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Endolides A and B, Vasopressin and Serotonin-Receptor Interacting *N*-Methylated Peptides from the Sponge-Derived Fungus *Stachylidium* sp.

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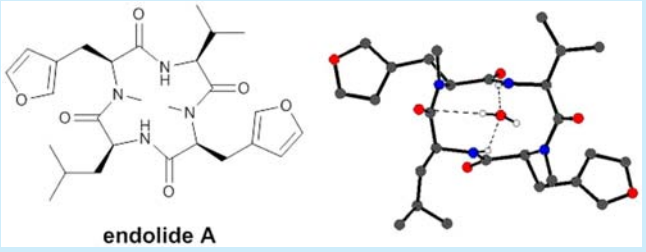
Supporting Information

ABSTRACT: The marine-derived fungus *Stachylidium* sp. was isolated from the sponge *Callyspongia* sp. cf. *C. flammea*. Culture on a biomalt medium supplemented with sea salt led to the isolation of two new, most unusual *N*-methylated peptides, i.e., the tetrapeptides endolide A and B (**1** and **2**). Both of these contain the very rare amino acid 3-(3-furyl)-alanine. In radioligand binding assays endolide A (**1**) showed affinity to the vasopressin receptor 1A with a K_i of 7.04 μM, whereas endolide B (**2**) exhibited no affinity to the latter receptor, but was selective toward the serotonin receptor $5HT_{2b}$ with a K_i of 0.77 μM.



The marine sponge-derived fungus *Stachylidium* sp. produces most unusual, novel peptides, i.e., endolides A and B (**1** and **2**; Figure 1), retrieved from a culture on agar-

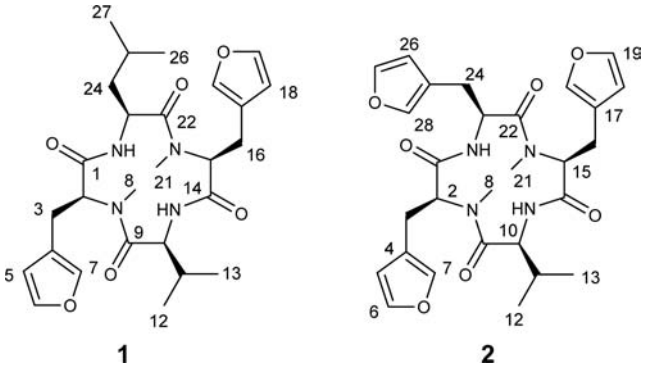


Figure 1. Structures of compounds **1** and **2**.

biomalt media supplemented with sea salt. The striking feature of these peptides is the very rare amino acid 3-(3-furyl)-alanine. The latter residue was only reported before in two heptapeptides isolated from the fungus *Rhizopus microsporus*,[1] and interestingly, these peptides were later found to be produced by symbiotic endobacteria associated with the fungus.[2] The only other report on 3-(3-furyl)-alanine relates to the cytotoxic bingchamides from *Streptomyces bingchenggensis*,[3] indicating a strong bacterial biosynthetic background for the origin of this rare amino acid. Endolide A (**1**) showed affinity to the vasopressin receptor 1A with a K_i of 7.04 μM, whereas the structurally related endolide B (**2**) exhibited affinity toward the serotonin receptor $5HT_{2b}$ with a K_i of 0.77 μM.

The ^{1}H NMR spectrum of **1** showed resonances for four α protons at δ_H 4.29, δ_H 4.35, δ_H 4.37, and δ_H 4.67, characteristic for amino acid residues (Table 1). Additionally, two resonances (δ_H 2.74, 2.78) indicated the presence of two *N*-methyl groups. Two further signals at δ_H 7.81 and δ_H 7.91 were attributed to amide protons (NH). The presence of four carbonyl carbon resonances in the ^{13}C NMR spectrum between δ_C 170.0 and δ_C 173.0 led us to conclude that compound **1** is a peptide, composed of four subunits, two of them methylated at the nitrogen atom.

