New Biologically Active Compounds from the Nematode-Trapping Fungus *Arthrobotrys oligospora* Fresen.

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Dedicated to Professor Dr. Wolfgang Steglich on the occasion of his 60th birthday

Arthrobotrys oligospora, Nematode-Trapping Fungi, Oligosporon, Oligosporols A and B, Cytotoxic Activities

The isolation, structural elucidation and biological activities of oligosporon (**1**), oligosporol A (**2**) and oligosporol B (**3**), three new antibiotics from cultures of the predacious deuteromycete, *Arthrobotrys oligospora*, are reported. The structures were elucidated by means of high resolution mass and high field NMR spectroscopy. The compounds exhibited weak antimicrobial, cytotoxic and hemolytic effects, but were not active towards the nematode *Caenorhabditis elegans*. Other *Arthrobotrys* species were also found to produce these or similar compounds.

Introduction

Nematode-trapping fungi have been studied in great detail, due to their unique abilities to catch nematodes and other invertebrates by means of special trapping organs [1]. Trapped nematodes are never colonized by microorganisms other than the predators themselves [2]. This finding led to some studies on the biological activities of extracts obtained from these fungi [3, 4]. The compounds, however, involved in these processes have never been isolated and identified. Therefore a screening of predacious fungi for the production of bioactive compounds was carried out. Nematicidal activities were detected in the mycelial extracts of *Arthrobotrys brochopaga*, *A. conoides*, *A. dactyloides* and *A. oligospora*, and linoleic acid was isolated as the nematicidal principle [5]. In contrast, extracts from the culture fluid of *A. oligospora* and the other *Arthrobotrys* species showed antimicrobial activities towards *Nematospora coryli* and gram-positive bacteria but no nematicidal activity. Consequently, three new structurally related antibiotics were isolated from the culture fluid. In this report, we describe the production, isolation and structural elucidation and biological activities of these compounds.

Reprint requests to Prof. Dr. H. Anke.

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Experimental

General

Nematode-trapping fungi (*A. brochopaga* strain 218.61, *A. conoides* strain 265.83, *A. dactyloides* strain 264.83, *A. oligospora* strain 115.81, *Dactylaria candida* strain 220.54, *Dactylella astenopaga* strain 262.83, *Monacrosporium doedycoides* strain 155.75 and *M. parvicollis* strain 219.61) were obtained from CBS, Baarn/Netherlands, except *A. conoides* strain HA Bit 1, which was isolated from a soil sample collected near Bitterfeld, F.R.G. [5]. Materials for chromatography, solvents and chemicals (p.A. quality) were purchased from Merck, Darmstadt, F.R.G. Preparative HPLC and MPLC columns measured 250 × 25 mm.

Biological assays

Assays for the evaluation of biological activities were carried out as described previously (cytotoxic activity [6], nematicidal activity [7], antimicrobial and phytotoxic activities [8]). In the Ames test [9], *Salmonella typhimurium* strains TA 97, TA 98, TA 100 and TA 102 were assayed in a "pour plate test" without S9 Mix and 50 µg/ml of compounds **1–3.** Hemolytic activities were determined with bovine erythrocytes (10^5 cells/ml in phosphate buffered saline) after incubation for 1 h at 37 °C. Triton X-100 (10 µl/l) was used as a standard, causing 100% hemolysis.

Fermentation

The culture medium for the production of the antimicrobial compounds was composed of (g/l): corn meal 20, glucose 4, pH 5.5 was adjusted with HCl (1 M) before autoclaving. Fermentations were carried out at 24 °C in a 20 l Braun Biostat U fermentation apparatus (Braun Melsungen F.R.G.) with agitation (160 rpm) and aeration (4 l/min). The inoculum was 400 ml of a five days old culture in the same medium.

Oxygen consumption was measured using a Magnos 4 G magnopneumatic oxygen analyser (Hartmann & Braun, Frankfurt), carbon dioxide production was followed using an ADC carbon dioxide analyser type SG-305 and oxygen saturation of the culture broth was measured with an Ingold oxygen electrode. The data were registered on line, using the Micro MFCS Process control system software equipment.

