J. Antibiot. 61(9): 563–567, 2008

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Sterelactones: New Isolactarane Type Sesquiterpenoids with Antifungal Activity from *Stereum* sp. IBWF 01060

Till Opatz, Heinz Kolshorn, Heidrun Anke

Received: June 24, 2008 / Accepted: August 29, 2008

Abstract Four members of a new family of tetracyclic sesquiterenoids possessing the isolactarane skeleton have been isolated from mycelial cultures of *Stereum* sp. IBWF 01060. Their structure elucidation and their antifungal activity against several plant pathogens as well as other microorganisms are reported.

Keywords sesquiterpenoids, isolactaranes, fungicide, structure elucidation, NMR spectroscopy

Introduction

Extracts from mycelial cultures of the basidiomycete *Stereum* sp. IBWF 01060 exhibit strong activity against several phytopathogenic fungi such as *Magnaporthe grisea* (rice blast fungus), *Fusarium graminearum* (wheat head blight fungus), and *Phytophthora infestans* (potato late blight fungus). The search for the active principle resulted in the isolation of the sterelactones A~D, the structure elucidation and the biological evaluation of which are reported.

The basidiomycete was grown in YMG-medium at ambient temperature until the glucose was consumed (11 days). The mycelia were removed by filtration and the culture filtrate (15 liters) was extracted with EtOAc (2×6.0 liters). Removal of the solvent *in vacuo*, fractionation and purification by silica gel column chromatography followed by separation by preparative reversed phase HPLC furnished four pure single compounds of different polarity but highly similar spectroscopic properties. Sterelactones A (**1**, 6.4 mg), B (**2**, 47 mg), C (**3**, 38 mg), and D (**4**, 15.6 mg) were obtained as yellowish or slightly amber oils. APCI-MS revealed mass differences of 28 units, in each case attributable to two additional methylene groups as judged by NMR and ESI-HRMS. The sterelactones exhibited almost identical UV-spectra with a pronounced absorption maximum at 304 nm ($\log \varepsilon = 3.9 \sim 4.0$) in MeOH. In combination with the consistent IR absorption at 1667 cm^{-1} and a singlet signal in the ^{1}H-NMR spectra at 9.82 ppm, this suggests the presence of a 2,4-dienal moiety, which is further supported by the presence of four olefinic carbon atoms with alternating ^{13}C chemical shifts: α (C-3,

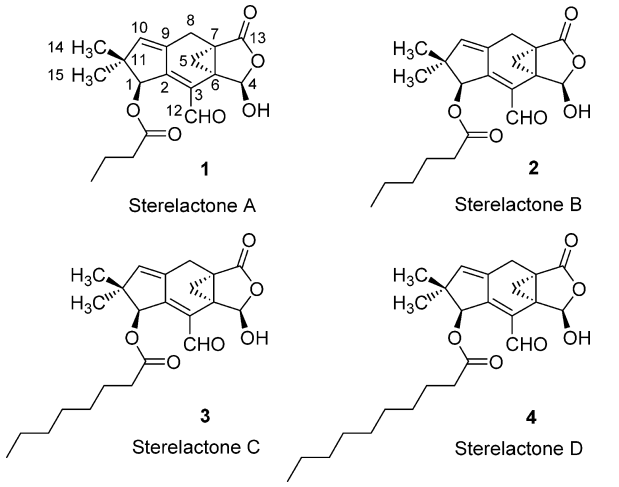

Fig. 1 Structures of the Sterelactones including the atomic numbering scheme.

Till Opatz (Corresponding author)**:** Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany, E-mail: opatz@chemie.uni-hamburg.de

Heinz Kolshorn: Institute of Organic Chemistry, University of Mainz, Duesbergweg 10-14, 55128 Mainz, Germany

Heidrun Anke: Institute of Biotechnology and Drug Research, Erwin-Schrödinger-Str. 56, 67663 Kaiserslautern, Germany