The molecular formula of **1** was deduced by accurate mass measurement (HREIMS) to be $C_{27}H_{38}N_4O_6$ implying 11 degrees of unsaturation. The ^{13}C NMR spectrum together with a DEPT135 spectrum indeed revealed 27 resonances resulting from six methyl, three methylene, six sp^3 methine, six sp^2 methine groups, and six quaternary carbons. Following the preliminary assignment of the compound as an *N*-methylated tetrapeptide, further 2D NMR spectroscopic data disclosed the structure and sequence of the four amino acid residues of **1** (Table 1). We found in the COSY spectrum a spin system assigned to valine, i.e., correlations ranging from the methyl groups H_3-12 and H_3-13 to NH (Val, δ_H 7.81) through H-11 and αH-10, indicating also that the valine residue was not *N*-

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 1 and 2 in Acetone-d_6 (^{1}H: 300 MHz; ^{13}C: 75 MHz)

		1				2	
amino acid	no.	δ_C, mult.	δ_H (J in Hz)	amino acid	no.	δ_C, mult.	δ_H (J in Hz)
N-Me-	1	170.3, qC		*N*-Me-	1	170.1, qC	
L-Fu Ala (A)	2	62.7, CH	4.29, dd (3.3, 11.7)	L-Fu Ala (A)	2	62.6, CH	4.29, dd (3.3, 11.3)
	3	24.5, CH_2	a: 3.36, dd (3.3, 15.0)		3	24.4, CH_2	a: 3.31, dd (3.3, 15.3)
			b: 2.91, dd (11.7, 15.0)				b: 2.83, dd (11.3, 15.3)
	4	122.1, qC			4	122.0, qC	
	5	111.0, CH	6.37, br s		5	111.0, CH	6.34, br s
	6	144.5, CH	7.53, t (1.6)		6	144.5, CH	7.51, t (1.6)
	7	141.0, CH	7.43, br s		7	141.1, CH	7.41, br s
	8	30.3, CH_3	2.74, s		8	30.3, CH_3	2.72, s
	N				N		
L-Val	9	172.1, qC		L-Val	9	172.1, qC	
	10	56.2, CH	4.35, br t (9.2)		10	56.2, CH	4.36, br t (9.2)
	11	30.2, CH	2.12, m		11	30.1, CH	2.11, m
	12	20.8, CH_3	0.83, d (6.6)		12	20.8, CH_3	0.82, d (6.6)
	13	18.5, CH_3	0.85, d (6.6)		13	18.4, CH_3	0.85, d (6.6)
	NH-10		7.81, d (9.2)		NH-10		7.83, d (9.5)
N-Me-	14	170.7, qC		*N*-Me-	14	170.7, qC	
L-Fu Ala (B)	15	62.9, CH	4.37, dd (3.3, 11.7)	L-Fu Ala (B)	15	62.8, CH	4.37, dd (3.3, 11.3)
	16	24.5, CH_2	a: 3.38, dd (3.3, 15.0)		16	24.7, CH_2	a: 3.37, dd (3.3, 15.3)
			b: 2.94, dd (11.7, 15.0)				b: 2.95, dd (11.3, 15.3)
	17	122.0, qC			17	122.0, qC	
	18	110.9, CH	6.37, br s		18	111.0, CH	6.33, br s
	19	144.4, CH	7.53, t (1.6)		19	144.5, CH	7.46, t (1.6)
	20	141.1, CH	7.43, br s		20	141.0, CH	7.41, br s
	21	30.6, CH_3	2.78, s		21	30.6, CH_3	2.80, s
	N				N		
L-Leu	22	172.9, qC		L-Fu Ala	22	172.5, qC	
	23	49.2, CH	4.67, td (9.2, 5.9)		23	51.3, CH	4.85, dt (9.5, 6.9)
	24	42.0, CH_2	1.30, m		24	28.0, CH_2	a: 2.95, dd (6.9, 14.9)
			1.65, m				b: 2.59, dd (6.9, 14.9)
	25	25.1, CH	1.54, m		25	121.7, qC	
	26	22.5, CH_3	0.87, d (6.6)		26	112.8, CH	6.29, br s
	27	23.3, CH_3	0.83, d (6.6)		27	143.2, CH	7.40, t (1.6)
					28	141.3, CH	7.27, br s
	NH-23		7.91, d (9.2)		NH-23		7.96, d (9.5)

