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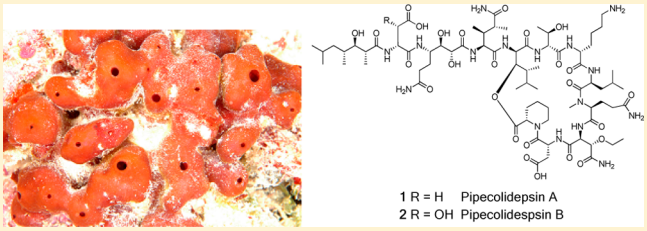
Isolation and Structures of Pipecolidepsins A and B, Cytotoxic Cyclic Depsipeptides from the Madagascan Sponge *Homophymia lamellosa*

Laura Coello, Fernando Reyes,[†] María Jesús Martín, Carmen Cuevas,* and Rogelio Fernández*

Medicinal Chemistry Department, PharmaMar S. A., Pol. Ind. La Mina Norte, Avenida de los Reyes 1, 28770, Colmenar Viejo (Madrid), Spain

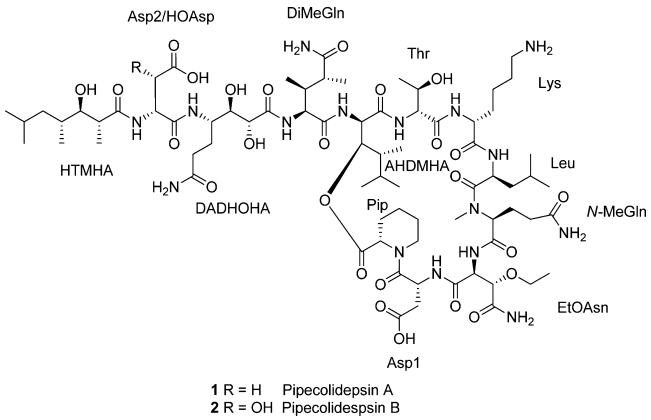
Supporting Information

ABSTRACT: Two new cyclic depsipeptides, pipecolidepsins A and B (**1** and **2**), have been isolated from the sponge *Homophymia lamellosa* collected off the coast of Madagascar. Their structures were determined by a combination of NMR experiments and by LC-MS analysis of the amino acid fragments obtained by hydrolysis and derivatization using Marfey's reagent. In addition to several common amino acids, these peptides contain unusual residues, including 2-amino-3-hydroxy-4,5-dimethylhexanoic acid, 3-ethoxyasparagine, 3,4-dimethylglutamine, 4,7-diamino-2,3-dihydroxy-7-oxoheptanoic acid, and 3-hydroxyaspartic acid as well as a terminal 3-hydroxy-2,4,6-trimethylheptanoic acid residue. Pipecolidepsins A and B displayed cytotoxic activity against a panel of different human tumor cell lines.



Marine sponges continue to be an abundant source of new molecules with interesting biological properties. Among them, cyclic depsipeptides constitute a structurally complex class, with many such compounds incorporating novel amino acid residues. Particularly, a number of complex sponge peptides including homophymines A–E and A1–E1 from *Homophymia* sp.,[1] callipeltins A–M from *Callipelta* sp. and *Latrunculia* sp.,[2] mirabamides A–H from *Siliquariaspongia mirabilis*,[3] papuamides A–F and theopapuamide from *Theonella mirabilis* and *T. swinhoei*,[4,5] and neamphamides A and B from *Neamphius huxleyi*[6] all share one or more of the unusual residues (2*S*,3*S*,4*R*)-3,4-dimethylglutamine (3,4-diMeGln), (2*S*,3*R*)-*β*-methoxytyrosine (*β*OMeTyr), (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (AGDHA), (2*R*,3*R*,4*S*)-4-amino-2,3-dihydroxy-1,7-heptandioic acid, (2*R*,3*R*,4*R*)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid, (2*R*,3*R*,4*R*)-3-hydroxy-2,4,6-trimethylheptanoic acid (HTMHA), and (2*R*,3*R*,4*R*)-3-hydroxy-2,4,6-trimethyloctanoic acid (HTMOA). Interestingly, many of these molecules have been reported to display strong anti-HIV, antifungal, or antitumor properties.

