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Stellatolides, a New Cyclodepsipeptide Family from the Sponge *Ecionemia acervus*: Isolation, Solid-Phase Total Synthesis, and Full Structural Assignment of Stellatolide A

María Jesús Martín,[†] Raquel Rodríguez-Acebes,[†] Yésica García-Ramos,[‡,§] Valentín Martínez,[†] Carmen Murcia,[†] Isabel Digón,[†] Isabel Marco,[†,▽] Marta Pelay-Gimeno,[‡,§] Rogelio Fernández,[†] Fernando Reyes,[†,▲] Andrés M. Francesch,[†] Simon Munt,[†] Judit Tulla-Puche,*[,‡,§] Fernando Albericio,[‡,§,∥,⊥] and Carmen Cuevas*[,†]

[†]Research and Development, PharmaMar, S. A., Avenida de los Reyes 1, 28770 Colmenar Viejo, Madrid, Spain

[‡]Institute for Research in Biomedicine, Baldiri Reixac 10, 08028 Barcelona, Spain

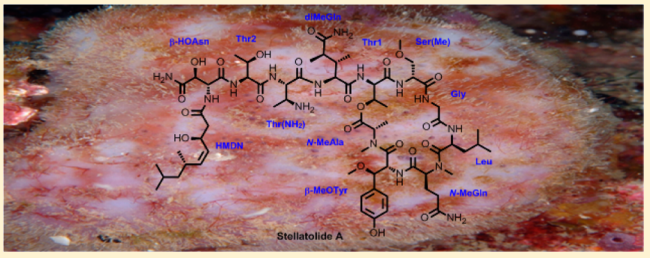
[§]Biomedical Research Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain

[∥]Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

[⊥]School of Chemistry & Physics, University of Kwazulu-Natal, Durban 4001, South Africa

Supporting Information

ABSTRACT: The marine environment is a rich source of metabolites with potential therapeutic properties and applications for humans. Here we describe the first isolation, solid-phase total synthesis, and full structural assignment of a new class of cyclodepsipeptides from the Madagascan sponge *Ecionemia acervus* that shows in vitro cytotoxic activities at submicromolar concentrations. Seven structures belonging to a new family of compounds, given the general name stellatolides, were characterized. The sequence and stereochemistry of all the amino acids in these molecules were established by a combination of spectroscopic analysis, chemical degradation, and derivatization studies. Furthermore, the complete structure of stellatolide A was confirmed by an efficient solid-phase method for the first total synthesis and the full structural assignment of this molecule, including the asymmetric synthesis of the unique β-hydroxy acid moiety (*Z*)-3-hydroxy-6,8-dimethylnon-4-enoic acid.



INTRODUCTION

Cyclodepsipeptides from marine organisms have emerged as a key class of bioactive compounds.[1] A range of biological activities, including cytotoxic, antiviral, and/or antifungal properties, have been observed for cyclodepsipeptides isolated from various marine sponges. These compounds include callipeltins A,[2] B–C,[3] D–E,[4] F–I,[5] and J–M,[6] papuamides A–D[7] and E–F,[8] neamphamides A[9] and B,[10] theopapuamides A[11] and B–D,[12] mirabamides A–D[13] and E–H,[14] homophymines A,[15] B–E and A1–E1,[16] and pipecolidepsins A–C.[17] From a chemical point of view, these cyclodepsipeptides show intriguing structures and their synthesis poses a challenge.

During the course of our ongoing screening program to identify new antitumor agents from marine organisms, extracts of the Madagascan sponge *Ecionemia acervus* were found to display cytotoxic activity against human tumor cell lines. Bioassay-guided fractionation of the aqueous extract of a specimen of this organism yielded a novel family of cyclic depsiundecapeptides named stellatolides as bioactive components. Of these, stellatolide A (**1**) (Figure 1) was the most abundant peptide isolated and was found to have a number of unique structural features compared to other marine cyclodepsipeptides. The 22-membered macrocycle of stellatolide A is

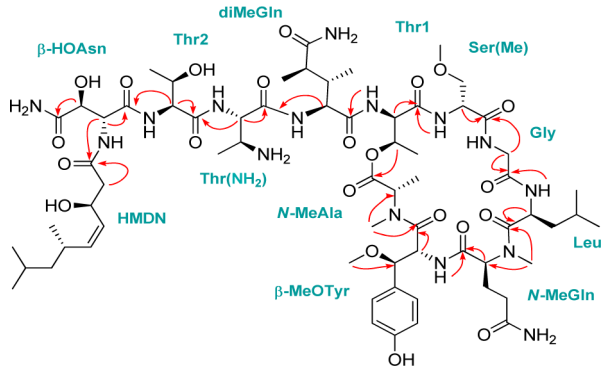


Figure 1. Chemical structure and selected HMBC correlations for stellatolide A (**1**).

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Table 1. ^{1}H and ^{13}C NMR Data[a] for Stellatolide A

position	δ_C, ppm	δ_H, ppm (mult, J in Hz)[b]	position	δ_C, ppm	δ_H, ppm (mult, J in Hz)[b]
	N-MeAla			diMeGln	
1	171.6		1	173.9	
2	51.8	5.44 (q, 7.1)	2	59.0	4.11 (d, 9.2)
3	13.2	1.29 (d, 7.1)	3	37.0	2.46 (m)
NMe	29.5	2.69 (s)	3-Me	17.2	1.31 (d, 6.9)
	β-MeOTyr		4	44.9	2.71 (m)
1	169.9		4-Me	14.0	1.33 (d, 7.2)
2	53.7	4.93 (d, 9.5)	5	182.1	
3	84.3	4.55 (d, 9.5)	NH		
4	129.4		NH_2		7.84 (br s), 7.10 (br s)[c]
5/5′	131.4	7.18 (d, 8.6)		Thr(NH_2)	
6/6′	116.0	6.80 (d, 8.6)	1	171.2	
7	158.7		2	56.4	4.49 (m)
MeO	56.9	3.11 (s)	3	49.1	3.97 (m)
NH		8.15 (d, 10.2)	4	16.8	1.46 (d, 6.9)
	N-MeGln		NH		
1	170.7		NH_2		
2	55.6	4.79 (dd, 9.5, 6.6)		Thr2	
3	25.0	1.64 (m), 1.27 (m)	1	173.3	
4	32.0	1.68 (m)	2	60.0	4.35 (d, 2.4)
5	177.7		3	67.4	4.48 (m)
NH_2		7.78 (br s), 7.34 (br s)	4	20.4	1.21 (d, 6.4)
NMe	30.3	2.93 (s)	NH		8.49 (d, 6.2)
	Leu			β-HOAsn	
1	174.0		1	171.9	
2	49.2	4.73 (dd, 10.8, 3.5)	2	59.1	4.75 (d, 4.2)
3	40.5	1.67 (m), 1.25 (m)	3	72.5	4.61 (d, 4.2)
4	26.1	1.67 (m)	4	176.5	
5	23.6	0.96 (d, 6.6)	NH		
5-Me	21.4	0.91 (d, 6.0)	NH_2		7.07 (br s), 6.80 (br s)[c]
NH		7.21 (d, 8.4)		HDMN	
	Gly		1	174.8	
1	172.2		2	45.3	2.59 (dd, 13.8, 9.4), 2.37 (dd, 13.7, 3.9)
2	44.1	3.96 (d, 17.1), 3.51 (d, 17.1)	3	66.5	4.88 (m)
NH		9.08 (m)	4	130.9	5.37 (dd, 10.8, 9.0)
	Ser(Me)		5	139.4	5.22 (dd, 10.8, 10.8)
1	172.7		6	31.3	2.64 (m)
2	55.2	4.51 (m)	6-Me	22.0	0.94 (d, 6.6)
3	71.5	3.80 (dd, 10.0, 7.1), 3.77 (dd, 10.0, 7.1)	7	48.1	1.15 (m)
MeO	59.5	3.40 (s)	8	26.9	1.57 (m)
NH		7.83 (d, 8.9)	8-Me	23.5	0.90 (d, 6.5)
	Thr1		9	23.0	0.90 (d, 6.4)
1	172.8				
2	57.3	5.21 (d, 3.1)			
3	71.6	5.60 (qd, 6.3, 3.1)			
4	14.8	1.19 (d, 6.3)			
NH		8.92 (d, 10.3)			