Table 1 ^{1}H Chemical shifts (ppm, $CDCl_3$) of the sterelactones

Atom No.	Sterelactone A	Sterelactone B	Sterelactone C	Sterelactone D
1-H	5.93 (s)	5.93 (s)	5.93 (s)	5.93 (s)
4-H	6.08∼6.20 (br s)[a]	6.10∼6.24 (br s)[a]	6.10∼6.23 (br s)[a]	6.11∼6.26 (br s)[a]
5-H_2	1.57[b]/0.98 (br d, ≈5 Hz)	1.55/0.99 (br d, 4.8 Hz)	1.55/0.99 (br d, 4.8 Hz)	1.55/0.99 (br d, 4.8 Hz)
8-H_2	3.11/2.98 (br d, 18.0 Hz)	3.11/2.98 (br d, 18.0 Hz)	3.11/2.98 (br d, 18.0 Hz)	3.11/2.97 (br d, 18.0 Hz)
10-H	6.08∼6.20 (br s)[a]	6.10∼6.24 (br s)[a]	6.10∼6.23 (br s)[a]	6.11∼6.26 (br s)[a]
12-H	9.83 (s)	9.82 (s)	9.82 (s)	9.82 (s)
14-H_3	1.05 (s)	1.04 (s)	1.04 (s)	1.04 (s)
15-H_3	1.21 (s)	1.21 (s)	1.21 (s)	1.21 (s)
Alkyl chain	2.30∼2.42 (m, α-CH_2), 1.68 (sextet, 7.4 Hz, β-CH_2), 0.96 (t, 7.4 Hz, CH_3)	2.31∼2.43 (m, α-CH_2), 1.64 (mc, β-CH_2), 1.25∼1.36 (m, 2×CH_2), 0.89 (t, 6.9 Hz, CH_3)	2.31∼2.43 (m, α-CH_2), 1.63 (mc, β-CH_2), 1.23∼1.34 (m, 4×CH_2), 0.87 (t, 6.9 Hz, CH_3)	2.31∼2.43 (m, α-CH_2), 1.63 (mc, β-CH_2), 1.22∼1.34 (m, 6×CH_2), 0.88 (t, 6.8 Hz, CH_3)

[a] Overlap.
[b] Broad signal, no coupling observable.

Table 2 ^{13}C Chemical shifts (ppm, $CDCl_3$) of the sterelactones

Atom No.	Sterelactone A	Sterelactone B	Sterelactone C	Sterelactone D
C-1	76.1	76.1	76.1	76.1
C-2	157.6[a]	157.6[a]	157.6[a]	157.6[a]
C-3	128.1	128.1	128.1	128.1
C-4	97.6	97.5	97.4	97.4
C-5	27.8[a]	27.9[a]	27.9[a]	27.8[a]
C-6	32.7	32.7	32.7	32.7
C-7	26.5[a]	26.6[a]	26.6[a]	26.6[a]
C-8	20.0	20.0	20.0	20.0
C-9	133.4	133.3	133.3	133.3
C-10	152.1	152.1	152.1	152.1
C-11	49.2	49.2	49.2	49.2
C-12	189.5	189.4	189.4	189.4
C-13	175.0[a]	175.1[a]	175.0[a]	175.1[a]
C-14	21.3	21.3	21.3	21.4
C-15	27.6	27.6	27.6	27.6
Ester-CO	172.9	173.1	173.1	173.1
Alkyl chain	36.0 (α), 18.4 (β), 13.7 (CH_3)	34.1 (α), 24.5, 22.2, 31.2, 13.9 (CH_3)	34.2 (α), 31.6, 29.1, 28.8, 24.9, 22.6, 14.0 (CH_3)	34.2 (α), 31.8, 29.4, 29.2 (2×), 29.1, 24.9, 22.6, 14.1 (CH_3)

[a] Very broad resonance.

128.1 ppm), β (C-2, 157.6 ppm), γ (C-9, 133.4 ppm), and δ (C-10, 152.1 ppm), and an upfield-shifted aldehyde carbon (C-12, 189.4 ppm). For sterelactone B ($C_{21}H_{26}O_6$), the structure of the tetracyclic core could be established to be identical to that of other isolactaranes by two-dimensional NMR spectroscopy (COSY, HSQC, HMBC, NOESY) [1, 2]. The β,γ-bond of the dienal joins a five- and a six-membered ring to a 2,4,5,6-tetrahydro-1*H*-indene substructure. Its five-membered ring carries a geminal dimethyl group and a secondary hydroxyl group, which is esterified with *n*-hexanoic acid. Sterelactone A is a *n*-butyrate while the less polar sterelactones C and D are

esters of *n*-octanoic and *n*-decanoic acid, respectively (Fig. 1).