During the course of our ongoing screening program to find new antitumor agents from marine organisms, extracts of the Madagascan sponge *Homophymia lamellosa* were found to display cytotoxic activity against the human tumor cell lines A-549 (lung), HT-29 (colon), and MDA-MB-231 (breast). Bioassay-guided fractionation of the 2-propanol extract of a specimen of this organism yielded pipecolidepsins A and B as the bioactive components. The structures of both compounds, their cytotoxic properties, and a total solid-phase synthesis of pipecolidepsin A have been previously disclosed.[7,8] Herein, we report the isolation and the rationale followed in their structural characterization. The chemical structures of these new compounds incorporate some of the unusual amino acids present in the depsipeptides cited above, as well as a 3-ethoxyasparagine residue, which is unprecedented in natural peptides.



The molecular formula of pipecolidepsin A (**1**) was established as $C_{74}H_{126}N_{16}O_{26}$ by HRMALDIMS of the $[M + H]^+$ molecular ion at m/z 1655.90918. The peptidic nature of pipecolidepsin A was evident from the abundance of signals in the amide NH proton (δ 9.00–6.49 ppm) and α-amino proton (δ 5.42–3.82 ppm) regions of its ^{1}H NMR spectrum and the presence of numerous carbonyl carbons (δ 180.2–169.7 ppm) in the ^{13}C NMR spectrum obtained in CD_3OH. Additionally, the ^{1}H NMR spectrum showed signals corresponding to an *N*-methyl group at δ 2.96 ppm (3H, s) and several other methyl

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Table 1. NMR Data of Pipecolidepsins A (1) and B (2) in CD_3OH

