Halipeptins A and B: Two Novel Potent Anti-inflammatory Cyclic Depsipeptides from the Vanuatu Marine Sponge *Haliclona* species

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Received January 2, 2001

Abstract: Two new metabolites, named halipeptins A and B, have been isolated from the marine sponge *Haliclona* sp. Their structures were determined by extensive use of one- and two-dimensional NMR experiments, mass spectrometry, and UV and IR spectroscopy. Halipeptin A is a novel 17-membered cyclic depsipeptide, consisting of five residues including two alanines (with L stereochemistry) and three new residues that appear to be previously undescribed from natural sources: 1,2-oxazetidine-4-methyl-4-carboxylic acid, 3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid (HTMMD), and *N*-methyl-δ-hydroxyisoleucine. The HTMMD residue is substituted with 3-hydroxy-2,2,4-trimethyl-7-hydroxydecanoic acid in halipeptin B. Halipeptin A was found to possess very potent anti-inflammatory activity in vivo, causing about 60% inhibition of edema in mice at the dose of 300 μg/kg (i.p.).

Introduction

Sponges continue to be a rich source of new bioactive natural products. Indeed, some of the most interesting natural products, often characterized by novel molecular architectures, have been isolated from Porifera. Among them, jaspamides,[1] geodiamolides,[2] discodermines,[3] swinholides,[4] spongistatin,[5] callipeltines,[6] and theonellamides[7] represent unique examples in terms of their complex structures, potent bioactivities, and biogenesis. In this paper we report the isolation, structure characterization, and pharmacological activity of two novel metabolites, named halipeptins A (**1**) and B (**2**), from the marine sponge *Haliclona* sp. (order Haplosclerida, family Chalinidae) collected in the waters off the Vanuatu Islands. Halipeptin A showed a very strong anti-inflammatory activity in vivo, causing a 60% reduction of edema in mice at the dose of 300 μg/kg.

Chemically, both halipeptins A and B are mixed-biogenesis metabolites consisting of a peptidic portion connected to a polyketide framework and characterized by the presence of unusual residues along with conventional proteinogenic amino acid units (Figure 1). In particular, the 1,2-oxazetidine-4-methyl-4-carboxylic acid (OMCA) residue appears to be a rather intriguing moiety, being an unprecedented four-membered N−O cyclic form of an α-methyl-isoserine. In addition, both **1** and **2** feature an *N*-methyl-δ-hydroxyisoleucine (NMeOHIle), two L-Ala residues, and a polysubstituted β-hydroxydecanoic acid [3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid (HTMMD) in **1** and 3-hydroxy-2,2,4-trimethyl-7-hydroxydecanoic acid (HTMHD) in **2**, see Figure 1) linked to the N-terminal Ala residue through its carboxyl group on one side and to the C-terminal Ala through its β-hydroxy group on the other side, giving rise to a 17-membered macrolactone ring.

Halipeptin A has raised intriguing speculations on the origin of its potent and, in a way, unexpected anti-inflammatory activity. In fact, compounds displaying anti-inflammatory activity typically belong to very different chemical classes, such as flat aromatic or heteroaromatic molecules, steroids, and terpenoids, with only two recent reports of anti-inflammatory despipeptides, cyclomarins,[8] and salinamides.[9] In addiction, geldanamycin,[10] another mixed biogenesis natural product isolated from a strain of *Streptomyces hygroscopicus* var. geldanus and that has recently attracted the attention of many

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10.1021/ja010015c CCC: $20.00
Published on Web 10/13/2001

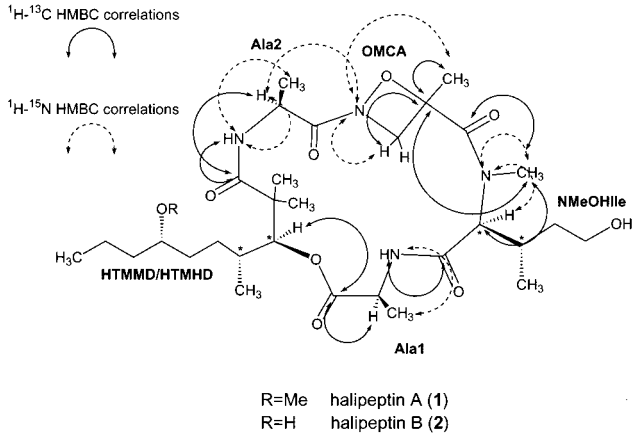

Figure 1. Structures of halipeptines A (**1**) and B (**2**), along with selected ^{1}H−^{13}C and ^{1}H−^{15}N HMBC correlations. Asterisks indicate relative configuration.

researchers for its ability to interfere with heat shock proteins (HSPs), also shows potent anti-inflammatory activity.[11]

Detailed pharmacological investigations on halipeptin A will be needed to determine its biomolecular target and therefore its mechanism of action at the molecular level. However, the occurrence of novel molecules with strong anti-inflammatory properties and peptidic chemical nature, including the present work, seems to indicate that new kinds of interference (at least from a chemical viewpoint) can be exerted in the complex signaling network of inflammation. Furthermore, although the intact halipeptin A appears to be a rather complicated synthetic target, its oligomeric nature may open the possibility to investigate new simplified pharmacophores that, in principle, may prove useful in the identification of new leads for developing a new generation of anti-inflammatory drugs.

