolites II*; Academic Press: New York, 1983, p 459.

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Table 2. ^{1}H NMR Spectral Data for the Phomacins (δ in ppm, J in hertz)

proton no.	phomacin A (**1**)	phomacin B (**4**)	phomacin C (**5**)
2	7.02 (brs)	6.87 (brs)	6.92 (brs)
3	3.16 (bd, J = 9.58)	3.16 (bd, J = 9.53)	3.14 (m)
4	2.86 (m)	2.85 (m)	2.98 (m)
5	3.03 (m)	3.05 (m)	2.47 (m)
7	5.26 (bs)	5.29 (bs)	5.41 (brs)
8	3.77 (bd, J = 12.62)	3.83 (bd, J = 10.11)	2.90 (bd, J = 10.00)
10	1.24 and 1.49 (m)	1.24 and 1.49 (m)	1.18 (m)
11	1.17 (d, J = 7.2)	1.22 (d, J = 7.08)	1.22 (d, J = 7.07)
12	1.69 (brs)	1.72 (brs)	1.75 (brs)
13	6.10 (bd, J = 10.60)	6.20 (bd, J = 10.3)	5.99 (bd, J = 10.80)
15	2.06 (m), 1.88 (m)	1.99 (bd, J = 12.89)	1.83 and 1.89 (m)
16	1.93 (m)	1.70 (m)	1.89 (m)
17	3.71 (dd, J = 6.21, 1.91), 1.70 (m)	2.20 (bdd, J = 13.01, 4.99)	1.89 and 2.17 (m)
18	4.57 (bs)	4.29 (bs)	4.59 (brs)
19	7.13 (bd, J = 15.32, 2.17)	7.19 (dd, J = 15.40, 1.91)	7.18 (d, J = 16.46)
20	5.96 (dd, J = 15.32, 2.11)	5.89 (bd, J = 15.38)	6.60 (dd, J = 16.46, 4.66)
22	1.61 (m)	1.66 (m)	1.55 (m)
23	0.89 (d, J = 7.27)	0.91 (d, J = 6.23)	0.89 (d, J = 6.60)
24	0.89 (d, J = 7.27)	0.90 (d, J 6.23)	0.86 (d, J 6.60)
25	1.36 (brs)	1.43 (brs)	1.35 (brs)
26	0.97 (d, J = 7.26)	3.28 (brt, J = 9.60)	3.29 (brt, J = 9.2)
	3.46 (m)	3.54 (dd, J = 8.74, 3.29)	

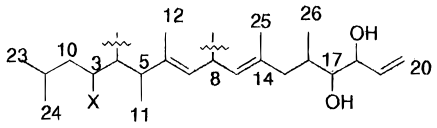

Figure 1. ^{1}H−^{1}H COSY correlation for partial structure A.

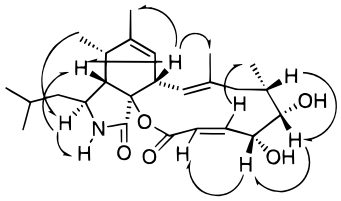

Figure 2. Key NOE correlations observed for phomacin A (**1**).

shift considerations allowed us to place the nitrogen atom at C-2 and the oxygen atoms at C-17 and C-18. The chemical shift of C-9 indicated that an ester oxygen was attached to this carbon atom. The overall structure was assembled by analysis of the long-range ^{1}H−^{13}C connectivities gleaned from an HMBC experiment using C-9 as the anchor point. Thus, a two-bond ^{1}H−^{13}C correlation was observed between H-4 and C-9 as well as H-8 and C-9; the HMBC spectrum also displayed cross peaks from the NH proton to C-9 and H-3 to C-9 (Table 3).

The stereochemical assignment of the cyclohexene ring of **1** made it possible to use H-4, H-5, and H-8 as stereo anchor points. Thus, a NOESY correlation from H-8 to H-5 and from H-5 to H-4 indicated a cis orientation between these three protons. Similarly, the NOE between H-3 and the C-11 methyl protons suggested their cis relationship. Furthermore, NOE cross peaks between H-17 to H-18 and H-18 to H-16 also require a cis orientation between these protons (Figure 2).