		1				2	
amino acid		δ_C, mult	δ_H, m, J (Hz)	amino acid		δ_C, mult	δ_H, m, J (Hz)
Pip	1	170.4, C		Pip	1	170.6, C[c]	
	2	53.9, CH	5.27, m		2	53.9, CH	5.26, m
	3	27.6, CH_2	2.19, m		3	27.6, CH_2	2.20, m
			1.64, m				1.63, m
	4	22.6, CH_2	1.74, m		4	22.6, CH_2	1.75, m
			1.25, m				1.23, m
	5	26.3, CH_2	1.63, m		5	28.0, CH_2	1.62, m
			1.53, m				1.56, m
	6	44.6, CH_2	3.70, m		6	44.6, CH_2	3.69, m
			3.12, m				3.12, m
Asp1	1	170.5, C		Asp	1	170.4, C[c]	
	2	47.2, CH	5.34, m		2	47.3, CH	5.32, m
	3	36.5, CH_2	2.90, dd (17.0, 8.0)		3	36.6, CH_2	2.85, m
			2.46, dd (16.5, 4.0)				2.46, m
	4	173.8, C			4	[h]	
	NH		8.39, d (9.0)		NH		8.43, d (7.6)
EtOAsn	1	169.7, C		EtOAsn	1	169.8, C	
	2	56.7, CH	4.92, m		2	56.8, CH	4.91[g]
	3	78.5, CH	4.62, br s		3	78.5, CH	4.60, br s
	4	174.1, C			4	[h]	
	5	68.0, CH_2	3.67, m		5	68.0, CH_2	3.67, m
			3.48, m				3.48, m
	6	16.0, CH_3	1.19, t (7.0)		6	15.9, CH_3	1.18, t (7.0)
	NH		6.49, d (9.0)		NH		6.48, d (8.5)
	NH_2		7.53, br s		NH_2		[i]
			7.31, br s				
N-MeGln	1	172.2, C		*N*-MeGln	1	172.3, C	
	2	58.2, CH	5.42, d (8.0)		2	58.2, CH	5.43, m
	3	23.5, CH_2	2.46, m		3	23.3, CH_2	2.48, m
			1.81, m				1.78, m
	4	31.9, CH_2	2.19, m		4	31.7, CH_2	2.22, m
			2.08, m				2.12, m
	5	177.8, C			5	177.7, C	
	NMe	31.4, CH_3	2.96, s		NMe	31.4, CH_3	2.95, s
	NH_2		7.21, br s		NH_2		[i]
			6.75, br s				
Leu	1	177.0, C		Leu	1	177.0, C	
	2	51.3, CH	4.58, m		2	51.2, CH	4.58, m
	3	38.9, CH_2	2.39, m		3	38.9, CH_2	2.38, m
			1.55, m				1.55, m
	4	26.1, CH	2.03, m		4	26.1, CH	2.04, m
	4-Me	20.8, CH_3	1.03, d (6.5)		4-Me	20.8, CH_3	1.01, d (6.5)
	5	24.1, CH_3	1.09, d (6.5)[a]		5	24.0, CH_3	1.09, d (6.9)[d]
	NH		7.60, d (6.0)		NH		7.57, d (5.5)
Lys	1	174.4, C[b]		Lys	1	[h]	
	2	53.1, CH	4.49, m		2	53.4, CH	4.44, m
	3	30.4, CH_2	2.02, m		3	30.5, CH_2	2.02, m
			1.55, m				1.58, m
	4	23.5, CH_2	1.48, m		4	23.6, CH_2	1.50, m
			1.36, m				1.35, m
	5	26.3, CH_2	1.66, m		5	27.9, CH_2	1.63, m
							1.59, m
	6	41.0, CH_2	2.95, m		6	41.1, CH_2	2.95, m
	NH		7.85, br d (8.5)		NH		7.88, br s
	NH_2		n.o.[j]		NH_2		n.o.
Thr	1	171.8, C		Thr	1	171.9, C	
	2	64.3, CH	3.82, br s		2	64.2, CH	3.82, br s
	3	67.6, CH	4.39, m		3	67.6, CH	4.38, m
	4	20.1, CH_3	1.34, d (6.0)		4	20.2, CH_3	1.33, d (6.5)