Isolation and Structure Elucidation

A specimen of the *Haliclona* sp. (0.7 kg of lyophilized powder) was extracted three times with MeOH at room temperature, and the methanol-soluble material was partitioned according to a modified Kupchan procedure[12] (see Experimental Section), affording four extracts of increasing polarity: *n*-hexane (5.7 g), CCl_4 (13.2 g), $CHCl_3$ (67.5 g), and *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 which contained up to 3% MeOH in $CHCl_3$ were further purified by reverse-phase HPLC on a μ-Bondapack C-18 column eluting with a linear gradient, H_2O/CH_3OH, 75:25−0:100 in 30 min, to yield pure halipeptins A (**1**, 30.9 mg) and B (**2**, 3.9 mg).

Halipeptin A showed a pseudomolecular ion peak at *m*/*z* 627.4073 [$(M + H)^+$] in the HRFABMS (positive ions) spectrum, consistent with a molecular formula $C_{31}H_{54}N_4O_9$ (calculated 627.3969 for $C_{31}H_{55}N_4O_9$), requiring seven degrees of formal unsaturation. The peptidic nature of the compound was suggested by the molecular formula and from the presence of two exchangeable amide NH signals at 7.01 and 7.21 ppm and an *N*-methyl signal at 2.8 ppm in the ^{1}H NMR spectrum ($CDCl_3$, 500 MHz). Moreover, characteristic IR bands at 3421 and 1635 cm^{-1} (*NH*CO and NH*CO* stretching) gave further support to this hypothesis. An additional IR band at 1751 cm^{-1} was indicative of the presence of an ester/lactone function, while a characteristic stretching at 1446 cm^{-1} suggested the presence of a N−O linkage in the molecule.[13] The combined analysis of ^{13}C and DEPT-135° NMR spectra revealed the presence of 10 methyls, 7 methylenes, 7 methines, 2 quaternary carbons, and 5 carbonyls that could be grouped into 4 amide groups (δ_C 169.2, 172.4, 173.3, 177.3) and 1 ester carbonyl (δ_C 169.6) on the basis of the observed NMR connectivities (see below). In-depth analysis of DQF-COSY, TOCSY, HMQC, and HMBC data (see Table 1) allowed us to easily identify spin systems for two alanines [δ_H 7.01d (NH), 4.76 quintet (Hα), 1.40d (Me) and 7.21d (NH), 4.82 quintet (Hα), 1.49d (Me), respectively]. Moreover, a spin system for a δ-hydroxyisoleucine residue was also characterized [δ_H 5.07d (Hα), 2.50m (Hβ), 0.97d (Me-β), 1.28m (Hγ_1), 1.56m (Hγ_2), 3.65m (Hδ_1), 3.77m (Hδ_2)]. An *N*-methyl signal at δ_H 2.8s (3H) was assigned to the latter residue on the basis of an intense cross-peak in the HMBC spectrum (δ_H 2.8 NMe/δ_C 64.6 Cα). Further analysis of the NMR spectra of **1** revealed the presence of a polysubstituted long-chain carboxylic acid moiety. The carbonyl at δ_C 173.3 was followed by a quaternary carbon (δ_C 45.7) substituted with two methyls (δ_H 1.12s/δ_C 26.1 and δ_H 1.19s/δ_C 22.3), as revealed by the key HMBC correlations: δ_H 1.12s (Me′-2)/δ_C 173.3 (C1), 45.7 (C2), 22.3 (Me″-2) and δ_H 1.19s (Me″-2)/δ_C 173.3 (C1), 45.7 (C2), 26.1 (Me′-2). The latter fragment was in turn linked with a well-defined CH(O)−$CH(CH_3)$−$(CH_2)_2$−$CH(OCH_3)$−$(CH_2)_2$−CH_3 spin system (DQF-COSY and TOCSY), as indicated by the HMBC long-range connectivities: δ_H 1.19s (Me″-2)/δ_C 82.5 (C3), δ_H 1.12s (Me′-2)/ δ_C 82.5 (C3). Therefore, this moiety was a 3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid residue that, to the best of our knowledge, has never been described before in natural products. Four (Ala1, Ala2, NMeOHIle, and HTMMD) out of the five constituents comprising halipeptin A were characterized up to this point. Subtraction of the formula that accounts for the sum of all residues so far described ($C_{27}H_{49}N_3O_7$) from the molecular formula of **1** ($C_{31}H_{54}N_4O_9$) gave the formula $C_4H_5NO_2$ for the fifth residue (OMCA). The cyclic nature of this unit was evident from its molecular formula $C_4H_5NO_2$, which requires two degrees of formal unsaturation, of which only one could be accounted for by the presence of an amide carbonyl. ^{1}H and ^{13}C NMR spectra contained signals for an isolated, highly diasterotopic methylene adjacent to a nitrogen (δ_C 44.4/δ_H 3.29d and 4.14d, AB system), a methyl singlet (δ_C 23.1/δ_H 1.46s), an oxygen-bearing quaternary carbon (δ_C 83.8), and an amide carbonyl (δ_C 172.4). These functional groups were interconnected by several key HMBC correlations via $^{2,3}J_{CH}$ (Figure 1 and Table 1), ^{13}C and ^{15}N chemical shift arguments, and signal multiplicity analysis, thus assembling a 1,2-oxazetidine-4-methyl-4-carboxylic structure. The presence of a N−O linkage was inferred from a characteristic IR band at 1446 cm^{-1} (stretchings in this range have already been observed in 1,2-oxazetidine systems[13]). ^{1}H−^{15}N HMBC spectrum analysis immediately revealed a strongly downfield-shifted nitrogen resonating at −89.3 ppm ($Me^{15}NO_2$ as reference). In particular, correlations at δ_H 1.49 (*CH*$_3$−Ala2)/δ_N −262.4 (*N*-Ala2), δ_H 4.82 (*H*α-Ala2)/δ_N −262.4 (*N*-Ala2), δ_H 2.80 (NMe)/δ_N −265.5 (*N*-NMeOHIle), and δ_H 1.40 (*CH*$_3$−Ala1)/δ_N −260.9 (*N*-Ala1) involved regular amide nitrogens (Figure 1), whereas correlations at δ_H 4.82 (*H*α-Ala2)/δ_N −89.3 (*N*-OMCA), δ_H 3.29 (*CH*$_2$−OMCA)/δ_N −89.3 (*N*-OMCA), and δ_H 1.46 (*CH*$_3$−OMCA)/δ_N −89.3 (*N*-OMCA) were indicative of the presence

