Scopularides A and B, Cyclodepsipeptides from a Marine Sponge-Derived Fungus, *Scopulariopsis brevicaulis*

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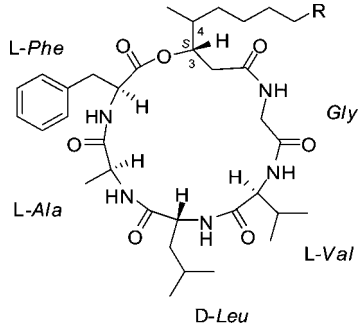
Two novel cyclodepsipeptides, scopularides A and B, were found in the fungus *Scopulariopsis brevicaulis*, which was isolated from the marine sponge *Tethya aurantium*. In addition, the known fungal metabolite paxilline was identified. The structures of the scopularides were elucidated by NMR, MS, and chemical derivatization methods as *cyclo*-(3-hydroxy-4-methyldecanoyl−Gly−L-Val−D-Leu−L-Ala−L-Phe) and *cyclo*-(3-hydroxy-4-methyloctanoyl−Gly−L-Val−D-Leu−L-Ala−L-Phe) for scopularide A and B, respectively. Antibiotic activity against Gram-negative bacteria was absent and against Gram-positive bacteria was weak, but activity against several tumor cell lines was significant at 10 μg/mL.

Within our program aimed at the isolation of biologically active natural products from marine-derived microorganisms, we have investigated the fungus *Scopulariopsis brevicaulis* isolated from the marine sponge *Tethya aurantium*. It is notable that despite the wide distribution of *Scopulariopsis* spp., including the marine habitat,[1] and their significance as human pathogens,[2] there is only limited knowledge about natural products from this genus. The only reported natural product from this genus is a pyranol derivative patented for its antifungal activity.[3] Another aspect of *Scopulariopsis* secondary metabolism is the ability to form volatile methylarsines and -stibines from inorganic arsenic and antimony salts, respectively.[4–6]



1: R = C_2H_5
2: R = H

Scopularides A (**1**) and B (**2**) were isolated from a mycelium extract of the fungus by preparative HPLC as white microcrystalline powders along with the known tremorgenic alkaloid paxilline.[7] HR-FTICRMS of **1** displayed a pseudomolecular ion $[M + H]^+$ at *m/z* 672.4331 corresponding to a molecular formula of $C_{36}H_{57}N_5O_7$ (calcd for $C_{36}H_{58}N_5O_7$ 672.4336). This was consistent with 34 signals observed in the ^{13}C NMR spectrum, taking into account the symmetry of one phenyl residue in the molecule. The ^1H NMR spectra showed features characteristic of a peptidic structure, particularly the signals of the α-protons with chemical shifts between δ 3.50 and 4.80. By detailed evaluation of COSY, HSQC, and HMBC spectra (Table 1) five different amino acid residues were identified: glycine, with characteristic signals of a diastereotopic methylene group at δ 3.53 and 4.24, phenylalanine, alanine, valine, and leucine. These substructures left 11 carbon signals unaccounted for, one carbonyl carbon (δ 174.5), two methyl carbons (δ 14.41 and 14.42), six methylene carbons (δ 40.9, 33.6, 33.0, 30.6, 28.2, and 23.7), and two methine carbons, one of them oxygenated (δ 39.0 and 78.6). The 2D NMR data revealed that these carbons formed a 3-hydroxy-4-methyldecanoic acid (HMDA) group. This result was derived particularly from the ^1H spin system [H-2 ↔ H-3 ↔ H-4 ↔ H-11] extracted from the $^1H-^1H$ COSY along with the long-range H,C-correlations of H-2 to C-1, C-3, and C-4 as well as of the methyl group H-11 to C-3, C-4, and C-5.

The sequence of amino acids and HMDA in **1** was partially determined by long-range H,C-correlations from amino acid α-protons to the carbonyl carbon of the preceding residue. The correlations [Gly-2 → HMDA-1] and [Val-2 → Gly-1] revealed the substructure [HMDA−Gly−Val], while the correlation [Ala-2 → Leu-1] showed the presence of a leucyl−alanine substructure. Furthermore, the molecular formula required the peptide to be cyclic. The cyclic nature was also corroborated by the low-field chemical shift of the proton in the β-position of the HMDA residue (HMDA-3: δ 4.77), suggesting that the oxygen bound to C-3 was part of an ester group. This left two possibilities for the overall sequence of the peptide: *cyclo*-(HMDA−Gly−Val−Leu−Ala−Phe) or *cyclo*-(HMDA−Gly−Val−Phe−Leu−Ala). These two possible structures could not be distinguished solely using the NMR data. On fragmenting the $[M + H]^+$ pseudomolecular ion at *m/z* 672.4 in an MS/MS experiment, fragment ions corresponding to neutral losses of [Phe], [Ala−Phe], and [Leu−Ala−Phe] were observed. Since an [Ala−Phe] substructure is present only in *cyclo*-(HMDA−Gly−Val−Leu−Ala−Phe), this was concluded to be the correct structure of scopularide (**1**).

