HHS Public Access

Author manuscript

J Org Chem. Author manuscript; available in PMC 2019 March 11.

Published in final edited form as:

J Org Chem. 2017 October 06; 82(19): 10181–10187. doi:10.1021/acs.joc.7b01659.

Cyclic Hexapeptide Dimers, Antatollamides A and B, from the Ascidian, *Didemnum molle*. A Tryptophan-Derived Auxiliary for L- and D-Amino Acid Assignments

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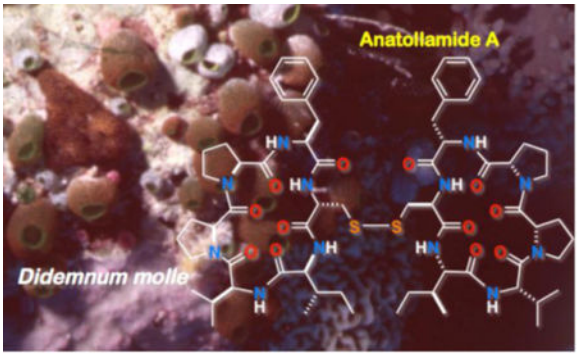
Abstract

Two dimerized cyclic hexapeptides, antatollamides A (**1**) and B (**2**) were isolated from the colonial ascidian *Didemnum molle* collected in Pohnpei. The amino acid compositions and sequences were determined by interpretation of MS and 1D and 2D NMR data. Raney Ni reduction of antatollamide A cleaved the dimer to the corresponding monomeric cyclic hexapeptide with replacement of Cys by Ala. The amino acid configuration of **1** was established, after total hydrolysis, by derivatization with a new chiral reagent, (5-fluoro-2,4-dinitrophenyl)-*N*$^{\alpha}$-L-tryptophanamide (FDTA), prepared from L-Trp, followed by LCMS analysis; all amino acids were found to be L-configured except for D-Ala.

Graphical Abstract



Ascidians are well-known sources of diverse peptides, peptolides and depsipeptides,[i] many with profound biological activities. For example, the antiproliferative aplidine (plitidepsin),

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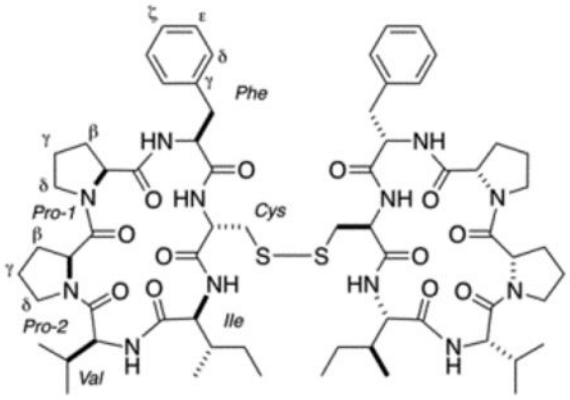
Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI: xxxx. 1D and 2D NMR spectra of **1** and **2**, ^{1}H NMR spectrum of **4** and ^{1}H and ^{13}C NMR spectra of **8**.

Notes.

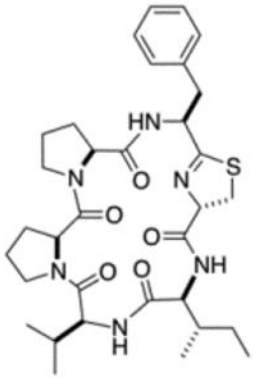
The authors declare no competing financial interest

discovered from a temperate Atlantic species of *Aplidium*, is now in phase II and III trials for treatment of multiple myeloma and T-cell lymphoma.[ii] Various species of *Lissoclinum* and *Didemnum* are the sources of a unique family of ribosomal RNA-derived peptides, including thiazoline-, thiazole-, oxazoline-, and oxazole-containing peptides lissolinamides, patellamides, and ulithiacyclamides: all are now collectively called cyanobactins.[iii] *Didemnum molle*, a common Indo-Pacific colonial tunicate known for its symbiotic relationship with the cyanobacterium, *Prochloron*, is the source of prenylated azole-containing peptides mollamides,[iv,v] and oligomeric phenethylamine/*N*-phenethylureas.5

In our search for antiproliferative and antiinfective natural products from marine invertebrates, we examined *D. molle*, collected from Pohnpei, Federated States of Micronesia and discovered the antatollamides A (**1**) and B (**2**). Peptides **1** and **2**, which are closely related to mollamide F (**3**),[5] are isomeric dimers of cyclic hexapeptides linked through a Cys-Cys disulfide bond and differ only in configuration at the restricted amide bonds of the Pro residues. In the course of these discoveries, we developed a chiral auxiliary; a variant of Marfey's reagent that resolved hitherto difficult or impossible stereoassignments of amino acid residues and was used to determine that the Cys residues of **1** are of the D-configuration while the remaining residues are L-.