[a]NMR data were taken in CD_3OD at 500 and 125 MHz. [b]NH and NH_2 chemical shifts, multiplicities, and correlations were determined through experiments in CD_3OH. [c]Assignments of NH_2 are interchangeable.

made up of seven subunits and is connected through an amide bond to a linear side chain comprising a complex tetrapeptide terminated by a β-hydroxy acid. Four unusual nonproteinogenic α-amino acid residues are present in the molecule, namely, β-methoxytyrosine, 3,4-dimethylglutamine, 2,3-diaminobutanoic acid, and 2,4-diamino-3-hydroxy-4-oxobutanoic acid (β-hydroxyasparagine). Its structure also contains a unique β-hydroxy acid moiety that blocks the N-terminus, while the C-terminus is lactonized via a D-*allo*-Thr residue. Here we report the isolation and structural elucidation of stellatolides. In addition, as part of our efforts to improve the availability of stellatolide A and explore its potential therapeutic applications, we describe an efficient solid-phase method for the first total synthesis of this compound in multigram scale. This last achievement allowed full stereochemical assignment, as well as the development of an asymmetric synthesis of the unique β-hydroxy acid moiety, (*Z*)-3-hydroxy-6,8-dimethylnon-4-enoic acid.

RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Stellatolide A. The molecular formula of **1** was established as $C_{66}H_{107}N_{15}O_{22}$ by positive-ion high-resolution electrospray ionization time-of-flight mass spectrometry [(+)-HR-ESI-TOF-MS], where the $[M + H]^+$ molecular ion was observed at m/z 1462.7764 (calcd for $C_{66}H_{108}N_{15}O_{22}$, 1462.7787). The peptidic nature of this molecule was evident from the presence of numerous carbonyl carbons (δ_C 174.8−169.9 ppm) in the ^{13}C NMR spectrum obtained in CD_3OD (see Table 1). In addition, the ^{1}H NMR spectrum showed signals corresponding to two methoxy groups at δ_H 3.40 and 3.11 ppm, signals for two N-methyl groups at δ_H 2.93 and 2.69 ppm, and several other methyl groups between δ_H 1.46 and 0.90 ppm. Extensive analysis of the ^{1}H and ^{13}C NMR data of **1**, including correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra, revealed the presence of the common amino acids Leu, Gly, and two Thr residues, together with the following seven nonproteinogenic amino acid moieties: *N*-methylalanine (N-MeAla), β-methoxytyrosine (β-MeOTyr), *N*-methylglutamine (N-MeGln), *O*-methoxyserine [Ser(Me)], 3,4-dimethylglutamine (diMeGln), 2,3-diaminobutanoic acid [Thr(NH_2)], and β-hydroxyasparagine (β-HOAsn). The presence of the amino-linked (*Z*)-3-hydroxy-6,8-dimethylnon-4-enoic acid (HDMN) was also inferred from the NMR spectra. Thus, a contiguous spin system comprising an oxymethine signal (δ_H 4.88 ppm, δ_C 66.5 ppm), a double bond at δ_H 5.37 and 5.22 ppm (δ_C 130.9 and 139.4 ppm), two methylene groups (δ_H 2.59/2.37 ppm, δ_C 45.3 and δ_H 1.15 ppm, δ_C 48.1 ppm), two methines at δ_H 2.64 (δ_C 31.3 ppm) and 1.57 ppm (δ_C 26.9 ppm), and three methyl groups at δ_H 0.94, 0.90, and 0.90 ppm (δ_C 22.0, 23.5, and 23.0 ppm, respectively) was deduced. The sequence of this residue from C_2 to C_9 was established by COSY and HMBC correlations. The C_4−C_5 olefin was assigned a *Z* geometry on the basis of a coupling constant of 10.8 Hz between H_4 and H_5. Correlations in the HMBC spectrum between the methylene at 2.59/2.37 ppm and the carbon resonance at 174.8 ppm indicated the presence of the carbonyl group at C_1 of this residue.

These units were sequenced by use of a combination of HMBC and rotating spectroscopy (ROESY) data. Long-range

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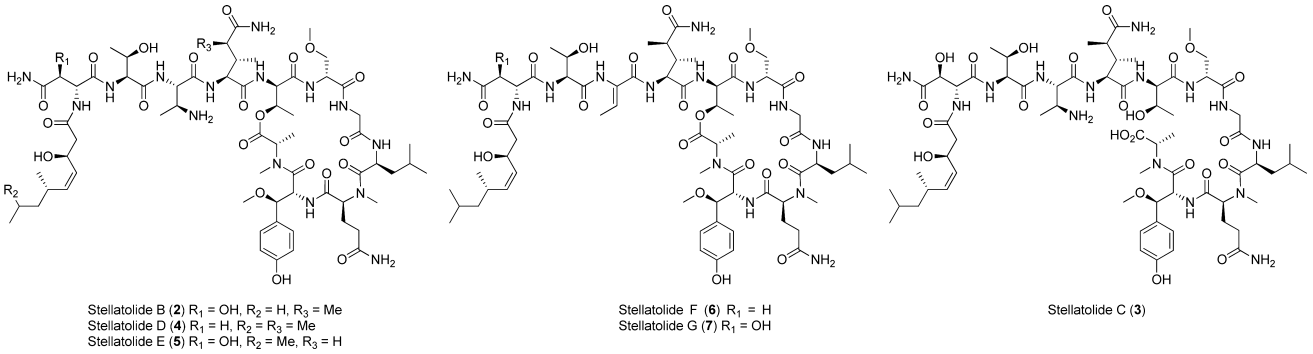

Figure 2. Chemical structures of stellatolides B–G (**2**–**7**).

correlations observed between α-protons and NH protons or N-Me protons and carbonyl carbons of adjacent amino acids (Figure 1) allowed us to establish the sequence as N-MeAla–β-MeOTyr–N-MeGln–Leu–Gly–Ser(Me)–Thr1–diMeGln–Thr(NH_2)–Thr2–β-HOAsn. The presence of an ester bond between the carbonyl group of N-MeAla and the hydroxyl group of the Thr1 residue was suggested by the downfield chemical shift of the H_3 oxymethine proton (δ_H 5.60 ppm) of this last residue[7] and confirmed by the presence of an HMBC correlation between this proton and the N-MeAla carbonyl carbon at δ_C 171.6 ppm. In addition, the acylation of β-HOAsn by HDMN was evidenced by HMBC correlation between the H_2 signal of β-HOAsn at δ_H 4.75 ppm and the carbonyl group of HDMN at δ_C 174.8 ppm.

