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Jaspamides H–L, new actin-targeting depsipeptides from the sponge *Jaspis splendans*

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ABSTRACT

Continued investigation of cytotoxic extracts of the marine sponge *Jaspis splendans* has resulted in the discovery of jaspamides H–L (**2**–**5**). Their structures were determined by interpretation of their NMR and ESIMS data. All compounds exhibited potent cytotoxic activities and were shown to cause microfilament disruption. Biological activity and structural elucidation are reported here.

1. Introduction

Jaspamide (jasplakinolide, **1**), a cyclodepsipeptide isolated from marine sponges of the *Jaspis* genus,[1] is one of the most studied marine natural products. Jaspamide has many interesting biological properties including antifungal,[2] anthelminthic, insecticidal, and ichthyotoxic activities.[1] It has been shown to be active against 36 human solid tumor cell cultures.[3] It is known that jaspamide binds to F-actin competitively with phalloidin,[4] a well-known fungal toxin affecting actin dynamics, promotes actin polymerization under non-polymerizing conditions and lowers the critical concentrations of actin assembly in vitro.[5] The biological proprieties and structural features of jaspamide attracted attention for total synthesis[6] and structural modification.[7]

The isolation of further natural jaspamide derivatives represents an alternative to investigate the structure–activity relationships. Several natural variants of jaspamide, geodiamolides,[8] neosiphoniamolide,[9] and seragamides,[10] often characterized by distinct biological proprieties have been reported. Recently we reported the isolation of new jaspamide derivatives, jaspamides B–G, from the sponge *Jaspis splendans*.[11,12] In order to fully characterize the secondary metabolites of this species, we decided to look further into its minor constituents. After careful sequential reversed-phase HPLC separation, we obtained further very minor jaspamide derivatives, named jaspamides H–L (**2**–**5**).

In this paper we describe the isolation, the determination of their structures and the biological activity of the new compounds.

2. Results and discussion

The lyophilized sponge was extracted with methanol, and the crude methanolic extract was subjected to a modified Kupchan's[13,14] partitioning procedure. Chromatography of the $CHCl_3$ extract (ca. 3.2 g) by silica gel MPLC followed by repeated reversed-phase HPLC yielded jaspamides H–L (**2**–**5**).

The molecular formula of jaspamide H (**2**), $C_{35}H_{43}BrN_4O_6$, suggested that it is a demethyl analogue of jaspamide. The NMR signals of **2**, relative to the tripeptide portion, were almost superimposable with the corresponding signals in **1** (Table 1). As for the polypropionate unit, the C-5 vinyl proton (δ_H 4.98) was found slightly downfield shifted with respect to the parent compound and appears as a triplet. In the COSY spectrum it showed cross-peak correlation with a diasterotopic methylene at δ_H 1.48, 1.80. The analysis of the COSY data and of the HMBC correlations clearly evidenced that jaspamide H is the 6-demethyl jaspamide. The lack of a methyl group determines a variation of the chemical shifts of some nuclei of the polypropionate subunit. Therefore the stereochemistry depicted in Figure 1 was tentatively assigned by analogy with the parent compound. The absolute configurations of the amino acid residues were determined by LC–MS analysis of the acid hydrolyzate derivatized with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide; L-FDAA)[15] and comparison with appropriate amino acid standards. Both aromatic residues, β-Tyr and *N*-methylabrine decompose during acid hydrolysis. Therefore they were transformed to Asp and to *N*-Me Asp, respectively, via ozonolysis with oxidative work-up,[16] prior to acid hydrolysis and Marfey's derivatization. Ion selective monitoring for FDAA–MeAsp (*m*/*z* 400) and for FDAA–Asp (*m*/*z* 386) indicated that both L-FDAA derivatives from jaspamide H coeluted with the corresponding derivatives

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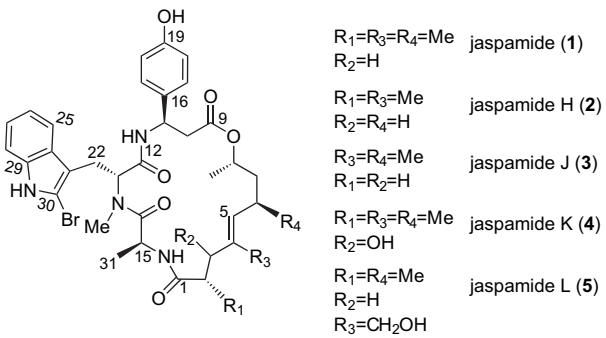
Table 1
^{1}H and ^{13}C NMR data (700 MHz, $CDCl_3$) for compounds **2** and **3**

