Mode of Action of Epoxyphomalins A and B and Characterization of Related Metabolites from the Marine-Derived Fungus *Paraconiothyrium* sp.

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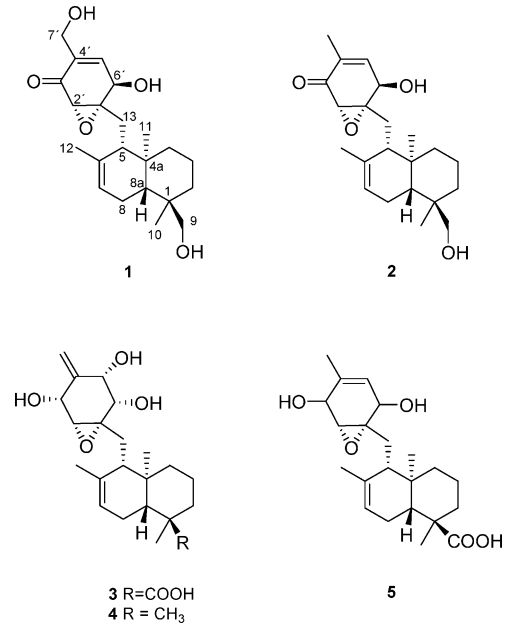
Epoxyphomalins A (**1**) and B (**2**) are highly potent cytotoxic fungal metabolites. During the course of purifying larger quantities of **1** and **2** from *Paraconiothyrium* sp. fermentation extracts, three new epoxyphomalins (**3**−**5**) were isolated and characterized, showing modifications to the oxidation pattern of the cyclohexene moiety or of C-9 of the decalin system. IC_{50} values for cytotoxicity against a panel of 36 human tumor cell lines were determined for all new compounds. Compound **4** was found to be cytotoxic particularly toward prostate PC3M (IC_{50} = 0.72 μM) and bladder BXF 1218 L (IC_{50} = 1.43 μM) cancer cell lines. In addition, inhibition of chymotrypsin-, caspase-, and trypsin-like activity of purified 20S proteasomes was determined for epoxyphomalins A (**1**) and B (**2**). The results indicate that compounds **1** and **2** exert their cytotoxic effect through potent inhibition of the 20S proteasome.

Epoxyphomalins A (**1**) and B (**2**) represent a new family of natural products that have been isolated previously by the authors from a marine-derived fungus which at that time was classified as a *Phoma* species.[1] The basic skeleton is composed of an isoprenoid decalin ring system that is fused to an epoxydon moiety[2] and shows an unprecedented and distinctive substitution and oxidation pattern. Strikingly, the congener epoxyphomalin A (**1**) showed superior cytotoxicity at nanomolar concentrations toward several tumor cell lines.

In order to provide sufficient material of epoxyphomalin A (**1**) for preclinical studies and for the investigation of the mode of action, we initiated a second cultivation batch of the putative *Phoma* strain, whose taxonomy was recently reexamined and was revised to a *Paraconiothyrium* sp. (*Paraconiothyrium* cf *sporulosum*). Scrutiny of the ^{1}H and ^{13}C NMR data of the resulting extract fractions indicated the presence of additional epoxyphomalin derivatives. We now describe the mode of action of epoxyphomalins A (**1**) and B (**2**) as well as the isolation, structure elucidation, and bioactivity of three new analogues, epoxyphomalins C (**3**), D (**4**), and E (**5**), from this extract.

Extensive column chromatography of the EtOAc extract, followed by reversed-phase HPLC of the resulting subfractions, afforded **1** and **2** as the major components and the new compounds **3**−**5** as minor metabolites. Spectroscopic analysis and comparison with literature data revealed that compound **3** is closely related to epoxyphomalin A (**1**). Differences in the ^{1}H and ^{13}C NMR spectra were the absence of CH_2-9, C-3′, C-4′, CH-5′, and CH_2-7′ in **3** and the appearance of two oxygenated methines ($\delta_{H/C}$ 4.57/69.9 and 3.85/75.1), an exomethylene ($\delta_{H/C}$ 5.21 + 5.25/107.7 and 147.2), and a carboxylic acid group ($\delta_{H/C}$ 179.6 and IR 1704 cm^{-1}). The ^{13}C NMR resonance of the latter functionality exhibited HMBC cross-correlations to H_2-2, H-8a, and H_3-10, indicating the presence



