Tamandarins A and B: New Cytotoxic Depsipeptides from a Brazilian Ascidian of the Family Didemnidae

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The structures of two new, naturally occurring cytotoxic depsipeptides, tamandarins A and B (**1** and **2**), are presented. The tamandarins were isolated from an unidentified Brazilian marine ascidian of the family Didemnidae. The structures of the new cytotoxins were assigned by interpretation of FABMS data and by extensive 2D NMR analyses. The absolute configurations of the tamandarins were assigned by acid and alkaline hydrolysis to yield their corresponding amino acids, which were then analyzed as their Marfey derivatives. The cytotoxicity of tamandarin A (**1**) was evaluated against various human cancer cell lines and shown to be slightly more potent than didemnin B. A qualitative discussion of the conformation of tamandarin A (**1**) in solution, obtained from NMR *J*-value data, variable temperature experiments, and NOESY/ROESY data, is included.

Marine invertebrates of the class Ascidiacea (phylum Chordata, subphylum Urochordata) have emerged as a rich source of nitrogen-containing metabolites that often exhibit potent anticancer activities. Studies of the Caribbean mangrove ascidian *Ecteinascidia turbinata*, whose potent anticancer activities were first discovered by Sigel et al.,[1] led to the isolation of ecteinascidin 743, a drug candidate currently in clinical trials. Ecteinascidin 743 showed significant activities against murine (L1210) leukemia in vitro (IC_{50}= 0.5 ng/mL) and in vivo activities against P-388 lymphocytic leukemia and human mammary tumors. Chemical studies of another Caribbean ascidian, *Trididemnum solidum*, led to the discovery of the didemnin depsipeptides, of which didemnin B is the most well-known member.[3,4] Although didemnin B showed significant in vivo activity in mice,[3,5] human clinical testing showed didemnin B to be toxic at doses near those required for therapeutic applications. Ascidians of the genus *Lissoclinum* have also been prolific sources of cytotoxic secondary metabolites such as patellamide A and ulithiacyclamide.[6,7]

As part of our program to explore marine ascidians for their applications in cancer, we encountered an unidentified didemnid[8] ascidian on a shallow-water reef near the Brazilian village of Tamandaré. The crude extract of this animal showed potent growth inhibitory activity in our primary cytotoxicity assay employing the human colon tumor cell line HCT 116. Subsequent bioassay-guided fractionation using LH-20 column chromatography and RP (C_{18}) HPLC yielded two cytotoxic depsipeptides, the major metabolite tamandarin A (**1**) and a minor metabolite, tamandarin B (**2**, Chart 1).

Analysis of spectral data revealed that tamandarins A (**1**) and B (**2**) are closely related to didemnin B and nordidemnin B, respectively. The molecules were found to differ only by the presence of hydroxyisovaleric acid (Hiv^2), instead of the hydroxyisovalerylpropionic acid (Hip^2) unit which is present in all other naturally occurring didemnin congeners. Tamandarin B (**2**) was found to contain a norstatine (Nst^1) residue instead of the isostatine (Ist^1) residue of tamandarin A (**1**). In this paper, we report the isolation and structure elucidation of tamandarins A (**1**) and B (**2**) and provide preliminary pharmacological information on tamandarin A (**1**). A

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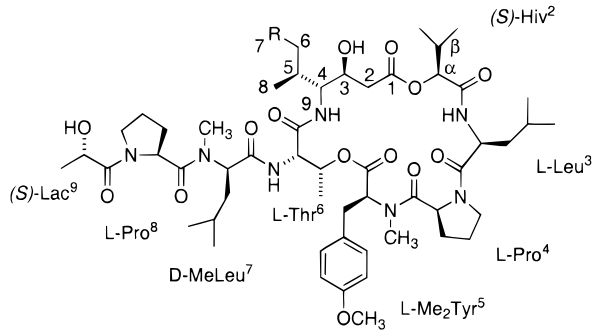
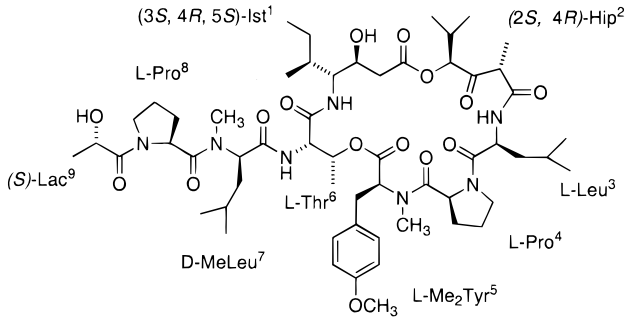
Chart 1



Tamandarin A (1): R = CH_3; (3*S*, 4*R*, 5*S*)-Ist[1]

Tamandarin B (2): R = H; (3*S*, 4*R*)-Nst[1]



Didemnin B

comparison of the conformation of this metabolite in solution versus that of didemnin B is also presented.

Tamandarin A (**1**) was obtained as a greenish white solid. Mass spectral analysis (FABMS) of this metabolite showed a pseudomolecular ion consistent with the molecular formula $C_{54}H_{87}N_7O_{14}$. The ^{1}H NMR spectrum of tamandarin A (**1**, Table 1) showed several characteristic features that strongly resembled those of didemnin B. Three NH protons were visible in the amide hydrogen region (δ 7.3−7.8 ppm), and a *para*-substituted phenyl ring was apparent (δ 7.07, d, 2H, J = 8.4 Hz; δ 6.84, d, 2H, J = 8.7 Hz). However, only 10 well-resolved apparent α-hydrogens were visible (δ 3.8−5.5 ppm), whereas didemnin B possesses 11. Three methyl groups on heteroatoms (δ 3.79, s, 3H, OCH_3; δ 3.11, s, 3H, NCH_3; δ 2.58, s, 3H, NCH_3) were observed, but only 10 additional methyl groups could be accounted for (δ 0.8−1.5 ppm), while didemnin B has 11. These findings indicated that tamandarin A (**1**) is a novel didemnin congener. Tandem mass spectral analysis (MS/MS, Figure 1) was undertaken to establish the relationship between these two molecules. The fragmentation pattern of the parent ion at 1056 amu ($M + H^+$) showed the loss of a lactic acid (Lac[9]) and a proline (Pro[8]) as one unit, 169 (*m*/*z* 887), and loss of 127 (*m*/*z* 760) for an *N*-methylleucine (MeLeu[7]) unit, indicating that tamandarin A (**1**) possesses the same side chain as didemnin B. Hence, the noted modifications must be within the macrocyclic ring. The ^{13}C NMR spectrum of tamandarin A (**1**) showed 54 carbon resonances, including nine carbonyl carbons (δ 168−175 ppm). Unlike didemnin B, tamandarin A did not possess a ketone, the carbonyl shift of the former being observed at approximately δ 200 ppm. Six carbon resonances appeared in the aromatic region, with the phenyl ring carbons overlapping (δ 114−160 ppm). The remaining 39

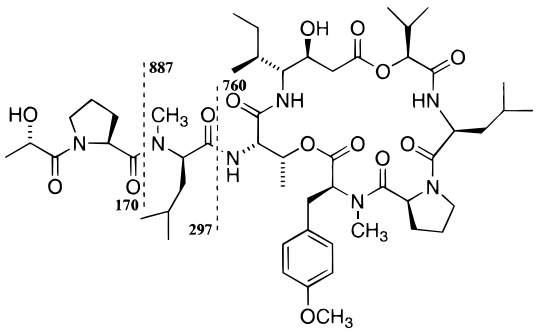


Figure 1. Structure of tamandarin A (**1**) showing fragment ions obtained by MS/MS (*m*/*z* 1057 [M^+ + H]).

carbon resonances were all in the aliphatic region. DEPT and HMQC experiments established the multiplicities of each carbon and all one-bond ^{1}H−^{13}C correlations, in agreement with the molecular formula. The spin systems of Lac[9], Pro[4], and Pro[8] units, a leucine (Leu[3]) and MeLeu[7] unit, a threonine (Thr[6]) unit, and parts of the Ist[1] and *N*,*O*-dimethyltyrosine (Me_2Tyr[5]) were identified by correlations observed in a double-quantum filtered COSY experiment (DQF-COSY). In addition, a Hiv[2] unit was identified, but the propionic acid of Hip[2] of didemnin B was clearly absent in tamandarin A.

As was pointed out by Kessler et al. for didemnin B, several correlations within Ist[1] (C5H to C6H) and Leu[3] and MeLeu[7] (CβH to CγH) do not appear in the COSY spectrum.[9] These correlations were not observed for tamandarin A (**1**), but they were obtained from a TOCSY experiment which also confirmed the proposed units.

A ROESY experiment established the sequence of the amino acids and confirmed the absence of the propionic acid in the Hip[2] unit. An HMBC experiment provided sequence data and allowed all carbonyl functionalities, except for the Leu[3] CO carbon, to be assigned. This experiment also confirmed the ester bonds between the Ist[1] and Hiv[2] units, and between the Thr[6] and Me_2Tyr[5] units. On the basis of these data, tamandarin A (**1**) was assigned with confidence as possessing a hydroxyisovaleric acid (Hiv[2]) unit instead of the hydroxyisovaleryl-propionic acid (Hip[2]) unit found in didemnin B.

