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A cyclic carbonate and related polyketides from a marine-derived fungus of the genus *Phoma*

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Dedicated to the memory of Prof. Jeffrey B. Harborne

Abstract

Two metabolites, phomoxin and phomoxide, as well as the previously synthesized antibiotic eupenoxide, have been isolated from the fermentation broth of a marine-derived fungus of the genus *Phoma* (strain CNC-651). The new compounds are highly oxygenated polyketides of a new structural class. Phomoxin contains an unusual cyclic carbonate functionality that is rare among natural products. The structures of the new metabolites were assigned by spectroscopic methods that relied heavily on 2D NMR spectroscopic analysis.

Keywords: *Phoma* sp.; Phomoxin; Phomoxide; Eupenoxide; Marine fungus; Carbonate; Polyketides

1. Introduction

Terrestrial fungi belonging to the genus *Phoma* are well known phytopathogens that cause significant losses in commercial crops such as the oilseeds rapeseed and canola (Gugel and Petrie, 1992). In culture, these fungi produce structurally diverse secondary metabolites including the highly selective and potent depsipeptide phytotoxin phomalide (Pedras and Taylor, 1993), unusual phytotoxic dioxopiperazines (Pedras et al., 2001), and the host selective sesquiterpene phomalairdenone (Pedras et al., 1999), among many others. Overall, fungi of the genus *Phoma* are among the most prolific producers of secondary metabolites.

Phoma species occur not only on land but are also frequently found in tropical marine environments (Hyde, 1986), from which a number of obligate marine species have been described. Chemical studies of marine-derived *Phoma* strains have led to the isolation of a series of diterpenoid platelet activating factor (PAF) antagonists, the phomactins (Sugano et al., 1991, 1994, 1995) suggesting that, like their terrestrial counterparts, marine strains are a productive source of structurally unique and biologically active secondary metabolites. This concept is supported by a recent HPLC study in which it was concluded that marine and terrestrial *Phoma* species differed significantly with respect to their secondary metabolite content (Osterhage, 2000).

2. Results and discussion

As part of our continuing effort to discover new secondary metabolites from marine microorganisms (Fenical, 1993; Fenical and Jensen, 1993, 2002) we have been studying the secondary metabolites produced by marine-derived *Phoma* species. This paper reports the isolation and structural elucidation of two new, highly oxygenated polyketides, phomoxin (**1**) and phomoxide (**3**), that are produced in the liquid culture of a *Phoma* sp. isolated from a marine microbial mat collected in the Bahamas. Phomoxin (**1**) contains an unusual, naturally occurring, cyclic carbonate functionality reminiscent of a similar structure in the aldgarose sugar moiety of the glycoside antibiotic swalpamycin (Chatterjee et al., 1987). Both phomoxin (**1**) and phomoxide (**3**) represent previously unpublished natural product carbon skeletons.

Fungal isolate, strain number CNC-651, was isolated using agar plating methods from a microbial mat col-

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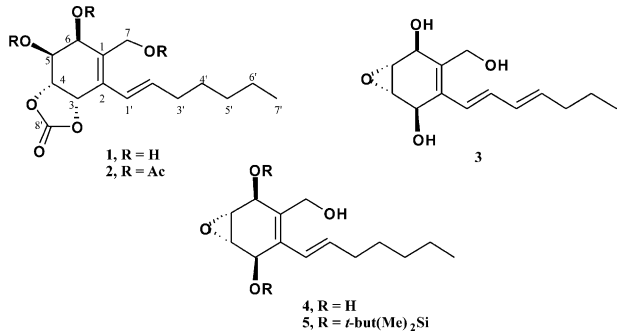

Fig. 1. Structures of the natural products phomoxin (**1**), phomoxide (**3**) and eupenoxide (**4**).