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Table 1. continued

		1				2	
amino acid		δ_C, mult	δ_H, m, J (Hz)	amino acid		δ_C, mult	δ_H, m, J (Hz)
	NH		8.33, br s		NH		8.39, br s
AHDMHA	1	174.6, C[b]		AHDMHA	1	[h]	
	2	55.6, CH	5.29, m		2	55.6, CH	5.29, m
	3	77.2, CH	5.62, dd (11.0, 2.0)		3	77.2, CH	5.57, br d (11.0)
	4	39.1, CH	1.91, m		4	39.1, CH	1.93, m
	4-Me	8.8, CH_3	0.74, d (7.5)		4-Me	8.7, CH_3	0.73, d (7.0)
	5	27.8, CH	1.93, m		5	27.9, CH	1.92, m
	5-Me	21.3, CH_3	0.94, d (6.5)		5-Me	21.3, CH_3	0.94, d (6.5)
	6	15.3, CH_3	0.73, d (7.5)		6	15.2, CH_3	0.71, d (7.5)
	NH		8.96, d (10.0)		NH		8.91, br d
DiMeGln	1	174.6, C		DiMeGln	1	[h]	
	2	59.0, CH	4.33, dd (11.0, 5.5)		2	59.0, CH	4.28, dd (10.5, 5.5)
	3	37.2, CH	2.30, m		3	36.9, CH	2.31, m
	3-Me	14.3, CH_3	1.09, d (7,1)[a]		3-Me	14.1, CH_3[e]	1.05, d (6.5)[d]
	4	42.5, CH	2.75, qd (7.0, 2.5)		4	42.7, CH	2.76, m
	4-Me	14.4, CH_3	1.24, d (7.0)		4-Me	14.4, CH_3	1.23, d (7.0)
	5	180.2, C			5	180.6, C	
	NH		9.00, d (4.5)		NH		9.19, br d
	NH_2		7.45, br s		NH_2		[i]
			6.94, br s				
DADHOHA	1	176.4, C		DADHOHA	1	176.3, C	
	2	72.9, CH	3.84, m		2	72.8, CH	3.92, m
	3	75.7, CH	3.59, m		3	75.6, CH	3.57, m
	4	51.1, CH	4.10, m		4	51.0, CH	4.10, m
	5	28.5, CH_2	1.92, m		5	28.5, CH_2	1.92, m
			1.78, m				1.75, m
	6	32.6, CH_2	2.23, m		6	32.7, CH_2	2.20, m
	7	178.6, C			7	178.7, C	
	NH		7.66, d (9.0)		NH		7.53[f]
	NH_2		7.53, br s		NH_2		[i]
			6.83, br s				
Asp2	1	174.8, C		HOAsp	1	[h]	
	2	51.7, CH	4.69, dd (12.0, 6.5)		2	58.2, CH	4.86[g]
	3	36.6, CH_2	2.92, m		3	72.2, CH	4.38, m
			2.84, dd (17.0, 5.0)				
	4	174.7, C			4	[h]	
	NH		8.35, d (8.0)		NH		8.28, d (7.0)
HTMHA	1	179.1, C		HTMHA	1	179.3, C	
	2	44.9, CH	2.62, qd (9.5, 7.0)		2	44.9, CH	2.69, qd (9.0, 7.0)
	2-Me	14.4, CH_3	1.07, d (6.9)[a]		2-Me	14.3, CH_3[e]	1.06, d (6.5)[d]
	3	79.6, CH	3.57, m		3	79.9, CH	3.55, m
	4	33.5, CH	1.74, m		4	33.4, CH	1.75, m
	4-Me	17.4, CH_3	0.99, d (6.5)		4-Me	17.4, CH	0.98, d (7.0)
	5	39.3, CH_2	1.16, m		5	39.1, CH_2	1.16, m
	6	26.2, CH	1.66, m		6	26.2, CH	1.63, m
	6-Me	21.5, CH_3	0.87, d (6.5)		6-Me	21.5, CH_3	0.86, d (6.5)
	7	24.7, CH_3	0.94, d (6.5)		7	24.7, CH_3	0.93, d (6.5)

[a−f]Assignment for these signals may be interchanged. [g]Under solvent. [h]Carbonyl signals at these positions (δ_C 174.7, 174.4, 174.2, 173.8 ppm) were not assigned due to the failure of obtaining $^1H-^{13}C$ long-range connectivities. Three carbonyl signals were overlapped/not detected. [i]Assignments of NH_2 at these positions (δ_H 7.54[f]/6.81, 7.50/6.96, 7.53[f]/7.30 and 7.16/6.79 ppm) are interchangeable. [j]Not observed.

groups between 1.34 and 0.73 ppm. Extensive analysis of the ^1H and ^{13}C NMR data of **1**, including COSY, TOCSY, HMQC-TOCSY, HSQC, and HMBC spectra (Table 1), established the presence of the common amino acids threonine, lysine, leucine, and two aspartic acids, together with six unusual amino acid moieties: pipecolic acid (Pip), 3-ethoxyasparagine (EtOAsn), *N*-methylglutamine (*N*-MeGln), 2-amino-3-hydroxy-4,5-dimethylhexanoic acid (AHDMHA), 3,4-dimethylglutamine (3,4-diMeGln), and 4,7-diamino-2,3-dihydroxy-7-oxoheptanoic acid (DADHOHA). The presence of the amino-linked 3-hydroxy-2,4,6-trimethylheptanoic acid (HTMHA), also present as the end group in callipeltin B, neamphamide A, and homophymines B and B1,[1,2,6] was also inferred from the analysis of the NMR spectra. The previously unreported amino acid 3-ethoxyasparagine was identified as one amino acid component by long-range correlations from the 3-oxymethine proton at

