Two New Metabolites, Epoxydine A and B, from *Phoma* sp.

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The new compounds, epoxydines A and B (**1** and **2**, resp.), along with the known and related metabolites, **3**–**6**, were isolated from the fungal endophyte *Phoma* sp. The structures of the new compounds were elucidated by detailed spectroscopic analysis, and the relative configuration of **1** was confirmed by ROESY experiments. Preliminary studies indicated that compounds **2**–**5** possess good antibacterial, antifungal, and algicidal properties. Similarly, compound **1** showed antifungal and algicidal, and compound **6** antibacterial and algicidal properties.

Introduction. – The term 'endophyte' includes all organisms that, during a variable period of their life, asymptomatically colonize the living internal tissues of their hosts [1]. Plant endophytic fungi are an important source of many structurally diverse and pharmacologically active natural products. Research on endophytes from different plant sources has yielded intriguing new structures with prominent biological activities [2–7]. In connection with our ongoing search for biologically active metabolites from fungi [8], we investigated the constituents of an endophytic fungus, *Phoma* sp. internal strain number 8889, isolated from the plant *Salsola oppostifolia*. The crude extract showed antifungal, antibacterial, and anti-algal activities. Extensive column and preparative thin-layer chromatography of the AcOEt culture extract afforded two new epoxydon derivates, named epoxydines A and B (**1** and **2**, resp.), together with four known related compounds, **3**–**6**. Here, we describe the isolation and structural elucidation of these compounds and their biological activities.

Results and Discussion. – The fungus *Phoma* sp. was cultivated on a barley-malt extract agar medium for four weeks, and the cultures were subsequently extracted with AcOEt. The crude extract was fractionated on a silica-gel column, followed by *Sephadex LH-20* column chromatography to yield pure compounds **1**–**6**. The structures were elucidated by careful spectroscopic analysis (*Fig. 1*).

The known compounds were readily identified as epoxydon (**3**) [9], (4*R*,5*R*,6*S*)-6-acetoxy-4,5-dihydroxy-2-(hydroxymethyl)cyclohex-2-en-1-one (**4**) [10], 2-chloro-6-(hydroxymethyl)benzene-1,4-diol (**5**) [11], and the antibiotic ES-242-1 (**6**) [12] by analysis of their NMR spectra and by comparison with the data reported in the literature.

Epoxydine A (**1**) was isolated as an optically active colorless powder ($[\alpha]_D = +71.9$ ($c = 1.6$ mg/ml, MeOH)). The UV spectrum of **1** displayed a maximum at 239 nm in agreement with the presence of an α,β-unsaturated C=O group. Its molecular formula