1: *s-trans*-Pro-1, *s-cis*-Pro-2
2: *s-cis*-Pro-1, *s-trans*-Pro-2



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RESULTS AND DISCUSSION

Separation of the MeOH-soluble extract of lyophilized *D. molle* by solvent partitioning and reversed phase HPLC gave two new peptides, **1** and **2**. HRMS analysis of **1** showed two prominent sodium adduct molecular ions: $[M+Na]^+$ at *m/z* 1333.6433, and the reduced monomeric form at *m/z* 679.3250 $[M/2+H+Na]^+$, corresponding to formulas $C_{66}H_{94}N_{12}O_{12}S_2$ and $C_{33}H_{48}N_6O_6S$, respectively. Analysis of 1D and 2D NMR data of **1** - including the presence of four amide proton signals (δ 7.04 (d, 9 Hz), 8.43 (d, 6.6 Hz), 7.69 (d, 7.8 Hz) and 8.15 (d, 7.8 Hz)), six α protons (δ 4.24 (t, 7.5), 4.50 (m), 4.39 (t, 8.4), 3.84 (t, 6.6), 4.46 (m) and 4.53 (m)), and six expected amide carbonyl ^{13}C NMR signals (δ 170.6, 170.4, 170.3, 170.2, 169.71 and 169.67) – supported a peptide structure. Exhaustive analysis of HSQC, HMBC, COSY, TOCSY and NOESY data identified six spin systems assigned to the amino acid residues as Cys, two Pro, Ile, Phe, and Val. Due to congestion of the ^{13}C NMR carbonyl signals, which hampered analysis of long-range heteronuclear correlations, the amino acid sequence was established from the NOESY data; specifically, observation of nOe crosspeaks (Figure 1) from the amide NH signals of each residue, *i*, to the adjacent CH_α proton signals of the adjacent residue, *i*−1.

A second dimeric cyclohexapeptide **2**, isomeric with **1**, was separated from the latter by reversed phase HPLC. Analysis of the 1D and 2D NMR data for **2** (Table 1) revealed that both **1** and **2** shared the same amino acid sequence, but exhibited significant differences in the ^1H and ^{13}C NMR chemical shifts of the Pro residues. Pro residues, in particular those involved in oligo-Pro sequences in conformationally restricted cyclic peptides, are known to exist in stable s-*cis* and s-*trans* configurations which interchange on slow time scales. The Pro amide bond configuration is empirically assigned from differences in the ^{13}C NMR chemical shifts (Δδ) of their respective β- and γ-carbons.[vi] Small and large chemical shift differences in the Pro-1 and Pro-2 residues of **1**, respectively ($\Delta\delta_{\beta\gamma}$ = 3.3 and 9.2 ppm), confirmed a *trans-cis* Pro-1–Pro-2 configuration. Conversely, **2** adopts the *cis-trans* Pro-1–Pro-2 configuration ($\Delta\delta_{\beta\gamma}$ = 9.5 and 4.2 ppm). The two diastereomers were stable; no interconversion between **1** and **2** or other isomers was observed over the time frame of these measurements (weeks).

The conformational changes introduced by s-*cis*/s-*trans* isomerism in **1** and **2** dramatically influenced their ECD spectra. The ECD spectrum of **1** (Figure 2) is dominated by three Cotton effects – one positive and two negative (λ 196 nm (Δε +18.7), 204 (−28.4), 221 (−24)) – that arise largely from π-π* exciton transitions of the amide carbonyl groups. In contrast, the signature features in the ECD spectrum of **2** are two considerably weaker positive Cotton effects (λ 196 nm (Δε +13.8), 204 (−5.5) 213 (+7.7)). We have noted earlier that similarly constrained medium ring peptide macrocycles, with embedded Pro residues – for example, syllisamides G and H from the Caribbean sponge, *Stylissa caribica*,[vii] and sanguinamides A and B from the Spanish dancer nudibranch, *Hexabranchus sanguineus*[viii] – display ECD spectra that are exquisitely sensitive to s-*cis*/s-*trans* isomerism.