Chemical shift arguments fully supported this stereochemistry. A similar substitution pattern exists in aspochalasin C (**2**) and aspochalasin D (**3**). The structure of 17,18-di-*O*-acetylaspochalasin C (**2a**) had been confirmed by X-ray analysis.[4] It was clear from the ^{13}C NMR data of **2a** and 17,18-di-*O*-acetyl aspochalasin D (**3a**) that the chemical shifts of C-18 are strongly influenced by a change in stereochemistry (δ 74.9 in **2a** versus δ 78.6 in **3a**).[5] For a direct comparison, **1** was converted to its diacetate (**1a**) with acetic anhydride and triethyl amine. As expected, the ^{1}H and ^{13}C NMR data of **1a** were very similar to those reported for **3a**[5] (Table 4). This result along with the NOESY data substantiates the relative configurations proposed for **1**.

Table 3. HMBC Correlation Data of Phomacin A (1)

carbon no.	phomacin A (**1**)	long range correlation to H no.
1	174.2	H-2 and H-3
3	51.4	H-2, H-4, H-5, and H-10
4	52.3	H-2, H-5, H-10, and H-11
5	34.2	H-3, H-4, H-7, H-11, and H-12
6	140.2	H-4, H-5, H-7, H-8, H-11, and H-12
7	124.1	H-5, H-8 H-12, and H-13
8	39.4	H-7 and H-13
9	88.6	H-2, H-3, H-4 H-5, and H-8
10	48.4	H-3, H-4, H-23, and H-24
11	13.9	H-4 and H-5
12	19.7	H-5 and H-7
13	122.0	H-7, H-8, H-15, and H-25
14	138.9	H-8, H-13, H-15, and H-25
15	50.9	H-13, H-16, H-25, and H-26
16	28.3	H-15, H-17, H-18, and H-26
17	79.9	H-16, H-18, and H-26
18	74.5	H-17, H-19, and H-20
19	157.5	H-17, H-18, and 20
20	118.5	H-18 and H-19
21	167.7	H-19 and H-20
22	24.9	H-10, H-23, and H-24
23	21.3	H-10 and H-22
24	23.7	H-10 and H-22
25	15.4	H-13, and H-25
26	20.6	H-15, H-16, and H-17

Structure of Phomacin B (4). The molecular formula for phomacin B (**4**) was determined to be the same as for **1**: $C_{25}H_{38}NO_5$ ($M^+ + H$: 432.2742). Although the ^{1}H and ^{13}C NMR spectra of **4** were almost coincident with those of **1**, there were some differences. Phomacin A showed a doublet for a secondary methyl group at δ 0.97 (H-26), but the corresponding signal was not observed in the ^{1}H NMR spectrum of **4** (Table 2). Instead two downfield-shifted methylene proton signals appeared at δ 3.28 and 3.46. A similar trend was observed in the ^{13}C NMR spectrum of phomacin B. The ^{13}C NMR spectrum

(4) Neupert-Laves, K.; Dobler, M. *Helv. Chim. Acta* **1982**, *65*, 1426.

(5) Keller-Schierlein, W.; Kupper, E. *Helv. Chim. Acta* **1979**, *62*, 1501.

Table 4. ^{13}C NMR Spectral Data for the 17,18-Di-*O*-acetyl Derivatives of Phomacin A (1a), Aspochalasin C (2a), and Aspochalasin D (3a)

carbon no.	1a	2a	3a
1	173.0	175.1	174.8
3	51.7	51.7	51.4
4	51.4	49.5	48.9
5	34.2	35.2	35.1
6	140.1	140.8	140.3
7	124.1	126.0	125.2
8	39.2	43.5	43.5
9	88.5	68.5	68.4
10	48.4	48.7	48.5
11	13.7	13.7	13.5
12	19.7	19.5	19.9
13	122.9	125.5	125.2
14	138.0	137.4	136.3
15	50.2	35.9	38.7
16	28.2	35.2	28.0
17	78.9	74.9	78.6
18	73.0	74.0	73.8
19	147.1	133.1	136.3
20	121.0	126.2	130.8
21	166.8	197.9	196.7
22	25.0	24.8	24.7
23	21.4	21.1	21.2
24	23.6	24.0	23.6
25	15.5	13.7	15.6
26	20.8		
OCO*CH*$_3$	19.7, 20.6	20.1, 20.6	20.9, 20.9
O*CO*CH$_3$	170.2, 169.7	169.9, 169.4	169.5, 169.5