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Table 1. ^{1}H, ^{13}C, and ^{15}N NMR Data of of Halipeptin A **(1)** ($CDCl_3$, 500 MHz)

residue	δ_H,[a] mult, *J* in Hz	δ_C[a]	δ_N[a]	$^{1}H-^{13}C$ HMBC	$^{1}H-^{15}N$ HMBC	ROESY (t_{max} 400 ms)
				Ala1		
CO		169.6				
α	4.76, quintet, 7.7	49.5		Me*β*, CO,		NH, *β*1-OMCA, NMe-NMeOHIle
β	1.40, d, 7.7	18.0		Cα	N-Ala1	
N	7.01, d, 7.7		−260.9	Cα, CO, CO-HTMMD		Hα, Hα-NMeOHIle
				NMeOHIle		
CO		169.2				
α	5.07, d, 10.5	64.6		CO, Me-*β*, C*β*, NMe, CO-OMCA	N-NMeOHIle	NH-Ala1, NH-Ala2, Me′2-HTMMD, Meα-OMCA
β	2.50, m	28.1		CO, Cα, Me-*β*, C*γ*, C*δ*		Hα
Me-*β*	0.97, d, 6.01	18.4		Cα, C*β*, C*γ*,		
γ	1.28, m; 1.56, m	35.1		Cα, C*β*, Me-*β*, C*δ*		NMe
δ	3.65, m; 3.77, m	60.8		C*β*, C*γ*		
NMe	2.80, s	30.7	−265.5	Cα, CO-OMCA	N-NMeOHIle	Hα-Ala1, Hα, H*γ*, *β*2-OMCA
				OMCA		
CO		172.4				
α		83.8				
Me-α	1.46, s	23.1		C*β*, CO	N-OMCA	*β*1-OMCA, Hα-NMeOHIle
*β*1	3.29, d, 12.0	44.2		Cα, CO-Ala2, Me-α	N-OMCA	Me-α, NH−Ala2, Hα-Ala1
*β*2	4.14, d, 12.0			Cα, CO, Me-α		NMe-NMeOHIle
N			−89.3			
				Ala2		
CO		177.3				
α	4.82, quintet, 7.6	48.5		Me*β*, CO, CO-HTMMD	N-Ala2, N-OMCA	NH, Me*β*, *β*1-OMCA
β	1.49, d, 7.6	22.0		Cα, CO	N-Ala2	
N	7.21, d, 7.6		−262.4	Cα, CO, CO-HTMMD		Hα, Hα-NMeOHIle, *β*1-OMCA
				HTMMD		
1		173.3				
2		45.7				
Me′-2	1.12, s	26.1		Me″-2, C1, C2, C3		H3, Hα-NMeOHIle
Me″-2	1.19, s	22.3		Me′-2, C1, C2, C3		H3, H4
3	4.69, d, 2.5	82.5		C1, C2, Me′-2, C4, Me-4,C5, CO-Ala1		Me′-2, Me″-2, H4
4	1.90, m	34.1		C2, Me-4, C5		H3, Me″-2
Me-4	0.79, d, 6.4	14.2		C3, C4, C5		
5	1.30, m	31.9		C3, C4, C6		
	1.34, m			C3		
6	1.45, m	31.2		C5		
	1.50, m					
7	3.07, quintet, 5.5	80.5		OMe-7, C8		
OMe-7	3.28, s	56.4		C7		
8	1.32, m 1.45, m	35.6		C7, C9 C7, C9		
9	1.30	18.4				
10	0.89, t, 6.0	14.4		C8, C9		

[a] Chemical shift values are referred to $CHCl_3$ ($\delta_H = 7.26$), $^{13}CHCl_3$ ($\delta_C = 77.0$), and $Me^{15}NO_2$ ($\delta_N = 0$) as internal standards.