Position	Jaspamide H (**2**)		Jaspamide J (**3**)	
	δ_H[a]	δ_C	δ_H	δ_C
1	—	174.4	—	170.7
2	2.38 m	40.2	2.05, 2.30 m	38.1
3	1.85 m, 2.37 m	43.6	2.38, 2.48 m	31.2
4	—	134.4	—	133.5
5	4.98 t (6.4)	124.6	4.71 ovl	126.6
6	1.48 m, 1.80 m	23.5	2.23 m	29.0
7	1.30 m, 1.51 m	35.8	1.22 m	43.3
8	4.83 m	70.3	4.60 m	70.6
9	—	170.5	—	170.7
10	2.63 dd (7.8, 15.8), 2.76 dd (4.0, 15.8)	40.0	2.63 dd (5.0, 15.0), 2.63 dd (5.1, 15.0)	39.5
11	5.20 m	49.3	5.30 m	48.5
12	—	169.3	—	170.7
13	5.80 dd (6.2, 10.3)	55.4	5.87 dd (5.8, 10.8)	55.1
14	—	175.0	—	174.1
15	4.64 m	46.0	4.74 m	45.6
16	—	133.0	—	132.2
17	7.09 d (8.4)	127.7	7.10 d (8.5)	127.4
18	6.75 d (8.4)	115.7	6.77 d (8.5)	115.2
19	—	155.2	—	154.6
20	6.75 d (8.4)	115.7	6.77 d (8.5)	115.2
21	7.10 d (8.4)	127.7	7.10 d (8.5)	127.4
22	3.29 ovl	23.5	3.24 dd (10.8, 15.4), 3.38 dd (5.8, 15.4)	22.7
23	—	110.9	—	108.7
24	—	127.8	—	127.3
25	7.25 ovl	110.6	7.26 ovl	110.3
26	7.12 t (7.6)	120.6	7.13 t (7.4)	120.3
27	7.14 t (7.4)	122.7	7.16 t (7.6)	122.7
28	7.56 d (7.8)	118.4	7.56 d (8.0)	118.1
29	—	136.5	—	136.0
30	—	109.1	—	109.3
31	0.72 d (6.7)	18.0	0.73 d (6.8)	17.6
Me-2	1.08 d (6.5)	20.3	—	—
Me-4	1.46 s	16.5	1.60 s	18.7
Me-6	—	—	0.82 d (6.6)	22.2
Me-8	1.08 d (6.5)	20.4	1.07 d (6.4)	18.7
Me–Trp	2.95 s	31.0	2.97 s	30.6
NH–Ala	6.53 d (6.3)	—	6.60 d (6.7)	—
NH–Tyr	7.11 ovl	—	7.65 d (8.5)	—
NH–Trp	8.07 s	—	8.00 s	—

Ovl: overlapped.
[a] Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, TOCSY, HSQC, and HMBC experiments.

from jaspamide. The results revealed the amino acids to be L-Ala, D-β-Tyr, and D-*N*-methylabrine.

As evidenced by mass data, jaspamide J (**3**) is an isomer of jaspamide H (HRESIMS calcd for $C_{35}H_{44}BrN_4O_6$: 695.2444–697.2424; found 695.2438 $[M+H]^+$). Also in this case the signals relative to the tripeptide portion were almost superimposable to the corresponding signals in jaspamide (Table 1). The analysis of the COSY data revealed a spin system composed by two adjacent methylene groups: δ_H 2.05–2.30 (δ_C 38.1) and 2.38–2.48 (δ_C 31.2). Key HMBC



R_1=R_3=R_4=Me, R_2=H — jaspamide (**1**)
R_1=R_3=Me, R_2=R_4=H — jaspamide H (**2**)
R_3=R_4=Me, R_1=R_2=H — jaspamide J (**3**)
R_1=R_3=R_4=Me, R_2=OH — jaspamide K (**4**)
R_1=R_4=Me, R_2=H, R_3=CH_2OH — jaspamide L (**5**)

Figure 1. Jaspamide derivatives from *Jaspis splendans*.

cross-peaks between the methylene group at δ_H 2.05–2.30 and the acyl group at δ 170.7 and between the vinylic methyl Me-4 and the carbon at δ_C 31.2 allowed to assign the CH_2–CH_2 unit as C2–C3 in 8-hydroxy 4,6-dimethylnon-4-enoic acid subunit. Therefore, jaspamide J (**3**) is the 2-demethyl analogue of jaspamide. Absolute configurations of the amino acid residues were determined by Marfey's method, as described before.