of **1** showed a signal due to the C-17 methine carbon atom bearing a hydroxyl group at δ 79.9 which was missing in the ^{13}C NMR spectrum of **4** (Table 1); instead a signal for a methylene group was observed at δ 41.2. Therefore, it was evident that the C-26 methyl group was now oxidized to a primary alcohol and a secondary alcohol at C-17 was reduced to a methylene group. Furthermore, C-15 and C-18 were shifted upfield ($\Delta\delta$ 5.2 and $\Delta\delta$ 6.1) and C-16 moved downfield ($\Delta\delta$ 6.1). This hypothesis was further confirmed by a HMBC experiment, which showed two- and three-bond correlations between H-26 and C-15, C-16, and C-17.

Structure of Phomacin C (5). The molecular formula of phomacin C (**5**) was established to be $C_{25}H_{38}NO_4$ ($M^+ + H$: 416.2805), indicating that phomacin C lacks one oxygen atom. Most of the spectroscopic properties of **5** were very similar to **4**. However, there were significant shift differences of carbon atoms C-21 and C-9. The ^{13}C NMR spectrum of **4** showed a carbonyl carbon signal at δ 167.7 (C-21), but the corresponding signal was not observed in the ^{13}C NMR spectrum of **5** (Table 1). Instead a new carbonyl signal appeared at δ 197.5 and the signal for C-9 moved upfield ($\Delta\delta$ 20.6). These data were in agreement with the presence of an α,β-unsaturated ketone group at C-21 instead of an α,β-unsaturated ester moiety. The overall framework again was confirmed by analysis of a $^1H-^1H$ COSY spectrum and HMQC and HMBC NMR spectra.

The relative stereochemistry of **4** and **5** was the same as in **1** based upon coupling constants and chemical shift comparisons and was further confirmed by NOESY data. It is important to note that in all cytochalasans the stereochemistry of the cyclohexene and isoindole moieties are the same and that the absolute stereochemistry of the isoindole system has also been established.[6]

Table 5. Cellular Proliferation and Cytotoxicity Analysis in HT29 cells

compound	3H-TdR (IC_{50} μg/mL)	Alamar blue (IC_{50} μg/mL)
control (0.5% DMSO)	nontoxic	nontoxic
phomacin A (**1**)	0.6	17.4
phomacin B (**4**)	1.4	10.1
phomacin C (**5**)	7.4	nontoxic
cytochalasin A	2.4	11.4

The biosynthesis of cytochalasans has been studied extensively[8] and like other cytochalasans, aspochalasans are assembled by the same biosynthetic pathway from leucine, acetate, and methionine. Similarly, phomacins should derive from the same building blocks. However, phomacins contain a substitution at C-16 which could originate from an additional methionine moiety as proven in the case of cytochalasin D.[9] The isolation of phomacin C (**5**) strongly suggests that the lactone ring present in phomacins A (**1**) and B (**4**) should be derived by oxidation of a carbocyclic precursor. A similar relationship exists between cytochalasins A and B and desoxaphomin. The latter was successfully incorporated into cytochalasin B.[10]

Cellular Proliferation Assay with HT29 Cells. All three compounds were tested for their efficacy in inhibiting the growth of the colonic adenocarcinoma cell line HT29 as determined by (^3H) thymidine incorporation as well as alamar blue cytotoxicity analysis. Based on the data presented in Table 5, the three metabolites are potent inhibitors of HT29 cell proliferation and exhibited effects that were comparable to the known compound cytochalasin A. In the case of phomacin A (**1**) and B (**4**), this effect could also be attributed to the associated cytotoxicity. However, phomacin C (**5**) exhibits potent inhibition of cellular proliferation as monitored by (^3H) thymidine incorportion, but is not toxic to the cells as determined by the alamar blue assay. Hence, phomacin C (**5**) would be the most attractive candidate as an antitumor compound.