By use of liquid chromatography/mass spectrometry (LC-MS) and after derivatization with Marfey's reagent, L-FDAA [N^{α}-(4,5-dinitrophenyl-5-fluoro)-L-alaninamide],[18] the absolute configurations of the amino acid residues were determined by chromatographic comparison of the acid hydrolysate of **1** (6 N HCl, 110 °C, 15 h) with appropriate amino acid standards. We assigned the L configuration for N-MeAla, Leu, and N-MeGln residues and the D configuration for Ser(Me) and the two D-*allo*-Thr residues. The configurations of the nonproteinogenic amino acids diMeGln, Thr(NH_2), and β-HOAsn were assigned as (2*S*,3*S*,4*R*), (2*S*,3*S*), and (2*R*,3*S*), respectively, by synthetic preparation of the corresponding standards.[19] To define the chirality of the β-MeOTyr residue, an aliquot of **1** was ozonized. This residue was then transformed to the corresponding β-MeOAsp derivative after work-up by hydrogen peroxide.[20] Hydrolysis, analysis by Marfey's method and comparison with synthetic sample revealed (2*R*,3*R*) as the stereochemistry. The remaining stereochemical uncertainty of **1** concerns the configuration of the two chiral centers in the moiety of HDMN.

Structural Elucidation of Stellatolides B–G. Stellatolide B (**2**) (Figure 2) has a molecular formula of $C_{65}H_{105}N_{15}O_{22}$, according to the (+)-HR-ESI-TOF-MS analysis (*m*/*z* 1448.7653 $[M + H]^+$; calcd for $C_{65}H_{106}N_{15}O_{22}$, 1448.7637). This molecular formula and comparison of ^1H and ^{13}C NMR spectra with those of **1** revealed that the only difference between the compounds was the absence of a methyl group at C_8 in the HDMN moiety of the latter. This observation was supported by analysis of the 1D and 2D NMR data. HPLC analysis of the acid hydrolysate derivatized with Marfey's reagent and comparison with appropriate amino acid samples indicated that all the amino acid residues in **2** had the same absolute configuration as in **1**. The configurations at C_3 and C_6 of the (*Z*)-3-hydroxy-6-methylnon-4-enoic acid (HMN) were assumed to be identical to those of the similar residue found in **1**, whereas the C_4–C_5 olefin was assigned a *Z* geometry on the basis of a coupling constant of 10.9 Hz between H_4 and H_5.

Stellatolide C (**3**) is closely related to **1**. The $[M + H]^+$ at *m*/*z* 1480.7898 (calcd for $C_{66}H_{110}N_{15}O_{23}$, 1480.7899) in the (+)-HR-ESI-TOF-MS mass spectrum, 18 mass units higher than 1, together with ^1H and ^{13}C NMR data pointed to **3** being the acyclic derivative of **1**. The ^1H and ^{13}C NMR spectra were very similar to those of **1**, except for the signals assigned to H_3 and C_3 of the Thr1 residue. In **3** these signals shifted upfield to δ 4.44 and 67.5 ppm (CD_3OD) respectively, relative to the same signal of **1** (δ_H 5.60 ppm and δ_C 71.6 ppm).

As deduced from (+)-HR-ESI-TOF-MS and NMR data, stellatolide D (**4**) (*m*/*z* 1446.7846 $[M + H]^+$; calcd for $C_{66}H_{108}N_{15}O_{21}$, 1446.7844) differed from **1** in that β-HOAsn was replaced by an Asn residue. In the ^1H NMR spectrum of **4**, the signal assigned to the β-hydroxy group of β-HOAsn (δ_H 4.61 ppm) was absent, and a new methylene group at δ_H 2.96/2.73 ppm in the ^1H spectrum and δ_C 37.2 ppm in the ^{13}C data was observed. The stereochemistry of the amino acid residues was established by Marfey's analysis, as described for **1**, and resulted to be the same as **1** for the similar amino acids. An *R* configuration was established for the new Asn residue after Marfey's derivatization and comparison with both *R* and *S* standards.

Stellatolide E (**5**) was concluded to have a molecular formula of $C_{65}H_{105}N_{15}O_{22}$, as deduced from (+)-HR-ESI-TOF-MS data (*m*/*z* 1448.7630 $[M + H]^+$; calcd for $C_{65}H_{106}N_{15}O_{22}$, 1448.7637). NMR data indicated that **5** differs from **1** in the absence of a methyl group at the 3,4-dimethylglutamine residue. The analysis of COSY, TOCSY, and HSQC experiments allowed us to infer that the missing methyl group was at C_4, and the compound has therefore a 3-methylglutamine residue (also present in callipeltins J and K).[6] The absolute configuration of the amino acid residues was determined by Marfey's method and was found to be the same as in **1**, except for 3-methylglutamine, whose absolute configuration was not determined due to the unavailability of appropriate standards.

The molecular formula $C_{66}H_{104}N_{14}O_{21}$ of stellatolide F (**6**), deduced from (+)-HR-ESI-TOF-MS data (*m*/*z* 1429.7636 $[M + H]^+$; calcd for $C_{66}H_{105}N_{14}O_{21}$, 1429.7579), indicated the loss of one ammonia molecule from **4**. The NMR spectra for **6** were consistent with those of **4**, except for the presence of a signal corresponding to a methyl group shifted downfield to δ 1.65 ppm and an olefinic signal present at δ_H 6.79 ppm, indicating that the Thr(NH_2) in **4** was replaced by didehydroaminobutyric acid (Dhb) in **6**. A cross-peak observed in the ROESY experiment (in CD_3CN–H_2O 5:1) between the NH at δ_H

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8.98 ppm and the methyl group at δ_H 1.65 ppm indicated a *Z* geometry for the double bond of the latter residue.

Similarly, and as deduced from (+)-HR-ESI-TOF-MS data and NMR spectra, stellatolide G (**7**) (*m*/*z* 1445.7607 $[M + H]^+$; calcd for $C_{66}H_{105}N_{14}O_{22}$, 1445.7528) differs from **1** by the replacement of Thr(NH_2) by a Dhb residue. The close resemblances of the ^1H and ^{13}C NMR chemical shifts of all the amino acid residues of **6** and **7** with the corresponding values of **1** suggest that chiral centers in the two latter molecules have the same absolute configuration as in **1**.