of an electron-poor nitrogen connected to the C-terminus of the Ala2 residue. This ^{15}N chemical shift value, well away from amide resonances that are typically observed in the range −270 → −260 ppm, lies at the edge of the expected range for an oxygen-bearing sp^2 nitrogen. A meaningful comparison of the latter ^{15}N chemical shift with literature values of suitable reference compounds proved a rather difficult task due to both the unusual structure of the OMCA residue and the scarcity of nitrogen shifts reported for natural and synthetic products. In particular, ^{15}N shifts for hydroxamic acid derivatives obtained from literature data[14] and from a direct measurement of ours[15] were found in the −190 → −170 ppm range, therefore downfield shifted in comparison with amides, yet at higher fields than those found for the OMCA nitrogen. On the other hand, the effect of ring strain on these ^{15}N shifts still remained to be taken into account. In this context, oxaziridine derivatives have been reported to resonate up to −90 ppm,[14] surprisingly downfield shifted in comparison with regular hydroxylamine derivatives. Thus, the unusually low-field chemical shift of OMCA nitrogen[16] could be explained in terms of a similar effect exerted by ring strain on the oxazetidine system. Further support for the presence of this uncommon four-membered ring system came from $^{1}J_{CH}$ measurements at the OMCA methylene obtained

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(15) The derivative we used for ^{15}N chemical shift measurement (by means of a $^{1}H-^{15}N$ HMBC experiment) was *N*-benzoyl-1-oxa-2-aza-bicyclo[2.2.1]hept-4-ene. The measured shift was $\delta_N = -192$ ppm ($CDCl_3$, 500 MHz).

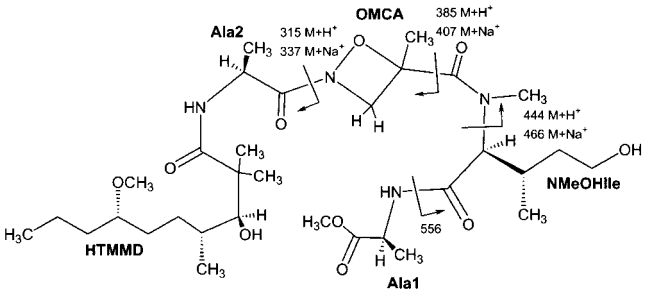

Figure 2. Acyclic methyl ester (**3**) with FABMS fragmentations.

through a {^{1}H,^{13}C} coupled HSQC experiment. Indeed, the large $^1J_{CH}$ values of 147.4 and 149.4 Hz observed for the two OMCA methylene protons are in excellent agreement with couplings reported for these ring systems, taking into account the electronegativity of the substituents.[17]

The plain structure of **1** could be obtained by linking all the residues on the basis of long-range HMBC correlations. Cross-peaks in the HMBC spectrum at δ_H 7.21 (*NH*-Ala2)/ δ_C 173.3 (*CO*-HTMMD), δ_H 4.82 (*H*α-Ala2)/ δ_C 173.3 (*CO*-HTMMD), δ_H 4.69 (*H3*-HTMMD)/ δ_C 169.6 (*C*O-Ala1), and δ_H 7.01 (*NH*-Ala1)/δ_C 169.2 (*C*O-NMeOHIle) (Figure 1) provided the sequence for the fragment Ala2-HTMMD-Ala1-NMeOHIle, where HTMMD and Ala1 were linked to each other through an ester bond. The following HMBC correlations provided the complete sequence of **1**: δ_H 2.8 (NMe)/δ_C 172.4 (*CO*-OMCA), δ_H 2.8 (NMe)/δ_C 83.8 (*C*α-OMCA), δ_H 5.07 (Cα-NMeOHIle)/ δ_C 172.4 (*CO*-OMCA), and δ_H 3.29 (*Hβ1*-OMCA)/δ_C 177.3 (*CO*-Ala2) (Figure 1). The structure of **1** was definitively confirmed by its treatment with sodium methoxide in dry methanol. This reaction furnished the acyclic methyl ester **3** (Figure 2). In addition to the pseudomolecular ions at *m*/*z* 659 [M + H$^+$] and 681 [M + Na$^+$], corresponding to the introduction of 32 mass units in the molecule, the FAB mass spectrum showed several fragment ion peaks, which were consistent with the structure and the sequence of halipeptin A (Figure 2). A similar pattern of ion fragments was also obtained by ESMS/MS experiments.

Along with halipeptin A, a closely related minor metabolite, named halipeptin B, was isolated from the same extract (Figure 1). Halipeptin B showed a pseudomolecular ion peak at *m*/*z* 613 [M + H$^+$] in the FABMS (positive ions) spectrum, 14 mass units less than that for **1**. Structure elucidation of **2** was accomplished in a straightforward manner. The ^{1}H and ^{13}C NMR of **2** showed signals that were almost superimposable with those of **1**, except in the C6−C8 fragment of the β-hydroxydecanoic acid residue (HTMHD). Indeed, the lack of the OMe singlet at δ_H 3.28, the upfield shift of the oxygen-bearing carbon at δ_C 71.9 (δ_C 80.5 in **1**), and the downfield shift of the proton of the same methine at δ_H 3.53 (δ_H 3.07 in **1**) suggested with no ambiguity that halipeptin B is the *O*-demethyl derivative of **1**.