of a carboxylic acid group instead of an oxymethylene functionality at C-1 of the decalin skeleton. The remaining unassigned signals formed a continuous spin system in the ^{1}H−^{1}H COSY spectrum in which an oxygenated methine proton singlet (δ 4.57) was coupled to exomethylene proton resonances (δ 5.21 and 5.25), which in turn showed couplings to another oxygenated methine doublet (δ 3.85). Further ^{1}H−^{1}H COSY couplings, this time from the signal at δ 4.57 (H-3′) to H-2′ and from the resonance at δ 3.85 (H-5′) to H-6′, proved the above-mentioned structural fragment to be embedded into an epoxidated cyclohexane ring system as depicted in compound **3**. HMBC correlations from H_2-7′ to C-3′, C-4′, and C-5′ as well as from H-6′ to C-1′, C-4′, and C-5′ and from H-2′ to C-1′, C-3′, and C-4′ confirmed the connectivity of the cyclohexane ring deduced from the COSY data, while HRMS data supported the resulting molecular formula of $C_{22}H_{32}O_6$.

Diagnostic NOE correlations between the resonances of H_3-10 to H-2_{eq}, H-8_{ax}, and H_3-11 and NOEs from H-4_{ax} to H-2_{ax}, H-5, and H-8a were indicative of the relative configuration of the decalin

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Table 1. ^{1}H and ^{13}C NMR Data for Epoxyphomalins C (**3**), D (**4**), and E (**5**) in d_6-Acetone (δ in ppm, J in Hz)

	3		**4**		**5**	
position	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]
1	46.7, C		33.5, C		46.7, C	
2	38.0, CH_2	1.60, m[d]	42.9, CH_2	1.19, m[d]	38.0, CH_2	1.59, m[d]
		1.78, m[c]		1.40, m[c]		1.77, m[c]
3	18.7, CH_2	1.55, m	19.4, CH_2	1.47, m	18.7, CH_2	1.55, m
4	39.1, CH_2	1.02, m[c]	39.9, CH_2	0.91, m[c]	39.1, CH_2	1.05, td (12.1, 4.4)[c]
		1.83, m[d]		1.78, m[d]		1.85, m[d]
4a	36.3, C		36.7, C		36.3, C	
5	47.7, CH	1.61, m	47.3, CH	1.49, m	47.8, CH	1.69, m
6	136.7, C		136.4, C		136.5, C	
7	121.6, CH	5.32, brs	122.0, CH	5.34, brs	121.7, CH	5.33, brs
8	26.1, CH_2	1.66, m[d]	24.4, CH_2	1.88, m	26.1, CH_2	1.69, m[d]
		1.96, m[c]				1.96, m[c]
8a	45.8, CH	2.04, m	50.8, CH	1.20, m	45.8, CH	2.07, m
9	179.6, C		33.4, CH_3	0.85, s	179.5, C	
10	17.3, CH_3	1.19, s	22.1, CH_3	0.87, s	17.3, CH_3	1.19, s
11	14.5, CH_3	0.82, s	14.2, CH_3	0.77, s	14.5, CH_3	0.82, s
12	22.2, CH_3	1.65, s	22.2, CH_3	1.64, s	22.3, CH_3	1.67, s
13	25.8, CH_2	1.91, m	25.9, CH_2	1.91, m	26.8, CH_2	1.90, dd (8.1, 15.7)
		2.09, m		2.06, m		2.13, d (15.7)
1′	60.9, C		61.0, C		61.7, C	
2′	61.4, CH	3.17, brs	61.2, CH	3.13, brs	58.3, CH	3.23, d (1.8)
3′	69.9, CH	4.57, d (1.5)	69.9, CH	4.55, brs	67.9, CH	4.32, brs
4′	147.2, C		147.3, C		135.0, C	
5′	75.1, CH	3.85, d (7.7)	75.1, CH	3.85, d (7.7)	124.0, CH	5.38, brs
6′	76.8, CH	3.53, d (7.7)	76.8, CH	3.51, d (7.7)	67.8, CH	4.12, d (4.4)
7′	107.7, CH_2	5.21, d (2.2)	107.6, CH_2	5.21, d (2.2)	19.4, CH_3	1.73, s
		5.25, d (2.2)		5.24, d (2.2)		