Experimental Section

Spectral Analysis. ^1H and ^{13}C NMR spectra were recorded at 300 K on a Bruker 300 AC spectrometer operating at 300 MHz and 75 MHz, respectively. The samples for NMR characterization were dissolved in chloroform-*d* at a concentration of approximately 5−10 mg/mL. Mass spectra were obtained on a PE Sciex API III triple-quadrupole mass spectrometer interfaced with a Sciex Ion-Spray probe. Exact mass measurements were performed on a VG 70SEQ spectrometer at high resolution (HRFAB) using peg300/thiogly as an internal standard. UV spectra were run on a Perkin-Elmer Lambda 6 spectrometer. IR spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer. Optical rotations were measured with a Perkin-Elmer 243B polarimeter in $CHCl_3$. Melting points were determined using an Electrothermal Engineering Ltd melting point apparatus and are uncorrected.

Microorganism. The fungal strain no. 14078 was isolated from a soil sample obtained from Raymond Terrace, Australia. Colonies on yeast-malt extract agar attained a diameter of approximately 18 mm in 14 days of dark incubation at 25 °C and 70% relative humidity. Colonies were typically round, wooly with a ciliate margin, and grey with numerous minute, globose black pycnidia. Conidiogenous cells were ampulliform, apparently phialidic. Conidia were hyaline, and ovate, with prominent oil droplets. Diffusible pigments were not produced.

(6) Binder, M.; Tamm, C. J. *Chem. Soc., Perkin Trans. 1* **1973**, 1146−1147.

(7) Alvi, K. A; Peterson, J.; Hofmann, B. *J. Ind. Microbiol.* **1995**, *15*, 80.

(8) Turner, W. B; Aldridge, D. C. In *Fungal Metabolites II*; Academic Press: London, 1983; p 464.

(9) Graf, W.; Robert, J.; Vederas, J. C.; Tamm, C.; Solomon, P. H.; Miura, I.; Nakanishi, K. *Helv. Chim. Acta* **1974**, *57*, 1801.

(10) Robert, J.-L.; Tamm, C. *Helv. Chim. Acta* **1975**, *58*, 2501.

The fungus was identified as a *Phoma* species. The culture was deposited with the American Type Culture Collection (ATCC) and given the accession number 74342.

Fermentation. The fermentation procedure utilized was a two-step process in which the suspension of spores or mycelium is inoculated into 250 mL flasks containing 30 mL of a nutrient seed medium having a composition as follows: glucose 20 g, Pharmamedia (Trader's Protein) 15 g, ammonium sulfate 3 g, zinc sulfate 0.03 g, calcium carbonate 4 g, and yeast extract (Difco) 5 g, per liter final volume. After inoculation, the flasks were incubated with agitation at 28 °C for two days. The agitation rate was 250 rpm. After two days of incubation, a 2 mL aliquot of the culture was used to inoculate flasks (250 mL flasks with 30 mL medium) of a production medium having a composition as follows: glucose 30 g, maltose 10 g, oatmeal 20 g, and yeast extract (Difco) 4 g, per liter final volume. After inoculation, the flasks of production medium were incubated with agitation at 28 °C for six days. The agitation rate was 250 rpm. After incubation, the fermentation flasks were harvested, and the fermentation mixture from each flask was pooled into a single vessel for extraction.

Extraction and Isolation. The pooled fermentation mixture was homogenized and then extracted with three equal volumes of EtOAc. The combined extracts were evaporated under reduced pressure to dryness to yield an oily residue (1.377 g). This oily residue was fractionated by dual mode high-speed countercurrent chromotagraphy (PC Inc., Potomac, MD). Solvent and full chromatographic conditions were used as previously described.[7] The activity was concentrated into peaks eluting at 60−69 (fractions 21−24), 108−120 (fractions 37−39) and 219−231 (fractions 74−78) min. The active fractions were pooled and evaporated under reduced pressure to dryness.