(16) In principle, there could be another structural hypothesis for the OMCA residue which also contains an electron-poor nitrogen and which would be fairly consistent with NMR connectivities. In this alternative hypothesis, the OMCA residue would be a 2-substituted 5-methyl-2-oxazoline-5-carboxylic acid *N*-oxide, with its C-2 being the Ala2 C-terminus and the C-5 carboxyl group linked to the NMe-δOH-Ile N-terminus. This hypothesis was ruled out on the basis of the C-2 chemical shift (expected at higher fields around 120 ppm) and chemical considerations. In fact, halipeptin A did not display any of the chemical properties required for an *N*-oxide. For instance, **1** remained intact after treatement with Zn in 0.2 M HCl at room temperature, whereas the reduction product was readily observed in the same conditions on a commercially available *N*-oxide compound, namely 5,5-dimethyl-1-pyrroline *N*-oxide.

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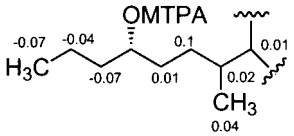

Figure 3. Δδ ($\delta_S - \delta_R$) values in ppm obtained at 500 MHz for MTPA esters of **2**.

As a result, the HTMMD moiety was substituted with a HTMHD in **2**.

Stereochemical Studies on Halipeptins A (1) and B (2)

Studies to determine the stereochemistry of these metabolites commenced with determination of the absolute configuration at C-7 of the HTMHD residue of halipeptin B through the modified Mosher's method.[18] The (*R*)- and (*S*)-2-methoxy-2-(trifluoromethyl)-2-phenylacetic (MTPA) esters of **2** were prepared, and Δδ values ($\delta_S - \delta_R$) were determined at 500 MHz. Positive Δδ values were found for protons on the C3−C6 side, while negative Δδ values were found for protons on the C8−C10 side (Figure 3), which allowed us to assign *S* configuration to C-7 in **2**. On the basis of biogenetic considerations, we propose that **1** possesses the same configuration at the C-7 in HTMMD.

The absolute stereochemistry of the two Ala residues was determined by HPLC analysis of the acid hydrolisate of **1** and **2** after derivatization with the Marfey reagent[19] (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, FDAA). Both alanine residues were found to possess an L absolute configuration at Cα in **1** and **2** (see Experimental Section).

Full stereochemical knowledge of the molecule also required study of the spatial relationships at the NMeOHIle and HTMMD residues. A method of configurational analysis for flexible structures, relying on the use of carbon−proton spin-coupling constants, has recently been proposed by Murata et al. for the stereochemical determination of natural products.[20] In contrast to NOE-based techniques, in which it is possible to observe large NOE contributions from minor conformers, the carbon−proton and the proton−proton coupling constants are detected as weighted averages of the *J* values produced from each conformer. The combined use of the *J* values allows the identification of the predominant rotamer between six main conformers, three for each relative configuration of a two stereogenic carbon systems. Halipeptins A and B are characterized by 17-membered rings, and they show a predominance of staggered conformers for both the HTMMD (HTMHD in **2**) and the NMeOHIle centers which makes them suitable for this kind of analysis. The proton−proton coupling constants were obtained from the ^{1}H NMR spectrum, while accurate measurements of $^{2,3}J_{C,H}$ have been carried out by 2D gradient-enhanced hetero half-filtered TOCSY (HETLOC)[21] and 2D phase-sensitive HMBC (PS-HMBC).[22] Carbons belonging to the HTMMD and HTMHD systems have a pattern of substitution particularly appropriate for this analysis. In particular, C-3 in both the HTMMD and HTMHD fragments bears an alkoxy substituent,

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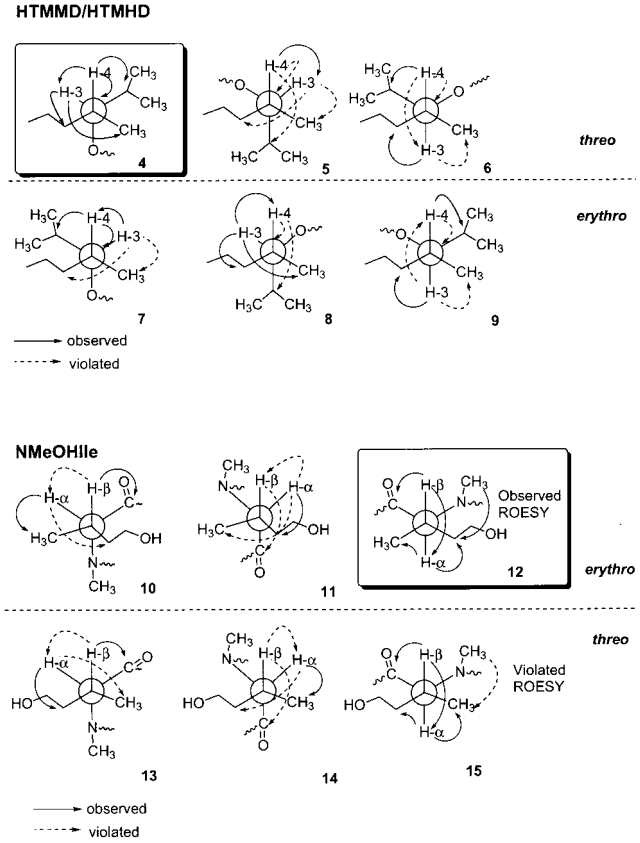