[a] Recorded at 300 MHz. [b] Recorded at 75 MHz, multiplicity determined by DEPT. [c] Axial. [d] Equatorial.

portion of **3** as given in epoxyphomalin A (1*R**, 4a*R**, 5*S**, 8a*R**).[1] The configuration of the cyclohexane moiety was inferred from a 2D-NOESY experiment in combination with coupling constant analysis. Cross-peaks were observed in the NOESY spectrum between H-3′ and H-2′ and H-5′ and between H-5′ and H-6′. Inspection of MM2 energy minimized models[3] of all 16 possible isomers indicated that these NOE interactions were only possible for the configurations 1′*R*,2′*S*,3′*S*,5′*R*,6′*S* and 1′*R*,2′*S*,3′*S*,5′*R*,6′*R* and their corresponding enantiomers 1′*S*,2′*R*,3′*R*,5′*S*,6′*R* and 1′*S*,2′*R*,3′*R*,5′*S*,6′*S*, respectively (see Supporting Information). In order to identify the correct stereoisomer, the coupling constants $^3J_{2'3'}$ and $^3J_{5'6'}$ of compound **3** were analyzed and compared with those of the model compounds. The latter coupling constant values were deduced from transformation of the respective dihedral angles of the model compounds into ^{1}H−^{1}H coupling constants through a modified Karplus equation.[4] While all four remaining isomers were in agreement for a small $^3J_{H2',H3'}$ ($\angle$H2′−H3′ = 67−70° ≡ 2−2.5 Hz), only 1′*S*,2′*R*,3′*R*,5′*S*,6′*S* ($\angle$H5′−H6′ = 44° ≡ 7.6 Hz) and its enantiomer 1′*R*,2′*S*,3′*S*,5′*R*,6′*R* ($\angle$H5′−H6′ = 46° ≡ 7.1 Hz) showed an excellent fit with the measured $^3J_{H5',H6'}$ value of 7.7 Hz. Because epoxyphomalins A and B, whose absolute configurations were deduced by CD studies, possess the 1′*S*,2′*S*,6′*R* configuration, for biogenetic reasons the equivalent configuration 1′*S*,2′*R*,3′*R*, 5′*S*,6′*S* is provisionally suggested for **3**. In comparison with epoxyphomalin A (**1**), during biosynthesis, several atoms of compound **3** changed their oxidation state and a double-bond shift occurred from $\Delta^{4',5'}$ to $\Delta^{4',7'}$. Thus, compound **3** represents a new member of the epoxyphomalin family with a different substitution pattern for which the trivial name epoxyphomalin C is proposed.

Compound **4** analyzed for $C_{22}H_{34}O_4$ by accurate mass measurement. Comparison of ^{1}H, ^{13}C, and HMBC NMR data of **4** with those of **3** (Table 1) indicated the two molecules to be very closely related. The obvious differences between the two compounds resulted from the presence of a methyl group ($\delta_{H/C}$ 0.85/33.4) at C-1 in **4** instead of the carboxylic acid group as found in **3**. Interpretation of the NOE data and coupling constants demonstrated that the relative configuration of **4** and epoxyphomalin C (**3**) is identical. For **4**, epoxyphomalin D is proposed as the trivial name.

Compound **5** displayed a pseudomolecular ion $[M + Na]^+$ at m/z 399.2140, allowing a molecular formula of $C_{22}H_{32}O_5$ to be assigned. The ^{1}H and ^{13}C NMR spectroscopic data of **5** showed strong similarities with those of epoxyphomalin B (Table 1). However, the NMR spectra of **5** present signals for an additional oxymethine group ($\delta_{H/C}$ 4.32/67.9) and a carboxylic acid group ($\delta_{H/C}$ 179.5 and IR 1704 cm^{-1}), while resonances for CH_2-9 and the ketonic carbon C-3′ were absent. HMBC correlations between the new signal at 179.5 ppm and H_2-2, H-8a, and H_3-10 revealed that, as in compound **3**, the oxymethylene functionality at C-1 had been replaced by a carboxylic acid moiety. The other differences can be accounted for by the presence of a hydroxy group instead of a ketone group at C-3′, as indicated by cross-peaks observed between the resonances of the oxygenated methine group (H-3′) and H-2′ and H_3-7′ in the ^{1}H−^{1}H COSY spectrum and two- and three-bond correlations with H-2′ and H_3-7′ in the ^{1}H−^{13}C-HMBC spectrum, respectively.