lected from a hypersaline pond located near Eleuthera Point, Eleuthera Island, Bahamas in August of 1995. The salinity of this pond was 11.5%, approximately three times that of ambient seawater. The strain was identified as a *Phoma* sp. based on morphological characteristics. Strain CNC-651 was cultured without shaking in six 2.8 l Fernbach flasks each containing 1 l of a seawater-based fermentation medium. After 13 days, the mycelium was separated from the culture broth by filtration and the broth was extracted with EtOAc. The EtOAc extract was concentrated under vacuum and subjected to reversed-phase C_{18} Sep-Pak chromatography eluting with mixtures of MeOH/H_2O to yield **1**, **3** and **4** in several subsequent fractions (Fig. 1). Final purification was achieved using reversed-phase C_{18} HPLC to give phomoxin (**1**, 6 mg) and phomoxide (**3**, 7 mg). The previously synthesized (Duke and Rickards, 1984), but unpublished fungal metabolite, eupenoxide (**4**, 20 mg), was also obtained. Spectral data for eupenoxide (**4**) are included in this paper.

The molecular formula of phomoxin (**1**) was established as $C_{15}H_{22}O_6$ by HRFAB mass spectrometry ($[M+Na]^+$ $m/z = 321.1295$, Δ 1.9 mmu). The ^{13}C NMR spectrum of **1** (Table 1) showed all 15 carbons, which consisted of 4 olefinic carbons, 5 oxygen-bearing carbons, one methyl group, 5 methylene carbons and one unusual carbon at 154.7 ppm. Broad IR absorptions at 3436 and 1637 cm^{-1} indicated the presence of hydroxyl and carbonyl groups. Proton and carbon NMR experiments, including results from COSY, HMQC and HMBC experiments (Fig. 2), allowed the entire structure of phomoxin to be assigned. The unusual quaternary carbon at 154.7 ppm was assigned to a carbonate carbonyl functionality (C-8′) involving two oxygen bearing carbons at C-3 and C-4 on the basis of specific NMR HMBC correlations (a correlation of C-8′ to H-4) and the characteristic carbonate infrared absorption recorded (CO = 1637 cm^{-1}).

Acetylation of **1** with Ac_2O in pyridine provided phomoxin triacetate (**2**), the molecular formula of which was determined to be $C_{21}H_{28}O_9$ by HRFAB mass spectrometry ($[M+Na]^+$ $m/z = 447.1631$ Δ 0 mmu). The downfield shifts of H-5, H-6 and H_2-7 in the ^1H-NMR spectrum of **2** showed that C-5, C-6 and C-7 were the sites of three hydroxyl groups in **1**. The relative stereochemistry of phomoxin (**1**) was obtained by interpretation of proton NOE data for phomoxin triacetate (**2**), Fig. 2. Irradiation of H-3 (δ 5.52) enhanced the H-4 proton (δ 5.00). Likewise, irradiation of H-5 enhanced H-6, indicating that these protons are in a *cis* configuration. The pseudo-axial orientations of H-4 and H-5 were confirmed by their large coupling constant of 10 Hz. Accordingly, H-6 was assigned an equatorial orientation due to small vicinal coupling constant of 3 Hz between H-5 and H-6.

Table 1
NMR spectral data for compounds **1**, **3** and **4**[a]