The configurations of the amino acid residues were established by acid hydrolysis of the peptide, derivatization with N^{α}-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA),[8] and HPLC analysis of the derivatives. Comparison with amino acid standards derivatized with L- and D-FDVA showed the valine, alanine, and phenylalanine residues to have the L-configuration, while the leucine was D-configured.

The configuration of the stereocenter at C-3 of scopularide A (**1**) of the HMDA unit was elucidated by the modified Mosher's method.[9] By hydrolysis with 1 M ethanolic KOH a ring-opened intermediate was obtained, of which one-half each was derivatized with (*R*)-MTPA-Cl (1-methoxy-1-phenyl-1-trifluoromethylacetic acid chloride) and (*S*)-MTPA-Cl, respectively. Comparison of the determined ^1H NMR shifts showed the stereocenter at C-3 to be *S*-configured.

From its HRMS spectrum the molecular formula of scopularide B (**2**) was determined to be $C_{34}H_{53}N_5O_7$. The similarity of the NMR

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Table 1. NMR Spectroscopic Data (600 MHz, MeOH-d_4) for Scopularide A (**1**)

position		δ_C, mult.	δ_H (*J* in Hz)	COSY	HMBC
Phe	1	173.6, qC			
	2	55.3, CH	4.79, m	3a, 3b	1, 3
	3	39.4, CH_2	3.18, dd (13.5, 7.5)	2, 3b	1, 2, 4, 5/9
			3.03, dd (13.5, 8.2)	2, 3a	1, 2, 4, 5/9
	4	137.8, qC			
	5/9	130.3, CH	7.24, m	6/8	3, 7, 5/9
	6/8	129.6, CH	7.28, m	5/9	4, 6/8
	7	128.0, CH	7.21, m		5/9
Ala	1	174.1, qC			
	2	50.3, CH	4.29, q (7.3)	3	1, 3, Leu-1
	3	17.6, CH_3	1.27, d (7.3)	2	1, 2
Leu	1	175.1, qC			
	2	54.7, CH	4.19, m	3	1, 3, 4
	3	40.3, CH_2	1.58, m	2, 4	1, 2, 4
	4	26.0, CH	1.68, m	3, 5, 6	2, 3, 5, 6
	5	23.1, CH_3	1.00, d (6.6)	4	3, 4, 6
	6	22.3, CH_3	0.95, d (6.6)	4	3, 4, 5
Val	1	173.4, qC			
	2	59.9, CH	4.20, m	3	1, 3, 4, Gly-1
	3	31.0, CH	2.19, m	2, 4, 5	1, 2, 4, 5
	4	19.0, CH_3	0.98, d (6.7)	3	2, 3, 5
	5	19.7, CH_3	0.97, d (6.7)	3	2, 3, 4
Gly	1	171.9, qC			
	2	44.0, CH_2	4.24, d (16.8)		1, HMDA-1
			3.53, d (16.8)		1, HMDA-1
HMDA[a]	1	174.5, qC			
	2	40.9, CH_2	2.42, m	3	1, 3, 4
	3	78.6, CH	4.77, m	2, 4	2, 4, 4-Me
	4	39.0, CH	1.54, m	3, 4-Me	
	5	33.6, CH_2	1.22, m		
			0.95, m		
	6	30.6, CH_2	1.30, m		
	7	28.2, CH_2	1.29, m		
	8	33.0, CH_2	1.26, m		
	9	23.7, CH_2	1.31, m	10	
	10	14.42[b], CH_3	0.91, t (7.2)	9	9, 8
	4-Me	14.41[b], CH_3	0.83, d (6.9)	4	3, 4, 5

[a] HMDA: 3-hydroxy-4-methyldecanoic acid. [b] Assignment may be interchanged.

spectra of **1** and **2** suggested that **2** was a homologue with two methylene groups less than **1**. More detailed analysis of the NMR data showed that the amino acid residues are identical in both peptides; however, **2** had a 3-hydroxy-4-methyloctanoic acid unit in place of the HMDA residue in **1**.