The observed NOE correlations of **5** were virtually identical to those of **3** and **4** regarding the decalin portion of the molecule, thus indicating that **5** also possessed a 4a*R**, 5*S**, 8a*R** configuration. Concerning the relative configuration of the epoxidated cyclohexene ring moiety, only cross-peaks in the NOESY spectrum between H-3′ and H-2′ and H_3-7′ and between H-5′ and H-6′ and H_3-7′ were observed. Analysis of the possible ^{1}H−^{1}H through-space interactions of the energy-minimized models of all eight possible stereoisomers showed that all models fulfilled the required through-space interactions. Because the coupling constant is correlated with the dihedral angle via the Karplus equation, the information from measured coupling constants ($^3J_{H2',H3'}$ and $^3J_{H5',H6'}$) as well as from literature data of several model compounds[5] was incorporated into the analysis. This strategy narrowed down the number of possible options but did not allow an unambiguous assignment of the relative configuration of the cyclohexene portion. However, based on biogenetic reasons, a 1′*S*,2′*R* configuration is suggested for **5**. Compound **5** represents the fifth member of the epoxyphomalin family and is thus named epoxyphomalin E.

The cytotoxicity of compounds **3**−**5** was investigated using a monolayer cell survival and proliferation assay in a panel of 36

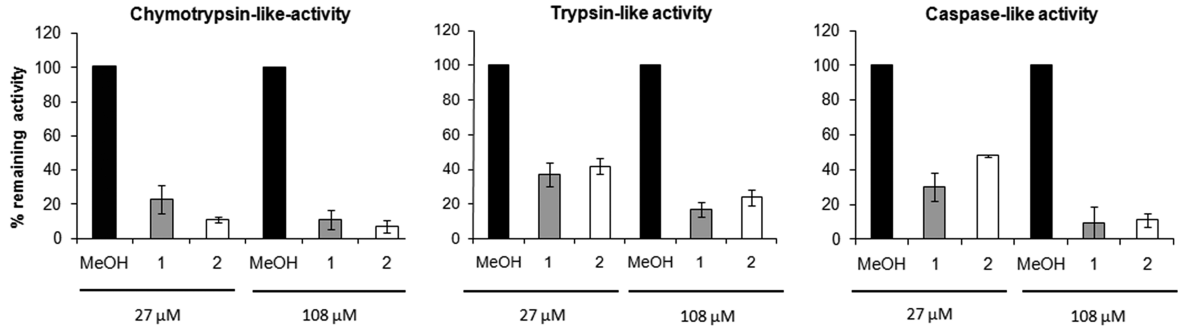

Figure 1. Epoxyphomalins A (**1**) and B (**2**) inhibit the proteasome. Purified human erythrocyte-derived 20S proteasome was incubated with the indicated amounts of epoxyphomalins A (**1**) and B (**2**), and the chymotrypsin-, trypsin-, and caspase-like proteasome activities were measured using fluorogenic peptide substrates specific for the different catalytic activities.