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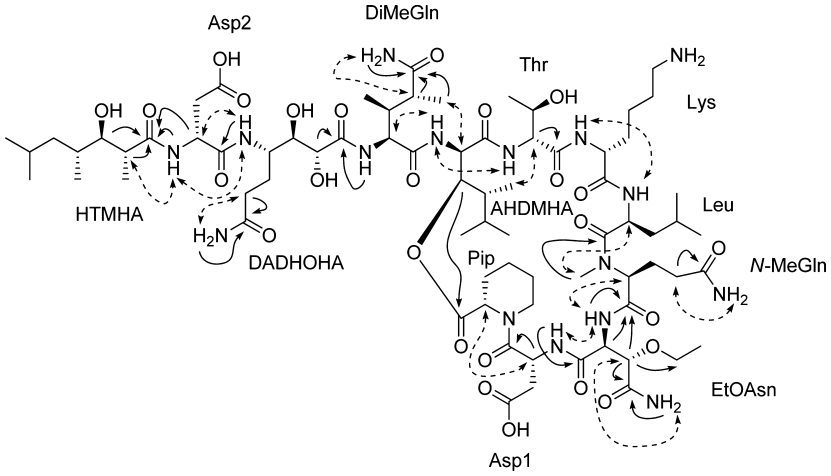

Figure 1. Selected NOE (dashed) and HMBC (plain) correlations for **1**.

4.62 ppm to a methylene at 3.67 and 3.48 ppm corresponding to an ethoxy group, coupled in turn in the COSY spectrum to a triplet methyl group at 1.19 ppm.

The sequencing of these units was carried out using a combination of HMBC and ROESY data. Long-range correlations from α-protons to carbonyl carbons of adjacent amino acids plus ROESY correlations between α-protons and NH protons of adjacent amino acids (Figure 1) allowed us to establish the sequence as Pip-Asp1-EtOAsn-*N*-MeGln-Leu-Lys-Thr-AHDMHA-DiMeGln-DADHOHA. The presence of an ester bond between the carbonyl group of pipecolic acid and the hydroxy group of AHDMHA was suggested by the downfield chemical shift of the H-3 oxymethine proton (δ_H 5.62 ppm) of this last residue[5] and confirmed by the presence of an HMBC correlation between this proton and the Pip carbonyl carbon at δ_C 170.4 ppm. Additionally, ROESY cross-peaks between the DADHOHA-NH (δ_H 7.66 ppm) and the Asp2 H-2 proton (δ_H 4.69 ppm) established an amide linkage between these two amino acids, and acylation of Asp2 by HTMHA was evidenced by HMBC correlations from the NH and H-2 signals of Asp2 to the carbonyl group of HTMHA at 179.1 ppm and a ROESY cross-peak between the Asp2 NH (δ_H 8.35 ppm) and proton H-2 of HTMHA at δ_H 2.62 ppm.

The absolute configurations of *S*-Pip, *R*-Asp, (2*S*,3*S*)-EtOAsn, *S*-*N*-Me-Gln, *S*-Leu, *R*-Lys, *R*-*allo*-Thr, and *R*-Asp were determined by comparing the hydrolysis products of **1** (6 N HCl, 110 °C, 15 h), after derivatization with Marfey's reagent (*N*-(3-fluoro-4.6-dinitrophenyl)-L-alaninamide, L-FDAA), with appropriate amino acid standards using HPLC-MS chromatography.[9] Commercially available samples of Pip, Asp, *N*-Me-Glu, Leu, and Thr and synthetic samples of 3-EtOAsp[10] were used in these analyses.