Hydrolysis of tamandarin A (**1**) under mild alkaline conditions (1 N NaOH in MeOH, 1 h, rt, Chart 2) yielded the northern and southern peptide fragments **3** and **4**, which were converted to their methyl esters by reaction with diazomethane. In a similar reaction, didemnin B was hydrolyzed to yield the analogous peptide fragments. Comparison of the ^{1}H NMR spectra, optical rotations, and tandem mass spectral results of the tamandarin A peptides with those obtained from didemnin B[10,11] revealed that the northern fragments, **3**, from both peptides are identical ($[\alpha]_D$ = +41°, c = 0.07). The chiralities of the amino acid residues in the northern fragment (**3**) were thus established as (*S*)-Lac[9], L-Pro[8], D-MeLeu[7], L-Thr[6], and (3*S*,4*R*,5*S*)-Ist[1]. The chiralities of the amino acids constituting the southern peptide fragment (**4**) of tamandarin A (**1**) were obtained by acid-catalyzed hydrolysis, preparation of the corresponding Marfey's de-

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Table 1. NMR Assignments for Tamandarin A (1)

structural unit	position	^{13}C ($CDCl_3$) δ (mult)[a]	^{1}H ($CDCl_3$) δ (mult, J, integ)[a]	COSY	gHMBC
(3*S*,4*R*,5*S*)-Ist[1]	1	174.7 (C)			5.03, 3.25, 2.44
	2	39.9 (CH_2)	(a) 2.44 (dd, 17.0, 8.1 Hz, 1H)	3.25, 3.91	4.03
			(b) 3.25 (br d, 17.0 Hz, 1H)	2.44, 3.91	
	3	69.1 (CH)	3.91 (br tr, 9.6, 8.1 Hz, 1H)	2.44, 3.25	2.44,
	3-OH		n.o.[b]		
	4	55.4 (CH)	4.03 (tr d, 9.6, 9.6, 3.4 Hz, 1H)	7.36, 1.96	3.91, 3.25, 1.20
	5	33.8 (CH)	1.96 (m, 1H)	4.03, 0.88	1.40, 1.20
	6	27.6 (CH_2)	1.20 (dd, 13.8, 6.9 Hz, 1H)	1.40, 0.92	
			1.40 (m, 1H)	1.20, 0.92	
	7	12.0 (CH_3)	0.92 (tr, 6.9, 6.9 Hz, 3H)	1.20, 1.40	1.40, 1.20
	8	14.3 (CH_3)	0.88 (d, 6.3 Hz, 3H)	1.96	4.03, 1.40, 1.20
	9		7.36 (br d, 9.6 Hz, 1H)	4.03	
(*S*)-Hiv[2]	CO	169.8 (C)			5.03
	α	79.1 (CH)	5.03 (d, 4.8 Hz, 1H)	2.26	
	β	30.3 (CH)	2.26 (m, 1H)	1.03, 1.04	5.03
	β-CH_3	17.8 (CH_3)	1.04 (d, 6.6 Hz, 3H)	2.26	5.03, 2.26
	β-CH_3	19.1 (CH_3)	1.03 (d, 6.6 Hz, 3H)	2.26	5.03, 2.26
L-Leu[3]	CO	170.6 (C)*			
	α	48.5 (CH)	4.88 (tr d, 9.6, 9.6, 1.6 Hz, 1H)	1.25, 1.50, 7.77	
	β	39.6 (CH_2)	1.25 (m, 1H)	1.50, 4.88	0.95, 0.91
			1.50 (m, 1H)	1.25, 4.88	
	γ	25.0 (CH)	1.55 (m, 1H)	0.95, 0.91	0.95, 0.91
	γ-CH_3	21.1 (CH_3)	0.95 (d, 6.3 Hz, 3H)	1.55	
	γ-CH_3	24.0 (CH_3)	0.91 (d, 6.3 Hz, 3H)	1.55	
	NH		7.77 (br d, 9.6 Hz, 1H)	4.88	
L-Pro[4]	CO	171.3 (C)			2.10, 1.77
	α	57.2 (CH)	4.65 (dd, 7.8, 3.6 Hz, 1H)	1.77, 2.10	
	β	28.2 (CH_2)	1.77 (m, 1H)	2.10, 4.65	4.65, 3.65
			2.10 (m, 1H)	1.77, 4.65	
	γ	25.1 (CH_2)	2.10 (m, 2H)	3.65	4.65, 3.65, 2.10
	δ	46.9 (CH_2)	3.65 (m, 2H)	2.10	1.77
L-Me_2Tyr[5]	CO	168.7 (C)			5.42, 3.59, 3.13
	α	66.4 (CH)	3.59 (dd, 10.8, 4.1 Hz, 1H)	3.13, 3.40	3.40, 3.13
	β	34.1 (CH_2)	3.13 (dd, 14.6, 10.8 Hz, 1H)	3.40, 3.59	7.07, 3.59
			3.40 (dd, 14.6, 4.1 Hz, 1H)	3.13, 3.59	
	γ	130.3 (C)			3.59, 3.40, 3.13
	δ	130.5 (CH)	7.07 (d, 8.6 Hz, 2H)	6.84	3.40, 3.13
	ϵ	114.3 (CH)	6.84 (d, 8.6 Hz, 2H)	7.07	7.07
	ζ	158.8 (C)			7.07, 3.79
	γ-CH_3	55.5 (CH_3)	3.78 (s, 3H)		
	N-CH_3	38.9 (CH_3)	2.58 (s, 3H)		
L-Thr[6]	CO	170.3 (C)			7.36, 4.03
	α	58.1 (CH)	4.26 (dd, 5.4, 1.4 Hz, 1H)	7.47, 5.42	5.42
	β	70.9 (CH)	5.42 (q d, 6.0, 1.4 Hz, 1H)	1.35, 4.26	7.47, 4.26, 1.35
	β-CH_3	16.7 (CH_3)	1.35 (d, 6.0 Hz, 3H)	5.42	5.42, 4.26
	NH		7.46 (br d, 5.4 Hz, 1H)	4.26	
D-MeLeu[7]	CO	170.8 (C)			7.47, 1.88
	α	55.1 (CH)	5.30 (dd, 11.4, 3.6 Hz, 1H)	1.88, 1.66	3.11, 1.66
	β	35.9 (CH_2)	1.88 (m, 1H)	1.66, 1.35, 5.30	5.30
			1.66 (m, 1H)	1.88, 1.35, 5.30	
	γ	25.0 (CH)	1.35 (m, 1H)	0.84, 0.91	5.30
	β-CH_3	23.7 (CH_3)	0.91 (d, 6.9 Hz, 3H)	1.35	
	β-CH_3	21.5 (CH_3)	0.84 (d, 6.9 Hz, 3H)	1.35	1.88, 1.66
	N-CH_3	31.5 (CH_3)	3.11 (s, 3H)		5.30
L-Pro[8]	CO	172.9 (C)			5.30, 3.11
	α	56.9 (CH)	4.72 (br t, 7.5, 7.5 Hz, 1H)	2.22, 1.96	3.59
	β	28.6 (CH_2)	1.96 (m, 1H)	2.22	4.72, 1.96
			2.22 (m, 1H)	1.96	
	γ	26.2 (CH_2)	1.96 (m, 1H)	2.22	3.59
			2.22 (m, 1H)	1.96	
	δ	47.2 (CH_2)	3.65 (m, 1H)	3.65, 2.22, 1.96	2.22
			3.59 (dd, 10.8, 3.9 Hz, 1H)	3.59, 2.22, 1.96	
(*S*)-Lac[9]	CO	174.0 (C)			4.38
	α	66.3 (CH)	4.38 (br q, 6.9, 1H)	1.43	1.43
	α-CH_3	20.5 (CH_3)	1.43 (d, 6.9, 3H)	4.38	4.38
	α-OH		n.o.[b]		

[a] ^{1}H and ^{13}C NMR shifts were referenced to $CDCl_3$ (^{1}H δ 7.27 and ^{13}C δ 77.2 ppm). [b] Not observed.

rivatives, and HPLC analysis using D- and L-amino acid (Marfey's derivatives) standards.[12] All comparable amino acids in this fragment were shown to have absolute configurations identical with those in didemnin B: L-Me_2-Tyr[5], L-Pro[4], and L-Leu[3]. Acid hydrolysis of the southern peptide fragment (**4**) did not cleave the methyl ester link in L-Me_2Tyr. Therefore, a mixture of the Marfey derivatives of D- and L-Me_2Tyr methyl ester (Me_3Tyr) was prepared and used in the HPLC analysis (Chart 2).