In theory, dimerism and Pro s-*cis*/s-*trans* isomerism in **1** and **2**, may generate up to 16 diastereomers, however, no evidence of isomers other than **1** and **2** were detected in the extracts of *D. molle*. From this we surmise that the **1** and **2** are not stereorandom isomers,

but the consequence of a thermodynamically favorable “well” resulting from specific non-bonded constraints in the rigid hexapeptide motif.

The absolute configurations of all the individual amino acids of **1** were secured by Marfey’s analysis[ix] using a new variant of his eponymous reagent. Dimeric **1** was cleaved to the monomeric cyclic peptide **4** by desulfurization of the Cys-S-S-Cys unit to two D-Ala residues (Raney Ni, H_2O-MeOH, 65 °C). Inspection of the ^{1}H NMR spectrum of **4** revealed essentially the same signals as **1** with the appearance of a new methyl signal (δ 1.03, d, J = 6.6 Hz, Hβ, Ala). After hydrolysis of **4** (6 M HCl, 110 °C), the amino acid residues were derivatized with FTDA, a versatile, new L-tryptophanamide variant of Marfey’s reagent (see below) and their configurations assigned by comparative analysis by LCMS (reversed phase HPLC, H_2O (0.1% HCOOH)-CH_3CN gradient, see Experimental for details) along with standards. The following residues were identified: L-Pro, L-Val, L-Ile, D-Ala and L-Phe, and confirmed by coinjection with standards. It is notable that the latter conditions failed to separate Ile and L-*allo*-Ile DTA derivatives, however successful resolution (t_R) was obtained with salt buffered mobile phase (Zorbax SB-Aq, H_2O (NH_4OAc)-CH_3CN gradient: L-Ile (34.41), and L-*allo*-Ile (33.38). See Experimental for details). The configurations of the amino acid residues in **2** are presumed to be the same as those of **1**; insufficient sample remained available to analyze **2** independently.

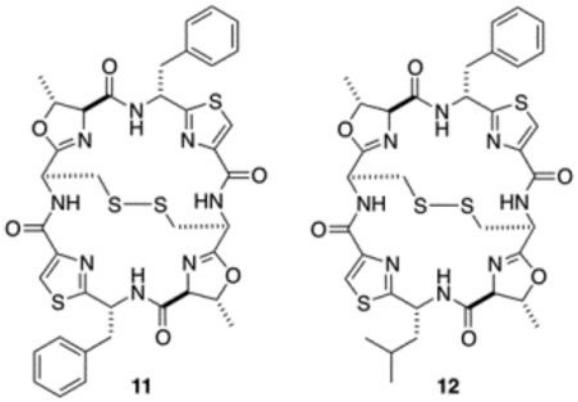
The 5-fluoro-2,4-dinitrophenyl-N^{α}-L-tryptophanamide reagent, FDTA (**8**), was developed as an alternate reagent to the conventional Marfey’s reagent, FDAA,9 for the determination of the absolute configuration of amino acids. Compound **8** was synthesized using a variant of the literature method (Scheme 1).9 L-Trp was converted to the corresponding methyl ester **6** that, in turn, was ammonialysed to the primary amide, L-Trp-NH_2 (**7**, NH_4OH, MeOH, quantitative). S_NAr coupling of **7** (1 equiv) with 1,5-difluoro-2,4-dinitrobenzene delivered FDTA (**8**, m.p. 220 – 222 °C). Reagent **8** was stable at room temperature in the dark with no detectable signs of decomposition after four months. The extinction coefficient of the long-wavelength UV-vis transition of **8** (λ_{max} 339 nm, ε log 4.12) is comparable to Marfey’s reagent (λ_{max} 338 nm, ε log 4.18),9 but the indole chromophore confers π-π* transitions to **8** of comparable intensity at shorter wavelength (e.g. λ_{max} 252, ε log 4.04) that may find utility in detection.

While Marfey’s analysis using the conventional DNP-alaninamide provides good resolution for differentiation of L- and D-enantiomers of non-polar amino acids, it notably performs poorly with some polar amino acids and fails entirely to discriminate certain *N*-Me-amino acids (e.g. L- and D-*N*-Me-Ala, see Table 2),[x,xi] but the corresponding L-DTA derivatives are well-resolved.

Other methods to overcome resolution problems in Marfey’s analysis include variants of the original reagent synthesized by replacements of the L-Ala-NH_2 group in FDAA with more non-polar amino acid amides (e.g. L-Val-$NH_2$11a, and L-Leu-NH_2),11[a] which increase the hydrophobic properties of the analytes, or conventional analysis using a C_3 reversed phase column under combined “C3 and 2D C3” HPLC conditions.[xii] As mentioned above, derivatives of L-Ile and L-*allo*-Ile prepared from **8** are resolved with a difference in t_R of more than 60 seconds.