In the NMR spectra, many carbon and proton resonances are strongly broadened, which particularly holds true for the western part of the molecule. Presumably, the dynamics of the 5-hydroxy-dihydrofuran-2-one moiety is responsible for this effect that has already been reported by Erkel *et al.* for the closely related hyphodontal [3]. As a consequence of this line broadening in both dimensions, several expected HMBC correlations could not be detected. Nevertheless, the oxidation state of carbons 4 and 13, *i.e.* the position of the lactone and the lactol center, could be established by a significant HMBC correlation between the slightly deshielded 8-H_2 methylene proton and C-13 to be identical to the arrangement in hyphodontal. The relative configuration of the sterelactones was deduced from weak NOE contacts between 4-H and 5-H_a as well as between 1-H and 5-H_b (Fig. 2). Thus, the cyclopropane ring is located on the same side as both the carbinol proton at C-1 and the lactol proton. The absolute configuration of the tetracyclic core was tentatively assigned in analogy to the isolactaranes merulidial [4, 5] and hyphodontal.

The sterelactones showed antibacterial, antifungal and cytotoxic activities. The obtained amounts of sterelactone A were not sufficient to allow broad testing—the activities measured were slightly lower than those of the other sterelactones as shown in Table 3. The cytotoxic activity of all compounds was moderate with compound **1** showing the lowest activity. The antifungal activities were more pronounced than the antibacterial activities. The yeast *Nematospora coryli* turned out to be the most sensitive organism in the agar diffusion assay. The length of the side chain seems to play an important role, the optimal length being eight carbon atoms, *i.e.* sterelactone C was the most active compound. In addition to the activities given in Table 3, sterelactone D exhibited weak nematicidal activity

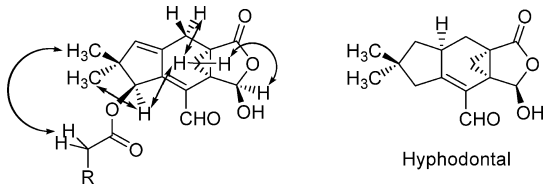

Fig. 2 Important NOE contacts in the sterelactone skeleton and the structure of hyphodontal.

Table 3 Antimicrobial activities of sterelactones A~D as measured in a germination assay using fungal conidia, in the disk diffusion assay with 50 μg per filter disk (6 mm diameter) and cytotoxic activity in a cell proliferation assay

Biological activity	Sterelactone A	Sterelactone B	Sterelactone C	Sterelactone D
Conidia germination IC_{100} (μg/ml)				
Magnaporthe grisea	5.0	5.0	1.0	1.0
Fusarium graminearum	2.5	1.0	1.0	1.0
Botrytis cinerea	nt*	>50	>50	>50
Phytophthora infestans	5.0	2.5	1.0	1.0
Penicillium notatum	nt	50	10	10
Paecilomyces variotii	nt	>50	20	50
Mucor miehei	nt	50	20	50
Agar diffusion assay Inhibition zone with 50 μg/disk (mm)				
Bacillus brevis	nt	14	17	16
Bacillus subtilis	nt	12	17	15
Micrococcus luteus	nt	—	7	7
Staphylococcus aureus	0	0	7	7
E. coli K12	nt	>50	>50	>50
Enterobacter dissolvens	nt	>50	>50	>50
Proteus vulgaris	13	16	18	15
Nematospora coryli	10	28	34	14
Candida albicans	0	11	24	17
Cytotoxic activity IC_{50} (μg/ml)				
HeLa S3 cells	20	10	10	10
Hep G2 cells	20	10	10	10

* nt=not tested.

towards *Caenorhabditis elegans,* at 100 µg/ml more than 50% of the nematodes were dead. Phytotoxic activity towards *Setarica italica* and *Lepidium sativum* were not observed up to 300 µg/ml. The pronounced antimicrobial activity exhibited by the sterelactones presumably originates from their masked dialdehyde structure and the unsaturated aldehyde moiety, which makes them potential crosslinking agents by means of direct and/or vinylogous addition.