The absolute stereochemistry of the Hiv[2] unit could not be obtained from the Marfey derivative HPLC analysis. Instead, the stereochemistry of the Hiv[2] unit was deter-

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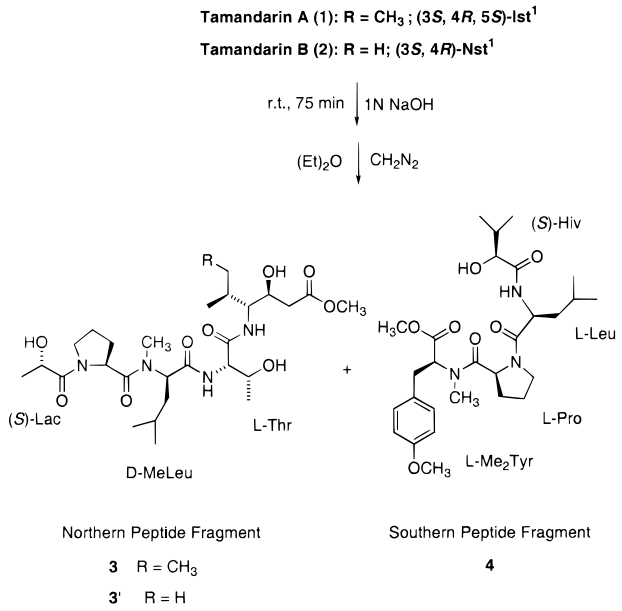
Chart 2



mined by analysis of the ^{1}H NMR spectra of the diastereomeric (*R*)- and (*S*)-Mosher ester derivatives of the southern peptide fragment (**4**).[13] In the (*S*)-MTPA ester, all protons in the Hiv[2] unit were more highly shielded (appeared further upfield), whereas the α-hydrogen and amide hydrogens in the neighboring leucine unit were less highly shielded (and appeared farther downfield). The reverse was true for the (*R*)-MTPA ester of the southern peptide fragment (**4**), permitting the identification of the absolute stereochemistry of the hydroxyisovaleric acid as (*S*)-Hiv[2].

Tamandarin B (**2**), obtained as an amorphous white solid, showed a FABMS pseudomolecular ion consistent with the molecular formula $C_{53}H_{82}N_7O_{14}$. The lower mass of this metabolite, shifted by 14 units as compared with tamandarin A (**1**), suggested that the difference was the absence of a methylene group. The ^{1}H NMR spectrum of tamandarin B (**2**, Table 2) was almost identical to that of tamandarin A (**1**), except for a small downfield shift ($\Delta\delta$ = 0.2 ppm) in one of the hydrogens in the amide region (δ 7.2−7.8 ppm) and slight upfield shifts ($\Delta\delta$ = 0.05 ppm) of two hydrogens in the α-hydrogen region (δ 3.8−5.5 ppm). Perhaps more importantly, the latter protons showed a change in coupling pattern. In addition, a hydrogen at δ 1.20 ppm, showing an obvious multiplet structure in the ^{1}H spectrum of tamandarin A (**1**), was lacking. These observations indicated that tamandarin B (**2**) contains a Nst[1] residue instead of the Ist[1] residue in tamandarin A (**1**).

With some difficulty from overlapping bands, the replacement of the triplet methyl signal of tamandarin A (**1**) at δ 0.92 ppm with a doublet methyl in tamandarin B (**1**) at δ 0.97 ppm was resolved. The ^{13}C NMR spectrum of tamandarin B (**2**) was nearly identical to that of tamandarin A (**1**), except for the lack of one methylene resonance in the aliphatic region. DEPT and HMQC experiments confirmed the multiplicity of each carbon and showed all one-bond ^{1}H−^{13}C correlations in agreement with the molecular formula. COSY and TOCSY experiments established the connectivities within the Nst[1] residue and confirmed the presence of the Lac[9] unit and the same remaining amino and hydroxy acids as in tamandarin A. Determination of the amino acid sequence and assignment of all carbonyl functionalities in tamandarin B (**2**) was accomplished by ROESY and HMBC NMR experiments. Tamandarin B (**2**) was assigned with confidence as differing from tamandarin A (**1**) by the presence of the amino acid Nst[1] instead of the Ist[1] unit of tamandarin A (**1**).

Similar to tamandarin A, hydrolysis of **2** yielded the northern and southern peptide fragments (**3′** and **4**), which were also converted to their methyl esters with diazomethane. Likewise, hydrolysis of nordidemnin B[14] yielded peptide fragments which were compared to those from tamandarin B. The northern peptide fragment (**3′**) of tamandarin B (**2**) ($[\alpha]_D = +24°$, $c = 0.05$) was found to be identical to the northern fragment of nordidemnin B, thus indicating the absolute configurations of the amino acids to be identical: (*S*)-Lac,[9] L-Pro[8], D-MeLeu[7], L-Thr[6], and (3*S*,4*R*)-Nst[1]. The southern peptide fragment (**4**) from tamandarin B (**2**) showed the same ^{1}H NMR spectrum and optical rotation as the southern peptide fragment (**4**) from tamandarin A (**1**), indicating the chiralities of the amino acids to be identical (L-Me_2Tyr[5], L-Pro[4], L-Leu[3], and (*S*)-Hiv[2]).

Given the comparison of tamandarin A (**1**) with didemnin B, studies were undertaken to document the tertiary structure of this molecule. Several independent studies of the conformation of didemnin B showed that it exhibits conformational constancy from crystal to solution structure and in a range of polar and apolar solvents.[4g,9b,11,15] Each study showed the molecule to exist in a globular shape, stabilized by at least three intramolecular hydrogen bonds. The strongest hydrogen bond is a transannular interaction linking Leu[3] CO and Ist[1] NH. This gives the macrocyclic ring the shape of a distorted "figure eight" rather than the flat antiparallel β-sheet-type arrangement more commonly observed in cyclic depsipeptides. The linear portion of didemnin B is involved in a β II-type turn, a structural feature often observed in linear peptides, in which four amino acids are stabilized in a well-defined conformation by hydrogen bonding between amino acids i (H donor) and $i + 3$ (H acceptor). The β turn in didemnin B encompasses residues Thr[6], MeLeu[7], Pro[8], and Lac[9], with hydrogen-bonding interaction consistently found between Thr[6] NH and Lac[9] CO. The third hydrogen bond in didemnin B was identified between MeLeu[7] CO in the linear portion of the molecule and Leu[3] NH in the macrocycle, orienting the folded linear chain back over the macrocyclic ring, resulting in its overall globular shape.

To assess the effects of the ring modifications in tamandarin A, conformational studies were undertaken using J values from 1D ^{1}H or 2D ECOSY NMR spectra, NH chemical shifts and their temperature dependence (Table 4), and NOE/ROE correlations obtained from 2D NOESY and ROESY experiments (Table 5). An effort was

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Table 2. NMR Assignments for Tamandarin B (2)