For determination of biological activities of the culture fluid, aliquots (100 ml) were taken under sterile conditions. After centrifugation, the supernatant was extracted with ethyl acetate (100 ml). The extracts were dried *in vacuo* and the oily residues were redissolved in methanol (1 ml). 10 µl of these concentrated extracts were tested in the agar diffusion assay towards *Nematospora coryli* and *Bacillus subtilis*.

Detection and isolation of compounds **1–3**

After centrifugation (10 min at 3000 × *g*) of the culture broth, the fluid (18.5 l) was applied onto Mitsubishi DIAION HP 21 resin. The HP 21 column (500 g resin) was washed with H_2O-acetone (8:2) and eluted with acetone (3 l), and the combined eluates were concentrated to an aqueous residue (1 l), which was subsequently extracted with ethyl acetate (3 × 500 ml). The organic solvent was evaporated *in vacuo* to yield 2.3 g of an oily crude product, which was treated as described in Fig. 2 in order to isolate the bioactive compounds **1–3.** The analytical HPLC system consisted of a Merck LiChroSpher RP 18 column (5 µm, 125 mm × 4 mm) eluted with 1.5 ml/min of a H_2O/methanol gradient (linear from 100% to 30% water between $t = 0$ and $t = 20$ min, and from 30% to

Table I. ^1H (500 MHz) NMR data for oligosporon (**1**), oligosporol A (**2**), oligosporol B (**3**) and triacetyloligosporol B (**4**). The spectra were recorded in $CDCl_3$, the signal for $CHCl_3$ (7.26 ppm) was used as reference, and the coupling constants *J* are given in Hz.

H	**1** δ; mult.; *J*	**2** δ; mult.; *J*	**3** δ; mult.; *J*	**4** δ; mult.; *J*
1	–	4.49; m	4.30; m	5.65; dd; 2, 2
3	6.69; ddd; 1.4, 1.4, 4.8	5.75; dddd; 1, 1, 1, 4.6	5.75; dm; 5.0	5.88; dm; 5
4	5.00; dm; 5	4.67; m	4.65; dm; 5	5.75; dm; 5
6	3.28; d; 1.4	3.35; dd; 0.9, 3.0	3.23; m	3.08; dd; 1.7, 1.7
1′	5.14; d; 9.1	4.96; d; 8.9	4.81; d; 8.9	6.25; d; 9.4
2′	5.39; d; 9.1	5.42; d; 8.9	5.48; d; 8.9	5.21; d; 9.4
3′–CH_3	1.87; d; 1.2	1.85; d; 1.2	1.85; d; 1	1.92; d; 1.1
4′	6.15; d; 15.3	6.15; d; 15.2	6.16; d; 15.2	6.11; d; 15.3
5′	6.52; dd; 10.9, 15.3	6.49; dd; 10.9, 15.2	6.48; dd; 10.8, 15.2	6.52; dd; 10.9, 15.3
6′	5.89; d; 10.9	5.88; d; 10.9	5.88; d; 10.8	5.88; d; 10.9
7′–CH_3	1.80; d; 1.1	1.79; d; 1.0	1.79; d; 1	1.80; d; 0.9
8′	2.1; m	2.1; m	2.1; m	2.1; m
9′	2.1; m	2.1; m	2.1; m	2.1; m
10′	5.09; m	5.08; m	5.08; m	5.08; m
11′–(E)–CH_3	1.68; d; 0.8	1.67; d; 0.8	1.67; d; 0.7	1.67; s
11′–(Z)–CH_3	1.60; d; 0.8	1.60; d; 0.7	1.60; d; 0.9	1.60; s
1″-Ha	4.78; ddd; 1, 1, 14.1	4.78; dd; 0.9, 13.3	4.62; m	4.55; d; 13.7
1″-Hb	4.73; ddd; 1.3, 1.4, 14.1	4.56; d; 13.3	4.62; m	4.45; d; 13.7
1″-Ac	2.08; s	2.07; s	2.08; s	2.06; s
1-Ac	–	–	–	2.12[a]; s
4-Ac	–	–	–	2.10[a]; s
1′-Ac	–	–	–	2.02[a]; s

[a] Interchangable.