Experimental

Producing Organism

Strain IBWF 01060 was obtained from the spore print of a fruiting body of a *Stereum* species growing on a dead woody twig of *Spartium junceum* in France. The strain is maintained on YMG agar (4.0 g yeast extract (Hartge Ingriedients, Hamburg), 10 g malt extract (Difco Laboratories, Detroit), 4.0 g glucose and 15 g agar (Difco Agar, Granulated, Becton Dickinson and Co., Sparks, USA) in 1.0 liter distilled water). It is deposited in the strain collection of the IBWF (Institute of Biotechnology and Drug Research, Kaiserslautern).

Fermentation and Isolation

The producing organism was cultivated in YMG medium containing per liter H_2O: yeast extract 4.0 g, malt extract 10 g, glucose 4.0 g in 1.0 liter tap water. Fermentations were carried out in a 20-liter fermentor (Biostat A-20, Braun, Melsungen) at 22°C, with aeration 3.0 liters/minute and agitation 120 rpm. A well-grown shake culture (250 ml, 9 days old) in the same medium was used as inoculum. After 11 days when the glucose was used up (measured with the Diabur-test 5000®, Roche Diagnostics, Mannheim), the culture fluid was separated from the mycelia by filtration. The culture fluid (15 liters) was extracted with EtOAc (2×6.0 liters) and the organic phase concentrated *in vacuo*. The crude extract (1.8 g) was applied onto a silica gel column (Merck 60, 0.063~0.2 mm, size 4.5×24 cm). Elution with cyclohexane/EtOAc 4 : 1~7 : 3 resulted in an intermediate product (482 mg) which was further purified by preparative HPLC (Merck Lichrosorb® RP18, 5.0 µm, column 25×250 mm, flow 20 ml/minute; gradient H_2O/MeCN 20 to 100% MeCN in 20 minutes) yielded sterelactones B (47 mg, eluted with 87% MeCN), C (38 mg, eluted with 97% MeCN) and D (15.6 mg, eluted with 100% MeCN). Along with these pure compounds, an intermediate product (85 mg, eluted with 78% MeCN) was obtained, from which sterelactone A (6.4 mg) could be isolated after a second preparative HPLC run (Merck Lichrosorb® RP18, 5.0 µm, column 25×250 mm, flow 20 ml/minute; isocratic MeCN/H_2O 50 : 50) The isolation of the antifungal compounds was guided by the antifungal activity in the agar diffusion assay with *N. coryli* as test organism.

Biological Assays

Hela S3 (ATCC CCL 2.2 human cervix carcinoma) and Hep G2 (DSMZ ACC 180, human liver carcinoma) cells were grown in DMEM-medium with 65 µg/ml of penicillin G and 100 µg/ml of streptomycin sulfate. The cells (10^5 cells/ml) were incubated in microtiter plates with the compounds at 37°C in a humidified atmosphere containing 5.0% CO_2. Viable cells were counted under the microscope after 24 and 48 hours [6]. The antifungal activity was measured in a spore germination assay with conidia of phytopathogenic as well as saprophytic fungi. The test was carried out in 96 well microtiterplates holding 200 µl per well. Condidia (5×10^4 conidia/ml) were suspended in distilled water (*M. grisea*), or in water with 0.4% glucose (*F. graminearum*) or YMG medium for the other strains. The compounds were added dissolved in MeOH. The maximum concentration of MeOH in the assay was 1.0%. The plates were incubated at 27°C. After 18 hours the test was evaluated under the microscope. Germination of the conidia was compared to the controls containing only MeOH. In each well 100 condidia were counted. The IC_{100} value are given as the concentrations at which the germination of the conidia was completely inhibited. The tests were carried out in triplicates.

The antimicrobial spectrum of the compounds was evaluated using the conventional agar diffusion assay. Yeasts were assayed in YMG agar and bacteria in nutrient broth (Difco Laboratories, Detroit). Nematicidal activity towards *Meloidogyne incognita* and *C. elegans* was determined as described earlier [7].