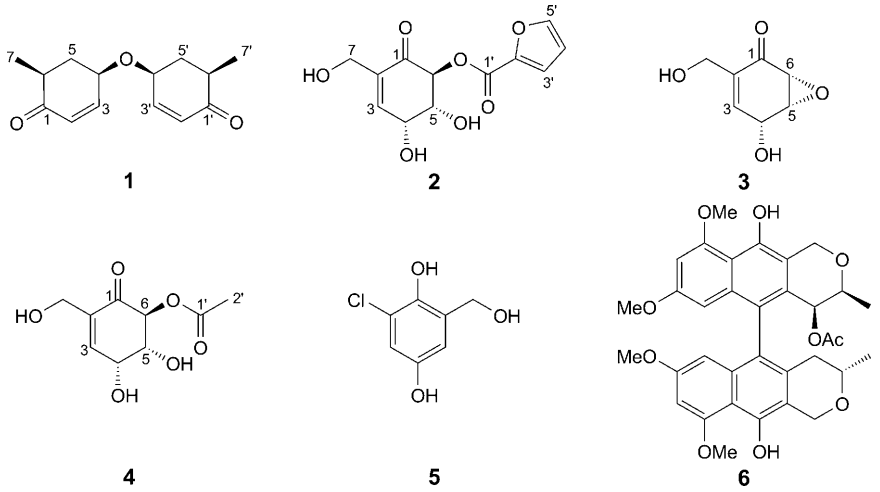

Fig. 1. *Chemical structures of compounds* **1**–**6**

$C_{14}H_{18}O_3$, deduced from the molecular ion peak at *m/z* 234.1256 (M^+), indicated six degrees of unsaturation. The ^{1}H-NMR spectrum of **1** showed the signals of one Me group at δ(H) 1.15 (*d*, *J* = 5.9 Hz, Me(7)) and four olefinic signals at δ(H) 5.94 (*d*, *J* = 10.2, H–C(3), H–C(3′)) and 6.85 (*m*, H–C(2), H–C(2′)). The ^{1}H-NMR spectrum also displayed O–CH signals at δ(H) 4.54 (*dd*, *J* = 4.5, 8.9, H–C(4), H–C(4′)). The ^{13}C-NMR (*Table 1*) and DEPT spectra revealed the presence of two C=O C-atoms (δ(C) 201.9 (*s*) and 201.0 (*s*)), four secondary olefinic C-atoms (δ(C) 152.7 (*d*), 148.4 (*d*), 129.2 (*d*), and 128.8 (*d*)), two secondary O-bearing C-atoms (δ(C) 67.5 (*d*) and 63.4 (*d*)), and two Me groups (δ(C) 15.2 (*q*) and 14.9 (*s*)). In the absence of any other sp and

Table 1. *^{1}H- and ^{13}C-NMR Data*[a]) *of Compounds* **1** *and* **2**. δ in ppm, *J* in Hz.

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1		201.9 (*s*)		192.5 (*s*)
2	5.94 (*d*, *J* = 10.2)	129.2 (*d*)		139.1 (*s*)
3	6.85–6.85 (*m*)	148.4 (*d*)	7.07 (*dt*, *J* = 6.0, 1.5)	140.7 (*d*)
4	4.54 (*dd*, *J* = 4.5, 8.9)	63.4 (*d*)	4.56 (*t*, *J* = 4.0)	65.8 (*d*)
5	2.13–2.14 (*m*), 2.07–2.08 (*m*)	38.8 (*t*)	4.16 (*dd*, *J* = 4.0, 10.8)	69.8 (*d*)
6	2.75–2.76 (*m*)	37.8 (*d*)	5.79 (*d*, *J* = 10.8)	75.7 (*d*)
7	1.17 (*d*, *J* = 7.1)	15.2 (*q*)	4.23(*dq*, *J* = 1.0, 15.3)	57.8 (*t*)
1′		201.0 (*s*)		158.3 (*s*)
2′	5.94 (*d*, 10.2)	128.8 (*d*)		144.1 (*s*)
3′	6.84–6.85 (*m*)	152.7 (*d*)	7.35 (*dd*, *J* = 3.5, 0.8)	118.5 (*d*)
4′	4.54 (*dd*, *J* = 4.5, 8.9)	67.5 (*d*)	6.66 (*dd*, *J* = 3.5, 1.7)	111.7 (*d*)
5′	2.36–2.38 (*m*), 1.73–1.74 (*m*)	41.5 (*t*)	1.26 (*dd*, *J* = 1.7, 0.8)	147.1 (*d*)
6′	2.38–2.39 (*m*)	40.1 (*d*)		
7′	1.15 (*d*, *J* = 5.9)	14.9 (*q*)		

[a]) Assignment made on the basis of DEPT, ^{1}H,^{1}H-COSY, HMQC, HMBC, and ROESY data.

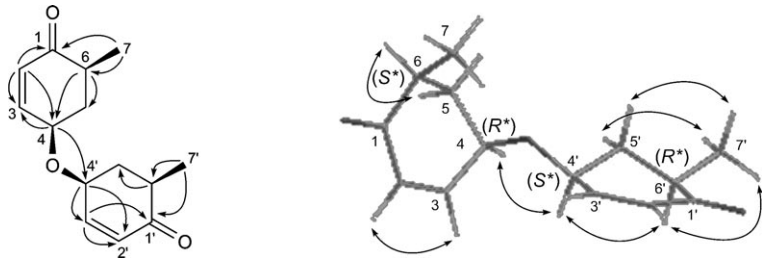

Fig. 2. *Key HMBC* (H → C) *and ROESY* (H ↔ H) *correlations of epoxydine A* (**1**)

sp^2 C-atoms, the gross structure of **1** must be bicyclic. Interpretation of the ^{1}H,^{1}H-COSY, HMQC, and HMBC (*Fig. 2*) data readily suggested that **1** was a compound with two 6-methylcyclohex-2-en-1-one moieties, connected by an O-atom.