The molecular formula of jaspamide K (**4**) was established by HRESIMS as $C_{36}H_{45}BrN_4O_7$. Compared with the molecular formula of **1**, there was the gain of one oxygen atom. The analysis of the NMR data clearly indicated that the tripeptide portion of **4** is the same of jaspamide (Table 2). The ^{1}H NMR spectrum showed a methine carbinol signal at δ 3.98 (d, J=10.3 Hz). In the COSY spectrum this signal was found to correlate with an allylic methine at δ 2.41, in turn coupled with the methyl protons at δ 1.27. Key HMBC correlations between the carbinol carbon signal at δ 77.3 with the vinylic proton at C-5, with the vinylic methyl protons at C-4 and with the methyl protons at δ 1.27, in turn long range coupled with the C-1 acyl carbon at δ 173.3 allowed the straightforward localization of the extra hydroxyl group on the C-3 of the polypropionate unit. The introduction of a hydroxyl group determines, as expected, a downfield shift of the β-carbon atoms C2, C4 with respect to the parent compound (δ_C 47.6 vs 40.1 in **1**; 138.6

Table 2
^{1}H and ^{13}C NMR data (700 MHz, $CDCl_3$) for compounds **4** and **5**

Position	Jaspamide K (**4**)		Position	Jaspamide L (**5**)	
	δ_H[a]	δ_C		δ_H[a]	δ_C
1	—	173.3	1	—	175.8
2	2.41 m	47.6	2	2.66 m	41.2
3	3.98 d (10.3)	77.3	3	2.10, 2.40 m	35.4
4	—	138.6	4	—	137.0
5	5.20 d (9.3)	130.9	5	5.01 d (10.0)	132.7
6	2.25 m	29.0	6	2.40 m	28.8
7	1.24 m, 1.49 m	43.4	7	1.30 m, 1.37 m	43.1
8	4.68 m	70.2	8	4.55 m	70.2
9	—	170.3	9	—	170.1
10	2.62 dd (7.7, 15.4), 2.72 dd (4.3, 15.4)	40.7	10	2.60 dd (7.5, 15.7), 2.76 dd (4.4, 15.7)	39.8
11	5.27 m	49.2	11	5.23 m	48.9
12	—	168.6	12	—	168.6
13	5.65 dd (7.0, 9.6)	55.5	13	5.70 dd (7.0, 9.5)	54.9
14	—	174.2	14	—	173.8
15	4.57 m	46.1	15	4.66 m	45.8
16	—	132.3	16	—	132.1
17	6.96 d (8.3)	127.7	17	7.02 d (8.1)	127.2
18	6.70 d (8.3)	115.7	18	6.73 d (8.1)	115.2
19	—	155.2	19	—	154.8
20	6.70 d (8.3)	115.7	20	6.73 d (8.1)	115.2
21	6.96 d (8.3)	127.7	21	7.02 d (8.1)	127.2
22	3.19 dd (9.6, 15.2), 3.29 dd (7.0, 15.2)	23.8	22	3.22 dd (9.5, 15.0), 3.31 dd (7.0, 15.0)	23.0
23	—	109.9	23	—	109.5
24	—	127.1	24	—	127.3
25	7.23 ovl	110.5	25	7.26 ovl	110.3
26	7.09 t (7.4)	120.6	26	7.11 t (7.9)	120.1
27	7.14 t (7.4)	122.7	27	7.16 t (7.1)	122.3
28	7.51 d (7.9)	118.5	28	7.54 d (7.7)	118.0
29	—	136.2	29	—	135.8
30	—	109.3	30	—	110.0
31	0.78 d (6.2)	18.1	31	0.82 d (6.5)	17.6
Me-2	1.27 d (7.0)	16.7	Me-2	1.19 d (7.1)	20.0
Me-4	1.69 s	16.5	CH_2OH-4	3.87 d (11.8), 4.18 d (11.8)	61.7
Me-6	0.88 d (6.7)	21.6	Me-6	0.88 d (6.5)	22.1
Me-8	1.11 d (6.8)	20.0	Me-8	1.12 d (6.2)	18.6
Me–Trp	2.98 s	31.0	Me–Trp	2.98 s	30.5
NH–Ala	6.57 d (6.5)	—	NH–Ala	6.55 d (6.5)	—
NH–Tyr	7.03 d (7.6)	—	NH–Tyr	n.o.	—
NH–Trp	8.03 s		NH–Trp	8.16 s	—