structural unit	position	^{13}C ($CDCl_3$) δ (mult)[a]	^{1}H ($CDCl_3$) δ (mult, J, integ)[a]	COSY	[g]HMBC
(3*R*,4*S*)-Nst[1]	1	174.2 (C)			3.25, 2.48
	2	39.7 (CH_2)	(a) 2.48 (dd, 17.0, 7.5 Hz, 1H)	3.96, 3.34	
			(b) 3.25 (dd, 17.0, 2.7 Hz, 1H)	3.96, 3.48	
	3	69.2 (CH)	3.96 (m, 1H)	3.86, 3.25, 2.48	2.48
	3-OH		3.34 (m, 1H)	3.96	
	4	58.8 (CH)	3.86 (dd, 8.8, 5.0 Hz, 1H)	7.56, 3.96, 2.13	0.97, 0.91
	5	27.3 (CH)	2.13 (m, 1H)	3.86, 0.97, 0.91	0.97, 0.91
	6	17.3 (CH_3)	0.91 (d, 6.9 Hz, 3H)	2.13	0.97
	7				
	8	20.7 (CH_3)	0.97 (d, 6.9 Hz, 3H)	2.13	0.91
	9		7.56 (br d, 8.8 Hz, 1H)	3.86	
(*S*)-Hiv[2]	CO	169.9 (C)			5.04
	α	78.9 (CH)	5.04 (d, 4.5 Hz, 1H)	2.25	1.04
	β	30.4 (CH)	2.25 (m, 1H)	1.05, 1.04	5.04, 1.05, 1.04
	β-CH_3	17.7 (CH_3)	1.05 (d, 6.9 Hz, 3H)	2.25	1.04
	β-CH_3	19.2 (CH_3)	1.04 (d, 6.9 Hz, 3H)	2.25	1.05
L-Leu[3]	CO	171.3 (C)			
	α	48.5 (CH)	4.89 (tr d, 9.8, 9.8, 1.6 Hz, 1H)	7.78	
	β	39.5 (CH_2)	1.33 (m, 1H)	1.51	0.96, 0.92
			1.51 (m, 1H)	1.33	
	γ	25.1 (CH)	1.59 (m, 1H)	0.96, 0.92	0.96, 0.92
	γ-CH_3	21.7 (CH_3)	0.96 (d, 6.9 Hz, 3H)	1.59	0.92
	γ-CH_3	24.0 (CH_3)	0.92 (d, 6.9 Hz, 3H)	1.59	0.96
	NH		7.78 (br d, 9.9 Hz, 1H)	4.89	
L-Pro[4]	CO	170.9 (C)			3.60, 2.59, 1.84
	α	57.2 (CH)	4.65 (dd, 7.5, 4.1 Hz, 1H)	2.05, 1.84	
	β	28.2 (CH_2)	1.84 (m, 1H)	2.05	4.65
			2.05 (m, 1H)	1.84	
	γ	25.1 (CH_2)	2.05 (m, 2H)	3.67	4.65
	δ	46.9 (CH_2)	3.67 (m, 2H)	2.05	
L-Me_2Tyr[5]	CO	168.8 (C)			5.42, 3.60
	α	66.5 (CH)	3.60 (dd, 10.8, 4.0 Hz, 1H)	3.40, 3.16	3.41, 3.16, 2.59
	β	34.2 (CH_2)	3.16 (dd, 14.1, 10.5 Hz, 1H)	3.40, 3.60	3.60
			3.40 (dd, 14.1, 4.0 Hz, 1H)	3.60, 3.16	
	γ	130.0 (C)			6.85, 3.60, 3.41
					3.16
	δ	130.6 (CH)	7.08 (d, 8.3 Hz, 2H)	6.85	6.85, 3.41, 3.16
	ϵ	114.3 (CH)	6.85 (d, 8.3 Hz, 2H)	7.08	6.85
	ζ	158.8 (C)			7.08, 6.85, 3.80
	γ-CH_3	55.5 (CH_3)	3.80 (s, 3H)		
	N-CH_3	38.9 (CH_3)	2.59 (s, 3H)		3.60
L-Thr[6]	CO	170.8 (C)			7.56
	α	58.0 (CH)	4.31 (dd, 5.6, 1.4 Hz, 1H)	7.49, 5.42	1.38
	β	71.0 (CH)	5.42 (qd, 6.5, 1.4 Hz, 1H)	4.32, 1.38	7.49, 4.31, 1.38
	β-CH_3	16.8 (CH_3)	1.38 (d, 6.5 Hz, 3H)	5.42	
	NH		7.49 (br d, 5.6 Hz, 1H)	4.31	
D-MeLeu[7]	CO	170.7 (C)			7.49
	α	55.1 (CH)	5.31 (dd, 11.4, 3.6 Hz, 1H)	1.88, 1.67	3.11
	β	36.8 (CH_2)	1.88 (m, 1H)	1.67	0.92, 0.85
			1.67 (m, 1H)	1.88	
	γ	25.1 (CH)	1.38 (m, 1H)	1.88, 1.67	0.92, 0.85, 0.92
					0.85
	β-CH_3	23.7 (CH_3)	0.92 (d, 6.9 Hz, 3H)	1.38	0.85
	β-CH_3	21.6 (CH_3)	0.85 (d, 6.9 Hz, 3H)	1.38	1.88, 1.67, 0.92
	N-CH_3	31.4 (CH_3)	3.11 (s, 3H)		5.31
L-Pro[8]	CO	172.9 (C)			5.31, 3.11
	α	57.0 (CH)	4.73 (br t, 7.8, 7.8 Hz, 1H)	2.13, 1.97	
	β	28.6 (CH_2)	1.97 (m, 1H)	2.13	
			2.13 (m, 1H)	1.97	
	γ	26.2 (CH_2)	1.97 (m, 1H)	2.13	4.73
			2.13 (m, 1H)	1.97	
	δ	47.3 (CH_2)	3.67 (m, 1H)	1.97	
			3.60 (dd, 10.8, 4.1 Hz, 1H)	2.13	
(*S*)-Lac[9]	CO	174.1 (C)			1.44
	α	66.3 (CH)	4.39 (br q, 6.9 Hz, 1H)	3.44, 1.44	1.44
	α-CH_3	20.5 (CH_3)	1.44 (d, 6.9 Hz, 3H)	4.39	
	α-OH		3.44 (m, 1H)	4.39	

made to identify the major differences in shape between tamandarin A and didemnin B brought about by the presence of a Hiv[2] group in tamandarin A. All NMR experiments (except the variable temperature experiments) were carried out in $CDCl_3$, in which one set of signals and therefore a single conformer of tamandarin A (**1**) was observed in solution. In this solvent, all ^{1}H and ^{13}C chemical shifts were assigned as described earlier. ^{1}H chemical shift values for tamandarin A (**1**) differed from those of didemnin B in the same solvent by no more than ±0.23 ppm. ^{13}C chemical shifts between the two molecules differed up to 2.7 ppm, except in Ist[1], where C1 appeared at δ 39.9 ppm in tamandarin A (**1**) versus δ 29.7 ppm in didemnin B.

Table 3. IC_{50} Cytotoxicity Values in Clonogenic (Colony-Forming) Assays

	tamandarin A (**1**) (ng/mL)	didemnin B (ng/mL)
pancreatic carcinoma BX-PC3	1.79	2.00
prostatic cancer DU-145	1.36	1.53
head and neck carcinoma UMSCC10b	0.99	1.76

Table 4. Temperature Dependence of NH Protons of Tamandarin A (1)

	Ist^1	Leu^3	Thr^6
$\Delta\delta/\Delta T$	9×10^{-4}	4×10^{-5}	2.8×10^{-3}

Tamandarin A (**1**) appeared rigid, as could be deduced from the vicinal coupling constants $^3J_{NH-C4H}$ in Ist^1 (9.6 Hz), Leu^3 (9.6 Hz), and Thr^6 (5.4 Hz), which indicated a single dominant conformation rather than freely interconverting residues for which average J values of 6−8 Hz would be seen. On the basis of these coupling constants, trans conformations were assigned to the NH−C4H and NH−CαH bonds in Ist^1 and Leu^3, respectively, versus a gauche conformation for Thr^6. Additional vicinal coupling constants for Thr^6, $^3J_{C\alpha H-C\beta H} = 1.4$ Hz and $^3J_{C\beta H-\beta-CH_3} = 6.0$ Hz, and ROE enhancements between Thr^6 NH and Thr^6 CβH and β-CH$_3$ determined that the conformation of this unit is practically identical to that of Thr^6 in didemnin B.[11] The diastereotopic protons Ist^1 C2H$_2$ were assigned on the basis of vicinal coupling constants $^3J_{C_2H_a-C_3H} = 8.1$ Hz and $^3J_{C_2H_b-C_3H} = \sim 0$ Hz, and their values together with the chemical shift difference between them is another indication of the rigidity of the molecule. For the Ist^1 unit, where one could expect significant conformational changes, additional coupling constants of $^3J_{C_2H_a-C_2H_b} = 17.0$ Hz, $^3J_{C_3H-C_4H} = 9.6$ Hz, $^3J_{C_4H-C_5H} = 3.4$ Hz, $^3J_{C_6H-C_7H} = 6.9$ Hz, and $^3J_{C_5H-C_8H} = 6.3$ Hz were observed. These values, together with NOE enhancements between Ist^1 NH and Ist^1 C8H$_3$ and C3H, and between Ist^1 C4H and Ist^1 C7H$_3$ and C5H, determined the conformation to be practically identical to that of Ist^1 in didemnin B as well.[11]

All remaining peptide bonds in tamandarin A (**1**) were assigned trans conformations, as in didemnin B, on the basis of NOE/ROE enhancements between NH or NCH$_3$ and the α protons of the preceding amino acids. In the case of Pro^4 and Pro^8, lacking such correlations, trans peptide bonds were assigned on the basis of NOE correlations between Pro^4 CβH$_2$ and Leu^3 CαH and between Pro^8 CβH$_2$ and Lac^9 CαH. A trans conformation was assigned with confidence to the bond Hiv^2−Leu^3, on the basis of NOE correlations between Hiv^2 CαH and Leu^3 NH. Based upon an NOE/ROE correlation between the MeLeu^7 NCH$_3$ and Thr^6 NH, a similar conclusion was reached for the MeLeu^7−Thr^6 peptide bond, where NOE/ROE correlations between MeLeu^7 NCH$_3$ and Thr^6 CαH were lacking.