Spectroscopy

^{1}H-NMR (400 MHz) and ^{13}C-NMR (100.6 MHz) were recorded at 25°C with a Bruker Avance-II spectrometer equipped with an inverse multinuclear 5 mm probehead and a z-gradient coil. The spectra were measured in $CDCl_3$ and the chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H=7.26, δ_C=77.0). Standard pulse sequences for gs-COSY, gs-HSQC, gs-HMBC, and gs-NOESY experiments were used. The refocussing delays for the inverse heterocorrelation experiments were set to 3.45 and 62.5 ms, corresponding to $^1J_{C,H}$=145 Hz and $^nJ_{C,H}$=8 Hz, respectively. The mixing time for the gs-NOESY was 1 second. Processing of the data was performed with the Mestre-C Software (Mestrelab Research). APCI-MS

spectra were measured from a solution of the analyte in MeCN/H_2O with a Hewlett Packard MSD 1100 using an evaporator temperature of 400°C, a drying gas temperature of 350°C at a flow of 6.0 liters/hour (N_2). In positive ionization mode, the capillary voltage amounted to 3.5 kV, the corona discharge current was 4.0 μA. In negative ionization mode, the capillary voltage amounted to 2.2 kV, the corona discharge current was 6.0 μA. ESI-HRMS data were measured from a solution of the analyte in MeCN with a Waters Q-TOF-Ultima 3 equipped with a LockSpray interface (tri-*n*-octylamine as external reference). IR and UV spectra were measured with a Bruker IFS48 FTIR spectrometer and a Perkin-Elmer Lambda-16 spectrophotometer, respectively. The optical rotations were measured with a Perkin-Elmer 241 polarimeter at 25°C.

Sterelactone A (**1**)

The title compound was obtained as a slightly amber oil. $[\alpha]_D^{25} = -19.4$ (*c* 0.37, $CHCl_3$). IR ν_{max} (KBr) cm^{-1} 3437 (br), 2931, 2865, 1735 (sh), 1667, 1455, 1402, 1368, 1159, 1096, 1053, 701. UV λ_{max}^{MeOH} nm (log ε) 304 (3.96). APCI-MS (negative) *m/z* (%) 345.1 (100) $[M-H]^-$, 257.0 (21), 213.0 (20). APCI-MS (positive) *m/z* (%) 259.0 (100) $[M-C_3H_7CO_2]^+$, 241.0 (54) $[M-C_3H_{17}CO_2-H_2O]^+$, 213.1 (16). ESI-HRMS calcd. for $C_{19}H_{22}O_6Na$ $[M+Na]^+$ 369.1314, found 369.1314.

Sterelactone B (**2**)

The title compound was obtained as a yellowish oil. $[\alpha]_D^{25} = -20.5$ (*c* 0.48, $CHCl_3$). IR ν_{max} (KBr) cm^{-1} 3432 (br), 2961, 2933, 2872, 1774, 1739, 1667, 1467, 1403, 1366, 1165, 1095, 1052, 947, 883, 803, 763, 735. UV λ_{max}^{MeOH} nm (log ε) 304 (3.93). Nontrivial NOESY contacts: 1-H∼15-H_3, 5-H_b; 5-H_b∼8-H_b; 4-H∼5-H_a; CH_2CO∼14-H_3. APCI-MS (negative) *m/z* (%) 373.2 (100) $[M-H]^-$. APCI-MS (positive) *m/z* (%) 259.1 (100) $[M-C_5H_{11}CO_2]^+$, 241.0 (57) $[M-C_5H_{11}CO_2-H_2O]^+$, 213.1 (16). ESI-HRMS calcd. for $C_{21}H_{26}O_6Na$ $[M+Na]^+$ 397.1627, found 397.1616.

Sterelactone C (**3**)