C#	Phomoxin (**1**)		Phomoxide (**3**)		Eupenoxide (**4**)	
	^{13}C[b]	^1H (m, *J*, int.)[b]	^{13}C[c]	^1H (m, *J*, int.)[c]	^{13}C[c]	^1H (m, *J*, int.)[c]
1	127.3 C		131.7 C		131.3 C	
2	127.3 C		132.8 C		131.5 C	
3	75.1 CH	5.46 (*d*, 8, 1H)	62.9 CH	4.73 (*s*, 1H)	63.1 CH	4.69 (*s*, 1H)
4	77.2 CH	4.92 (*t*, 8, 1H)	54.7 CH	3.54 (*s*, 1H)	54.5 CH	3.51 (*s*, 1H)
5	69.1 CH	3.87 (*dd*, 8, 3, 1H)	53.7 CH	3.54 (*s*, 1H)	53.8 CH	3.51 (*s*, 1H)
6	67.6 CH	4.49 (*d*, 3, 1H)	66.6 CH	4.73 (*s*, 1H)	66.2 CH	4.69 (*s*, 1H)
7	59.8 CH_2	4.39 (*m*, 2H)	58.8 CH_2	4.39 (*s*, 2H)	58.4 CH_2	4.36 (*s*, 2H)
1′	123.3 CH	6.33 (*d*, 16, 1H)	124.7 CH	6.35 (*d*, 16, 1H)	124.8 CH	6.28 (*d*, 16, 1H)
2′	137.4 CH	6.03 (*dt*, 16, 5, 1H)	132.8 CH	6.54 (*dd*, 16, 10, 1H)	134.9 CH	6.02 (*dt*, 16, 7, 1H)
3′	33.5 CH_2	2.18 (*m*, 2H)	130.7 CH	6.12 (*dd*, 15, 10, 1H)	33.5 CH_2	2.14 (*m*, 2H)
4′	28.6 CH_2	1.43 (*m*, 2H)	137.8 CH	5.84 (*dt*, 15, 7, 1H)	29.0 CH_2	1.41 (*m*, 2H)
5′	31.3 CH_2	1.28 (*m*, 2H)	35.0 CH_2	2.10 (*m*, 2H)	31.5 CH_2	1.28 (*m*, 2H)
6′	22.4 CH_2	1.31 (*m*, 2H)	22.3 CH_2	1.43 (*m*, 2H)	22.5 CH_2	1.30 (*m*, 2H)
7′	13.8 CH_3	0.89 (*t*, 7, 3H)	13.7 CH_3	0.91 (*t*, 7, 3H)	14.0 CH_3	0.89 (*t*, 7, 3H)
8′	154.7 C					

[a] Assignments are based upon COSY, DEPT, HMQC and HMBC experiments (^1H: 400 and 500 MHz, ^{13}C: 100 and 125 MHz).
[b] Recorded in $CDCl_3$ + trace CD_3OD.
[c] Recorded in $CDCl_3$.

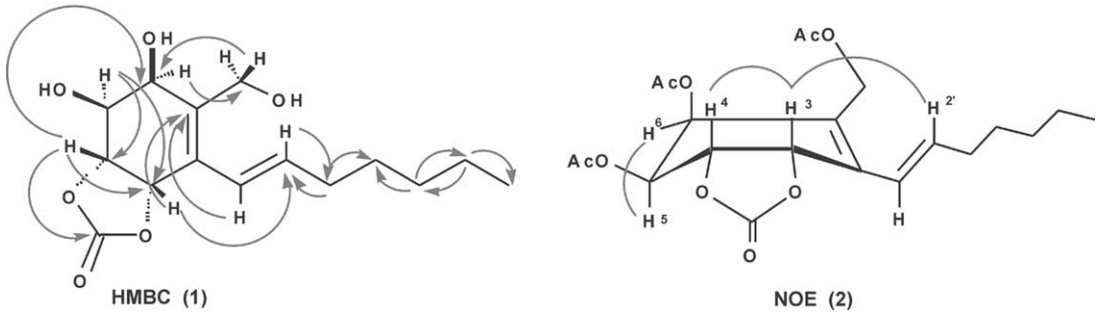

Fig. 2. Prominent NMR HMBC correlations for phomoxin (**1**) and NOE correlations for phomoxin triacetate (**2**).