human tumor cell lines. While epoxyphomalins C (**3**) and E (**5**) were not active at a level of 10 μg/mL (27.6 μM), expoxyphomalin D (**4**) exhibited a mean IC_{50} value of 6.12 μM and showed selectivity toward two of the 36 tested tumor cell lines (see Supporting Information). IC_{50} values in the above average sensitive cell lines were found to be 0.72 μM (prostate cancer PC3M) and 1.43 μM (bladder cancer BXF 1218 L). It is noteworthy that, despite the close structural similarity of the new compounds **3**−**5** to the highly cytotoxic metabolite epoxyphomalin A (**1**), only compound **4** was found to be cytotoxic. The three new epoxyphomalins differ structurally from **1**, apart from changes in the substitution pattern of the six-membered epoxidated ring, only in the substituents at C-1. Particularly remarkable in comparison with the cytotoxicity of compound **4** is that the closely related congener **3** was devoid of inhibitory activity toward the cancer cell lines. Therefore, this study revealed that the substituents at C-1 are critical components of the pharmacophore of the epoxyphomalins. Small substituents at C-1, i.e., hydroxymethyl and methyl groups, appear to be well tolerated, while the larger and polar carboxylic acid functionality at C-1 markedly decreases the cytotoxicity.

In our initial studies regarding the mode of action of epoxyphomalins A (**1**) and B (**2**) the COMPARE analysis of the cytotoxic selectivity pattern suggested that compound **2** might exert its cytotoxic effect by proteasome inhibition.[1] In order to test this hypothesis, the effect of compounds **1** and **2** on the 20S proteasome was investigated in detail. Incubation of purified human 20S proteasome with epoxyphomalins A (**1**) and B (**2**) *in vitro* led to a dose-dependent inhibition of chymotrypsin-, caspase-, and trypsin-like proteasome activities (Figure 1). Remarkably, epoxyphomalin B (**2**) preferentially inhibits the chymotrypsin subunit, whereas epoxyphomalin A (**1**) leads to equally reduced protease activities in the proteasome. The 20S proteasome is the catalytic core of the proteasome complex that provides the primary pathway for degradation of ubiquitin-tagged proteins in eukaryotic cells. Thus, it plays a pivotal role in the control of cell proliferation, apoptosis, and differentiation in a variety of healthy and tumor cells.[6] Proteasome inhibitors like the epoxyphomalins therefore offer potential new therapeutic options for the treatment of cancers, e.g., multiple myeloma, lymphoma, prostate, and lung cancers.[6]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP 140 polarimeter. UV and IR spectra were obtained using Perkin-Elmer Lambda and Perkin-Elmer spectrum BX instruments, respectively. All NMR spectra were recorded on Bruker Avance 300 DPX and Bruker Avance 500 DRX spectrometers. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 2.04/29.8 ($[CD_3]_2CO$). ESIMS and HRESIMS were recorded on a Bruker Daltonics micrOTOF-Q instrument. Semipreparative HPLC was carried out using a system composed of a Waters 515 solvent delivery system pump, a Knauer differential refractometer K-2300 detector, and a Linseis L250E chart recorder. Merck silica gel 60 (0.063−0.2 mm) was used for vacuum chromatography. All solvents used were distilled prior to use.

Biological Material, Collection, and Identification. The Caribbean marine sponge *Ectyplasia perox* Duch. & Mich. 1864 (coll. no. CT 193 H; see Supporting Information) was collected by scuba in 1993 at Lauro Club Reef, Dominica. The sponge sample was frozen after collection and stored at −18 °C until workup. Following transport to Germany, the fungal strain *Phoma* sp. 193H12 was isolated among other fungi from the sponge, freed from competing microorganisms and other contaminants as previously described,[7] and integrated into the in-house culture collection as strain number 71. While the fungal strain was initially identified as a *Phoma* species, a detailed analysis of the ITS region of the nuclear ribosomal operon performed by the Belgian Coordinated Collections of Microorganisms, Mycothèque de L′Université Catholique de Louvain (BCCM/MUCL), allowed the revision of the taxonomy to *Paraconiothyrium* cf *sporulosum* (see Supporting Information).

Cultivation. The fungal strain was cultivated at room temperature for three months in 120 Fernbach flasks, each containing 250 mL of solid media (= 30 L). The solid medium consisted of 4 g/L yeast extract, 10 g/L malt, 4 g/L glucose, and 15 g/L agar (Fluka Chemie AG). The pH of the medium was adjusted to 7.3 prior to sterilization.