0% water between $t = 20$ and $t = 30$ min. The injection volume was 20 µl, and the compounds were detected using Diode Array Detection (Hewlett Packard 1090 Series II).

The NMR spectra (see Table I and II) were recorded with a Bruker ARX 500 spectrometer, the UV spectra with a Perkin Elmer Lambda 16, the IR spectra with a Bruker IFS 48, and the mass spectra with a Jeol SX 102 spectrometer. Optical rotations were measured with a Perkin Elmer 1541 automatic polarimeter (10 cm cell).

Oligosporon (**1**) (3-acetoxymethyl-5-hydroxy-5a-[1-hydroxy-3,7,11-trimethyldodeca-2,4,6,10-tetraenyl]-oxabicyclo[4.1.0]hept-3-en-2-one) was obtained as a colourless oil. $[\alpha]_D = +87°$ (*c* 1.0 in $CDCl_3$). The HPLC retention time was 23.0 min. UV (methanol) λ_{max} (ε): 280 nm (17,200). IR (KBr): 3420, 2920, 1740, 1680, 1440, 1380, 1230 and 960 cm^{-1}. For NMR data, see Table I and II. MS (EI, 70 eV), *m/z:* 416.2206 (M^+, 36%, $C_{24}H_{32}O_6$ requires 416.2199), 347 (14%), 269 (10%), 186 (24%), 149 (37%), 121 (81%), 69 (100%).

Table II. ^{13}C (125 MHz) NMR data for oligosporon (**1**), oligosporol A (**2**), oligosporol B (**3**) and triacetyloligosporol B (**4**). The spectra were recorded in $CDCl_3$, and the solvent signal (77.0 ppm) was used as reference.

C	**1** δ; mult.	**2** δ; mult.	**3** δ; mult.	**4** δ; mult.
1	192.5; s	65.4; d	63.2; d	63.7; d
2	131.8; s	133.0; s	132.7; s	130.9; s
3	141.1; d	125,3; d	124.7; d	123.6; d
4	63.9; d	63.5; d	63.1; d	63.1; d
5	67.6; s	64.8; s	60.4; s	58.3; s
6	57.3; d	58.7; d	57.8; d	53,5; d
1′	67.5; d	68.1; d	69.4; d	67.8; d
2′	124.3; d	125.7; d	126.4; d	121.0; d
3′	140.5; s	139.2; s	138.9; s	141.4; s
3′−CH_3	13.3; q	13.0; q	13.2; q	13.2; q
4′	133.3; d	133.6; d	133.9; d	133.4; d
5′	127.0; d	126.2; d	126.0; d	127.0; d
6′	124.8; d	124.7; d	124.9; d	124.8; d
7′	141.2; s	140.5; s	140.4; s	141.2; s
7′−CH_3	17.0; q	16.7; q	16.9; q	16.9; q
8′	40.1; t	39.9; t	40.1; t	40.1; t
9′	26.6; t	26.4; t	26.6; t	26.6; t
10′	123.8; d	123.7; d	123.8; d	123.8; d
11′	131.9; s	131.6; s	131,8; s	131.8; s
11′−(E)−CH_3	25.7; q	25.5; q	25.7; q	25.7; q
11′−(Z)−CH_3	17.7; q	17.5; q	17.7; q	17.7; q
1″	60.4; t	64.0; t	65.0; t	63.9; t
1″−Ac−C=O	170.6; s	171.0; s	171,4; s	170.3; s
1″−Ac−CH_3	20.8; q	20.7; q	20.9; q	20.7; q
1−Ac−C=O	–	–	–	170.1[a]; s
1−Ac−CH_3	–	–	–	20.8; q
4−Ac−C=O	–	–	–	170.0[a]; s
4−Ac−CH_3	–	–	–	20.8; q
1′−Ac−C=O	–	–	–	169.9[a]; s
1′−Ac−CH_3	–	–	–	20.8; q

[a] Interchangable.