methylated. $^{1}H-^{13}C$ HMBC correlations of H-11 and αH-10 with the carbonyl group qC-9 concluded the assignment of the valine subunit. Another subunit was found to be leucine, based on a spin system observed in the COSY spectrum, ranging from the methyl groups H_3-27 and H_3-26 to NH (Leu, δ_H 7.91) through H-25, H_2-24, and αH-23, indicating also the leucine residue not to be *N*-methylated. HMBC correlations of H_2-24 and αH-23 with the carbonyl group qC-22 finalized this subunit assignment. The aromatic proton and carbon resonances were assigned to two magnetically equivalent furyl moieties (spin systems H-5, H-6, H-7, and H-18, H-19, H-20). From the three sp^2 methine protons of each furyl structure, two of them had downfield shifts caused by a common neighboring oxygen atom, revealing the furyl structure as CH−O−CH−CH, with the terminal sp^2 methines connected to a quaternary carbon, closing thus the furyl ring. In the HMBC spectrum sp^2 methines H-5 and H-7 showed correlations with the quaternary furyl carbon C-4. COSY and HMBC spectra correlated αCH-2 with CH_2-3, the carbonyl carbon qC-1, and the *N*-methyl group CH_3-8. This amino acid subunit was thus assigned as *N*-methyl-3-(3-furyl)-alanine, further on designated *N*-Me Fu Ala (A). In the second overposed aromatic spin system, sp^2 methines H-18 and H-20 had correlations with the quaternary furyl carbon C-17. COSY and HMBC spectra correlated αCH-15 with CH_2-16, carbonyl carbon qC-14, and the *N*-methyl CH_3-21, evidencing a second *N*-Me Fu Ala moiety, here designated *N*-Me Fu Ala (B). The sequence of the four residues in the cyclic structure was established by heteronuclear long-range correlations along the cyclic fragment, i.e., from the α proton of an amino acid to the carbonyl carbon of the neighboring amino acid, namely αH-10 (Val) to qC-14 [*N*-Me Fu Ala (B)], αH-2 [*N*-Me Fu Ala (A)] to qC-9 (Val), αH-23 (Leu) to qC-1 [*N*-Me Fu Ala (A)], and αH-15 [*N*-Me Fu Ala (B)] to qC-22 (Leu). The *N*-methyl substituents also had HMBC correlations with the neighboring carbonyl carbons and α protons, namely NCH_3-21 to both qC-22 (Leu) and αCH-15 [*N*-Me Fu Ala (B)], NCH_3-8 to both qC-9 (Val) and αCH-2 [*N*-Me Fu Ala (A)], hence confirming the sequence of the subunits. Compound **1** was thus the sequencial tetrapeptide cyclo-[*N*-methyl-3-(3-furyl)-alanyl, leucyl, *N*-methyl-3-(3-furyl)-alanyl, valinyl].[4]

The molecular formula of compound **2** was deduced by accurate mass measurement (HRESIMS) to be $C_{28}H_{34}N_4O_7$ implying 14 degrees of unsaturation. The ^{13}C NMR spectrum together with a DEPT135 measurement revealed 28 resonances resulting from four methyl, three methylene, five sp^3 methine,