Extraction and Isolation. Cultivation medium (30 L) and mycelium were extracted with EtOAc (2 × 30 L) after being homogenized using an Ultra Turrax T45. After evaporation of the organic solvent, 10.8 g of dark brown gum was obtained. The extract was subjected to normal-phase vacuum liquid chromatography (VLC) using stepwise gradient elution from petroleum ether (PE) containing increasing proportions of EtOAc followed by MeOH to produce 10 fractions. ^{1}H NMR profiling of these fractions indicated fractions 3 and 4 to be of further interest due to the presence of resonances characteristic for the epoxyphomalin skeleton. Further chromatography of fraction 4, using the previously described purification methods,[1] afforded compounds **1** (13.9 mg) and **2** (14.2 mg). The remaining fraction of interest, fraction 3, was further divided due to its size (2.5 g) into nine subfractions via a silica VLC employing different PE, EtOAc, and MeOH gradients. Subfraction 5 was further separated into three fractions using Sephadex LH-20 (Amersham Pharmacia Biotech AB) eluting with MeOH. The second of these fractions was rechromatographed by semipreparative reversed-phase HPLC (column: Knauer C_{18} Eurospher-100, 250 × 8 mm, 5 μm; MeOH/H_2O (70:30), 2 mL/min) to yield compounds **3** (7 mg), **4** (5.1 mg), and **5** (8 mg).

Epoxyphomalin C (3): white, amorphous powder; $[\alpha]_D^{20}$ −211 (*c* 0.18, acetone); UV (MeCN) λ_{max} (log ε) 197 (3.59) nm; IR (ATR) ν_{max} 3384, 2924, 1704, 1230, 1025, 607 cm^{-1}; ^{1}H and ^{13}C NMR data (d_6-acetone), see Table 1; HRESIMS *m*/*z* 415.2093 $[M + Na]^+$ (calcd for $C_{22}H_{32}NaO_6$, 415.2097).

Epoxyphomalin D (4): white, amorphous powder; $[\alpha]_D^{20}$ −235 (*c* 0.18, acetone); UV (MeCN) λ_{max} (log ε) 199 (3.78) nm; IR (ATR) ν_{max} 3378, 2924, 1364, 1026, 604 cm^{-1}; ^{1}H and ^{13}C NMR data (d_6-acetone), see Table 1; HRESIMS *m*/*z* 385.2345 $[M + Na]^+$ (calcd for $C_{22}H_{34}NaO_4$, 385.2355).

Epoxyphomalin E (5): white, amorphous powder; $[\alpha]_D^{20}$ −235 (*c* 0.18, acetone); UV (MeCN) λ_{max} (log ε) 198 (3.95) nm; IR (ATR) ν_{max} 3375, 2923, 1704, 1230, 1014 cm^{-1}; ^{1}H and ^{13}C NMR data (d_6-acetone),

see Table 1; HRESIMS *m/z* 399.2140 $[M + Na]^+$ (calcd for $C_{22}H_{32}NaO_5$, 399.2147).

Cytotoxicity Assay. Cytotoxic activity of the extract, fractions, and pure compounds **3**−**5** was tested in monolayer cytotoxicity and proliferation assays using human tumor cell lines as reported previously.[1]

***In Vitro* Proteasome Activity Assay.** Proteasome activity assays with purified 20S proteasome were performed as described previously[8] and were carried out in a 100 μL reaction volume containing 27 or 108 μM epoxyphomalin A or B, respectively (1 mg/mL = stock solution in MeOH), 2 μg of human erythrocyte-derived 20S proteasomes (Biomol International, LP), Tris/EDTA (20 mM Tris/HCl; 1 mM EDTA; pH 7.8) and 50 μmol/L fluorogenic substrate (chymotrypsin-, trypsin-, or caspase-like) at 37 °C. The assay buffer was supplemented with a final concentration of 0.05% SDS for the evaluation of the chymotrypsin-like and caspase-like activity.

The rate of cleavage of fluorogenic peptide substrates was determined by monitoring the fluorescence of released amido-4-methylcoumarin using a Victor 1420 multilabel counter (Wallac) at an excitation wavelength of 355 nm and emission wavelength of 460 nm over a period of 60 min.

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Supporting Information Available: Photomicrographs and a taxonomic analysis of the source organisms, ^1H and ^{13}C NMR and MS spectra of compounds **3**, **4**, and **5**, modeling data of compounds **3** and **5**, and detailed IC and T/C values for **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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