The temperature dependence of amide proton chemical shifts is generally considered to be well correlated with their inaccessibility to solvent and hence their potential to be involved in intramolecular hydrogen bonds. Many studies on amide proton temperature dependence in a variety of solvents have led to the view that $\Delta\delta/\Delta T$ coefficients higher than 5×10^{-3} ppm/K in polar solvents indicate amide protons that are solvated, as in the case of linear extended peptide conformations. Conversely, a coefficient of 4.0×10^{-3} ppm/K is considered the upper limit for internal protons that are inaccessible to solvent and thus might be involved in hydrogen bonds.[16]

The temperature coefficients (Table 4) for the amide proton chemical shifts of tamandarin A (**1**) were determined in DMSO-d_6. In this solvent, several conformers of **1** can be observed. The major conformer of **1** was assumed to be conformationally constant in solvents ranging from $CDCl_3$ to DMSO-d_6, analogous to what has been found for didemnin B.[11] On this assumption, the amide proton chemical shifts of the major conformer were assigned on the basis of their coupling constants. Temperature dependence was measured over the range 298−388 K. The Ist^1 and Leu^3 NHs were found to be practically temperature-independent, with very small $\Delta\delta/\Delta T$ coefficients of 9×10^{-4} and 4×10^{-5} ppm/K, respectively. The Thr^6 NH showed temperature dependence with the larger $\Delta\delta/\Delta T$ coefficient of 2.8×10^{-3} ppm/K. All these coefficients, however, are less than 4.0×10^{-3} ppm/K; hence, the amide protons of tamandarin A (**1**) exist in inaccessible environments and could be involved in hydrogen bonds. Since only a limited number of hydrogen bonds can be formed in the molecule, these results pointed toward a hydrogen bond situation very similar to that of didemnin B. A weak ROE correlation between Leu^3 NH and Ist^1 C2H$_b$, together with the small temperature coefficient for Ist^1, points to the involvement of Leu^3 in a transannular hydrogen-bonding interaction with Ist^1. Additional ROE correlations between Ist^1 C6H$_3$ and Me$_2$-Tyr^5 NCH$_3$, and between Ist^1 C6H$_3$ and Pro^4 CαH, are consistent with such a hydrogen bridge across the cyclic peptide ring. Transannular ROE correlations between Thr^6 CαH and Leu^3 CβH$_a$, and between Hiv^2 CγH$_3$ and MeLeu^7 CβH$_a$, confirmed the internal orientation of the Leu^3 NH. These ROE correlations, consistent with the solvent inaccessibility of the Leu^3 NH, suggest orientation of part of the linear portion of the molecule back over the cyclic backbone and possible stabilization by hydrogen bonding. Strong interresidue ROE and NOE correlations were observed in the linear portion of the molecule, particularly between Pro^8 CαH and Thr^6 NH, MeLeu^7 NCH$_3$ and Thr^6 NH, and MeLeu^7 NCH$_3$ and Pro^8 CαH. These correlations are indicative of a β II turn in the linear side chain of the molecule, stabilized by a hydrogen bond between Thr^6 NH and Lac^9 CO. The corner positions of the turn are occupied by Pro^8 in the ($i + 1$) position and MeLeu^7 in the ($i + 2$) position, bringing Pro^8 CO and MeLeu^7 CαH coplanar, as can been concluded from the low-field chemical shift, δ 5.30 ppm, of the latter.

The conformations of the side-chains in the amino acids of tamandarin A (**1**) were analyzed by means of vicinal coupling constants $^3J_{C\alpha H-C\beta H}$ and analysis of their corresponding dihedral angles χ_1. As was described earlier, based on coupling constants, the conformations of Ist^1 and Thr^6 were found identical to these units in didemnin B, which in both the crystal and solution structures adopt dihedral angles χ_1 of ~60° and ~155°, respectively. Hiv^2, in which one might expect the greatest changes to appear, showed a vicinal coupling constant $^3J_{C\alpha H-C\beta H}$ of 4.8 Hz (versus ~3.5 Hz in didemnin B), which indicated a gauche conformation about the Cα−Cβ bond. Although the sign of χ_1 could not be unambiguously determined, the identi-

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Table 5. Observed NOE/ROE Correlations for Tamandarin A (1)

Ist[1]	Hiv[2]	Leu[3]	Pro[4]	Me_2Tyr^5	Thr[6]	MeLeu[7]	Pro[8]	Lac[9]
NH - - C8H_3					NH -------	-------	---- CαH	
NH - - C3H		CαH ----	---- CβH_2				CβH_2 -----	----- CαH
C4H - - C7H_3	CαH ----	---- NH				NCH_3 ----	---- CαH	
C4H - - C5H			CαH ------	------ NCH_3	NH -----	----- NCH_3		
C2H_b -------	-------	---- NH		CαH -------	------- NH			
					NH - - CβH			
					NH - - β-CH_3			
		NH -------	-------	-------	------- CαH			
		CβH_a -------	-------	-------	------- CαH			
NH -------	-------	-------	-------	-------	------- CαH			
C6H_3 -------	-------	-------	-------	------- NCH_3				
C6H_3 -------	-------	-------	------- CαH					
	CγH_3 -------	-------	-------	-------	-------	------- CβH_3		

cal chemical shifts of the *pro-R* and *pro-S* methyl groups, and thus their equal proximity to the neighboring carbonyl, seem to favor $\chi_1 = 60°$, as in didemnin B. Vicinal coupling constants $^3J_{C\alpha H-C\beta H}$ and $^3J_{C\alpha H-C\beta H'}$ in Leu[3] (9.6 and 1.6 Hz), Pro[4] (3.6 and 7.8 Hz), Me_2Tyr^5 (10.8 and 4.1 Hz), and MeLeu[7] (3.6 and 11.4 Hz) were extracted from the 1D ^{1}H NMR spectrum of tamandarin A (**1**). These values are virtually identical with those found in didemnin B, indicating the conformations about the Cα−Cβ bonds to be identical in both molecules. Therefore, the side chain of Leu[3] in tamandarin A (**1**) appears to be highly restrained as in didemnin B, although the expected strong NOE correlations between Leu[3] CβH and Pro[4] CγH were not observed. Coupling constants for Pro[4] seemed to indicate an "*endo*" configuration with respect to ring puckering, in which Cγ and CO point in the same direction, as in didemnin B. Vicinal coupling constants $^3J_{C\alpha H-C\beta H}$ and $^3J_{C\alpha H-C\beta H'}$ for Pro[8], both 7.5 Hz, are quite different from those observed in Pro[4]. The measured values correspond to a dihedral angle and ring puckering identical to Pro[8] in didemnin B, which adopts an "*exo*" configuration (Cγ and CO pointing in opposite directions, Figure 3).

Tamandarin A (**1**) was evaluated for its cytotoxicity (Figure 2) against three cell lines: the pancreatic carcinoma BX-PC3 (a), the prostate carcinoma DU145 (b), and the head and neck carcinoma UMSCC10b (c). These bioassays were clonogenic (colony-forming) assays performed under continuous exposure to tamandarin A (**1**) and didemnin B. The concentrations causing a 50% reduction in overall cell survival (IC_{50}, Table 3) were 1.79, 1.36, and 0.99 ng/mL, respectively (versus 2.00, 1.53, and 1.76 ng/mL, respectively, for didemnin B). Although the cell lines differed slightly in sensitivity, the two compounds showed virtually the same patterns of activity and potency.

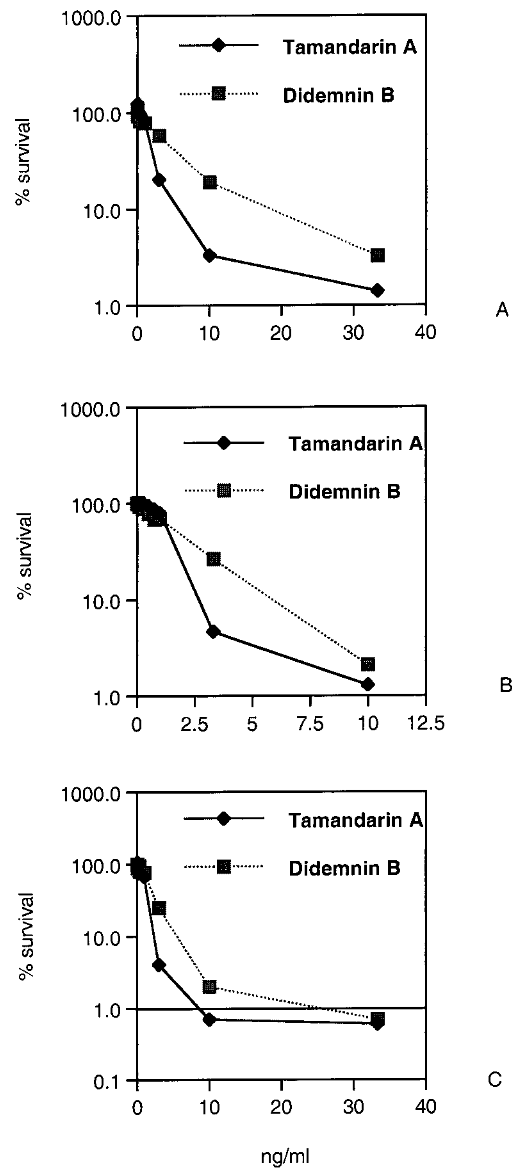

Figure 2. Dose−response curves for tamandarin A (**1**) and didemnin B, in clonogenic (colony-forming) assays, using (a) pancreatic carcinoma BX-PC3, (b) prostatic cancer DU-145, and (c) head and neck carcinoma UMSCC10b cell lines.