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and nine sp^2 methine groups as well as seven quaternary carbons. The ^{1}H NMR spectrum displayed four α proton signals at δ_H 4.29, δ_H 4.36, δ_H 4.37, and δ_H 4.85. Two singlets at δ_H 2.72 and δ_H 2.80 again indicated the presence of two *N*-methyl groups, whereas two doublets at δ_H 7.83 and δ_H 7.96 are characteristic for amide protons. The ^{13}C NMR spectrum included four carbonyl signals at δ_C 170.1, δ_C 170.7, δ_C 172.1, and δ_C 172.5 along with four α carbon signals at δ_C 51.3, δ_C 56.2, δ_C 62.6, and δ_C 62.8 all indicating compound **2** to be also a peptide composed of four amino acid residues, two of which are *N*-methylated. Comprehensive analysis of the 2D NMR spectroscopic data revealed the structures of the four amino acid residues of peptide **2** to be Fu Ala, Val and two times *N*-Me Fu Ala. The sequence of the four residues was again established by HMBC correlations from the α proton of an amino acid to the carbonyl carbon of the neighboring amino acid, namely αH-10 (Val) to qC-14 [*N*-Me Fu Ala (B)], αH-15 [*N*-Me Fu Ala (B)] to qC-22 (Fu Ala), αH-23 (Fu Ala) to qC-1 [N-Me Fu Ala (A)], and αH-2 [N-Me Fu Ala (A)] to qC-9 (Val). HMBC correlations of the *N*-methyl protons with the neighboring carbonyl carbons and α protons, namely NCH_3-8 to both qC-9 and αCH-2 and NCH_3-21 to both qC-22 and αCH-15 confirmed the structure of compound **2** to be the cyclic tetrapeptide cyclo-(*N*-methyl-3-(3-furyl)-alanyl, 3-(3-furyl)-alanyl, *N*-methyl-3-(3-furyl)-alanyl, valinyl).[5]

The configurational analysis of the amino acid residues in **1** and **2** was performed by means of the advanced Marfey method. By comparing the retention time of Marfey's reagent-derivatized residues with that of standard amino acids modified in the same way, the absolute configuration was established as L for almost all of the amino acid residues of compounds **1** and **2** (LC-MS data in the Supporting Information). However, the configuration of the 3-(3-furyl) alanine residues could not be assigned using this method, as 3-(3-furyl) alanine degraded during acidic hydrolysis of the peptides, even under mild conditions. As we obtained crystals from compound **1** we were able to determine the relative configuration from X-ray crystallographic data, which showed that all four α protons have the same relative configuration (Figure 2). Considering this together with the results of the Marfey analysis, the absolute configuration of the two *N*-methyl-3-(3-furyl)-alanine residues in **1** also has to be L. Regarding the 3-(3-furyl)-alanine residues in compound **2** we assume them also to have an L configuration due to biosynthetic considerations.

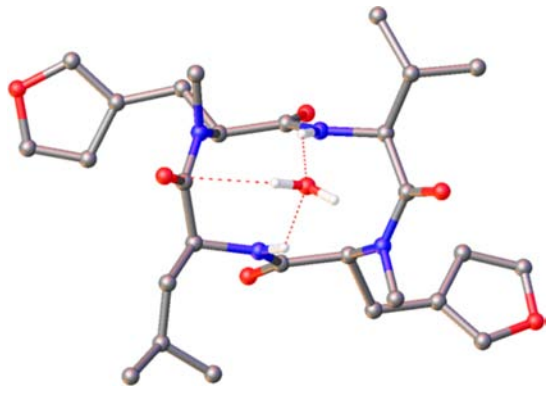

Figure 2. Structure of compound **1** as deduced from X-ray crystallographic data (compound **1** was found to contain a water molecule in the peptidic ring).

Compounds **1** and **2** were assayed in a vast array of bioassays, and most did not show activity (see the Supporting Information).