Oligosporol A (**2**) (4-acetoxymethyl-1a-[1-hydroxy-3,7,11-trimethyldodeca-2,4,6,10-tetraenyl]-oxabicyclo[4.1.0]hept-3-en-2,5-diol) was obtained as a colourless oil. $[\alpha]_D = +27°$ (*c* 3.0 in $CDCl_3$). The HPLC retention time was 22.5 min. UV (methanol) λ_{max} (ε): 280 nm (9,850). IR (KBr): 3400, 2920, 1730, 1440, 1380, 1240, 1030, 960 and 900 cm^{-1}. For NMR data, see Table I and II. MS (EI, 70 eV), *m/z:* 418.2359 (M^+, 41%, $C_{24}H_{34}O_6$ requires 418.2355), 331 (12%), 271 (19%), 149 (40%), 121 (83%), 69 (100%).

Oligosporol B (**3**) (4-acetoxymethyl-1a-[1-hydroxy-3,7,11-trimethyldodeca-2,4,6,10-tetraenyl]-oxabicyclo[4.1.0]hept-3-en-2,5-diol) was obtained as a colourless oil. $[\alpha]_D = -20°$ (*c* 1.3 in $CDCl_3$). The HPLC retention time was 22.3 min. UV (methanol) λ_{max} (ε): 280 nm (14,100). IR (KBr): 3370, 2920, 1740, 1440, 1380, 1240 and 1030 cm^{-1}. For NMR data, see Table I and II. MS (EI, 70 eV), *m/z:* 418.2371 (M^+, 32%, $C_{24}H_{34}O_6$ requires 418.2355), 271 (16%), 175 (22%), 149 (47%), 121 (85%), 69 (100%).

Triacetyloligosporol B (**4**) (2,5-diacetoxy-4-acetoxymethyl-1a-[1-acetoxy-3,7,11-trimethyldodeca-2,4,6,10-tetraenyl]-oxabicyclo[4.1.0]hept-3-ene) was obtained as a colourless oil after acetylation of oligosporol B in pyridine/acetic anhydride at room temperature over night, and chromatography on silica gel. $[\alpha]_D = +22°$ (*c* 0.7 in $CDCl_3$). The HPLC retention time was 26.3 min. UV (methanol) λ_{max} (ε): 283 nm (14,800). IR (KBr): 3450, 2930, 1750, 1370, 1230, 1020 and 960 cm^{-1}. For NMR data, see Table I and II. MS (EI, 70 eV), *m/z:* 544 (M^+, 42%), 475 (21%), 415 (21%), 313 (28%), 295 (45%), 253 (100%), 225 (59%), 159 (53%).

Results and Discussion

In Fig. 1, a fermentation diagram of *A. oligospora* in 20 l scale is depicted. Antimicrobial activities of the culture fluid extracts were detected after 55 h. The fermentation was terminated after six days, when the glucose in the culture broth was