The *S* configuration at C-4 of the 4,7-diamino-2,3-dihydroxy-7-oxoheptanoic acid (DADHOHA) was determined by cleavage of the 2,3-diol in **1** with sodium periodate, followed by oxidative workup, hydrolysis with 6 N HCl, derivatization with Marfey's reagent, HPLC-MS analysis of the resulting Glu residue, and comparison with the corresponding *S*- and *R*-Glu Marfey's derivatives. The configurations at C-2 and C-3 of the diol were assumed to be identical to those of the similar amino acid found in homophymine, callipeltins, and the theopapuamides and were confirmed by analysis of ^{1}H and ^{13}C NMR chemical shifts, their coupling constants, and solid-phase synthesis of pipecolidepsin A.[8]

In a similar manner, the absolute configurations of DiMeGln, AHDMHA, and HTMHA were assumed to be the same as those previously reported for other similar peptides due to the close resemblance of the ^{1}H and ^{13}C NMR chemical shifts and use of synthetic standards for Marfey's analysis.[11] Definitive confirmation of these configurations and the proposed structure of pipecolidepsin A came from the solid-phase synthesis of the compound.[8] Comparison of the data obtained for the synthetic compound and the natural pipecolidepsin A revealed a perfect overlap of the NMR spectra, identical analytical HPLC behavior, and similar *in vitro* activity.

Pipecolidepsin B (**2**) had a molecular formula of $C_{74}H_{126}N_{16}O_{27}$, according to the (+)-HRMALDIMS (*m*/*z* 1671.89954 $[M + H]^+$). This molecular formula and a comparison of ^{1}H and ^{13}C NMR spectra with those of pipecolidepsin A revealed that the only difference between the compounds was the absence of the methylene group present in the Asp2 of **1** and its substitution for an oxymethine (δ_H 4.38 and δ_C 72.2 ppm) to give a 3-hydroxyaspartic acid (HOAsp) in **2**. Further support for this proposal was provided by an extensive analysis of the 1D and 2D NMR data. Finally, hydrolysis of **2** with 6 N HCl, derivatization with Marfey's reagent, HPLC-MS analysis, and comparison with the four synthetic stereoisomers[12] of 3-hydroxyaspartic acid confirmed the presence of (2*R*,3*S*)-3-hydroxyaspartic acid in pipecolidepsin B.

Finally, a second test of the cytotoxic activity of the pipecolidepsin A against three human tumor cell lines, lung (A-549), colon (HT-29), and breast (MDA-MB-231), produced similar results to those previously reported[8] with GI_{50} values of 0.6, 1.12, and 0.7 μM, respectively. Values of 0.04, 0.01, and 0.02 μM, respectively, were obtained when **2** was assayed against the same cell lines. Interestingly, the replacement of the Asp2 residue in pipecolidepsin A with an HOAsp amino acid in pipecolidepsin B gives rise to a 10-fold (100-fold for the HT-29 cell line) increase in the bioactivity of the latter compound, revealing perhaps a key role for the substitution at C-3 and the hydrophilic nature of this amino acid residue in the mode of action of the compound.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined using a Jasco P-1020 polarimeter. UV spectra were performed using an Agilent 8453 UV−vis spectrometer. IR spectra

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were obtained with a Perkin-Elmer Spectrum 100 FT-IR spectrometer with ATR sampling. NMR spectra were recorded on a Varian "Unity 500" spectrometer at 500/125 MHz ($^1H/^{13}C$). Chemical shifts were reported in ppm using residual CD_3OH (δ 3.30 ppm for ^1H and 49.0 ppm for ^{13}C) as an internal reference. (+)-HRMALDIMS was performed on an Applied Biosystems 4700 Proteomics Analyzer spectrometer employing an α-cyano-4-hydroxycinnamic acid matrix. (+)-ESIMS were recorded using an Agilent 1100 Series LC/MSD spectrometer.

Animal Material. The sponge *Homophymia lamellosa* (order Lithistida, family Neopeltidae) was collected by hand using scuba diving near Saint Marie Island, Madagascar (17°07.436′ S/49°47.525′ E) at depths ranging between 3 and 7 m in May 2004 and frozen immediately after collection. A voucher specimen (ORMA028881) is deposited at PharmaMar.