The relative configuration of **1** was established by detailed analysis of its ROESY spectrum (*Fig. 2*). The clear ROESY correlations between H−C(4) and H−C(4′) indicated that the two H-atoms at the C-atoms of the O-bridge were both *cis*-oriented. The fact that Me(7) has no correlation with CH_2(5) and H−C(4) suggests that the Me group at C(6) was axially oriented on the six-membered ring and also on the opposite side of H−C(4). Thus, the relative configurations of C(6) and C(4) can be deduced to be (*S**) and (*R**), respectively. Likewise, the clear correlations between Me(7′) and CH_2(5′), and between H−C(4′) and H−C(6′) suggested that H−C(6′) was also axially oriented and on the same side of H−C(4′), and that the Me group at C(6′) was equatorially oriented on the ring. Thus, the relative configurations of C(4′) and C(6′) could be deduced as (*S**) and (*R**), respectively. In light of the above analysis, the structure of **1** could be readily determined as (4*R**,4′*S**,6*S**,6′*R**)-4,4′-oxybis(6-methylcyclohex-2-en-1-one), named epoxydine A.

Epoxydine B (**2**) was obtained as a colorless oil with the molecular formula $C_{12}H_{12}O_7$, as deduced from the molecular ion peak at *m*/*z* 268.0591 (M^+), indicating seven degrees of unsaturation. The IR spectrum of **2** with bands at 3387 and 1727 cm^{-1} indicated the presence of OH and ester groups. Analysis of the ^{1}H- and ^{13}C-NMR data of **2** (*Table 1*) revealed the presence of one primary O-bearing C-atom (δ(H) 4.23 (*dq*, *J* = 1.0, 15.3, 2 H), δ(C) 58.0 (*t*)), three secondary O-bearing C-atoms ((δ(H) 5.79 (*d*, *J* = 10.8, 1 H), δ(C) 75.7 (*d*); δ(H) 4.56 (*t*, *J* = 4.8, 1 H), δ(C) 65.8 (*d*); and δ(H) 4.16 (*dd*, *J* = 4.0, 10.7, 1 H), δ(C) 69.8 (*d*)), and an α,β-unsaturated enone moiety (δ(H) 7.07 (*dt*, *J* = 6.0, 1.5, 1 H), δ(C) 140.7 (*d*), δ(C) 139.1 (*s*), and δ(C) 192.5 (*s*)). Likewise, the presence of a furan-2-carbonyl moiety was suggested by the signals at δ(H) 7.35 (*dd*, *J* = 3.5, 0.8, 1 H, H−C(3′)), 6.66 (*dd*, *J* = 1.7, 3.5, H−C(4′)), and 7.80 (*dd*, *J* = 0.8, 1.7, 1 H, H−C(5′)), and δ(C) 158.3 (*s*, C(1′)), 144.1 (*s*, C(2′)), 118.5 (*d*, C(3′)), 111.7 (*d*, C(4′)), and 147.1 (*d*, C(5′)). On the basis of the above observations, the identity of a cyclohexene skeleton as 4,5,6-trihydroxy-2-(hydroxymethyl)cyclohex-2-en-1-one [10] could be easily deduced. In fact, the ^{1}H- and ^{13}C-NMR spectra of **2** showed essentially the same signals as those present in the spectra of **4**. The molecular weight of **2**, 52 mass units more than that of **4**, further supported structure **2** for this isolate. Analysis of the ^{1}H,^{1}H-COSY and HMBC data (*Fig. 3*) established the structure as **2**. Considering their close biogenetic relationship and the likewise negative optical-rotation value of **2**, the