Total Synthesis. In order to establish the configuration of **1** and to further explore its pharmaceutical development and in vivo evaluation, we undertook the development of a solid-phase synthetic strategy for its preparation. To the best of our knowledge, stellatolide A is one of the few complex *β*-OMeTyr-containing peptides whose synthesis has been attempted.[21] These kinds of peptide are not stable in moderate concentrations of acid, thus leading to the elimination of MeOH from the *β*-MeOTyr residue to give didehydrotyrosine. In addition, and as observed in other depsipeptides of related families, careful attention needs to be paid to transacylation, nitrile formation from Asn/Gln residues, and both pyro-diMeGln and diketopiperazine (DKP) formation.[17b]

Solid-Phase Strategies. First our efforts focused on determining the best methodology and devising the optimal anchoring, and thus the cyclization point (Figure 3). The

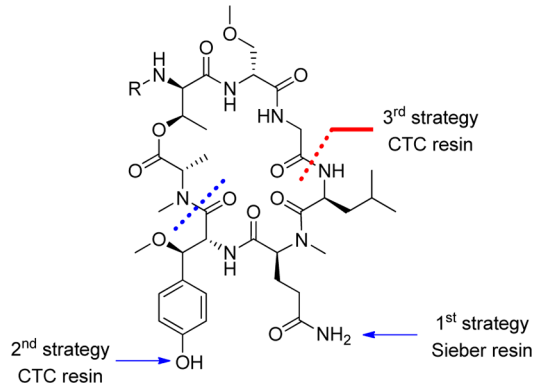
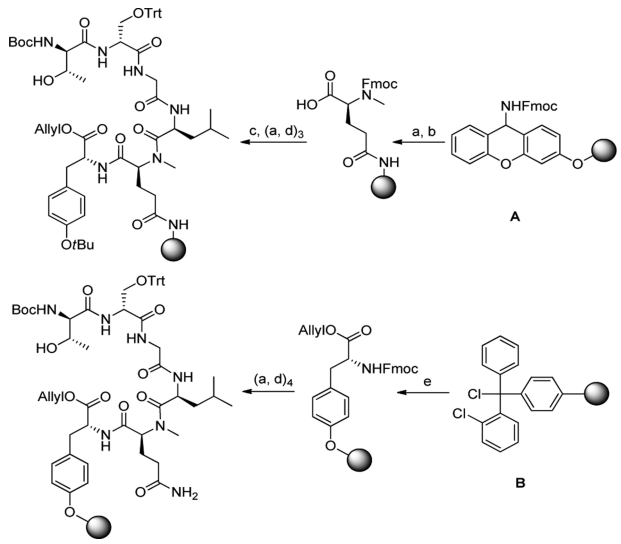


Figure 3. Anchoring and cyclization points (dashed lines).

presence of N-MeGln and the modified Tyr in **1** allows cyclization on solid phase through side-chain anchoring of these two residues (strategies 1 and 2), whereas Gly as a C-terminus entails performing the cyclization in solution (strategy 3). None of these candidate points considers the esterification reaction as the cyclization step because of the slower kinetics of the ester formation, especially in a cyclization reaction, which implies a major risk of racemization.

In the first strategy, following the work of Lipton and coworkers on callipeltin B,[21a] we introduced Fmoc-N-MeGlu anhydride (Fmoc = fluorenylmethyloxycarbonyl) on a Sieber resin in a regioselective manner. This approach allows the release of the Gln-containing peptide by mild trifluoroacetic acid (TFA) cleavage. As the cyclization between N-terminal Tyr and C-terminal Gln would be highly unfavorable because of competing DKP (MeAla–*β*-OMe-D-Tyr)[22] formation, H-D-Tyr-OAllyl[23] was coupled as a second residue to the *α*-carboxyl of Gln. After release of the Fmoc group, the peptide was elongated by standard C to N Fmoc chemistry until the hexapeptide was reached (Scheme 1). HPLC analysis of an aliquot after cleavage from the resin showed a purity of 88%.

Scheme 1. Hexapeptide Synthesis following (A) Strategy 1 and (B) Strategy 2[a]



[a]Conditions: (a) piperidine–DMF (1:4); (b) Fmoc-N-MeGln anhydride, DIPEA; (c) H-D-Tyr(*t*Bu)-OAllyl, HATU, DIPEA; (d) Fmoc-AA-OH, HATU, DIPEA; (e) Fmoc-D-Tyr-OAllyl, DIPEA, CH_2Cl_2.

In the second strategy, Fmoc-D-Tyr-OAllyl was loaded on a 2-chlorotrityl chloride (2-CTC) resin through the phenol, and the peptide was elongated until it reached the same hexapeptide as in the previous strategy. In both cases, Boc-D-Thr-OH was used at the branching position, since the linear arm would not be constructed at this point. In both strategies, after performing the esterification with Alloc-N-MeAla-OH **13**[24] and removing the allyloxycarbonyl (Alloc) and allyl groups, cyclization with benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate (PyBOP, 2 equiv), 1-hydroxy-7-azabenzotriazole (HOAt, 2 equiv), and *N*,*N*-diisopropylethylamine (DIPEA, 4 equiv) in *N*,*N*-dimethylformamide (DMF) was allowed to proceed for 12 h. In both strategies, the cyclic peptide, which was cleaved from the corresponding resins with <5% TFA, was obtained with acceptable purity.

In order to explore strategy 3, Fmoc-Gly-OH was anchored to 2-CTC resin, and the peptide was elongated by introducing D-Ser as the following residue, which was used instead of D-Ser(Me), and Boc-D-Thr, N-MeAla (through esterification), D-Tyr, and N-MeGln. The linear peptide was obtained as a main product but with considerable formation of DKP (N-MeAla–D-Tyr),[22] even when exposure to piperidine was minimized (1 × 3 min) and coupling of the following amino acid (N-MeGln) was performed under neutral conditions [*N*,*N*′-diisopropylcarbodiimide (DIPCDI)/HOAt]. In view of these results, the Alloc group, which is removed in the absence of base, was chosen for protecting the amino function of the D-Tyr residue.