Ovl: overlapped; n.o.: not observed.
[a] Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, TOCSY, HSQC, and HMBC experiments.

vs 131.1 in **1**, respectively) and the upfield shift of the γ-carbon nuclei C1, Me-2, Me-4 (δ_C 173.3 vs 175.1; 16.7 vs 20.3; 16.7 vs 18.5, respectively). The decomposition of the natural product under usual Mosher's derivatization conditions hampered the definition of the absolute configuration of the secondary alcoholic function in **4**. Absolute configurations of the amino acid residues were determined by Marfey's method, as described before.

Jaspamide L (**5**) was obtained as colorless amorphous solid and showed the pseudomolecular ion peak at *m*/*z* 725.2561 in the HRESIMS spectrum, corresponding to the molecular formula $C_{36}H_{45}BrN_4O_7$, indicating the presence of an additional oxygen atom compared to **1**. Inspection of 1D and 2D NMR data indicated that jaspamide shares the same tripeptide portion as **1** (Table 2). The vinyl methyl group (δ_H 1.60, δ_C 19.0) in **1** disappeared, replaced by an AB methylene system at δ 3.87 (d, J=11.8 Hz) and 4.18 (d, J=11.8 Hz). These two carbinol protons showed HMBC correlations with both olefinic carbon atoms at δ 137.0 and 132.7 and with the allylic carbon at δ 35.4. Therefore jaspamide L is a derivative of jaspamide in which the 4-methyl group is oxidized to an hydroxymethyl group. The *Z* configuration of 4,5-double bond was assigned on the basis of the ROESY correlation between the carbinol protons and the methyl group at C-6.

The IC_{50} values of jaspamides H–L against HT-29 and MCF-7 tumor cell lines were reported in Table 3. For all of the jaspamide compounds at the IC_{50} dose, there were extensive changes in microfilament morphology with many loci of actin aggregation and loss of organization in the stress fibers.

A comparison between the IC_{50} values of all jaspamides so far tested indicated that jaspamides F,[12] H, and J, lacking a methyl group in the polypropionate subunit, showed a reduced antiproliferative activity. This finding confirms the hypothesis formulated by Maier et al.[7c,d] that points the role of 1,3 dimethyl groups in imposing conformational constraints on the macrocycle conformation. On the contrary the introduction of a polar functional group (e.g., jaspamides G,[12] K, and L) in the polypropionate unit seems to influence in modest extent the antiproliferative and antimicrofilament activity.

3. Experimental

3.1. General experimental procedures

Specific rotations were measured on a Perkin–Elmer 243 B polarimeter. High-resolution ESIMS spectra were performed with a Micromass QTOF Micro mass spectrometer. ESIMS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 500 and Varian Inova 700 NMR spectrometers (^{1}H at 500 and 700 MHz, ^{13}C at 125 and 175 MHz, respectively) equipped with a Sun hardware, δ (ppm), J in hertz, spectra referred to $CDCl_3$ as internal standards (δ_H=7.26). HPLC was performed using a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401.

All reagents were commercially obtained (Aldrich, Fluka) at the highest commercial quality and used without further purification except where noted. All reactions were monitored by TLC on silica gel plates (Macherey-Nagel).

Table 3
Cytotoxic activity of jaspamide H–L (**2**–**5**)

Compound	Cell line IC_{50} (μM)[a]	
	MCF-7	HT-29
Jaspamide (**1**)	0.019±0	0.035±0
Jaspamide H (**2**)	30±0	Not tested
Jaspamide J (**3**)	5.0±0	Not tested
Jaspamide K (**4**)	0.48±0.09	0.90±0.07
Jaspamide L (**5**)	0.61±0	Not tested

[a] MCF-7: human breast adenocarcinoma; HT-29: colon carcinoma.