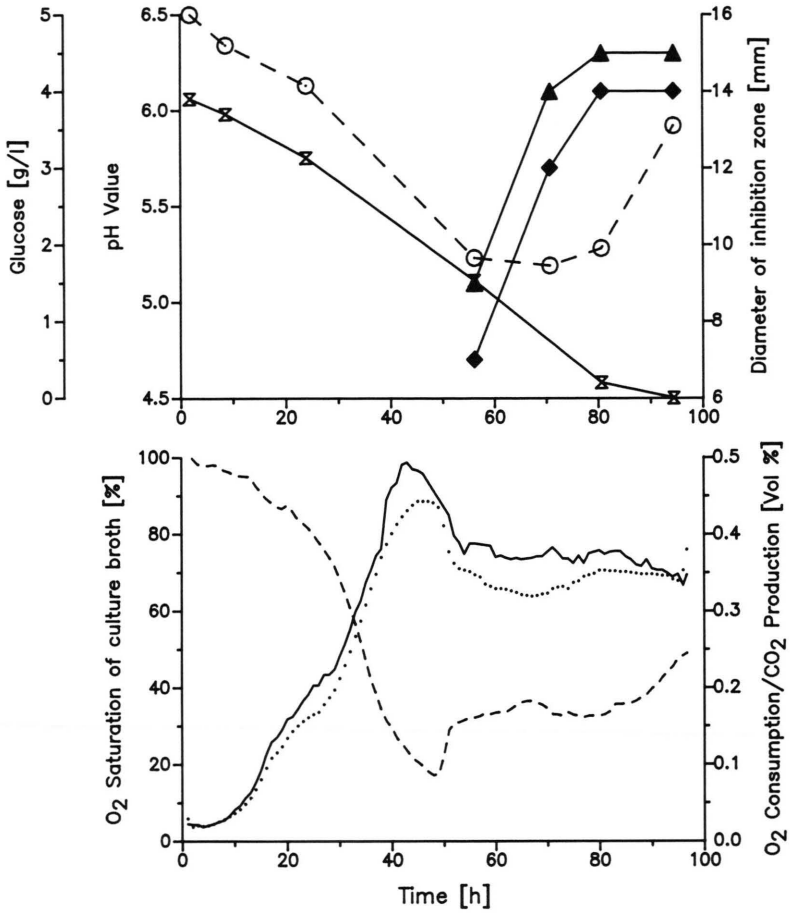


Fig. 1. Fermentation diagram of *Arthrobotrys oligospora* (20 l scale). Legends: O—O, pH value; x—x, glucose [g/l]; – –, oxygen saturation of culture broth [%]; ——, CO_2 [Vol %]; ···, O_2 [Vol %]. Diameter of inhibition zone [mm]: ▲—▲, *N. coryli;* ◆—◆, *B. subtilis.*

used up and the biological activities of the extracts showed no further increase.

Fig. 2 shows the isolation procedure for oligosporon (**1**), oligosporol A (**2**) and oligosporol B (**3**). The structures are depicted in Fig. 3.

Extracts from cultures of other *Arthrobotrys* species showed biological activities similar to those of *A. oligospora.* According to the results of HPLC analysis, peaks with t_r and UV spectra identical with **1–3** were also present in *A. brochopaga, A. conoides* and *A. dactyloides.* This would suggest a chemotaxonomical relationship between the different species, independent of the type of traps formed (constricting rings or adhesive nets). The identity of the metabolites detected in other *Arthrobotrys* species with **1–3** still remains to be clarified. In cultures of other predacious fungi like *Dactylaria, Dactylella* and *Monacrosporium* strains, no antimicrobial activities and no compounds similar to **1–3** were detected.

The antimicrobial and cytotoxic effects of compounds **1–3** are shown in Table III and IV. In general, oligosporon (**1**) showed higher activities than the oligosporols **2** and **3.** Whereas the growth of the filamentous fungi *Mucor miehei* and *Penicillium notatum* was not inhibited at 100 µg of **1–3**/disc in the agar diffusion assay, some yeasts and bacteria were sensitive.