The UV absorption of phomoxin (**1**) at 207 nm indicated that the olefinic bonds could not achieve the coplanarity required for a conjugated diene. Molecular models show that arranging the side-chain olefin coplanar to the ring olefin creates significant steric hindrance between the side-chain and adjacent functional groups. This hypothesis was confirmed by NOE enhancements of the H-3 proton (δ 5.46) when H-2′ (δ 6.03) was irradiated. These data defined phomoxin (**1**) as a novel polyketide possessing carbonate and three alcohol functionalities. Because of the presence of multiple hydroxyl functionalities, no attempts were made to establish the absolute configuration of this molecule.

Phomoxide (**3**) was isolated as a white amorphous solid that analyzed for $C_{14}H_{20}O_4$ by LRMS and ^{13}C NMR methods. The ^{13}C NMR spectrum revealed a structure similar to phomoxin, but **3** contained one additional double bond and lacked the carbonate functionality found in **1**. Broad infrared absorptions at ca. 3400 cm^{-1}, coupled with the presence of **5** oxygen-bearing carbons in the ^{13}C NMR spectrum, suggested the presence of multiple hydroxyl groups in this metabolite. The ^1H NMR spectrum, and data from COSY NMR experiments, showed the presence of two *E* olefinic bonds on the side chain at the $\Delta^{1'\text{-}2'}$ and $\Delta^{3'\text{-}4'}$ positions. Although the NMR spectral data showed a relationship with phomoxin (**1**), the simultaneous isolation of eupenoxide (**4**) in this study was key to establishing the structures of both **3** and **4**. The synthesis of eupenoxide (**4**) has been previously reported (Duke and Rickards, 1984), but inspection of the literature shows that the details of its isolation and structure elucidation as a natural product were never reported. In order to fully assign the structures of both molecules, the structure elucidation of **4** was also undertaken.

Eupenoxide (**4**) analyzed for the molecular formula $C_{14}H_{22}O_4$ by HRCI mass spectrometry and by interpretation of ^{13}C NMR spectroscopic data. The structure assignment of this metabolite followed by interpretation of 2D NMR spectral data and by comparison of appropriate ^1H NMR data with those reported for a silane-protected intermediate (**5**) in the synthesis of eupenoxide (Duke and Rickards, 1984). This intermediate is eupenoxide with the C-3 and C-6 hydroxyl groups protected as *t*-butyldimethyl silane derivatives. The spectral data reported for the synthetic intermediate, in particular the coupling constants between H-3 and H-4, H-4 and H-5, and H-5 and H-6, were identical to our sample of eupenoxide (**4**). Given this comparison, and our analysis of molecular models and predicted vicinal coupling values, we were able to make a confident assignment of the full stereostructure of eupenoxide (**4**). Comparison of the overall spectral data, including the specific coupling constants which define the stereochemistry of the cyclohexene ring, allowed the structure of phomoxide (**3**), with relative stereochemistry only, to be assigned. The presence of the additional olefinic bond in **3** was clearly defined by COSY analysis of the side chain diene system, and by the characteristic UV absorption at 275 nm (log ε 2.81) for the triene chromophore.

Phomoxin (**1**) and phomoxide (**3**) represent new carbon skeletons that appear to be derived via polyketide pathways. These molecules appear to be produced by the cyclization of a polyketide intermediate to form the cyclohexene ring. Phomoxin contains a rare carbonate functionality. Although eupenoxide (**4**) is reported as an antibiotic, we did not observed significant antibacterial, antifungal activity or cancer cell cytotoxity for any of these metabolites.

3. Experimental

3.1. Collection, fermentation, extraction, and isolation

Phoma sp. (strain CNC-651, on deposit at the Center for Marine Biotechnology and Biomedicine fungal strain repository) was isolated from a microbial mat collected from a hypersaline pond (11.5% salinity) on the eastern point of Eleuthera Island in the Bahamas in August of 1995. The fungal isolate was cultured in six 2.8 l Fernbach flasks containing 1 l of fermentation medium prepared from the following components: yeast extract 5 g, glucose 10 g, crab meal 2 g, and seawater 1 l. The flasks were allowed to sit undisturbed (static) for 13 days at 22 °C, after which the mycelium was removed