Among the cyanobactins, peptide **1** is most similar to mollamide F (**3**) reported by Ireland and coworkers from a *D. molle* collected from New Britain, Papua New Guinea.5b Both are cyclic peptides with two differences: **1** contains L-Phe and free D-Cys dimerically linked through a disulfide bridge (D-cystine) while mollamide F is monomeric, containing D-Phe and an (*S*)-thiazoline residue. The mechanism and gene clusters responsible for formation of thiazoline and oxazoline peptide residues in ascidians are now well-studied,[xiii] and it is clear that *Prochloron* in *D. molle* harbors the biosynthetic capacity for cyanobactin production. Mollamide F (**3**) appears to be formally related to **1** through heterocyclodehydration of D-Cys to the (*S*)-thiazoline residue and the epimerization of L-Phe to D-Phe. The unexpected D-thiazoline configuration of **3** – and by analogy, D-Cys in **1** – has drawn comment because the responsible biosynthetic gene cluster in *Prochloron*3 from *D. molle* reveals, “absence of any apparent epimerase enzymes in the sequenced pathways”.5

Disulfide-linked peptides are among the more cytotoxic cyanobactins. Each of the structures of ulithiacyclamide (**11**)[xiv] and ulithiacyclamide B (**12**) – highly cytotoxic cyanobactins from *Lissoclinum patella* (IC_{50} (KB cells) = 35 and 17 $ng.mL^{-1}$, respectively[xvi]) – has two Cys residues linked by an intramolecular disulfide bond.[xvii] To the best of our knowledge, **1** and **2** are the only cyanobactins that have intermolecular dimerization through Cys. In order to compare the antiproliferative properties of **1** and **11**, both were assayed against a chronic lymphocytic leukemia (CLL) primary cell line. While compound **11** was modestly potent (IC_{50} ~300 nM, or 400 $ng.mL^{-1}$), the open-disulfide dimeric analog **1** lacked substantial cell cytotoxicity (IC_{50} $>10^4$ nM).



CONCLUSION

In conclusion, two new dimeric hexapeptides, antatollamide A (**1**) and B (**2**) were isolated from the ascidian, *Didemnum molle* collected in Pohnpei, and characterized by NMR, chemical degradation to constituent amino acids, and derivatization with L-FDTA – a novel tryptophanamide variant of Marfey’s reagent to give DTA-amino acid derivatives. Under UHPLC analysis, DTA-derivatives of critical amino acids show superior resolution compared to conventional Marfey’s derivatives. NMR studies of the antatollamides revealed two configurations for the Pro-Pro dipeptide segment with the predominant natural product, **1**, bearing the *trans-cis* amide bond configuration.

EXPERIMENTAL SECTION

General Experimental Procedures.

UV-Vis spectra were measured on a JASCO V-630 double beam spectrometer. ECD spectra were recorded on a JASCO J-810 spectropolarimeter and optical rotations were measured on a JASCO P-2000 at the D-double emission line of Na. FTIR spectra were collected on thin film samples using a JASCO FTIR-4100 fitted with an ATR accessory (ZnSe plate). Direct-detected ^{1}H and inverse-detected 2D NMR spectra were measured on a Jeol ECA (500 MHz) spectrometer, equipped with a 5 mm $^{1}H\{^{13}C\}$ 5 mm probe, or a Bruker Avance III (600 MHz) NMR spectrometer fitted with a 1.7 mm $^{1}H\{^{13}C\}$ microcryoprobe. ^{13}C NMR spectra were collected on a Varian NMR spectrometer (125 MHz) equipped with a 5 mm Xsens $^{13}C\{^{1}H\}$ cryoprobe. NMR spectra were referenced to solvent signals (δ_H 2.50 ppm, residual CHD_2SOCD_3; δ_C 39.52 ppm, $(CD_3)_2SO$-d_6). High-resolution ESITOF analyses were carried out on an Agilent 1200 HPLC coupled to an Agilent 6350 TOFMS. Low-resolution MS measurements were made on a Thermoelectron Surveyor UHPLC coupled to an MSD single-quadrupole detector. Semi-preparative HPLC was performed on an Agilent 1100 HPLC system.

Biological Material.