In the microwell plate assay (Table IV), HL 60 cells were more sensitive than other mammalian cells. The effects of **1–3** on the incorporation of precursors into macromolecules of these cells were investigated, following the method described by Erkel *et al.* [6]. After preincubation for 15 min, all compounds were not active up to 50 µg/ml, although they caused total lysis of the cells at

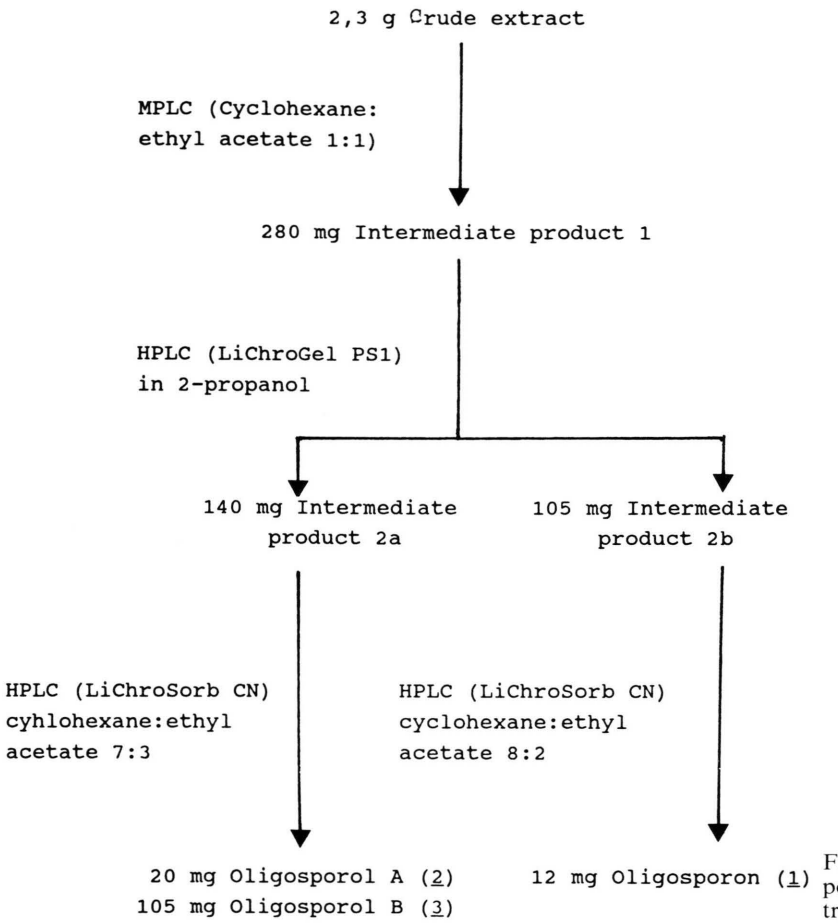
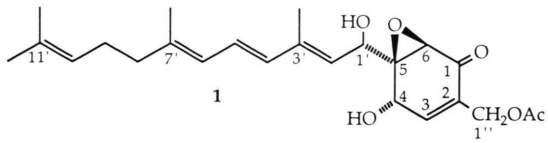


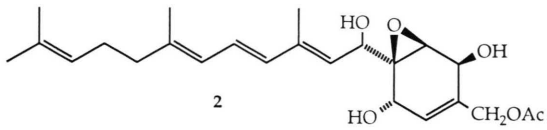
Fig. 2. Isolation of bioactive compounds from the culture filtrate extract of *Arthrobotrys oligospora*.

Table III. Minimal inhibitory concentrations (MIC) of the antibiotics from *A. oligospora* in the serial dilution assay towards yeasts and bacteria after 24 h.

Organism	MIC [µg/ml]		
	Oligosporol A (**2**)	Oligosporol B (**3**)	Oligosporon (**1**)
Bacteria (Nutrient Broth):			
Acinetobacter calcoaceticus	100	100	50
Bacillus brevis	25	50	10
B. subtilis	50	50	25
Micrococcus luteus	100	100	50
Yeasts (YMG medium):			
Candida albicans	>100	100	100
Nematospora coryli	100	100	25
Rhodotorula glutinis	>100	>100	>100
Saccharomyces cerevisiae	>100	>100	100



1



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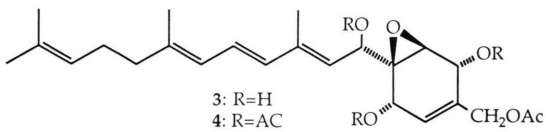
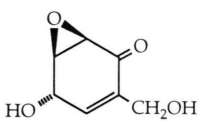


3: R=H
4: R=AC



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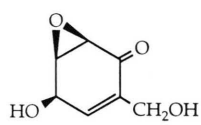

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Fig. 3. Structures of oligosporon (**1**), oligosporol A (**2**), oligosporol B (**3**), oligosporon B triacetate (**4**), isoepoxydon (**5**) and epoxydon (**6**).