Figure 4. Relative configurations of HTMMD/HTMHD and NMeOHIle residues obtained by the application of the *J*-based Murata methodology (see text). Solid and dashed lines refer to observed and violated homonuclear and heteronuclear $^{2,3}J$ values, respectively. Key ROESY correlations are also reported.

while C-4 has a methyl substitution. As we observed in other cases,[23] the best results for the *J*-based analysis of HTMMD were obtained at low temperature (268 K), where it is very likely to observe a dominant conformer. The H3−H4 coupling constant is (in both **1** and **2**) 2.5 Hz, suggesting their gauche orientation and ruling out the two anti conformers **6** and **9** (see Figure 4). Concerning the proton−carbon coupling constants, H-4 has a ^2J coupling constant with C-3 of −2.2 Hz (small in absolute value), indicating its anti orientation with regard to the oxygen bound to C-3. H-3 has a large ^3J coupling constant with the Me-4, indicating their relative anti orientation. Furthermore, H-4 shows a small ^3J coupling constant with C-2, and H-3 has a small ^3J with C-5, suggesting for both couples of substituents a gauche relationship. ROESY spectra contained very strong cross-peaks between H3 and H5a/H5b and further supported the assignments inferred from *J*-couplings. All these data are in agreement with conformer **4** and are violated, to different extents, for conformers **5**−**9**, establishing for residues HTMMD and HTMHD a *threo* relative stereochemistry. The analysis of the *J* values describing the α and β centers of *N*-methyl-δ-hydroxyisoleucine (NMeOHIle) also indicated a predominant staggered arrangement for the main conformer. The proton−proton and the proton−carbon ^3J values are sufficient to discard conformers **10**, **11**, **13**, and **14** (Figure 4). The observation of a strong ROESY effect between NMe protons at δ_H 2.80 and δ protons at δ_H 1.50 and 1.26 was indicative of an *erythro* relative stereochemistry (conformer **12**) of this residue, though it is noteworthy that both conformers **12** and **15** satisfy the experimental *J* values (but not the ROESY effect). Moreover, the *erythro* stereochemistry found for the NMeOHIle residue is in agreement with that usually observed for the conventional Ile amino acid, giving additional support to our assignment. Presently, we lack any stereochemical information on the OMCA residue.

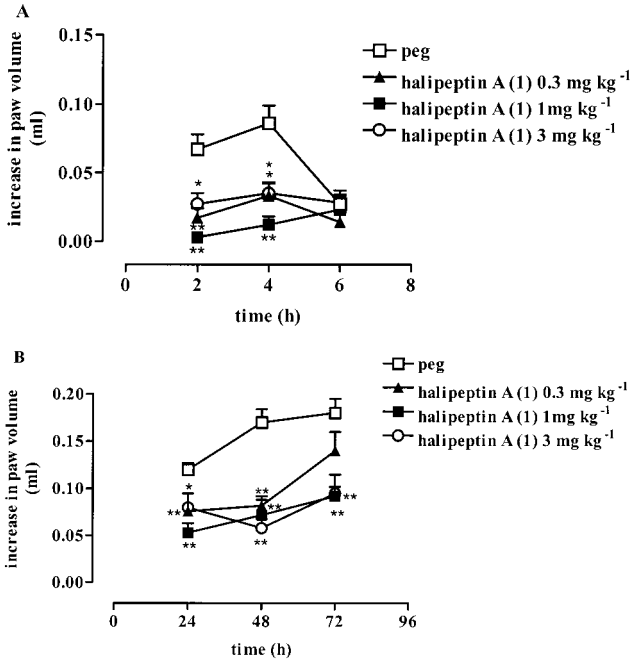

Figure 5. Dose-dependent inhibition on first (A) and second (B) phase of carrageenan-induced paw edema by halipeptin A (**1**).

Pharmacological Activity

Halipeptin A was tested for cytotoxic activity (SK-OV3 and L1210 cell lines) and for antifungal, antiviral (HSV1 and HIV1), and antimicrobial activities with no significant results over a wide range of concentrations. However, it is of particular interest that halipeptin A showed a dose-dependent inhibition of mouse paw edema (Figure 5) and that the inhibitory effect was already very significant at a very low systemic dose, i.e., 300 μg/kg. Indeed, a comparison of this value with those displayed in the same assay by indomethacin and naproxen (ED_{50} of 12 and 40 mg/kg, respectively[24]) indicates that halipeptin A (at least in these experimental conditions) is 40 and 130 times more potent than these standard anti-inflammatory drugs.