The countercurrent chromatography fractions contained substantially pure material. However, final purification was achieved using semipreparative HPLC. The chromatography system consisted of a Waters HPLC equipped with a Waters 600 system controller and a Waters 996 photodiode array detector. Separation was achieved on two semipreparative C_{18}-cartridges connected in series (25 × 100 mm, Nova-pak 6 μm). The mobile phase was pumped as a binary system at a rate of 10 mL/min and consisted of a 35 min linear gradient, starting with 70% H_2O, 30% CH_3CN, and ending with 100% CH_3CN. Phomacin A (19 mg), phomacin B (19 mg), and phomacin C (32 mg) were isolated from the CCC fractions 21−24, 74−78 and 37−39, respectively.

HT-29 Cellular Proliferation Assay (^{3}H-TdR incorporation for HT-29 cells). The HT-29 cell line used in the assay is a colon adenocarcinoma cell line (ATCC HTB 38). Individual samples were added to the wells in DMSO (final assay concentration of DMSO did not exceed 0.5%). The cells were added to each individual well of the microtiter plates containing eight different concentrations of samples. The resulting solutions were incubated for 42 h. ^{3}H-TdR was added to the wells at a final concentration of 1 μCi/mL. Plates were then incubated in an incubator for an additional 6 h. After the incubation period, the cells were rinsed twice with HBSS, and 200 μL of 0.25% trypsin was added. The cells were then allowed to detach for 15−30 min and then harvested on a printed filtermat B employing a Tomtec Harvester 96 using 0.9% NaCl as the rinse buffer with a preprogrammed cycle. After addition of scintillation fluid, the filter mats were counted on the Wallac 1205 BETAPLATE liquid scintillation counter.

Alamar Blue Cytotoxicity Assay for HT-29 Cells. Individual samples were added to the wells in DMSO (final assay concentration of DMSO did not exceed 0.5%). The cells were added to each individual well of the microtiter plates containing eight different concentrations of samples. The resulting solutions were incubated for 45 h. Alamar blue reagent was added in an amount equal to 10% of the culture volume. Plates were then incubated in an incubator for an additional 2.5 h. Fluorescence was measured with excitation wavelength at 560 nm and emission wave length at 590 nm on the Millipore Cytofluor 2300 system.

Phomacin A (1): white powder; mp 130−133 °C; $[\alpha]_{na}$ −91 (*c* 1.0, $CHCl_3$); IR ν_{max} 3389, 2900, 1702, 1680; ^{1}H NMR (Table 1); ^{13}C NMR (Table 2); ion-Spray MS (positive): *m*/*z* 432 (M^+ + H); HRFABMS calcd for $C_{25}H_{38}NO_5$ (M^+ + H) 432.2749; found 432.2753.

17,18-Di-*O*-acetylphomacin A (1a). To a solution of compound **1** (10 mg) in triethylamine (1 mL) was added Ac_2O (500 μL), and the reaction was left in the dark at room temperature for 24 h. The reaction mixture was dried *in vacuo* and purified by semipreparative HPLC using a linear gradient (water/acetonitrile 1:1 to 100% acetonitrile over 35 min) on a C_{18} Waters Novapak cartridge at the flow rate of 10 mL/min.

Phomacin B (4): white powder; mp 98−100 °C; $[\alpha]_{na}$ −51.2 (*c* 1.0, $CHCl_3$); IR ν_{max} 3400, 2900, 1706, 1680; ^{1}H NMR (Table 1); ^{13}C NMR (Table 2); ion-spray MS (positive): *m*/*z* 432 (M^+ + H), HRFABMS calcd for $C_{25}H_{38}NO_5$ (M^+ + H) 432.2749; found 432.2742.

Phomacin C (5): white powder; mp 112−114 °C; $[\alpha]_{na}$ −74.6 (c 1.0, $CHCl_3$); IR ν_{max} 3409, 2912, 1720, 1660; ^{1}H NMR (Table 1); ^{13}C NMR (Table 2); ion-ipray MS (positive): *m*/*z* 416 (M^+ + H); HRFABMS calcd for $C_{25}H_{38}NO_4$ (M^+ + H) 416.2801; found 416.2805.

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Supporting Information Available: NMR spectra of **1**, **4**, and **5** (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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