Discussion

Tamandarins A and B (**1** and **2**) are novel depsipeptides related to the didemnins, a well-known class of cyclic, highly active antiviral, antitumor, and immunosuppressive peptides. To date, 18 naturally occurring didemnin congeners have been isolated from the Caribbean tunicate *Trididemnum solidum* (class Ascidiacea, order Aplousobranchia, family Didemnidae), the Mediterranean species *Trididemnum cyanophorum* and *Aplidium albicans* (order Aplousobranchia, family Polyclinidae), and the unidentified Brazilian ascidian (family Didemnidae) in this study.[3,4] Didemnin B is among the most potent

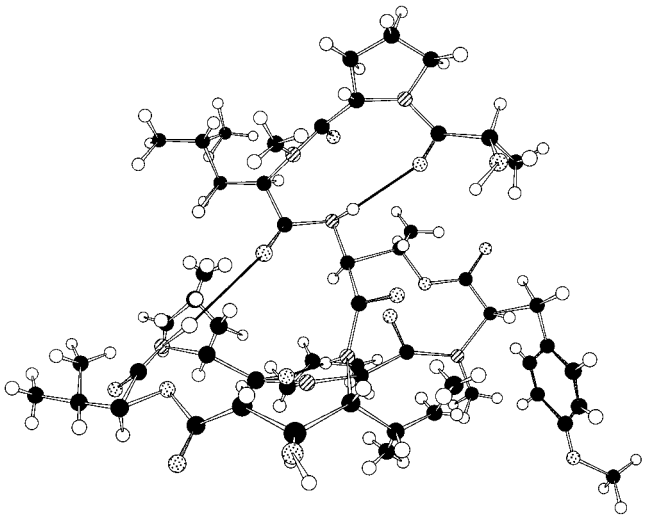

Figure 3. Computer-generated drawing of tamandarin A (**1**) in solution. Most hydrogens are omitted for clarity.

members of this class of molecules, rivaled by tamandarin A (**1**) in some assays. Didemnin B was early shown to be active against several DNA and RNA viruses in vitro and in vivo but is unfortunately ineffective against the AIDS-causing virus HIV.[3a,17,18]

In several in vitro and in vivo assays, didemnin B showed antiproliferative activity in T-lymphocytes with a potency stronger than that of cyclosporin A, at present the most important immunosuppressive agent used clinicically.[19] During further evaluation of the antiproliferative activity of didemnin B for the purpose of prolonging organ allograft survival, it was generally found that didemnin B displayed immunosuppressive activity in vivo but possessed a very narrow therapeutic index, as it displayed significant toxicity at therapeutic dosages.[20]

Didemnin B was also shown to be cytotoxic toward B16 melanoma, both in vitro and in vivo, and P388 lymphocytic leukemia in vivo and L1210 leukemia in vitro.[21] On the basis of these results and activity observed against a number of human tumor stem cell lines, didemnin B was the first marine cytotoxin to enter clinical trials as an antineoplastic agent.[22] Phase I and II clinical trials were conducted against melanoma and cancers of the ovaries, cervix, prostate, lung, breast, and kidney.[23] Invariably, didemnin B was found to be toxic but inactive in most of the trials completed. Didemnin B showed no significant antitumor activity against these cancers, and further clinical trials were canceled.[24]

Based on its structural similarity to didemnin B, tamandarin A (**1**) can be expected to show potent antiviral, immunosuppressive, and antitumor activities; however, the former have not been confirmed. Indeed, clonogenic cytotoxicity assays showed that cancer cell colony formation under continuous exposure is equally strongly inhibited by tamandarin A (**1**) and by didemnin B.

The possible mechanisms through which didemnin B may exert its antiviral, antitumor, and immunomodulating activities have been studied extensively. In both cellular (in vivo and in vitro)[25] and cell-free assays,[26] didemnin B was found to inhibit protein and DNA synthesis and, to a lesser extent, RNA synthesis. Evidence has been provided that inhibition of peptide synthesis by didemnin B occurs by stabilization of aminoacyl-tRNA binding to the ribosomal A-site, preventing translocation of phenylalanyl-tRNA^{phe} from the A- to the P-site, but not preventing peptide bond formation.[27] Consistent with these findings, it was previously found that didemnin B binds to elongation factor 1α in a GTP-dependent manner, and formation of the didemnin B-GTP-EF1α complex may be responsible for the observed inhibition of protein synthesis.[28] The structural similarity of tamandarin A (**1**) with didemnin B suggests that they may possess the same mechanism of action. Accordingly, in cell-free assays, tamandarin A (**1**) was

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found to be a more potent inhibitor of protein biosynthesis than didemnin B.

In view of their potent biological properties, numerous synthetic and structure−activity studies have been performed on the didemnins. The total syntheses of didemnins A, B, and C[29] and nordidemnin B[30] have been reported, as well as the (semi)synthesis of approximately 40 "unnatural" analogues of didemnin B and its parent structure didemnin A.[31] Antitumor, antiviral, and immunosuppressive activities of the structures were assessed in vitro and in vivo, and several compounds with increased potency over didemnin B were discovered. Structure−activity studies identified several parts of the molecule as potentially important in drug−receptor interactions. Whereas modifications of the linear side chain resulted in increased potency in certain cases, most stereocenters and functionalities of the cyclic depsipeptide core were deemed essential for bioactivity.[32] It was proposed that the keto group of the Hip[2] unit plays a major role in the bioactivities of the didemnins, since reduction to the alcohol resulted in loss of bioactivity.[3e] This proposal seems incorrect, however, since tamandarin A (**1**), which lacks this group, shows potent cytotoxicity and protein inhibition at levels comparable to those observed in didemnin B.

The solution conformation of tamandarin A (**1**), described in this paper, indicates that the lack of the propionic acid unit in didemnin B results in only minor conformational modifications. Whereas the cyclic peptide ring in didemnin B is 23-membered, the ring in tamandarin A (**1**) is 21-membered. The effect of this change seems to be spread out over the entire molecule, resulting in little overall change. The backbone configuration of tamandarin A (**1**) appears to be stabilized by the same three hydrogen bonds, Ist[1] NH−Leu[3] CO, Leu[3] NH−MeLeu[7] CO, and Lac[9] CO−Thr[6] NH, yielding the same "bent figure eight" shape of the cyclic peptide ring and the side-chain folding back over the cyclic portion of the molecule. The side-chain conformations of the amino acids in tamandarin A (**1**) are almost identical to those in didemnin B as well, indicating no major increase or decrease in local crowding due to the reduced size of the cyclic peptide.

Although the didemnin class of cyclic depsipeptides may not yield a clinically useful antitumor drug with utility in conventional chemotherapy, these molecules remain of great interest for the development of novel cancer treatments. In view of the exceptional potency of these cyclic peptides, they may become useful in future applications in which antitumor compounds are delivered directly to the tumor by means of an alternative transport system. Such targeted systems can potentially avoid the problem of toxicity toward normal tissues. Carrier systems that have been reported include monoclonal antibodies, liposomes, viral particles, and tumor-homing peptides.

Although the mechanism of cytotoxic action of tamandarin A has not been thoroughly studied, it appears that the molecule behaves similar to didemnin B. Recent research in the laboratory of Dr. Peter Toogood at the University of Michigan has shown that tamandarin A inhibits protein biosynthesis in rabbit reticulocyte cell lysates with an IC_{50} value of 1.3 μM. This is approximately 3 times more potent than didemnin B in the same assay.

Experimental Section

General. NMR spectra were recorded on a Varian Unity INOVA spectrometer at 300 MHz (^{1}H) and a Varian GEMINI spectrometer at 100 MHz (^{13}C) in $CDCl_3$. FAB mass spectrometry and tandem mass spectrometry were performed by K. S. Chatman at the Scripps Research Institute, La Jolla, CA. High-performance liquid chromatography was carried out using a reversed-phase (C_{18}) Rainin Dynamax 60 Å column (i.d. 10 mm) and a Waters R401 differential refractometer. Optical rotations were determined on an Autopol III automatic polarimeter (Rudolph Research, Flanders NJ). UV spectra were measured on a Perkin-Elmer Lambda 3B UV/vis spectrophotometer and IR spectra on a Perkin-Elmer 1600 Series FTIR spectrophotometer.

Isolation. Approximately 1123 g of wet ascidian was collected in March 1996, at a depth of approximately 10 m on a small reef off the coast of the village Tamandaré, Mamucabinha, Brazil. The specimen was immediately frozen and, upon lyophilization, yielded approximately 465 g of dry material. The animal material was extracted three times with a mixture of dichloromethane and methanol (1:1) to produce, after removal of solvents in vacuo, a crude extract. The crude extract (26.6 g) showed potent growth inhibitory activity at or below 0.03 μg/mL in the primary in vitro cytotoxicity assay using the HCT 116 cell line. The dark oil was partitioned between isooctane and methanol, and the methanol fraction was dried in vacuo and further partitioned between water and ethyl acetate, water and dichloromethane, and water and butanol. The ethyl acetate and dichloromethane fractions appeared very similar by TLC analyses and contained the bulk of the cytotoxic activity. Both solvent partitions were combined and then fractionated by size exclusion chromatography, employing Sephadex LH-20, using a mixture of hexane, toluene, and methanol (3:1:1). This method has proven to be an excellent technique for the separation of medium-sized peptides from complex mixtures. The procedure yielded 21 fractions, all of which showed potent inhibition in the cytotoxicity assay at or below 0.03 μg/mL. However, by ^{1}H NMR, one fraction appeared to be clearly enriched in peptide metabolites. This fraction yielded 75 mg of tamandarin A (**1**) accompanied by 10 mg of tamandarin B (**2**) after final purification by RP (C_{18}) HPLC using 23% water in methanol.