Table IV. Cytotoxic activities of the compounds from *A. oligospora* towards mammalian cell lines. IC_{100} = concentrations causing total lysis of the cells after 24 h.

Cell line	IC_{100} [µg/ml]		
	Oligosporol A (**2**)	Oligosporol B (**3**)	Oligosporon (**1**)
L 1210	50	50	25
HL 60	10	10	5
BHK 21	100	100	100

5–10 µg/ml after 24 h. With bovine erythrocytes, over 50% hemolysis was observed at 10–25 µg/ml, indicating that the biological activities of **1–3** were mainly caused by their interactions with the membranes of sensitive target organisms. The additional biological effects of **1** may be due to the presence of an unsaturated epoxide-linked carbonyl moiety.

The structure determination of the three new compounds was based on NMR data, which are summarized in Table I (^{1}H NMR data) and II (^{13}C NMR data), as well as in Fig. 4 [long-range ^{1}H-^{13}C correlations and NOESY correlations for oligosporon (**1**)]. High resolution mass spectroscopy in-

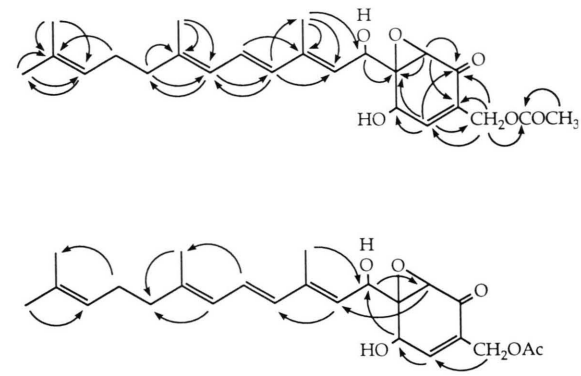

Fig. 4. Significant long-range ^{1}H-^{13}C correlations (top) and NOESY correlations (bottom) for oligosporon (**1**).

dicated that the composition of oligosporon (**1**) is $C_{24}H_{32}O_6$, while oligosporol A (**2**) and oligosporol B (**3**) both contain two additional hydrogens. The NMR data suggested that oligosporon (**1**) contains a keto functionality which has been reduced to a secondary alcohol in oligosporol A (**2**) and oligosporol B (**3**), although attempts to perform this reduction *in vitro* by reducing oligosporon (**1**) with KBH_4 in ethanol failed due to the chemical instability of the compound. The C_{15} side chain was found to be identical in all three compounds, although it was not possible to determine the relative configuration of C-1′ (see Fig. 3 for the numbering of the skeleton). The stereochemistry of the double bonds in the chain was determined by a combination of ^{1}H-^{1}H coupling constants (see Table I), long-range ^{1}H-^{13}C correlations and NOESY correlations (see Fig. 4). The elucidation of the stereochemistry of the six-membered ring in oligosporon (**1**) is based on the assumption that it prefers the boat conformation with the C-4 hydroxyl group axial and the C-5 C_{15} alkyl substituent equatorial, as this would allow the best possible overlap between the delocalized electrons in the oxirane ring and the carbonyl π-orbitals [10]. The coupling constants between 3-H and 4-H in similar compounds with the same stereochemistry [for example isoepoxydon (**5**)] is in all reported cases close to 5.0 Hz [10], while the J_{3-4} in epoxydon (**6**) (in which the oxirane ring and the C-4 hydroxyl group are *cis*) is 2.5 Hz [11]. The J_{3-4} in oligosporon (**1**) is 4.8 Hz, suggesting that it has the same relative stereochemistry as isoepoxydon (**5**), and this is also in accordance with the NOESY correlation