Once elongation of the sequence and cyclization had been addressed, we next studied formation of the central core: elongation of the exocyclic arm, paying special attention to diMeGln, in conjunction with esterification using a model system with Tyr and Ile in place of *β*-MeO–D-Tyr and diMeGln, respectively. First, the pentapeptide was constructed as in strategy 1, for which superior results had been obtained in construction of the cycle. Then Fmoc-D-*allo*-Thr-OH was used in place of Boc-D-Thr-OH to allow growth of the exocyclic arm. Nevertheless,

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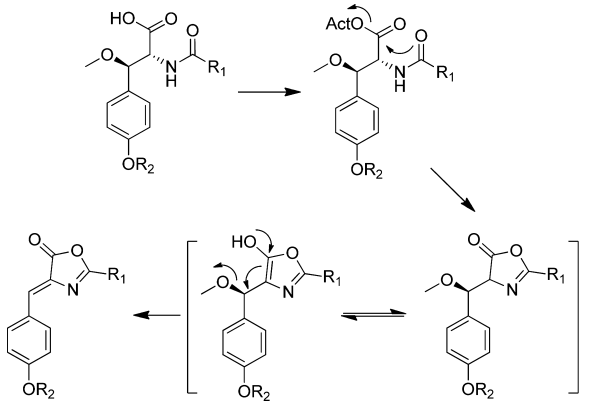
even if the cycle was first formed, when the Ile residue was deprotected, a negative ninhydrin was obtained, showing complete O to N transacylation. The only way it could be minimized was by performing the esterification with N-MeAla after the Fmoc-Ile-OH introduction. In this manner, and although ester formation was more difficult due to steric hindrance of the Fmoc group [10 equiv of N-MeAla, 5 equiv of DIPCDI, and 0.2 equiv of 4-dimethylaminopyridine (DMAP) were needed to show completion], the transacylation reaction was minimized and no racemization of N-MeAla was detected by Marfey's test.

This strategy (first strategy in Figure 3) was finally tested with the key amino acid Alloc-β-MeO-D-Tyr(Trt)-OH **8**. Although the elongation proceeded as planned, the main problem arose at the cyclization step. It was first attempted with PyBOP (2 equiv), HOAt (2 equiv) and DIPEA (4 equiv) in DMF. However, after 2 h, a peak with the same t_R as starting material but a mass that did not correspond to either linear or cyclic peptide (32 units less than the latter) appeared. Moreover, the UV profile was altered drastically from a smooth absorbance at 274.4 nm to an intense peak at 307.7 nm. Numerous attempts involving harsh conditions [(7-azabenzotriazol-1-yl)oxy]tris-(pyrrolidino) phosphonium hexafluorophosphate (PyAOP)/HOAt/DIPEA, Lipton's conditions, PyAOP/HOAt/collidine;[21a] *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/HOAt/DIEA microwave irradiation, heating] gave the same outcome. In contrast, milder conditions (DIPCDI/HOAt, DIPCDI/OxymaPure, Pfp activation) gave two main peaks, one identified as the activated peptide and the other lacking 18 units and upon first glance appearing to be the desired cyclic product. The UV profile, however, was completely different. The use of distinct solvents [DMF, DMF−CH_2Cl_2 (1:1), tetrahydrofuran (THF)] did not change the outcome of the reaction, and the reaction was driven to the byproduct (32 units less) previously observed when the harsher conditions were used.

These side products were formed when β-MeO-D-Tyr was activated, thus preventing reaction of the carboxylic acid and completion of the cycle. Scheme 2 shows the proposed

Scheme 2. Demethoxylation of β-MeO-Tyr



mechanism for the demethoxylation reaction. With activated carboxylate, an intermediate oxazolone is formed, which later undergoes demethoxylation to give the demethoxylated oxazolone.

As the demethoxylation reaction could not be prevented or minimized, it was not possible to obtain **1** by cyclizing at this position (first and second strategies in Figure 3). We envisaged that the solution would be to achieve the participation of the activated carboxyl of β-MeO-D-Tyr in a regular amide bond instead of the slower cyclization reaction. Coupling studies with a model tripeptide designed to simulate the actual peptide supported this notion. Reactivity of the β-MeO-D-Tyr carboxyl functionality was studied by testing coupling conditions to form a standard amide bond with Fmoc-N-MeGlu anhydride coupled to a Sieber resin and H-Gly-OMe as a third amino acid. A 97:3 ratio (product/side product) was obtained when the DIPCDI/HOAt system was used. On the basis of all these previous experiments, we concluded that strategy 3 was the method of choice for the synthesis of **1**.

Further fine-tuning was needed in order to suppress DKP formation, already devised in the simplified model. In addition to using Alloc protection for the β-MeO-D-Tyr residue, other conditions were tested, such as the tandem reaction with Fmoc-N-MeGln-OH. However, only prior elongation of the exocyclic arm before cycle completion effectively eliminated DKP formation. This observation can be explained by the presence of the exocyclic arm, which could imply a hindered environment that slows down reactions, and therefore certain selectivity between straight and side reactions is accomplished.[25]

Synthesis of Building Blocks and (Z)-3-Hydroxy-6,8-dimethylnon-4-enoic Acid. Before peptide assembly on solid phase, six of the component building blocks (Figure 4) were

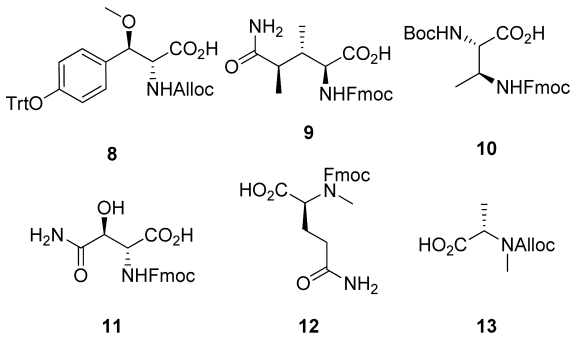


Figure 4. Chemical structures of amino acid building blocks.

stereoselectively synthesized and appropriately protected by an Fmoc strategy, by modification and/or optimization of protocols described in the literature: (2*R*,3*R*)-Alloc-β-MeO-Tyr(Trt)-OH **8**,[26] (2*S*,3*S*,4*R*)-Fmoc-3,4-diMeGln-OH **9**,[27] (2*S*,3*S*)-Fmoc-Thr(NHBoc)-OH **10**,[19b] (2*R*,3*S*)-Fmoc-3-OH-Asn-OH **11**,[28] Fmoc-N-Me-L-Gln **12**,[29] and N-Alloc-N-Me-L-Ala **13**.[30]

The stereoisomerism of the unprecedented HDMN moiety could not be determined by spectroscopic analysis, chemical degradation, or derivatization due to the lack of appropriate standards. Our group developed an asymmetric synthesis to enantioselectively prepare the four possible stereoisomers: (3*R*,6*R*), (3*S*,6*R*), (3*R*,6*S*), and (3*S*,6*S*). The synthesis involved the use of the chiral Evans' oxazolidinone chemistry to form the C_2/C_3 bond as the key step (Scheme 3).