The scopularides do not belong to any existing group of natural cyclodepsipeptides. However, 3-hydroxy-4-methylalkanoic acids are a structural element also found in the beauveriolides, fungal cyclodepsipeptides with antiatherogenic activity.[10,11] For beauveriolide III the 3-hydroxy-4-methyloctanoic acid residue has been shown to possess the (3*S*,4*S*)-configuration.[12]

Scopularides A and B showed similar inhibitory effects. Both did not inhibit Gram-negative bacteria and showed weak inhibition of Gram-positive bacteria. They significantly inhibited growth of several tumor cell lines. At a final concentration of 10 μg/mL the viability of the cell lines Colo357, Panc89 (pancreatic tumor cells), and HT29 (colon tumor cells) was reduced by 36% (A) and 26% (B), 42% (A) and 49% (B), and 37% (A) and 24% (B), respectively.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. NMR spectra were recorded on a 600 MHz spectrometer (Bruker AV600). For calibration of ^{13}C and ^1H NMR chemical shifts the carbon signals and the residual proton signals of the solvent were used (MeOD: δ_H 3.31 and δ_C 49.0). HPLC analyses were performed using a C18 column (Phenomenex Luna C18(2), 4.6 × 250 mm, 5 μm) applying a H_2O/MeCN gradient with 0.1% (v/v) HCOOH added to both solvents. For MS detection the HPLC was coupled to an ESI-ion trap system (Esquire 4000, Bruker). High-resolution mass spectra were acquired on a FT-ICR spectrometer (7 T Apex-Qe, Bruker) using positive electrospray ionization.

Fungus. The fungus was isolated from the inner tissue of a marine sponge, *Tethya aurantium*, collected in the Limski Fjord, Croatia, in August 2006. Scanning electron microscopy of the fungus showed single conidiophores, arising from hyphae below the agar surface, unbranched or once to twice verticillately branched. Conidiogenous cells were cylindrical, sometimes with a swollen base, and with an equally wide annellated zone of variable length. Conidia were globose to ovoid (4−6 × 6−7 μm), with a truncate base; their surface was coarsely verrucose (for scanning electron micrographs see Supporting Information). By these morphological characteristics the fungus was identified as *Scopulariopsis brevicaulis*. Sequence analysis of the 18S rDNA revealed the next relative to be *S. brevicaulis* strain NCPF 2177 (GenBank/EMBL acc. no. AY083220) with a similarity of 99.5%. A voucher specimen (LF580) has been deposited at the Zentrum für Marine Wirkstoffe. For chemical investigations the fungus was cultivated in liquid saline (3% NaCl) Wickerham medium[13] (6 × 700 mL) at 28 °C for 14 days in static cultures.

Extraction and Isolation. The mycelia were separated from the culture medium, macerated, and extracted with acetone (800 mL). The acetone extract was dried and the powdery residue washed with MeOH/H_2O (2:8) and *n*-hexane (100 mL each). The remaining extract (300 mg) was subjected to preparative HPLC (column: Phenomenex Luna C18, 21.2 × 250 mm, 5 μm; H_2O + 0.1% HCOOH (A), MeCN + 0.1% HCOOH (B); isocratic 70% B; 18 mL/min). Scopularide B (**2**), paxilline, and scopularide A (**1**) were eluted after 8.0, 12.0, and 13.0 min, respectively. The fractions containing these substances were concentrated to dryness *in vacuo* to give pure paxilline (1.8 mg), **1** (28.6 mg), and **2** (5.9 mg) as white powders.

Scopularide A (1): thin, colorless needles (Me_2CO); mp 229−230 °C; $[\alpha]^{25}_D$ −38 (*c* 0.5, MeOH); for NMR data (^1H, ^{13}C, COSY, and HMBC) see Table 1; ESIMS *m/z* 672.4 $[M + H]^+$; ESIMS/MS (fragmentation of *m/z* 672.4) 654.4 $[M - H_2O + H]^+$, 525.3 $[M -$ Phe $+ H]^+$, 507.3 $[M -$ Phe $- H_2O + H]^+$, 454.3 $[M -$ Phe $-$ Ala $+ H]^+$, 436.3 $[M -$ Phe $-$ Ala $- H_2O + H]^+$, 341.2 $[M -$ Phe $-$ Ala

− Leu + H]$^+$, 323.1 [M − Phe − Ala − Leu − H_2O + H]$^+$; HRESIMS *m/z* 672.4331 [M + H]$^+$ (calcd for $C_{36}H_{58}N_5O_7$ 672.4336).