observed between 3-H and 4-H in oligosporon (**1**). The same J_{3-4} and NOESY correlations were also observed in the spectra of oligosporol A (**2**), and oligosporol B (**3**), as well as for triacetyloligosporol B (**4**), which suggests that the two reduced derivatives exist in the same conformation as oligosporon (**1**) and that they have the same relative C-4/C-5 stereochemistry (differing only at C-1). If the C-4 hydroxyl group and the oxirane ring were cis, the axial position of the hydroxyl group (which would be required to give a J_{3-4} if 5 Hz) should result in a observable hydrogen bond to the oxirane oxygen, but this could not be seen. The hydroxyl protons of compounds **1–3** appear as broad singlets between 2 and 3.3 ppm. Comparison of Dreiding models of the two C-1 epimers show that one has an equatorial hydroxyl group cis to the oxirane ring, while the other has an axial trans hydroxyl group. NOESY correlations between 1-H and 6-H and especially between 1-H and 1″-H_2 were observed in the spectra of oligosporol B (**3**) and of its triacetate **4,** suggesting that their 1-H are equatorial. The corresponding NOESY correlation between 1-H and 1″-H was not observed in oligosporol A (**2**), suggesting that its 1-H is axial. Unfortunately, due to the small amounts available and the instability of the compounds, it was not possible to prepare a derivative in which the C-1′ and C-4 hydroxyl groups were locked for example as an acetal, in order to establish the configuration of C-1′.

Acetylation of oligosporol B with acetic anhydride in pyridine gave low yields of the triacetate **4,** with a molecular weight of 544 according to EIMS. The three methine protons 1-H, 4-H and 1′-H were shifted downfield in the ^1H NMR spectrum as expected (see Table III), and long range ^1H-^{13}C correlations were observed between these protons and the new carbonyl carbons. Oligosporol B triacetate (**4**) showed no hemolytic activities and no cytotoxic effects up to 100 μg/ml.

None of the compounds was nematicidal towards *Caenorhabditis elegans* up to 100 μg/ml and they showed no phytotoxic or mutagenic effects. The antibiotics **1–3** were produced in different culture media and under different culture conditions. The induction of trap formation by living nematodes or phenylalanyl-valine [5] had no influence on their production.

Whereas culture extracts of *Dactylaria candida* showed no biological activities, compounds with antimicrobial activities have been reported from cultures of saprophytic *Dactylaria* species. Dactylarin and dactylariol have been isolated from *D. lutea* [12, 13] and the dactylfungins were obtained from *D. parvispora* [14]. In both cases, the producing strains were isolated from soil and have not been shown to be nematode-trapping. Therefore the oligosporols and oligosporon are not only members of a new class of natural products, but may also be considered as the first antimicrobial metabolites from predacious Deuteromycetes.

A number of derivatives of isoepoxydon (**5**) and epoxydon (**6**) have been isolated from natural sources, especially Deuteromycetes [15], however **5** could not be detected in extracts of *A. oligospora*. It is believed that **5** and **6** are biosynthezised *via* polyketides [11]. The C_{15} substituent in the compounds isolated in this investigation obviously has a terpenoid origin, and the combination of these two biogenetic pathways is less common in this class of fungi. To our knowledge 6-farnesyl-5,7-dihydroxy-4-methylphthalide, a biogenetic precursor of mycophenolic acid, is the only example. Among metabolites from Basidiomycetes, grifolic acid and scutigeral from *Albatrellus* species are derived by farnesylation of orsellinic acid (tetraketide) [16]. Several pigments isolated from fruiting bodies for example helveticone from *Chroogomphus helveticus* and the boviquinones from *Suillus bovinus* contain a farnesyl side-chain whereas the other part of the compounds is derived from the shikimate pathway [16]. In panepoxydon and its derivatives [17] isopentyl substituents have been found.

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