The use of commercially available pseudoephedrine as a chiral auxiliary in the presence of LiCl allows highly diastereoselective alkylations with alkyl halides according to Myers' chemistry.[31] (1*R*,2*R*)-(−)- and (1*S*,2*S*)-(+)-pseudoephedrinepropionamides **14** and **15** were alkylated with 1-iodo-2-methylpropane **16** to give (*S*,*R*,*R*)-**17** and (*R*,*S*,*S*)-**18**, respectively. The latter two compounds were then converted to the corresponding primary alcohols with $LiH_2N{\cdot}BH_3$, and subsequent oxidation with pyridinium chlorochromate (PCC) yielded aldehydes (*S*)-**19** and (*R*)-**20**. A variation of the Horner−Wadsworth−Emmons reaction over (*S*)-**19** and (*R*)-**20** that

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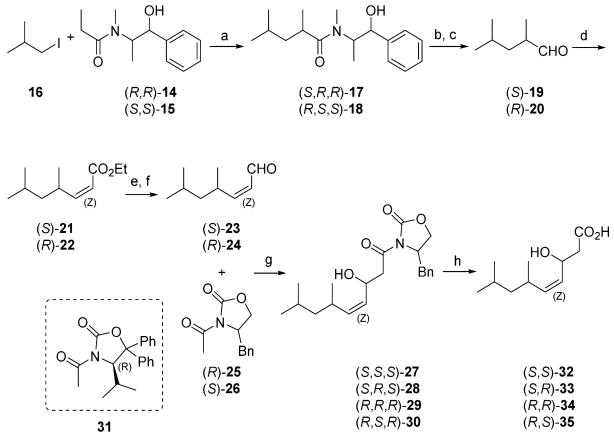
Scheme 3. Synthesis of Four Diastereoisomers of (*Z*)-3-Hydroxy-6,8-dimethylnon-4-enoic acid[a]



[a]Conditions: (a) LDA, LiCl, THF, from −78 to 25 °C, 2 h, 74%; (b) $BH_3{\cdot}NH_3$, LDA, THF, from 0 to 25 °C, 2 h, 95%; (c) PCC, CH_2Cl_2, 25 °C, 2 h, 100%; (d) $(CF_3CH_2O)_2P(O)CH_2CO_2Et$, KHMDS, 18-crown-6, THF, −78 °C, 2 h, 55%; (e) DIBAL, THF, −78 °C, 4 h, 95%; (f) MnO_2, CH_2Cl_2, 25 °C, 2 h, 90%; (g) $TiCl_4$, DIPEA, NMP, −78 °C, 1 h, 50%; (h) LiOH, H_2O_2, THF/H_2O, 0 °C, 2 h, 99%.

employed a phosphonate with electron-withdrawing groups, ethyl 2-[bis(2,2,2-trifluoroethoxy)phosphoryl]acetate, together with strongly dissociating conditions [potassium bis(trimethylsilyl)amide (KHMDS) and 18-crown-6 in THF], yielded exclusively *Z*-alkenes (*S*)-**21** and (*R*)-**22**. Ethyl esters were subjected to a sequence of reduction−oxidation with diisobutylaluminum hydride (DIBAL-H) and manganese(IV) oxide, respectively, to yield aldehydes (*S*)-**23** and (*R*)-**24**. Treatment of the two chiral aldehydes with (*R*)- and (*S*)-4-benzyl-2-oxazolidinone **25** and **26** provided alcohols (*S*,*S*,*S*)-**27**, (*S*,*R*,*S*)-**28**, (*R*,*R*,*R*)-**29**, and (*R*,*S*,*R*)-**30** with 70% enantiomeric excess (ee). The four Mosher esters were made to assign the absolute configuration of the alcohols. In our attempts to improve the enantioselectivity of preparation of the desired acid in bulk, we used various oxazolidinones. The best performance was achieved with (*R*)-3-acetyl-4-isopropyl-5,5-diphenyloxazolidin-2-one **31**, $TiCl_4$, and (−)-sparteine with 95% ee and 75% chemical yield. Hydrolysis with LiOH produced the desired four diastereoisomers of HDMN.

Solid-Phase Synthesis of Stellatolide A. With all the building blocks in hand, we undertook the challenging synthesis of **1**. Our optimized approach for solid-phase synthesis involved the following: (i) cyclization via Gly to Leu, which implied cyclization in solution; (ii) given the lability of *β*-MeOTyr in acidic conditions, the starting Gly was introduced on 2-CTC resin; (iii) Fmoc and Alloc were both used as temporary N^{α} protecting groups (the latter, which is removed under milder conditions than the former, is smaller and shows better coupling, thus minimizing side reactions); (iv) minimal side-chain protection was used: only trityl (Trt) for the Tyr derivative and *N*-*tert*-butoxycarbonyl (Boc) for the Thr(NH_2) incorporation,[32] whereas D-*allo*-Thr, L-Thr, (2*R*,3*S*)-Fmoc-3-OH-Asn **11**, and the terminated *β*-hydroxy acid were introduced without protection of the hydroxyl function; (v) sequential incorporation of N-MeAla and *β*-MeO-D-Tyr to prevent O to N transacylation and pyro-diMeGlu formation; (vi) elongation of the exocyclic arm before conclusion of the endocyclic sequence, in order to minimize DKP formation; (vii) use of HOAt as coupling additive for the most demanding coupling and HOBt to minimize dehydration reactions; (viii) cleavage of the protected peptide from the resins; and (ix) cyclization in solution.

Elongation of the peptide chain was accomplished by stepwise coupling of the corresponding Fmoc-protected amino acids; the amino acids were activated with HOBt or HOAt and DIPCDI in DMF for amide formation. Progress of the amino acid coupling was monitored by the ninhydrin test (Kaiser test), except when couplings were performed over N-Me amino acids. Fmoc removal before each coupling step was achieved by treatment of the resin-bound peptide with a 20% solution of piperidine in DMF. All coupling reactions and deprotections were monitored by cleavage of a small aliquot of resin with 2% TFA in CH_2Cl_2 and reverse-phase HPLC-MS analysis.

The synthesis began by limited anchoring of Fmoc-Gly-OH (0.38 equiv) to 2-CTC resin in the presence of DIPEA (3.3 equiv) **36**. Peptide elongation was accomplished with 4.0 equiv of the Fmoc-protected amino acid, furnishing pentapeptide **37** with a high coupling yield. By using DIPCDI/HOBt, we minimized dehydration of the amide group into a nitrile group (only 7%). An initial plan to proceed to esterification at this point was revised because of the low yield observed in further external chain elongation. As a result, we introduced the residue (2*S*,3*S*)-Fmoc-Thr(NHBoc) **10** before formation of the ester bond. This was achieved by first removing Fmoc-diMeGln, which was efficiently accomplished without intramolecular lactamization of the diMeGln into a pyroglutamic derivative, with only one 3 min deprotection cycle, followed by coupling with **10** in the presence of HOAt.