Scopularide B (2): white, amorphous powder; $[\alpha]^{25}_D$ −43 (*c* 0.5, MeOH); for NMR data (^{1}H, ^{13}C, COSY, and HMBC) see Table S5 in the Supporting Information; HRESIMS *m/z* 644.4007 [M + H]$^+$ (calcd for $C_{34}H_{54}N_5O_7$ 644.4023).

Preparation and Analysis of D- and L-FDVA Derivatives. Scopularide (**1**; 3 mg) was hydrolyzed by heating in HCl (6 M; 1 mL) for 20 h at 110 °C. The solution was then evaporated to dryness and redissolved in H_2O (250 μL). A 1% (w/v) solution (100 μL) of L-FDVA (N^{α}-(2,4-dinitro-5-fluorophenyl)-L-valinamide)[8] in acetone was added to an aliquot (50 μL) of the acid hydrolysate solution (or to 50 μL of a 50 mM solution of the respective amino acid). After addition of $NaHCO_3$ solution (1 M; 20 μL) the mixture was incubated (1 h at 60 °C). The reaction was stopped by addition of HCl (2 M; 10 μL). The reaction mixtures were then diluted with MeOH/H_2O (1:1; 820 μL) and analyzed by HPLC (Phenomenex Luna C18(2) 5 μm, 250 × 4.6 mm; solvents: A H_2O + 0.1% HCOOH, B MeCN + 0.1% HCOOH; linear gradient: 0 min 40% B, 30 min 65% B; 25 °C; 1 mL min^{-1}). The amino acid standards were derivatized with both D- and L-FDVA. Retention times (min) of the amino acid derivatives were as follows: L-FDVA-L-Ala (8.4), D-FDVA-L-Ala (11.5), L-FDVA-L-Phe (14.5), D-FDVA-L-Phe (19.2), L-FDVA-L-Leu (14.0), D-FDVA-L-Leu (20.8), L-FDVA-L-Val (11.0), and D-FDVA-L-Val (16.5).

Derivatization and Analysis of the (*S*)- and (*R*)-Mosher's Ester. Scopularide A (**1**; 16 mg) was hydrolyzed by stirring at room temperature in ethanolic KOH (1 M; 1 mL) overnight. The solution was then neutralized with HCl and directly used to isolate the ring-opened intermediate by preparative HPLC (column: Phenomenex Luna C18, 21.2 × 250 mm, 5 μm; H_2O + 0.1% HCOOH (A), MeCN + 0.1% HCOOH (B); isocratic 70% B; 18 mL/min). Subsequently, the dried ring-opened intermediate of **1** was resolved in 1 mL of dry CH_2Cl_2, and 200 μL of a 0.2 M solution of (*S*)- or (*R*)-MTPA-Cl was added as well as 40 μL of NEt_3.[14] After stirring overnight and addition of 2 mL of water the solutions were extracted with ether. The obtained extracts were then washed with diluted HCl, H_2O, and saturated Na_2CO_3. Finally the organic extracts were dried over $MgSO_4$ and evaporated to dryness.[15] The following NMR analyses were carried out in MeOD-d_4. Observed important ^{1}H chemical shift differences: HMDA H-2 −0.001, Gly H-2 −0.009, HMDA H-4 +0.002, HMDA H-5 +0.001, HMDA H-6 +0.002, HMDA H-7 +0.002, HMDA H-11 +0.001. The configuration of C-3 was determined as described by Kusumi et al.[9]

Biological Activities. Standard procedures used for the determination of antibiotic activities[16] of both scopularide A and B revealed the absence of activity against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas fluorescens* as well as the yeast *Candida glabrata* and no or weak activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus lentus*. Significant activities between 25% and 50% inhibition at 10 μg/mL were found against several tumor cell lines including the pancreatic tumor cell lines Colo357 and Panc89 and the colon tumor cell line HT29.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of scopularides A (**1**) and B (**2**), a table with 1D and 2D NMR data of **2**, and scanning electron micrographs of the fungus are available free of charge via the Internet at http://pubs.acs.org.

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