Conclusion

Two new metabolites, named halipeptins A (**1**) and B (**2**), have been isolated from a $CHCl_3$ extract of the marine sponge *Haliclona* sp. collected in the waters off the Vanuatu Islands. Halipeptins A and B resulted to be novel 17-membered cyclic depsipeptides, consisting of 5 residues. Halipeptin A contains, along with two common alanines, three new residues: 1,2-oxazetidine-4-methyl-4-carboxylic acid (OMCA), 3-hydroxy-2,2,4-trimethyl-7-methoxydecanoic acid (HTMMD), and *N*-methyl-δ-hydroxyisoleucine (NMeOHIle). The HTMMD residue is substituted with 3-hydroxy-2,2,4-trimethyl-7-hydroxydecanoic acid (HTMHD) in **2**. Above all, our attention focused on the OMCA residue. This was characterized by a particularly unusual ring system. A related, but not identical, system has been

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described only in a series of metabolites isolated from fungal coltures of *Streptomyces* sp. and named neopeptins.[25]

While a more exhaustive pharmacological investigation on halipeptin A is needed to characterize the exact target(s) of the drug, it is clear that halipeptin A displays a potent anti-inflammatory effect in vivo. In addition, even though halipeptin A itself cannot be considered an attractive lead compound for the complexity of its molecular framework, its oligomeric nature may open the possibility to delineate new simplified leads for the design and synthesis of new anti-inflammatory drugs.

Experimental Section

General Experimental Procedures. NMR spectra: Bruker AMX-500 (^{1}H at 500 MHz, ^{13}C at 125 MHz, ^{15}N at 50 MHz), δ (ppm), J in hertz, spectra referred to $CHCl_3$ ($\delta_H = 7.26$), $^{13}CHCl_3$ ($\delta_C = 77$), and $Me^{15}NO_2$ ($\delta_N = 0$) as internal standards. Standard pulse sequences were employed for DQF-COSY, TOCSY, HMQC, HMBC, and ROESY. Phase-sensitive ROESY spectra were measured with a mixing time of 400 ms, while HMQC and ^{1}H−^{13}C HMBC were optimized for $^1J_{C-H} = 135$ Hz and $^{2,3}J_{C-H} = 10$ Hz, respectively. ^{1}H−^{15}N HMBC was optimized for $^1J_{N-H} = 90$ Hz and $^{2,3}J_{N-H} = 10$ Hz.

Gradient-enhanced hetero half-filtered TOCSY (HETLOC)[21] and phase-sensitive gradient-enhanced (PS-HMBC)[22] spectra were recorded on a Bruker DRX600 at 300 and 268 K. $^3J_{H,H}$ values were measured from 1D ^{1}H NMR, while $^{2,3}J_{C,H}$ values were obtained from HETLOC and PS-HMBC. A total of 16 scans per t_1 value were acquired for the HETLOC spectrum, with a spin-lock of 35 ms and a t_{1max} of 50 ms. For the HMBC experiment, 64 scans per t_1 value were acquired, with a t_{1max} of 5 ms.

HRFABMS (in glycerol; Cs^+ ions bombardment) were obtained with a 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 a U6K injector and an UV detector.

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 as follows: the methanolic extract was dissolved in 1 L of a mixture of MeOH/H_2O containing 10% 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 semipreparative (7.8 × 300 mm) μBondapack C-18 column, eluting with CH_3OH:H_2O mixtures to afford pure compounds **1** and **2**. The purity of each compound was judged to be greater than 90% by HPLC and ^{1}H NMR.

Halipeptin A (1): white amorphous solid, $[\alpha]_D = -16.6°$ ($c = 0.029$ g/mL in $CHCl_3$); IR (KBr) ν_{max} 3421, 2931, 1751, 1635, 1616, 1539, 1512, 1446 cm^{-1}; HRFABMS *m*/*z* 627.4073 (M + H$^+$; calculated for $C_{31}H_{55}N_4O_9$, 627.396905, Δmmu = 10.4); $t_R = 22.68$ min, linear gradient elution, H_2O/CH_3OH, 75:25−0:100 in 30 min. For ^{1}H, ^{13}C, and ^{15}N NMR data, see text and Table 1. Heteronuclear coupling constant for HTMMD/HTMHD residues: $^2J_{C3,H4} = -2.2$ Hz, $^3J_{C5,H3} = 4.0$ Hz, $^3J_{Me4,H3} = 4.0$ Hz, $^3J_{C2,H4} < 2.0$ Hz (300 K), $^2J_{C3,H4} = -2.2$ Hz, $^3J_{C5,H3} = 2.8$ Hz, $^3J_{Me4,H3} = 5.5$ Hz, $^3J_{C2,H4} < 2.0$ Hz (268 K). Heteronuclear coupling constant for NMeOHIle residue: $^2J_{C\alpha,H\beta} = 6.2$ Hz, $^3J_{C\gamma,H\alpha} = 0.9$ Hz, $^3J_{Me,\ H\alpha} = 3.2$ Hz, $^3J_{CO,H\beta} = 3.5$ Hz. ROESY data: NMe/δH_2 (strong) for NMeOHIle residue; H3/H5a (very strong) and H3/H5b (very strong), H3-Me-4 (very weak). According to the Murata's method: heteronuclear couplings -3 Hz $< {}^2J < +1$ Hz and 0 Hz $< {}^3J < 3$ Hz have to be considered small and are indicative of anti (^2J) and gauche (^3J) relative orientation, respectively; heteronuclear couplings -7 Hz $< {}^2J < -5$ and 5 Hz $< {}^3J < 8$ Hz have to be considered large and are indicative of gauche (^2J) and anti (^3J) relative orientation, respectively.