At this stage and as mentioned before, ester bond formation required the use of a protecting group for the *α*-amino function, such as the Alloc group, which is orthogonal to the Fmoc, and the acid-labile protecting groups present in **37**. N-Alloc-N-Me-L-Ala **13** (10 equiv) was quantitatively incorporated in 2 h when DIPCDI (10 equiv) and DMAP (1.6 equiv) were used, to obtain hexapeptide **38**. Removal of the Alloc group was carried out with $Pd(PPh_3)_4$ in the presence of $PhSiH_3$, and (2*R*,3*R*)-Alloc-*β*-MeO-D-Tyr(Trt)-OH **8** (5 equiv) was coupled in 3 h with 5 equiv of DIPCDI and HOAt to afford heptapeptide **39** with neither racemization nor demethoxylation. Introduction of *β*-MeO-D-Tyr before growth of the exocyclic arm allowed minimization of didehydrotyrosine formation (Scheme 2). At this point, elongation of the exocyclic arm to the last *β*-hydroxy acid, instead of removal of the Alloc group of the Tyr derivative, allowed us to create a hindered environment in which DKP formation is minimized.[25]

The exocyclic arm was sequentially constructed by incorporation of the four hydroxy acids **32**−**35** with the less reactive HOBt coupling additive to give nonapeptides **40**−**43**. The Alloc group was removed from each of them, as described before, and Fmoc-N-Me-L-Gln **12** (4 equiv) and Fmoc-L-Leu (4 equiv) were anchored with a mixture of 4 equiv of HOAt and HATU in the presence of DIPEA (10 equiv) to obtain undecapeptides **44**−**47**, without DKP and dehydration formation.

After removal of the Fmoc group with washes of piperidine−DMF (1:4), the unstable linear peptides were cleaved from the resin [TFA−triisopropylsilane (TIS)−CH_2Cl_2 (1.5:2:96.5), 10 × 1 min] and precipitated over hexane at −78 °C. Macrolactamization of the dried crude product was successfully performed by use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)−HOAt (5:5 equiv) under high dilution conditions in CH_2Cl_2 for 5 h at 0 °C to afford the protected peptides. Subsequent treatment with TFA−TIS−CH_2Cl_2

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(10:2:88) for 45 min led to global deprotection, followed by reverse-phase HPLC purification of peptides **48**–**51** by use of a C18 X-Bridge column and water/MeOH as eluents.

Spectroscopic data of the four diastereoisomers **48**–**51** were compared with those of natural **1**. Disappointingly, they differed significantly from the spectrum of the natural compound. Furthermore, the four synthetic peptides showed a different HPLC retention time and, surprisingly, no in vitro cytotoxic activity. We suspected that the originally proposed structure for **1** was incorrect.

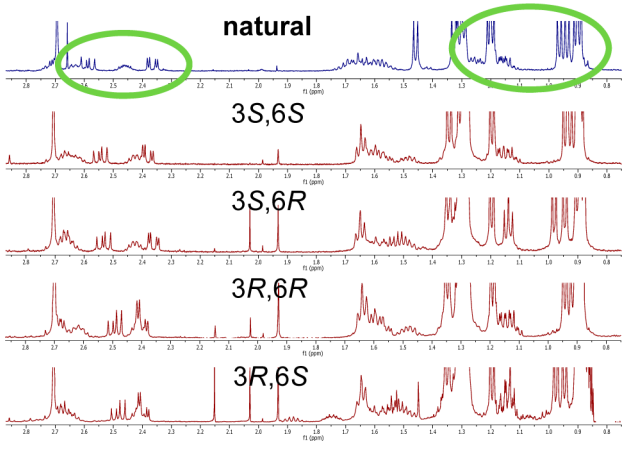
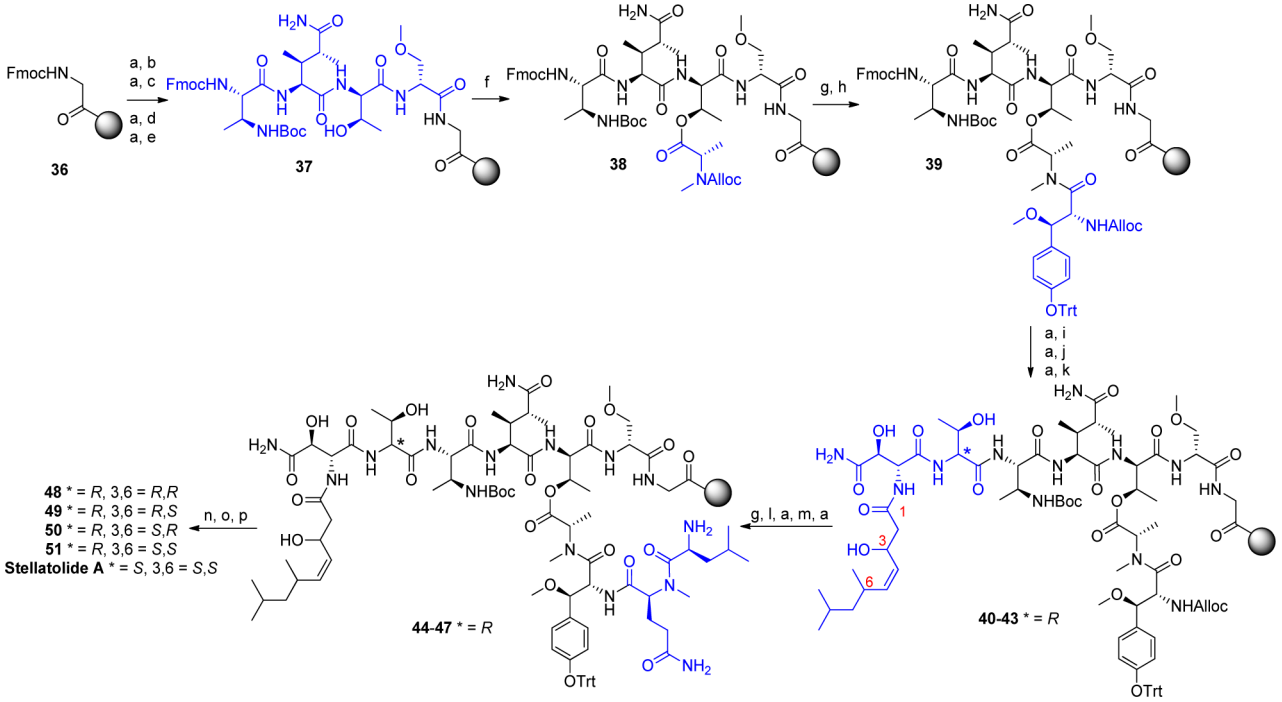


Figure 5. Partial ^{1}H NMR spectra of stellatolide A (**1**) and diastereoisomers **48**–**51**.

Careful examination of ^{1}H NMR spectrum of the synthetic products revealed that the (3*S*,6*S*) configuration is to be expected for the acidic residue. All ^{1}H resonances fell within the corresponding natural product values, and good correlation was also observed for the respective $J_{H,H}$ coupling constants (Figure 5). Having concluded that the absolute configuration of the two chiral centers of the acid moiety was (3*S*,6*S*), we focused our attention on finding the misassignment.