3.2. Sponge material and separation of individual peptides

J. splendans (order Choristidae, family Jaspidae) was collected at a depth of 15–20 m at Tonga, in the Vanuatu Island, in June 1996. The samples were frozen immediately after collection and lyophilized to yield 360 g of dry mass. Taxonomic identification was performed by Prof. John Hooper of Queensland Museum, Brisbane, Australia, and reference specimens are on file (R1646) at the ORSTOM Centre of Noumea.

The lyophilized material (360 g) was extracted with methanol (4×2.5 L) at room temperature and the crude methanolic extract (90 g) was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. The chloroform-soluble material (ca. 3.2 g) was chromatographed by silica gel MPLC (Macherey-Nagel 200–400 mesh, eluting with CH_2Cl_2/MeOH 0–10%), the fractions were collected on the basis of their TLC retention times.

Fraction 5 was purified by HPLC on a μ-Bondapack C18 column (10 μ, 300×7.8 mm, 4.0 mL/min) with 65% MeOH/H_2O as eluent to give 96.4 mg of jaspamide (t_R=20 min). The baseline was collected and further purified on a Phenomenex Luna C18 column (3 μ, 150×4.6 mm, 1.0 mL/min) with 65% MeOH/H_2O as eluent to give 1.7 mg of jaspamide H (t_R=25.4 min) and a peak at t_R=31.8 min. This latter was purified by HPLC on Phenomenex Luna C18 column (3 μ, 150×4.6 mm, 1.0 mL/min) with 62% MeOH/H_2O as eluent to give 0.5 mg of jaspamide J (t_R=75.7 min).

Fraction 6 was purified by HPLC on Phenomenex Luna C18 column (3 μ, 150×4.6 mm, 1.0 mL/min) with 65% MeOH/H_2O as eluent to give 23.2 mg of jaspamide (t_R=40.5 min) together with two peaks at t_R=12.6 min and 25.8 min containing jaspamide M and L, respectively. The compound at t_R=25.8 min was purified on Phenomenex Luna C18 column (3 μ, 150×4.6 mm, 1.0 mL/min) with 63% MeOH/H_2O as eluent to give 0.5 mg of jaspamide L (t_R=36.9 min) whereas compound at t_R=12.6 min was purified on Phenomenex Luna C18 column (3 μ, 150×4.6 mm, 1.0 mL/min) with 65% MeOH/H_2O as eluent to give 0.9 mg of jaspamide M (t_R=12.3 min).

Fraction 7 was purified by HPLC on a Phenomenex Luna C18 column (3 μ, 150×4.6 mm, 1.0 mL/min) with 65% MeOH/H_2O as eluent to give 1.8 mg of jaspamide K (t_R=12.1 min) and 2.5 mg of jaspamide (t_R=58 min).

3.3. Characteristic data for each compound

3.3.1. Jaspamide H

White amorphous solid; $[\alpha]_D^{25}$ +1.82 (*c* 0.1, chloroform); ^{1}H and ^{13}C NMR data in $CDCl_3$ given in Table 1; ESIMS: *m*/*z* (%) 695.6–697.6 (20) $[M+H]^+$, 717.3–719.3 (100) $[M+Na]^+$. HRMS (ESI): calcd for $C_{35}H_{44}BrN_4O_6$: 695.2444–697.2424; found 695.2452 $[M+H]^+$.

3.3.2. Jaspamide J

White amorphous solid; $[\alpha]_D^{25}$ −4.1 (*c* 0.02, chloroform); ^{1}H and ^{13}C NMR data in $CDCl_3$ given in Table 1; ESIMS: *m*/*z* (%) 695.5–697.6 (20) $[M+H]^+$, 717.5–719.6 (100) $[M+Na]^+$. HRMS (ESI): calcd for $C_{35}H_{44}BrN_4O_6$: 695.2444–697.2424; found 695.2438 $[M+H]^+$.

3.3.3. Jaspamide K

White amorphous solid; $[\alpha]_D^{25}$ −35.0 (*c* 0.02, chloroform); ^{1}H and ^{13}C NMR data in $CDCl_3$ given in Table 2; ESIMS: *m*/*z* (%) 725.7–727.6

(20) $[M+H]^+$, 747.5–749.5 (100) $[M+Na]^+$. HRMS (ESI): calcd for $C_{36}H_{46}BrN_4O_7$: 725.2550–727.2529; found 725.2562 $[M+H]^+$.