from the broth by filtration. EtOAc extraction of the broth, followed by removal of solvent under vacuum, produced 900 mg of dark brown syrup. This extract was subjected to C_{18} Sep-Pak (35 ml, 10 g cartridge) separation eluting with 20% methanol/water, 80% methanol/water, and EtOAc. From the 80% methanol/water fraction, semipure **1**, **3**, and **4** were obtained by reversed-phase C_{18} HPLC eluting with 50% methanol/water. Final purification by repeated C18 HPLC with 50 and 70% methanol/water provided phomoxin (**1**) (6 mg), phomoxide (**3**) (7 mg), and eupenoxide (**4**) (20 mg).

3.2. Phomoxin (*1*)

Phomoxin (**1**) was isolated as a white amorphous powder which showed the following spectral features: $[\alpha]_D = -24.4^\circ$ (*c* 0.045, MeOH), IR (neat) 3436 (*br*), 2919, 1783, 1637 cm^{-1}; UV λmax (MeOH): 207 nm (log ε 3.60); ^1H NMR and ^{13}C NMR (see NMR Table); LRCIMS: 316 $[M+NH_4]^+$, 298, 254, 237, 219, 203, 191, 147, 121, 107, 91, 78 amu; HRFABMS, $[M+Na]^+$ *m*/*z* 321.1295, calc. for $C_{15}H_{22}O_6Na$, 321.1314.

3.3. Phomoxin triacetate (*2*)

Phomoxin (**1**, 4 mg) in 0.5 ml pyridine and 0.5 ml Ac_2O were combined and allowed to stand at RT overnight without stirring. Ice water (10 ml) was added to the mixture and it was then extracted with EtOAc (3×10 ml). The combined EtOAc extracts were washed with water (3×10 ml), and concentrated under vacuum to yield the triacetate 2 in essentially quantitative yield (4 mg). The triacetate showed the following spectral features: ^1H NMR spectrum ($CDCl_3$, 400 MHz): δ 0.90 (3H, *t*, *J* = 7 Hz), 1.30 (2H, *m*), 1.32 (2H, *m*), 1.44 (2H, *m*), 2.22 (2H, *m*). 4.78 (2H, *m*), 5.00 (1H, *dd*, *J* = 10, 8 Hz), 5.14 (1H, *dd*, *J* = 10, 3 Hz), 5.52 (1H, *d*, *J* = 8 Hz), 5.90 (1H, *d*, *J* = 3 Hz), 6.18 (1H, *dt*, *J* = 16, 6 Hz), 6.32 (1H, *d*, *J* = 16 Hz). LRFABMS: 447 $[M+Na]^+$, 365, 219, 201; HRFABMS, $[M+Na]^+$ *m*/*z* 447.1631, calc. for $C_{21}H_{28}O_9Na$, 447.1631.

3.4. Phomoxide (*3*)

Phomoxide was isolated as a white amorphous powder which showed the following spectral features: $[\alpha]_D = -20.0^\circ$ (*c* = 0.050, MeOH), IR (neat) 3401 (*br*), 2919, 1719, 1661, 1461, 1384, 1096 cm^{-1}; UV λ_{max} (MeOH): 275 (log ε 2.81), 230 (3.18), 203 (3.45); LRMS (EI): M^+ (*m*/*z*) = 252, *m*/*z* 234, 216. For ^1H and ^{13}C NMR data, see the NMR Table.

3.5. Eupenoxide (*4*)

Eupenoxide was isolated as a white amorphous powder which showed the following spectral features: LRMS (CI) 272 $[M+NH_4]^+$, 254 $[M+NH_4-H_2O]^+$, 237, 219, 207, 121; HRMS: $[M+NH_4]^+$ *m*/*z* calc. for $C_{14}H_{26}NO_4$ 272.1862, found 272.1857. For ^1H and ^{13}C NMR data, see the NMR Table.

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