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Haliclamide, a novel cyclic metabolite from the Vanuatu marine sponge *Haliclona* sp.

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Abstract—A new cyclic metabolite, named haliclamide (**1**), has been isolated from the Vanuatu marine sponge *Haliclona* sp. Its structure was determined from 1D and 2D NMR studies and mass spectral data. Haliclamide (**1**) represents a new 16-membered cyclic depsipeptide containing, along with a *N*-methylphenylalanine, two residues never isolated from natural sources: 5-hydroxy-octanoic acid (HOA) and 6-amino-7-hydroxy-2-methylheptanoic acid (AHMA). Haliclamide (**1**) exhibited cytotoxicity against the NSCLC-N6 human bronchopulmonary non-small-cell-lung carcinoma cell lines with an IC_{50} value of 4.0 μg/mL.

1. Introduction

Sponges of the genus Haliclona are well-known for producing a variety of bioactive natural products such as alkaloids,[1–4] macrolides,[5] polyacetylenes,[6,7] polyketides,[8,9] and steroids.[10,11]

As part of our continuing efforts aimed towards the isolation of biologically active compounds from marine invertebrates, we had the occasion to examine the marine sponge *Haliclona* sp. (Order Haplosclerida, Family Chalinidae) collected in the waters off Vanuatu Islands.

In this paper we report the isolation, structure characterization and biological activity of a novel metabolite, named haliclamide (**1**). Haliclamide exhibited in vitro antitumor activity against human bronchopulmonary non-small-cell-lung-carcinoma lines (NSCLC-N6) with IC_{50} values of 4.0 μg/mL.

2. Results and discussion

A specimen of the *Haliclona* sp. (0.7 kg of lyophilized powder) was sequentially extracted three times with MeOH at room temperature and the methanol soluble material was partitioned according to a modified Kupchan procedure[12] (see Experimental Part) affording four extracts at increasing polarity: *n*-hexane (5.7 g), CCl_4 (13.2 g), $CHCl_3$ (67.5 g), *n*-BuOH (110.0 g). The $CHCl_3$ fraction was selected for the isolation work. This extract was first subjected to medium pressure silica gel flash chromatography (MPLC) eluting with $CHCl_3$-MeOH mixtures with increasing amounts of MeOH. Fractions up to 3% of MeOH in $CHCl_3$ were further purified by reverse phase HPLC on a μ-Bondapack C-18 column with a linear gradient elution, H_2O/CH_3OH, 75:25–0:100 in 30 min., to yield pure haliclamide (**1**, 6.6 mg).

Haliclamide (**1**, Fig. 1) showed a pseudomolecular ion peak at *m/z* 461.3072 [$(M+H)^+$] in the HRFABMS (positive ions) spectrum consistent with a molecular formula $C_{26}H_{41}N_2O_5$ (calculated 461.3015), requiring 8 degrees of formal unsaturation. The ^1H NMR spectrum ($CDCl_3$, 500 MHz), showing an exchangeable NH resonance at 6.82 ppm, a N–Me singlet at 2.83 ppm and several aromatic multiplets, implied that **1** is an *N*-methylated peptide containing both aromatic and aliphatic residues. Analysis of ^{13}C and DEPT-135° NMR spectral data revealed the presence of three methyls, ten methylenes, nine methines (five aromatic), one aromatic quaternary carbon and three

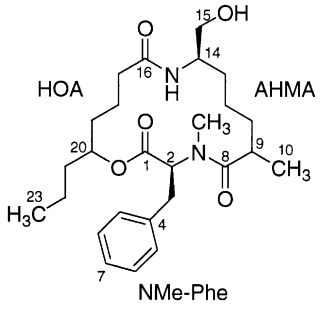

Figure 1. Structure of haliclamide (**1**).

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Table 1. ^{1}H and ^{13}C data of haliclamide (**1**)

Residue	δ_H, mult, *J* in Hz	δ_C	^{1}H–^{13}C HMBC
NMe-Phe			
1		170.7	
2	5.90, dd, 12.4, 5.6	56.2	C3, C8, NMe
3	2.94, dd, 12.4, 14.6; 3.51, dd, 14.6, 5.6	34.1	C2, C5/5', C7
4		136.5	
5/5′	7.27, t, 7.7	128.4	C6/6′
6/6′	7.18, t, 7.7	128.4	C5/5′
7	7.20, t, 7.7	126.7	
NMe	2.83, s	31.2	C1, C2, C8
AHMA			
8		178.2	
9	2.60, m	36.4	C8
10	0.63, d, 6.8	18.0	C9, C11
11	1.20, m, 1.72, m	31.3	C13, C9
12	1.02, m, 1.45, m	23.2	
13	1.25, m, 2.14, m	27.7	
14	3.4, brm	53.6	
15	3.68,brdd, 4.3, 11.6; 3.87, d, 11.6	65.7	C13, C14
NH	3.82, brd, 5.6		C16
HOA			
16		174.5	
17	2.18, m, 2.43, m	36.3	C16, C18, C19
18	1.60, m, 1.77, m	22.9	C20
19	1.45, m, 1.62, m	31.9	C20
20	4.98, m	77.0	C1
21	1.50, m, 1.55, m	37.1	C19, C20, C22, C23
22	1.32, m	18.5	C23
23	0.92, t, 7.3	13.9	C21, C22