After detailed comparisons of NMR spectra of the four synthetic peptides with those of the natural product, we observed that signal patterns were quite different for the external D-*allo*-Thr. Furthermore, we reconsidered the Marfey's analysis and detected a negligible peak that we considered could correspond to an L-Thr residue. For some unexplained reason, L-Thr in the natural product underwent racemization (or even chemical degradation) under the strong acidic conditions used for peptide hydrolysis before Marfey's derivatization. Furthermore, marine cyclodepsipeptides isolated from sponges with a *β*-hydroxy-*α*-amino acid placed at the branching position usually present a D-*allo* configuration, (2*R*,3*R*)-D-*allo*-Thr being the most frequent residue in callipeltins, neamphamides, theopapuamides, and pipecolidepsin C. In addition, D-*allo*-*β*-hydroxyleucine found in papuamides and mirabamides, and the (2*R*,3*R*,4*R*)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid (D-*allo*-AHDMHA) residue in homophymines and pipecolidepsins A and B also have a 2*R*,3*R* absolute configuration. These two observations strongly suggested that **1** has the same 2*R*,3*R* configuration at the branching position and thus has an L configuration at the second Thr residue (Thr2).

Scheme 4. Solid-Phase Synthesis of Stellatolide A[a]



[a]Conditions: (a) piperidine–DMF (1:4); (b) Fmoc-D-Ser(Me)-OH, HOBt, DIPCDI, DMF; (c) Fmoc-D-allo-Thr-OH, HOBt, DIPCDI, DMF; (d) (2S,3S,4*R*)-Fmoc-diMeGln-OH **9**, HOBt, DIPCDI, DMF; (e) (2*S*,3*S*)-Fmoc-Thr(NHBoc)-OH **10**, HOBt, DIPEA, DMF; (f) N-Alloc-N-Me-L-Ala-OH **13**, DIPCDI, DMAP, CH_2Cl_2; (g) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2; (h) (2*R*,3*R*)-Alloc-*β*-MeO-Tyr(Trt)-OH **8**, DIPCDI, HOAt, DMF; (i) Fmoc-D-Allo-Thr-OH or Fmoc-L-Thr-OH, HOBt, DIPCDI, DMF; (j) (2*R*,3*S*)-Fmoc-3-OH-Asn-OH **11**, HOBt, DIPCDI, DMF; (k) (*Z*)-3-hydroxy-6,8-dimethylnon-4-enoic acid **32**–**35**, HOBt, DIPCDI, DMF; (l) Fmoc-N-Me-L-Gln-OH **12**, HOAt, HATU, DIPEA, DMF; (m) Fmoc-L-Leu, HOAt, HATU, DIPEA, DMF; (n) (i) TFA–TIS–CH_2Cl_2 (1.5:2:96.5), 10 × 1 min, (ii) −78 °C, hexane; (o) HOAt, EDC, CH_2Cl_2, DMF, 0 °C, 5 h; (p) TFA–TIS–CH_2Cl_2 (10:2:88), 25 °C, 45 min.

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Therefore, we performed the synthesis, coupling **39** with the L-Thr residue instead of D-*allo*-Thr, and following the sequence depicted in Scheme 4, coupling with (3*S*,6*S*,*Z*)-3-hydroxy-6,8-dimethyl-4-nonenoic acid **32**. The corresponding synthetic compound and the natural **1** revealed a perfect overlap of the NMR spectra, identical analytical HPLC behavior, and the same in vitro activity (Table 2). This finding highlights the relevance of the chirality of the Thr amino acid in the exocyclic arm for the biological activities exerted by members of the stellatolide family.

Table 2. Cytotoxic Activity Data of Stellatolides A−G

	GI_{50}, μM		
stellatolide	lung, A549	colon, HT-29	breast, MDA-MB-231
A (natural)	0.08	0.43	0.21
A (synthetic)	0.08	0.38	0.26
B	0.64	1.15	0.70
C	4.73	>6.75	>6.75
D	0.21	0.71	0.31
E	0.90	2.69	1.60
F	1.23	>6.48	2.14
G	>6.41	>6.41	>6.41

On the basis of these results, we firmly established that the β-hydroxy acid has an (*S*,*S*) configuration and unequivocally confirmed the presence of the L-Thr residue located in the exocyclic arm, which was clearly difficult to determine by conventional methods. The presence of this residue was then validated by total synthesis.

Biological Evaluation. A bioassay was conducted in parallel during fractionation of the sponge extracts: cell-killing capacity was evaluated against a panel of three human tumor cell lines, including lung (A-549), colon (HT-29), and breast (MDA-MB-231). In vitro antiproliferative activity was assessed by the colorimetric SRB (sulforhodamine B) method, performed as described previously.[33] The GI_{50} (micromolar), a concentration that causes 50% growth inhibition, values obtained for stellatolides in this assay are shown in Table 2. It is interesting to note that stellatolides F and G containing didehydroaminobutyric acid (Dhb) lose much of their potency and the acyclic stellatolide C is also less potent. The other stellatolides showed significant in vitro antiproliferative activity with values of 0.08−2.7 μM.

CONCLUSION

In conclusion, here we isolated seven novel cytotoxic depsipeptides, stellatolides A−G, which contain various non-natural amino acids from extracts of the sponge *Ecionemia acervus*. Their structures were established by spectroscopic methods and corroborated by the first total synthesis of stellatolide A. This synthetic process allowed us establish that for these branched peptides, in addition to the choice of the protecting groups, resins, and coupling reagents, the order of sequence completion of the two arms is crucial for minimizing side reactions. Furthermore, the total synthesis of stellatolide A allowed us to unambiguously establish its absolute configuration, which was initially erroneously misassigned by advanced Marfey's analysis for one of the Thr residues. The strategy presented allows the synthesis of analogues of stellatolides for drug discovery purposes, thus permitting the establishment of the first structure−activity relationships of this promising antitumor family of compounds. Our findings reveal the key role of the absolute configuration of some chiral centers of the exocyclic arm. In this regard, for instance, activity is lost when L-Thr is replaced by D-*allo*-Thr, a finding that is valuable for future research purposes. In addition, the development of an efficient synthesis of stellatolide A on solid support has circumvented the issue of limited availability and allowed us to initiate advanced in vivo evaluation of this compound.

ASSOCIATED CONTENT

Supporting Information

Additional text, structures, and three tables with experimental procedures and spectral and other characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

judit.tulla@irbbarcelona.org
ccuevas@pharmamar.com

Present Addresses

▲(F.R.) Fundación MEDINA, 18016 Granada, Spain.
▽(I.M.) Clarke, Modet & Company, 28001 Madrid, Spain.

Notes

The authors declare the following competing financial interest(s): M.J.M., R.R.-A., V.M., C.M., I.D., R.F., A.M.F., S.M., and C.C. are employees and shareholders of PharmaMar. I.M. and F.R. were employees of PharmaMar.

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(32) First, Fmoc-Thr(N_3)-OH was used as building block **10** because of its easier elaboration. The reduction on solid phase was successful with $SnCl_2$, thiophenol, and DIPEA, but the protection of the new amine was not achieved in high yields. So the alternative was to carry out the reduction in solution after the cyclization step. Several conditions were tested, such as Staudinger reaction, $SnCl_2$ treatment, hydrogenation with Pd, and Lindlar catalysts and Zn, but all of them failed since the quality of the crude product decreased. Finally, the Thr(NH_2) residue was found to be the best choice to facilitate the synthesis of this complex natural depsipeptide.
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