3.3.4. Jaspamide L

White amorphous solid; $[\alpha]_D^{25}$ −4.0 (*c* 0.02, chloroform); ^1H and ^{13}C NMR data in $CDCl_3$ given in Table 2; ESIMS: *m*/*z* (%) 725.7–727.6 (20) $[M+H]^+$, 747.6–749.5 (100) $[M+Na]^+$. HRMS (ESI): calcd for $C_{36}H_{46}BrN_4O_7$: 725.2550–727.2529; found 725.2561 $[M+H]^+$.

3.4. Determination of absolute stereochemistry

3.4.1. Peptide hydrolysis

Peptide samples (200 μg) were dissolved in degassed 6 M HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed in vacuo and the resulting material was subjected to further derivatization.

3.4.2. LC–MS analysis of Marfey's (FDAA) derivatives

A portion of the hydrolyzate mixture (800 μg) or the amino acid standard (500 μg) was dissolved in 80 μL of a 2:3 solution of TEA/MeCN and treated with 75 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in 1:2 MeCN/acetone. The vials were heated at 70 °C for 1 h, and the contents were neutralized with 0.2 M HCl (50 μL) after cooling to room temperature. An aliquot of the L-FDAA (or D-FDAA) derivative was dried under vacuum, diluted with MeCN/5% HCOOH in H_2O (1:1), and separated on a Vydac C18 (25×1.8 mm i.d.) column by means of a linear gradient from 10% to 50% aqueous acetonitrile containing 5% formic acid and 0.05% trifluoroacetic acid, over 45 min at 1 mL/min. The RP-HPLC system was connected to the electrospray ion source by inserting a splitter valve and the flow going into the mass spectrometer source was set at a value of 100 μL/min. Mass spectra were acquired in positive ion detection mode (*m*/*z* interval of 320–900) and the data were analyzed using the suite of programs Xcalibur (ThermoQuest, San José, California); all masses were reported as average values. Capillary temperature was set at 280 °C, capillary voltage at 37 V, tube lens offset at 50 V, and ion spray voltage at 5 V.

Retention times of authentic FDAA-amino acids (min): L-Ala (16.6 min), D-Ala (20.5 min).

The hydrolyzate of Jaspamides H–L contained L-Ala (16.6 min).

3.4.3. Determination of the absolute stereochemistry of aromatic amino acids in jaspamides H–L

To determine the absolute configuration of *N*-methylabrine and β-Tyr, an authentic sample of Jaspamide was used as standard.

A stream of ozone in O_2 was bubbled through cooled solutions of jaspamides H–L (0.2 mg) or of Jaspamide (0.5 mg) in MeOH (0.5 mL) at −78 °C for 1 h. Hydrogen peroxide (35%, 10 drops) was added to the reaction mixture, which was then allowed to stand at room temperature overnight. The solvent was removed under a stream of N_2. The ozonolysis product of jaspamides H–L and jaspamide were then dissolved in degassed 6 M HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed in vacuo. The resulting material from jaspamide was subjected to Marfey's derivatization with L- and D-FDAA and LC–MS analysis.

Retention times of authentic FDAA-amino acids from jaspamide: L-MeAsp (14.2 min), D-MeAsp (16.3 min), L-Asp (15.6 min), D-Asp (16.8 min).

The hydrolyzate of jaspamides H–L contained D-Asp (16.8 min) and D-MeAsp (16.3 min).

3.5. Cytotoxicity assays

The human tumor cell lines MCF-7 (breast adenocarcinoma) and HT-29 (colon carcinoma) were obtained from the American Type Culture Collection and grown in DMEM medium containing 10% fetal bovine serum and 50 μg/mL gentamycin following standard techniques. To determine the effects of the test compounds on proliferation, cells were plated into 96-well microtiter plates and allowed to attach for 24 h. Varying concentrations (0.05–50 μM) of the test compounds were added to individual wells and the cells were incubated for an additional 72 h. At the end of this period, the number of viable cells was determined using the sulforhodamine-binding assay.

3.6. Immunofluorescence assays

A-10 cells were grown to near-confluency on glass coverslips and treated with the indicated compounds for 24 h. Microtubules and microfilaments were stained with monoclonal anti-β-tubulin and monoclonal anti-actin antibodies, respectively, and visualized with fluorescein-conjugated anti-mouse IgG.

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