carbonyls, that on the basis of molecular formula and their chemical shifts could be assigned to two amides (δ_C 178.2 and 174.5) and one ester carbonyl (δ_C 170.7). Only seven out of eight degrees of formal unsaturation required by the molecular formula were accounted for by functional groups (two amide carbonyls, one ester carbonyl and a monosubstituted phenyl group), suggesting the cyclic nature of **1**. Following the interpretation of DQF-COSY, TOCSY, HMQC and HMBC data (Table 1), we easily identified a spin system for one phenylalanine: δ_H 5.90dd (H-2), 2.94dd (H-3′) and 3.51dd (H-3″). The *N*-methyl signal at δ_H 2.83 (3H, s) was assigned to the latter residue on the basis of intense cross peaks in the HMBC spectrum at δ_H 2.83 (NMe)/δ_C 56.2 (C-2), 170.7 (C-1) and δ_H 5.90 (H-2)/δ_C 31.2 (NMe). Further analysis of the NMR spectra of **1** revealed the presence of two substituted long chain moieties. In particular, sequential COSY correlations were observed between signals at δ_H 3.68, 3.87 (CH_2-15), 3.40 (CH-14), 1.25, 2.14 (CH_2-13), 1.02, 1.45 (CH_2-12), 1.20, 1.72 (CH_2-11), 2.60 (CH-9) and 0.63 (CH_3-10). The connection between the carbonyl carbon at δ_C 178.2 (C-8) and C-9 was inferred by the HMBC correlation between proton at 2.60 ppm (H-9) and δ_C 178.2 (C-8) itself. Therefore, this moiety resulted to be a 6-amino-7-hydroxy-2-methylheptanoic acid (AHMA) residue. Similarly, we identified a third residue in the molecule. The carbonyl at δ_C 174.5 (C-16) was followed by a linear spin system [$(CH_2)_3$-CH(O)-$(CH_2)_2$-CH_3] (DQF-COSY, TOCSY and HMBC; see Table 1). This moiety was assigned to a 5-hydroxy-octanoic acid (HOA) derivative. The latter two residues, to the best of our knowledge, have never been isolated from natural sources.

The residue sequence for **1** was determined by HMBC data. Cross peaks at δ_H 2.83 (NMe-Phe)/δ_C 178.2 (C-8) and δ_H 5.90 (H-2)/δ_C 178.2 (C-8) indicated the amide linkage between NMe-Phe and AHMA residues. At the same time, the connectivity between AHMA and HOA was established by a cross peak between δ_H 6.82 (*NH*-AHMA) and δ_C 174.5 (C-16), indicating an amide linkage as well. Finally, correlation between δ_H 4.98 (H-20) and δ_C 170.7 (C-1) suggested the ester linkage between HOA and NMe-Phe and provided the complete sequence of **1** (Fig. 1).

The absolute stereochemistry of the NMe-Phe residue was determined by HPLC analysis of the acid hydrolisate of **1** after derivatization with the Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, FDAA).[13] HPLC analysis (see Experimental Section) of the FDAA derivatives in comparison with similarly derivatized L- and D-NMe-Phe standards established the L absolute stereochemistry of this residue.

The stereochemistry at C-14 was determined using Yamaguchi's method.[14] The (*R*)- and (*S*)-2-methoxy-2-(trifluoromethyl)-2-phenylacetic (MTPA)[15] esters of **1** were synthesised (see Experimental Section) and a 2:1 mixture of these esters was prepared. The latter solution was then titrated with a solution of the lanthanide shift reagent $Eu(fod)_3$. The observed 'lanthanide induced shift' (LIS) for the OMe group of the MTPA esters was greater for the (*R*)-MTPA derivative, implying an *R* configuration for C-14. The absolute configuration at C-9 and C-20 stereocenters, however, remains unassigned.

Among the wide variety of cyclodepsiptides that have been described to date, haliclamide (**1**) represents a rather new 16-membered cyclic molecule containing, along with a *N*-methylphenylalanine, two novel residues: 5-hydroxy-octanoic acid (HOA) and 6-amino-7-hydroxy-2-methylheptanoic

acid (AHMA). As concerning bioactivity, haliclamide (**1**) exhibited in vitro antitumor activity against human broncho-pulmonary non-small-cell-lung-carcinoma lines (NSCLC-N6) with an IC_{50} value of 4.0 μg/mL.