Halipeptin B (2): white amorphous solid, $[\alpha]_D = -22.7°$ ($c = 0.002$ g/mL in $CHCl_3$); IR (KBr) ν_{max} 3410, 2940, 1755, 1645, 1440 cm^{-1}; FABMS *m*/*z* 613 (MH$^+$; consistent with a molecular formula $C_{30}H_{52}N_4O_9$); $t_R = 34.7$ min, linear gradient elution, H_2O/CH_3OH, 75:25−0:100 in 30 min.

Hydrolysis and Derivatization of 1 and 2 (Marfey's Procedure).[19] Two 100 μg samples of **1** and **2** were dissolved in 6 N HCl (0.5 mL) under a nitrogen atmosphere in two sealed tubes at 130 °C for 16 h. After evaporation, the two residual hydrolysates were suspended in 100 μL of water, treated with 250 μL of a solution of FDAA (1%) in acetone and 300 μL of a 1 M $NaHCO_3$ solution, 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 derivatives in comparison with similarly derivatized amino acid standards established the stereochemistry of the two alanines that were found to be L. It is noteworthy that we observed a 15% racemization of alanine residues under the experimental conditions typically used for acidic hydrolysis. Therefore, for unambiguous results it is advisable to treat the amino acid standards in the same conditions used for the hydrolysis of the peptide before reaction with FDAA in order to take into appropriate account the extent of racemization produced by the strong acidic conditions.

Methanolysis of Halipeptin A (1). A solution of 1 mg of halipeptin A was treated with 1.2 mg of NaOMe in dry methanol (0.2 mL) at room temperature for 3 h. The reaction mixture was neutralized with 0.1 N HCl, poured into ice/water, and then extracted with *n*-BuOH. The *n*-BuOH phase was evaporated under reduced pressure, and the crude product (0.7 mg) was directly subjected to FABMS analysis.

***R* (+) and *S* (−) Mosher[18] Esters of 2.** One milligram of halipeptin B (**2**) was dissolved in 100 μL of dry pyridine. Freshly distilled (+)-2-methoxy-2-(trifluoromethyl)phenylacetic (MTPA) chloride (5 μL) was added, and this solution was 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 0.6 mg of (*R*)-(+)-MTPA ester of **2**. The same procedure was used to obtain the same amount of (*S*)-(−)-MTPA ester of **2**. In these experimental conditions both the primary alchool function of NMeOHIle and the C-7 secondary alchool function of HTMHD reacted.

Pharmacological Assays. (a) Mouse Paw Edema. Male Swiss mice (8−10 weeks of age) were divided in groups ($n = 7$) and lightly anesthetized with enflurane. Each group of animals received subplantar injection of 50 μL of λ carrageenan 1% w/v in H_2O.[10] Paw volume was measured using a hydroplethismometer specially modified for small volumes (Ugo Basile, Comerio, Italy) immediately before the subplantar injection and 2, 4, 6, 24, 48, and 72 h thereafter. The increase in paw volume was evaluated as the difference between the paw volume at each time point and the basal paw volume. Animals were divided into four groups: the control group was treated with vehicle (PEG), and the other three groups were treated with 0.3, 1, and 3 mg/kg of halipeptin A, respectively. All the administrations were intraperitoneal (i.p.), and the final volume injected was always 100 μL. Poly(ethylene glycol)400 and λ carrageenan were purchased from Sigma Chemical Co. (Milano, Italy).

(b) Statistical Analysis. All data are expressed as mean ± sem and analyzed by using ANOVA for handling multiple data comparison followed by Dunnett's test. Statistical significance is set at $P < 0.05$ ($n = 7-8$).

Acknowledgment. This contribution is part of the Marine Science and Technology (MAST-III) European project named "Bioactive Marine Natural Products in the Field of Antitumoral, Antiviral and Immunomodulant Activity". Therefore we thank the European Community for financial support (contract MAS3-CT95-0032). Additional financial support came from Universi-

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ties of Napoli and Salerno, MURST and CNR (Roma, Italy). NMR and FAB-MS spectra were recorded at C.R.I.A.S. "Centro Interdipartimentale di Analisi Strumentale", Faculty of Pharmacy, University of Naples "Federico II". We thank Dr. Quadrelli (University of Pavia) for providing us with an authentic sample of *N*-benzoyl-1-oxa-2-aza-bicyclo[2.2.1]hept-4-ene for ^{15}N NMR measurements. We also thank the diving team of IRD (ex-ORSTOM) of Nouméa (New Caledonia) for the collection of sponge specimens, Dr. John Hooper (Brisbane, Queensland) for the identification of the sponge, and the Vanuatu government through the Fisheries Department for allowing the collection of biological material. This paper is dedicated to the memory of Prof. Giacomino Randazzo.

Supporting Information Available: FABMS, ^{1}H NMR, ^{13}C NMR, DEPT-135°, DQF-COSY, TOCSY, HMQC, ^{1}H−^{13}C HMBC, ^{1}H−^{15}N HMBC, PS-HMBC, HETLOC, and ROESY spectra for **1**; FABMS, ^{1}H NMR, HMQC, and ^{1}H−^{13}C HMBC spectra for **2**; and FABMS for **3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA010015C