Tamandarin A (1): white amorphous solid or transparent glass; $[\alpha]_D = -35°$ ($c = 0.11$, MeOH); IR (KBr) 3495, 3342, 2966, 2872, 1743, 1649, 1537, 1514, 1455, 1249, 1173, 1079, 1032 cm^{-1}; UV (CH_2Cl_2) λ_{max} 228 (ϵ 24 500), 276 (ϵ 4000), 283 nm (3400); HRFABMS (NBA/CsI matrix) *m*/*z* 1187.5074

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$[MCs + 2H]^+$, calcd for $C_{54}H_{84}N_7O_{14}$, 1187.5131 ($\Delta = 4.8$ ppm); ^1H and ^{13}C NMR data ($CDCl_3$) are shown in Table 1.

Tamandarin B (2): white amorphous solid or transparent glass; $[\alpha]_D = -29°$ ($c = 0.11$, MeOH); IR (KBr) 3472, 3342, 2966, 2872, 1742, 1661, 1637, 1531, 1514, 1449, 1249, 1173, 1078, 1032 cm^{-1}; UV (CH_2Cl_2) λ_{max} 227 (ϵ 14 400), 277 (ϵ 1500), 283 nm (1200); HRFABMS (NBA/CsI matrix) *m/z* 1173.4916 $[MCs + 2H]^+$, calcd for $C_{53}H_{82}N_7O_{14}$, 1173.4974 ($\Delta = 4.9$ ppm); ^1H and ^{13}C NMR data ($CDCl_3$) are shown in Table 2.

Mild Alkaline Hydrolysis of Tamandarins A (1) and B (2). A sample of tamandarin A (**1**) (5 mg) was dissolved in cooled (4 °C) methanol (500 μL) and hydrolyzed by adding 30 μL of 1 N sodium hydroxide at room temperature. The disappearance of starting material was monitored by silica TLC. After 75 min, the reaction was quenched by acidification with 30 μL of 1 N hydrochloric acid. The crude reaction mixture was methylated by addition of diazomethane in ether until a yellow coloration persisted and gas liberation ceased (approximately 1.5 mL). The resulting solution was evaporated to dryness, and the residue was fractionated by silica column chromatography (1 g) using 5% methanol in dichloromethane. The two major peptide fragments produced (northern fragment **3** and southern fragment **4**) were purified by RP (C_{18}) HPLC using 23% water in methanol. The procedure was repeated with 5 mg of didemnin B to obtain two reference peptide fragments (northern fragment **3** and a southern fragment). Tamandarin B (**2**) (5 mg) was hydrolyzed using the same method, yielding two peptide fragments, a northern fragment (**3′**) and a southern fragment (**4**). Similarly, 1 mg of nordidemnin B was hydrolyzed to obtain reference peptide fragments (northern fragment **3′** and a southern fragment).

Northern Peptide Fragment of Tamandarin A (3): white, amorphous solid or transparent glass; $[\alpha]_D = +41°$ ($c = 0.07$, MeOH); HRFABMS (NBA/CsI matrix) *m/z* 719.2658 [MCs + $2H^+$], calcd for $C_{28}H_{50}N_4O_9$, 719.2632 ($\Delta = 3.6$ ppm); ^1H NMR ($CDCl_3$, determined by ^1H and COSY experiments) for (*S*)-Lac, δ 4.41 (m, 1H), 1.18 (d, 3H, $J = 6.3$ Hz), for L-Pro, δ 4.75 (br tr, 1H, $J = 6.6$ Hz), 3.64 (m, 2H), 2.23 (m, 2H), 1.97 (m, 2H), for D-MeLeu, δ 5.36 (dd, 1H, $J = 10.2$, 5.1 Hz), 3.08 (s, 3H), 1.96 (m, 1H), 1.90 (m, 1H), 1.43 (m, 1H), 0.97 (d, 3H, $J = 6.6$ Hz), 0.93 (d, 3H, $J = 6.6$ Hz), for L-Thr, δ 7.37 (d, 1H, $J = 7.8$ Hz), 4.37 (m, 2H), 1.37 (d, 3H, $J = 6.6$ Hz), for (3*S*,4*R*,5*S*)-Ist, δ 6.62 (br d, 1H, $J = 10.8$ Hz), 4.16 (d tr, 1H, $J = 6.0$, 5.3 Hz), 4.04 (m, 1H), 3.70 (s, 3H), 2.54 (s, 1H), 2.52 (d, 1H, $J = 6.3$ Hz), 1.91 (m, 1H), 1.37 (m, 1H), 1.20 (m, 1H), 0.93 (d, 3H, $J = 8.1$ Hz), 0.91 (tr, 3H, $J = 6.8$ Hz).

Southern Peptide Fragment of Tamandarins A and B (4): white amorphous solid or transparent glass; $[\alpha]_D = -100°$($c = 0.03$, MeOH); HRFABMS (NBA/CsI or NaI matrix) *m/z* 666.2179 [MCs + H^+], calcd for $C_{28}H_{43}N_3O_7$, 666.2155 ($\Delta = 3.6$ ppm), and *m/z* 534.3160 [M + H^+], calcd for $C_{28}H_{43}N_3O_7$, 534.3179 ($\Delta = 3.6$ ppm); ^1H NMR ($CDCl_3$, determined by ^1H and COSY experiments) for L-Me_3Tyr, δ 2.93 (s, 3H), 3.01 (dd, 1H, $J = 14.4$, 9.3 Hz), 3.27, (dd, 1H, $J = 14.7$, 6.0 Hz), 3.80 (s, 3H), 3.69 (s, 3H), 5.03 (dd, 1H, $J = 9.3$, 6.3 Hz), 6.83 (d, 2H, $J = 8.7$ Hz), 7.12 (d, 2H, $J = 8.4$ Hz), for L-Pro, δ 1.87 (m, 1H), 2.16 (m, 3H), 3.68 (m, 1H), 3.82 (m, 1H), 4.78 (m, 1H), for L-Leu, δ 0.98 (tr, 6H, $J = 6.3$ Hz), 1.67 (m, 1H), 1.71 (m, 2H), 4.78 (m, 1H), 6.99 (d, 1H, $J = 8.7$ Hz), for (*S*)-Hiv, δ 0.87 (d, 3H $J = 6.9$), 1.03 (d, 3H, $J = 6.9$ Hz), 2.16 (m, 1H), 3.96 (d, 1H, $J = 3.0$ Hz).

Northern Peptide Fragment of Tamandarin B (3′): white amorphous solid or transparent glass; $[\alpha]_D = +24°$ ($c = 0.05$, MeOH); HRFABMS (NBA/NaI matrix) *m/z* 595.3302 [MNa + $2H^+$], calcd for $C_{27}H_{46}N_4O_9$, 595.3319 ($\Delta = 2.9$ ppm); ^1H NMR ($CDCl_3$, determined by ^1H and COSY experiments) for (*S*)-Lac, δ 1.21 (d, 3H, $J = 6.3$ Hz), 4.45 (m, 1H), for L-Pro, 1.99 (m, 2H), 2.26 (m, 2H), 3.68 (m, 2H), 4.78 (br tr, 1H, $J = 7.2$ Hz), for D-MeLeu, δ 0.93 (d, 3H, $J = 6.6$ Hz), 0.97 (d, 3H, $J = 7.2$ Hz), 1.48 (m, 1H), 1.68 (m, 1H), 1.94 (m, 1H), 3.11 (s, 3H), 5.38 (dd, 1H, $J = 10.5$, 4.8 Hz), for L-Thr, δ 1.40 (d, 3H, $J = 6.9$ Hz), 4.41 (m, 2H), 7.41 (br d, 1H, $J = 7.2$ Hz), for (3*S*,4*R*)-Nst, δ 0.98 (tr, 3H, $J = 6.6$ Hz), 0.99 (d, 3H, $J = 6.9$ Hz), 1.99 (m, 1H), 2.52 (d, 1H, $J = 6.0$ Hz), 2.54 (s, 1H), 3.72 (s, 3H), 3.95 (m, 1H), 4.21 (m, 1H), 6.66 (br d, 1H, $J = 10.5$ Hz).