Extraction and Isolation. A group of specimens of *H. lamellosa* (382.5 g) was triturated and exhaustively extracted with 2-propanol (4 × 400 mL, 2 × 300 mL). The combined extracts were concentrated to yield a crude mass of 13.19 g, which was dissolved in 300 mL of H_2O and extracted with hexane (3 × 300 mL), EtOAc (3 × 300 mL), and *n*-butanol (3 × 100 mL).

The *n*-butanol extract was evaporated to yield 5.86 g of a crude product that was subjected to VLC on Lichroprep RP-18 with a stepped gradient from H_2O to MeOH and then MeOH/CH_2Cl_2 (50:50). Fractions eluted with MeOH/H_2O (75:25) and MeOH/H_2O (85:15) were pooled to give a fraction of 592.5 mg, which was subjected to RP-18 column chromatography with a stepped gradient from H_2O/MeOH (35:65) to MeOH. Fractions eluted with H_2O/MeOH (30:70, 223.4 mg) were subjected to preparative HPLC (Symmetry C_{18}, 7 μm, 19 × 150 mm, gradient H_2O + 0.1% TFA/CH_3CN + 0.1% TFA from 22% to 42% CH_3CN + 0.1% TFA in 25 min and then from 42% to 100% in 7 min, flow: 15 mL/min, UV detection) to yield a fraction (95.9 mg) containing a mixture of pipecolidepsins A and B (retention time from 24.2 to 26.2 min). This fraction was further purified by semipreparative HPLC (X-Bridge Prep C_{18}, 5 μm, 10 × 150 mm, isocratic H_2O + 0.1% TFA/CH_3CN + 0.1% TFA (65:35), flow 2.3 mL/min, UV detection) to obtain impure pipecolidepsin A (39.9 mg, retention time 26.49 min) and pure pipecolidepsin B (13.6 mg, 0.0035% w/w, retention time 24.99 min). Final purification of pipecolidepsin A (14.9 mg, 0.038% w/w) was achieved by semipreparative HPLC (Kromasil 100 C_8, 10 μm, 10 × 150 mm, gradient H_2O/CH_3CN from 30% to 45% in 30 min, flow 2.5 mL/min, UV detection, retention time 20.70 min).

*Pipecolidepsin A (**1**):* amorphous, white solid; $[\alpha]^{25}_D$ +14.4 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 196 nm; IR (KBr) ν_{max} 3345, 2961, 2875, 1664, 1525, 1460, 1203, 1141, 1013, 827, 801, 722 cm^{-1}; ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) see Table 1; (+)HRMALDIMS *m/z* 1655.90918 $[M + H]^+$ (calcd for $C_{74}H_{127}N_{16}O_{26}$, 1655.91020).

*Pipecolidepsin B (**2**):* amorphous, white solid; $[\alpha]^{25}_D$ +6.1 (*c* 0.2, MeOH); UV (MeOH) λ_{max} 195 nm; IR (KBr) ν_{max} 3326, 2961, 2931, 1663, 1525, 1449, 1204, 1138, 1013, 841, 801, 723 cm^{-1}; ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) see Table 1; (+)HRMALDIMS *m/z* 1671.89954 $[M + H]^+$ (calcd for $C_{74}H_{127}N_{16}O_{27}$, 1671.90511).

Evaluation of Cytotoxic Activity. The cytotoxic activity of pipecolidepsins was evaluated against a panel of three human tumor cell lines as previously described.[13]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tabulated NMR data, 1D and 2D NMR spectra, details of the biological activity and determination of the absolute configuration of amino acids, and LC-MS chromatogram charts of L-FDAA derivatives of pipecolidepsins A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +34 91 823 4664. Fax: +34 91 846 6001. E-mail: rfernandez@pharmamar.com.

Present Address

†Fundación MEDINA, Avenida del Conocimiento 3, 18016, Granada, Spain.

Notes

The authors declare no competing financial interest.

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