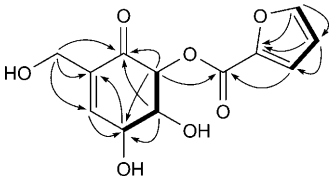

Fig. 3. *Key HMBC* (H → C) *and* $^1H,^1H$*-COSY* (—) *correlations of epoxydine B* (**2**)

same relative and absolute configuration is suggested for compounds **2** and **4**. In the ^{1}H-NMR spectrum, finding that $J(4,5) = 4.0$ and $J(5,6) = 10.8$ Hz was a further evidence for the *cis*,*trans*-configuration at C(4), C(5), and C(6) [13]. Thus, compound **2** was determined as (1*S*,5*R*,6*R*)-5,6-dihydroxy-3-(hydroxymethyl)-2-oxocyclohex-3-en-1-yl furan-2-carboxylate, named epoxydine B.

Bioactivity. – The biological activities of the substances were tested in an agar diffusion assay for antibacterial (*Escherichia coli* and *Bacillus megaterium*), antifungal (*Microbotryum violaceum*), and antialgal activities (*Chlorella fusca*) (*Table 2*). All six compounds, **1**–**6**, were biologically active exhibiting antibacterial and antialgal activities, and compounds **2**–**5** were also antifungal. Particularly noteworthy are the activities of compound **4**, which inhibited all four test organisms.

Table 2. *Biological Activity of Compounds* **1**–**6** *in an Agar Diffusion Assay.* Values are the radius of zone of inhibition [mm]. Application of pure substances at a concentration of 0.05 mg (50 µl of 1 mg/ml).

Compound	Antibacterial (*Ec*)[a])	Antibacterial (*Bm*)	Antifungal (*Mb*)	Antialgal (*Chl*)
1	0	0	pi 7[b])	10
2	0	pi 9	pi 7	6
3	pi 7	0	pi 8	9
4	pi 7	10	pi 7	7
5	0	pi 7	pi 10	7
6	pi 10	pi 7	0	6
Penicillin	14	18	0	0
Tetracycline	18	18	0	pi 10
Nystatin	0	0	20	0
Actidione	0	0	50	35
Acetone	0	0	0	0

[a]) *Escherichia coli* (*Ec*), *Bacillus megaterium* (*Bm*), *Microbotryum violaceum* (*Mb*), and *Chlorella fusca* (*Chl*). [b]) pi = Partial inhibition, *i.e.*, there was some growth within the zone of inhibition.

In summary, the epoxidone family is extended by two new derivatives, showing the interesting C_2-symmetric dimeric structure of epoxydine A and B (**1** and **2**, resp.), and the furancarboxylic acid derivative of epoxydon (**3**). Chemically, this compound can be derived *via* the epoxide opening of epoxydon (**3**) with furancarboxylic acid, while the known acetate **4** is the ring-opening product of **3** with AcOH. Coupling of monomeric units to 'dimers' with identical or similar units is increasingly recognized as a general principle in nature to produce chemical diversity of secondary metabolites [14][15]. This is also seen in the structure of ES-242-1 (**6**) [12].

Experimental Part

General. Anal. and prep. TLC: precoated silica gel plates (SiO_2; *Merck*, *G60 F_{254}* or *G50* UV-254). Column chromatography (CC): commercial SiO_2 (*Merck*, 0.040–0.063 mm) and *Sephadex LH-20* (*Amersham Biosciences*). Optical rotations: *Perkin-Elmer 241 MC* polarimeter at the Na D-line. UV Spectra: *Shimadzu UV-2101PC* spectrophotometer; λ_{max} nm ($\log \varepsilon$). IR Spectra: *Nicolet-510P* spectrophotometer; $\tilde{\nu}_{max}$ in cm^{-1}. ^1H- and ^{13}C-NMR Spectra: *Bruker Avance 500* (500 MHz for ^1H and 125 MHz for ^{13}C) spectrometer; δ in ppm rel. to Me_4Si as internal standard or residual solvent peak used for referencing, *J* in Hz. EI- and HR-EI-MS: *Finnigan MAT 8200* and *Micromass LCT* mass spectrometers, in *m*/*z* (rel. %).