Preparation of *N,O*-Dimethyltyrosine Methyl Ester (Me_3Tyr) Standard. *N,O*-Dimethyltyrosine methyl ester was obtained by deprotection of *N*-Cbz-*N,O*-dimethyltyrosine methyl ester (1 mg, see further) in refluxing TFA (1 mL) for 30 min. Reaction progress was monitored by silica TLC analysis. The reaction mixture was cooled and evaporated to dryness, and traces of TFA were removed by repeated evaporation from water. The crude *N,O*-dimethyltyrosine methyl ester was then converted to its Marfey derivative without further purification.

Preparation of *N*-Cbz-*N,O*-Dimethyltyrosine Methyl Ester. A mixture of 100 mg (0.32 mmol) of *N*-Cbz-tyrosine (D:L = 3:7) was dimethylated using methods described previously.[33] The protected amino acid was dissolved in 5 mL of DMF, and 10 mg of Bu_4NHSO_4 (20 wt %) was added. Portions of 180 mg of finely powdered KOH and 300 μL of dimethyl sulfate were added three times over a period of 16 h, during which time the reaction mixture was stirred vigorously at room temperature. The reaction mixture was cooled to 0 °C and diluted with 20 mL of diethyl ether, and the 30 mL of water was added. The aqueous layer was separated, and the organic layer was extracted twice with 30 mL of saturated $NaHCO_3$ solution. The aqueous layers were combined, acidified with $KHSO_4$ (1 M) to pH 1, and extracted three times with ethyl acetate. The organic layers were combined, dried over $MgSO_4$, filtered, and dried in vacuo. The crude reaction mixture was dissolved in 1 mL of absolute methanol, cooled in ice, and methylated with CH_2N_2 in ether. The solvent was evaporated under a stream of nitrogen, and *N*-Cbz-*N,O*-dimethyltyrosine methyl ester was purified by RP (C_{18}) HPLC using 23% water in methanol. Compound **5** was obtained as a colorless oil: ^1H NMR ($CDCl_3$) δ 2.85 and 2.83 (s, 3H, rotational isomers), 2.97 (dd, 1H, $J = 11.9$ Hz), 3.28 (tr d, 1H, $J = 14.2$, 5.7 Hz), 3.75 and 3.68 (s, 3H, rotational isomers), 3.79 (s, 3H), 4.77 and 4.98 (dd, 1H, $J = 5.1$, 10.2 Hz, rotational isomers), 5.05 (d, 1H, $J = 5.7$ Hz), 5.12 (d, 1H, 5.7 Hz), 6.78 (d, 1H, $J = 7.9$ Hz), 6.82 (d, 1H, $J = 8.0$ Hz), 7.04 (d, 1H, $J = 7.9$ Hz), 7.13 (d, 1H, $J = 8.0$ Hz), 7.24 (m, 1H), 7.33 (m, 4H).

Acid-Catalyzed Hydrolysis of Southern Peptide Fragment (4). The southern peptide fragment (**4**, 1 mg) in 0.5 mL of 6 N HCl was heated at 105 °C for 16 h in a sealed vial. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolysate by repeated evaporation from H_2O.

Amino Acid Analysis of Southern Peptide (4) Using Marfey's Method. The previously obtained crude hydrolysate of the southern peptide fragment (**4**), or the crude *N,O*-dimethyltyrosine methyl ester (Me_3Tyr), or a small amount of standard free amino acid (D:L = 3:7), in 50 μL of water/acetone was mixed with 100 μL of a 1% solution of FDAA (Marfey's reagent = 1-*f*luoro-2,4-*d*initrophenyl-5-L-*a*lanine *a*mide) in acetone. $NaHCO_3$ (20 μL, 1 M) was added to this mixture, and the resultant solution was heated at 40 °C for 1 h and then allowed to cool. After addition of 10 μL of 2 M HCl, the resulting solution was evaporated, dissolved in 0.5 mL of DMSO, and analyzed by diode array HPLC. The analysis used the following conditions: solvent A, 0.1% TFA in H_2O; solvent B, 0.1% TFA in methanol; gradient flow rate of A + B at 1 mL/min, 100/0 to 80/20 in 45 min and from 80/20 to 40/60 in 45 min; column, Hewlett-Packard ODS Hypersil 5μ, 200 mm × 4.6 mm; UV detection at 340 nm. The peaks were identified by comparison with a mixture of D/L-standard amino acid-DAA derivatives (D:L = 3:7). Retention times (min): L-Me_3Tyr-DAA, 56.5; L-Pro-DAA, 58.8; L-Leu-DAA, 70.5.

Mosher Ester Analysis of Hiv[2] in Southern Peptide Fragment (4). The southern peptide fragment (**4**, 1 mg) was dissolved in 200 μL of dichloromethane. Dry pyridine (100 μL,

(33) The method as described in the literature (Li, W.-R.; Ewing, W. R.; Harris, B. D.; Joullié, M. M. *J. Am. Chem. Soc.* **1990**, *112*, 7659−7672), followed by methylation with diazomethane, yielded only *O*-methyltyrosine methyl ester. For dimethylation to occur, the modified reaction scheme was used.

predried over 4-Å molecular sieves) was added, followed by 0.5 mg of 4-(dimethylamino)pyridine. Approximately 5 μL of (*R*)- or (*S*)-MTPA acid chloride ((*R*)- or (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride) was added, and the solution was left at room temperature. Reaction progress was monitored by silica TLC analysis. After 3 days, 3 mL of saturated $NaHCO_3$ solution and 3 mL of diethyl ether were added, and the solution was stirred vigorously for 30 min to hydrolyze excess MTPA acid chloride. The organic phase was separated, and the aqueous phase was extracted with 3 mL of diethyl ether. The organic phases were combined, washed three times with 3 mL of 5% aqueous $NaHCO_3$ to remove pyridine and three times with 3 mL of saturated NaCl solution, dried over $MgSO_4$, and dried in vacuo. The (*R*)- or (*S*)-MTPA ester of southern peptide fragment (**4**) was purified by taking the reaction mixture over a 1-in. silica flash column in a Pasteur pipet using 3:1 isooctane/ethyl acetate.

(*R*)-MTPA Ester of the Southern Peptide Fragment (4): ^{1}H NMR ($CDCl_3$, determined by ^{1}H and COSY experiments) for L-Me_3Tyr, δ 2.94 (s, 3H), 3.02 (dd, 1H, J = 14.1, 9.4 Hz), 3.25, (dd, 1H, J = 14.1, 6.5 Hz), 3.77 (s, 3H), 3.70 (s, 3H), 5.01 (dd, 1H, J = 9.4, 6.5 Hz), 6.82 (d, 2H, J = 9.2 Hz), 7.13 (d, 2H, J = 9.2 Hz), for L-Pro, δ 1.90 (m, 1H), 2.15 (m, 3H), 3.70 (m, 1H), 3.77 (m, 1H), 4.80 (m, 1H), for L-Leu, δ 0.90 (tr, 6H, J = 6.3 Hz), 1.40 (m, 1H), 1.52 (m, 1H), 1.56 (m, 1H), 4.72 (m, 1H), 6.35 (br d, 1H, J = 9.2 Hz), for (*S*)-Hiv-(*R*)-MTPA, δ 0.97 (d, 3H J = 7.5), 1.00 (d, 3H, J = 7.5 Hz), 2.35 (m, 1H), 3.58 (s, 3H), 5.20 (d, 1H, J = 3.9 Hz), 7.42 (m, 3H), 7.58 (d, 2H, J = 6.6 Hz).

(*S*)-MTPA Ester of the Southern Peptide Fragment (4): ^{1}H NMR data as determined by ^{1}H and COSY experiments for L-Me_3Tyr, δ 2.95 (s, 3H), 3.04 (dd, 1H, J = 14.1, 9.0 Hz), 3.27, (dd, 1H, J = 14.1, 6.3 Hz), 3.85 (s, 3H), 3.69 (s, 3H), 5.01 (dd, 1H, J = 9.0, 6.3 Hz), 6.81 (d, 2H, J = 9.4 Hz), 7.12 (d, 2H, J = 9.4 Hz), for L-Pro, δ 1.90 (m, 1H), 2.15 (m, 3H), 3.69 (m, 1H), 3.85 (m, 1H), 4.80 (m, 1H), for L-Leu, δ 0.90 (tr, 6H, J = 6.3 Hz), 1.40 (m, 1H), 1.50 (m, 1H), 1.58 (m, 1H), 4.76 (m, 1H), 6.45 (br d, 1H, J = 9.3 Hz), for (*S*)-Hiv-(*R*)-MTPA, δ 0.90 (d, 6H J = 7.5 Hz), 2.28 (m, 1H), 3.58 (s, 3H), 5.16 (d, 1H, J = 4.5 Hz), 7.44 (m, 3H), 7.63 (d, 2H, J = 7.5 Hz).

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Supporting Information Available: Spectra [NMR (^{1}H, ^{13}C, DEPT, COSY, TOCSY, ROESY, NOESY, g HMQC, g HMBC), UV, IR, and MS] for tamandarins A and B. This material is available free of charge via the Internet at http://pubs.acs.org.

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