Most importantly, the compounds were proven to have no *in vitro* cytotoxicity. In radioligand binding assays, however, endolide A (**1**) was found to have affinity to the vasopressin receptor 1A with a K_i of 7.04 μM, whereas endolide B (**2**) showed affinity toward the serotonin receptor $5HT_{2b}$ with a K_i of 0.77 μM. It is remarkable that compound **2** has no affinity toward 10 other serotonin subtype receptors such as $5HT_{1a}$, $5HT_{1b}$, $5HT_{1d}$, $5HT_{1e}$, $5HT_{2a}$, $5HT_{2c}$, $5HT_3$, $5HT_{5a}$, $5HT_6$, and $5HT_7$. $5HT_{2b}$ receptors have been reported to play an important role at cardiac, intestinal, and central levels, as well as in bone marrow formation and growth.[6] Interestingly, selective antagonism of $5HT_{2b}$ was shown to enhance hepatocyte growth in models of acute and chronic liver injury, showing potential application in liver regeneration.[7] As the endolides are noncytotoxic, their receptor interacting potential is of interest for further pharmacological evaluation.

Due to their cyclic nature as well as the presence of *N*-methylated amide bonds, a nonribosomal biosynthesis can be proposed for the endolides. Indeed, it is not unusual for fungi to produce this type of secondary metabolites, and many of them have prominent therapeutic value, e.g. cyclosporine, and enniatins.[8] A characteristic and almost unique chemical feature of the endolides is the presence of a 3-(3-furyl)-alanine moiety, an amino acid hitherto only encountered in two cases in bacteria, first in the strongly hepatotoxic heptapeptide rhizonin originally isolated from the mold fungus *Rhizopus microsporus*.[1] In this case the cytosolic bacterium *Burkholderia endofungorum* was recently shown to produce the rhizonin peptides.[2] The only other cyclic peptide harboring a 3-(3-furyl)-alanine residue is bingchamide from *Streptomyces bingchenggensis*, also showing some weaker cytotoxic activity toward a human colon carcinoma cell line.[3]

Regarding the hypothetical endobacterial biosynthetic origin of the endolides, we were not successful in isolating and culturing axenic bacteria from the fungus, although we were able to amplify 16S rDNA from the fungal metagenome, of likely (as yet) unculturable bacteria. Further studies in this direction are underway.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03553.

^{1}H, ^{13}C, 2D NMR and MS spectra as well as complete spectroscopic data for compounds **1** and **2**; results of Marfey's method; results of biological activities; Experimental section (PDF)

X-ray crystallographic data for **1** (CIF)

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Notes

The authors declare no competing financial interest.

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(4) **Endolide A** (**1**), tetrapeptide (−)-cyclo-[(*N*-methyl-L)-3-(3-furyl)-alanyl), (L)-leucyl, (*N*-methyl-(L)-3-(3-furyl)-alanyl), (L)-valinyl]: white amorphous solid (5 mg/L, 2.33%); $[\alpha]_D^{23}$ −69 (*c* 0.033, acetone); UV (CH_3CN) λ_{max} 202 nm (log ϵ 3.83); IR (ATR) ν_{max} 3330 (br), 2958, 2929, 1660 cm^{-1}; ^1H NMR and ^{13}C NMR (Table 1); LREIMS *m*/*z* 514.3 $[M]^+$; HREIMS *m*/*z* 514.2781 $[M]^+$ (calcd for $C_{27}H_{38}N_4O_6$, 514.2781).

(5) **Endolide B** (**2**), tetrapeptide (−)-cyclo-[(*N*-methyl-(L)-3-(3-furyl)-alanyl), (L)-3-(3-furyl)-alanyl, (*N*-methyl-(L)-3-(3-furyl)-alanyl), (L)-valinyl]: white amorphous solid (1.97 mg/L, 0.94%), $[\alpha]_D^{23}$ −95 (*c* 0.033, acetone); UV (CH_3CN) λ_{max} 202 nm (log ϵ 3.73); IR (ATR) ν_{max} 3345 (br), 2962, 2921, 1704, 1660 cm^{-1}; ^1H NMR and ^{13}C NMR (see Table 1); LRESIMS *m*/*z* 539.3 $[M+H]^+$; HRESIMS calcd *m*/*z* 539.2500 $[M + H]^+$ (calcd for $C_{28}H_{35}N_4O_7$, 539.2492).

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