Fungal Strains. The fungal endophyte was identified as *Phoma* sp. on the basis of morphology: conidia: colorless to pink, oval, and slimy; pycnidia: darkly pigmented; conidiogenic cells: conical [16].

Culture, Extraction, and Isolation. The fungal endophyte, *Phoma* sp. (internal strain no. 8889), was isolated from the halotolerant plant *Salsola oppostifolia*, of Gomera. It was cultivated at r.t. for 28 d in 12 l of a barley-malt extract (biomalt), 5% (*w*/*v*) solid agar medium in 576 plastic petri dishes (9 cm in diameter). (Biomalt is 30% H_2O; 100 ml contain 0.5 g of potassium gluconate, 3.8 g of protein, 50.3 g of carbohydrates of which 50.3 g are sugars, 0.8 g fats of which 0.5 g are sat. fats, 1.5 g of dietary fibers, 0.01 g of sodium; *Villa Natura Gesundprodukte GmbH*, D-Kirn). The culture media were then extracted with AcOEt (4 l) to afford a crude extract (1.86 g) after removal of the solvent under reduced pressure. The extract was chromatographed on a SiO_2 column using eluents of increasing polarity, from petroleum ether (PE) to acetone to MeOH. The fractions eluted with PE/acetone 7:3 were further purified by *Sephadex LH-20* CC using $CHCl_3$/MeOH 1:1 as eluent to afford compound **1** (14.3 mg), **2** (11.5 mg), and **3** (10.9 mg), and a mixture containing compounds **4**–**6**. Further CC (SiO_2; CH_2Cl_2/MeOH 98:2) gave compounds **4** (13.1 mg), **5** (9.7 mg), and **6** (15.2 mg) in pure form.

Epoxydine A (=*(4*R*,4′*S*,6*S*,6′*S*)-4,4′-Oxybis(6-methylcyclohex-2-en-1-one*); **1**). Amorphous powder. $[\alpha]_D^{25} = +71.9$ ($c = 1.6$ mg/ml, $CHCl_3$). UV (MeOH): 239 (3.71), 325 (2.10). IR (KBr): 2963, 2920, 1703, 1697 (C=O), 1465, 1061 (O–alkyl). ^1H- and ^{13}C-NMR: see *Table 1*. EI-MS (230°): 234.0 (11, M^+), 208.0 (7), 167.0 (24, $[M - C_4H_9]^+$), 148.9 (41, $[M - C_4H_9 - H_2O]^+$), 97.0 (65), 55.0 (100). HR-EI-MS: 234.1256 (M^+, $C_{14}H_{18}O_3^+$; calc. 234.1256).

Epoxydine B (=*(1*S*,5*R*,6*R*)-5,6-Dihydroxy-3-(hydroxymethyl)-2-oxocyclohex-3-en-1-yl furan-2-carboxylate*; **2**). Colorless needles. $[\alpha]_D^{25} = -72.7$ ($c = 1.1$ mg/ml, $CHCl_3$). UV (MeOH): 255 (3.73), 330 (2.35). IR (KBr): 3387 (OH), 2925, 2855, 1727, 1693 (C=O), 1478, 1397, 1293. ^1H- and ^{13}C-NMR: see *Table 1*. EI-MS (230°): 268.1 (8, M^+) 211.1 (13, $[M - C_4H_9]^+$), 167.0 (22, $[M - C_6H_{13}O]^+$), 149.0 (42), 98.9 (58), 69.1 (90), 43.0 (100). HR-EI-MS: 268.0591 (M^+, $C_{12}H_{12}O_7^+$; calc. 268.0583).

Tests for Biological Activity. For the agar diffusion assay [17], the compounds were dissolved in acetone at a concentration of 1 μg/μl. Fifty μl of the solns. were pipetted onto a sterile filter disc (0.05 mg/filter disc), which was placed onto an appropriate agar growth medium for the respective test organism (*Escherichia coli*, *Bacillus megaterium*, *Microbotryum violaceum*, and *Chlorella fusca*), and subsequently sprayed with a suspension of the test organism [17].

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