The title compound was obtained as a slightly amber oil. $[\alpha]_D^{25} = -23.8$ (*c* 0.45, $CHCl_3$). IR ν_{max} (KBr) cm^{-1} 3441 (br), 2959, 2931, 2859, 1775, 1740, 1668, 1467, 1402, 1365, 1258, 1203, 1158, 1097, 1052, 947, 882, 805, 762, 736. UV λ_{max}^{MeOH} nm (log ε) 304 (3.89). Typical HMBC-contacts for the sterelactones (sorted by decreasing intensity): 12-H: C-6, C-3; 10-H: C-11, C-1, C-2, C-9, C-8; 1-H: C-9, C-15, Ester-CO, C-10, C-3, C-2, C-11, C-6; 8-H_a: C-7, C-9, C-5, C-10, C-13, C-2; 8-H_b: C-9, C-7, C-6, C-5, C-2, C-10; α-CH_2: CO, β-C, γ-C; β-CH_2: γ-C, α-C, Ester-CO; 5-H_a: C-13, C-4, C-6, C-7, C-3, C-8; 15-H_3: C-1, C-11 C-14, C-10; 14-H_3: C-15, C-11, C-1, C-10; 5-H_b: C-8, C-13, C-3, C-7, C-4, C-6. APCI-MS (negative) *m/z* (%) 401.2 (100) $[M-H]^-$. APCI-MS (positive) *m/z* (%) 403.2 (7) $[M+H]^+$, 259.0 (100) $[M-C_7H_{15}CO_2]^+$, 241.1 (57) $[M-C_7H_{15}CO_2-H_2O]^+$, 213.1 (12). ESI-HRMS calcd. for $C_{23}H_{30}O_6Na$ $[M+Na]^+$ 425.1940, found 425.1942.

Sterelactone D (**4**)

The title compound was obtained as a slightly amber oil. $[\alpha]_D^{25} = -21.0$ (*c* 0.52, $CHCl_3$). IR ν_{max} (KBr) cm^{-1} 3435 (br), 2967, 2936, 2875, 1771, 1739, 1667, 1609, 1463, 1403, 1384, 1367, 1254, 1173, 1095, 1053, 946, 882, 802, 762, 735. UV λ_{max}^{MeOH} nm (log ε) 304 (3.97). APCI-MS (negative) *m/z* (%) 429.2 (100) $[M-H]^-$. APCI-MS (positive) *m/z* (%) 431.2 (9) $[M+H]^+$, 259.0 (100) $[M-C_9H_{19}CO_2]^+$, 241.0 (53) $[M-C_9H_{19}CO_2-H_2O]^+$, 213.1 (12). ESI-HRMS calcd. for $C_{25}H_{34}O_6Na$ $[M+Na]^+$ 453.2253, found 453.2250.

Acknowledgments We thank the Kompetenzzentrum der integrierten Naturstoff-Forschung at the University of Mainz. We are grateful to Prof. Dr. T. Anke, Kaiserslautern, for providing the mycelial culture of strain IBWF 01060. The expert technical assistance of K. Gries and A. Meffert is gratefully acknowledged.

References

1. Daniewski WM, Kocor M, Thoren S. Constituents of higher fungi. Part VIII. Isolactarorufin, a novel tetracyclic sesquiterpene lactone from *Lactarius rufus*. Heterocycles 5: 77–84 (1976)
2. Konitz A, Bogucka-Ledochowska M, Dauter Z, Hempel A, Borowski E. The structure of isolactarorufin. Tetrahedron Lett 38: 3401–3402 (1977)
3. Erkel G, Anke T, Velten R, Gimenez A, Steglich W. Hyphodontal, a new antifungal inhibitor of reverse transcriptases from *Hyphodontia* sp. (Corticiaceae, Basidiomycetes). Z Naturforsch C Biosciences 49: 561–570 (1994)
4. Quack W, Anke T, Oberwinkler F, Giannetti BM, Steglich W. Antibiotics from basidiomycetes. V. Merulidial, a new antibiotic from the basidiomycete *Merulius tremellosus* Fr. J Antibiot 31: 737–741 (1978)
5. Giannetti BM, Steffan B, Steglich W, Quack W, Anke T. Antibiotics from basidiomycetes. Part 23. Merulidial, an isolactarane derivative from *Merulius tremellosus*. Tetrahedron 42: 3579–3586 (1986)
6. Zapf S, Hossfeld M, Anke H, Velten R, Steglich W. Darlucins A and B, new isocyanide antibiotics from *Sphaerellopsis filum* (Darluca filum). J Antibiot 48: 36–41 (1995)
7. Schwarz M, Koepcke B, Weber RWS, Sterner O, Anke H. 3-Hydroxypropionic acid as a nematicidal principle in endophytic fungi. Phytochemistry 65: 2239–2245 (2004)