3. Experimental

3.1. General methods

NMR spectra: Bruker AMX-500 (^1H at 500 MHz, ^{13}C at 125 MHz), δ (ppm), *J* in Hz, spectra referred to $CHCl_3$ (δ_H=7.26) and $^{13}CHCl_3$ (δ_C=77) as internal standards. Standard pulse sequences were employed for DQF-COSY, TOCSY, HMQC, HMBC. HMQC and ^1H–^{13}C HMBC were optimized for $^1J_{C-H}$=135 Hz and $^{2,3}J_{C-H}$=10 Hz, respectively.

HRFABMS [in glycerol; Cs^+ ions bombardment] were obtained with VG AUTOSPEC mass spectrometer; optical rotations were measured with a Perkin-Elmer 141 polarimeter; reverse-phase HPLC, column μBondapack C-18 (300×7.8 mm i.d.; flow rate 5 mL min^{-1}) Waters Model 6000 A or 512 pump equipped with U6K injector and an UV detector.

3.2. Extraction and isolation

The organism (lyophilized material, 700 g) was extracted with MeOH (3×2.5 L) at room temperature. The methanolic extracts were filtered and concentrated under reduced pressure and successively extracted using a modified Kupchan partition[12] as follows: the methanolic extract was dissolved in 1 L of a mixture of MeOH/H_2O containing 10% of H_2O and partitioned against 1 L of *n*-hexane. The water content (% v/v) of the methanolic fraction was adjusted to 20% and 40% and partitioned against 1 L of CCl_4 and 1 L of $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. The crude chloroformic (67.5 g) extract was subjected to MPLC on a silica gel column (150 g) using a solvent gradient from $CHCl_3$ to $CHCl_3$/MeOH 80:20. MPLC fractions were further purified by HPLC on a semi preparative (7.8×300 mm) μBondapack C-18 column eluting with CH_3OH:H_2O mixtures to afford pure compound **1**. The purity of each compound was judged to be greater than 90% by HPLC and ^1H NMR.

3.2.1. Halciclamide (1). White amorphous solid, $[\alpha]_D$= −4.8 (c=0.006 g/mL in $CHCl_3$); HRFABMS: *m/z* 461.3072 ($M+H^+$; calculated for $C_{26}H_{41}N_2O_5$, 461.3015, Δmmu=5.7); t_R=20.07 min.: linear gradient elution, H_2O/CH_3OH, 75:25–0:100 in 30 min. ^1H, ^{13}C data see text and Table 1.

3.3. Hydrolysis and derivatization of (1) (Marfey's procedure)[13]

A 100 μg sample of **1** was dissolved in HCl 6N (0.5mL) under nitrogen atmosphere in a sealed tube at 130 °C for 16 h. After evaporation, the residual hydrolysates was suspended in 100 μl of water and treated with 250 μl of a solution of FDAA (1%) in acetone and 300 μl of a solution 1 M of $NaHCO_3$, and then heated at 50 °C for 1 h. HPLC analysis (Vydac C18, analytical column; linear gradient elution, H_2O (0.1% TFA)/MeCN (0.01% TFA), from 9:1 to 1:1 in 45 min.; UV detection at 340 nm) of FDAA derivative in comparison with similarly derivatized L- and D-NMe-Phe standards established the stereochemistry of the *N*-methylphenylalanine that was found to be L.

3.4. R (+) and S (−) Mosher[15] esters of (1)

1 mg of haliclamide (**1**) was dissolved in 100 μL of dry pyridine. Freshly distilled (+)-2-methoxy-2-(trifluoromethyl)phenylacetic (MTPA) chloride (5 μL) was added to this solution and allowed to stand at room temperature for 12 h under argon atmosphere. The residue obtained after evaporation of the solvent, was subjected to reverse-phase HPLC using a linear gradient from water to MeOH (100%), UV detector 260 nm, to obtain 0.8 mg of (*R*)-(+)-MTPA ester of **1**. The same procedure was used to obtain approximately the same amount of (*S*)-(−)-MTPA ester of **1**. (*S*)-(−)-MTPA ester of **1**: ^1H NMR ($CDCl_3$) δ: 6.76 (NH-AHMA), 4.76, 4.40 (H_2-15), 3.50 (H-14), 2.12, 1.20 (H_2-13), 1.20, 1.31 (H_2-12), 1.68, 1.12 (H_2-11), 2.57 (H-9), 0.64 (CH_3-10).(*R*)-(+)-MTPA ester of **1**: ^1H NMR ($CDCl_3$) δ: 6.71 (NH-AHMA), 4.77, 4.40 (H_2-15), 3.46 (H-14), 2.11, 1.19 (H_2-13), 1.20, 1.31 (H_2-12), 1.68, 1.11 (H_2-11), 2.59 (H-9), 0.67 (CH_3-10).

3.5. Cytotoxic assays

Experiments were performed in 96 well microtiter plates (2×10^5 cells/ml). Cell growth is estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product using live mitochondria.[16] Eight determinations were performed for each concentration. Control growth is estimated for 16 determinations. Optical density at 570 nm corresponding to solubilized formazan is read for each well on Titertek Multiskan MKII.

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