Didemnum molle (01-10-039) was collected in 2001 from a shallow reef near Ant Atoll, Pohnpei, Federated States of Micronesia (6° 53.963’ N, 158° 20.525’ E), using snorkel, at a depth of approximately −2 m. Voucher samples are archived in the Department of Chemistry and Biochemistry.

Extraction and Isolation.

The *D. molle* tunicate was lyophilized (dry wt. 35.5 g) and extracted twice by homogenization with CH_2Cl_2/MeOH (1:1, 2 × 400 mL) and the filtered extracts were combined and concentrated under reduced pressure to yield the crude extract which was dissolved in MeOH-H_2O (9:1) and separated starting with progressive solvent partitioning as follows. The aqueous MeOH solution (400 mL) layer was repeatedly extracted with hexane (2 × 400 mL) to provide the hexane-soluble ‘A’ layer (0.6493 g). Water was added to the MeOH-H_2O layer (6:4 final ratio of MeOH-H_2O) and the mixture twice partitioned against CH_2Cl_2 (2 × 400 mL). The combined lower layers were concentrated to deliver the CH_2Cl_2-MeOH soluble ‘B-layer’ (1.50 g).

The B-layer was separated by flash chromatography (SiO_2, step gradient of MeOH in CH_2Cl_2), and the fraction eluted with 1:9 MeOH-CH_2Cl_2 (0.2163 g) was further partitioned by size-exclusion chromatography (Sephadex LH-20, MeOH) into nine fractions according to TLC (UV-activity, *p*-anisaldehyde staining). The third fraction (73.8 mg) containing **1** and **2** was separated by preparative reversed-phase HPLC (Phenomenex, Kinetex C_{18} column, 5 μ, 150 × 21.2 mm, linear gradient, initial conditions 90:10 H_2O-0.1% TFA-CH_3CN for three minutes to CH_3CN over 18 minutes for a total of 30 minutes, flow rate = 11 mL·min^{-1}) to yield 22 fractions which included the known compounds *N,N*-diphenylurea and mollurea.5 The eighth fraction (6.2 mg) was purified by reversed-phase analytical HPLC (Luna Phenyl Hexyl column, 250 × 4.60 mm, step gradients, initial conditions 90:10 H_2O-0.1% TFA-

CH_3CN for 3 minutes, 50: 50 for 15 min, 30:70 for 15 min to 100% CH_3CN for the last 5 min; flow rate = 0.700 mL·min^{-1}) to antatollamide A (**1**, 2.2 mg, t_R = 24.80 min) and antatollamide B (**2**, 0.1 mg, t_R = 23.32 min).

Antatollamide A (1):

colorless solid; $[\alpha]^{23.5}_D$ −97 ± 1.6 (*c* 0.22, CH_3CN); UV (CH_3CN) λ 220 nm (ε $\log_{10}$ 4.19). ECD (CH_3CN, *c* 8.39 × 10^{-5} M, 23 °C) λ 196 nm (Δε +18.7), 204 (−28.4), 221 (−24); FTIR (ATR, ZnSe plate) ν 3484, 3300, 2965, 2877, 1653, 1520, 1456, 1318, 1205, 1139, 1027, 840, 802, 723 cm^{-1}; HRMS (ESI-TOF) *m/z*: $[M+Na]^+$ Calcd for $C_{66}H_{94}N_{12}O_{12}S_2Na$ 1333.6454; Found 1333.6433. $[M/2+H+Na]^+$ Calcd for $C_{33}H_{48}N_6O_6SNa$ 679.3248; Found 679.3250; ^{1}H NMR and ^{13}C NMR. See Table 1.

Antatollamide B (2):

colorless solid; $[\alpha]^{23.5}_D$ −22 ± 3 (*c* 0.080, CH_3CN); UV (CH_3CN) λ 219 nm (ε $\log_{10}$ 4.09). ECD (CH_3CN, *c* = 3.05 × 10^{-5} M, 23 °C) λ 196 nm (Δε +13.8), 204 (−5.5), 215 (+7.7); FTIR (ATR, ZnSe plate) ν 3445, 2968, 1678, 1633, 1523, 1440, 1331, 1204, 1138, 1028, 839, 802, 723 cm^{-1}; HRMS (ESI-TOF) *m/z*: $[M+Na]^+$ Calcd for 1333.6454; Found 1333.6448. $[M/2+H+Na]^+$ Calcd for $C_{33}H_{48}N_6O_6SNa$ 679.3248; Found 679.3248; ^{1}H NMR and ^{13}C NMR. See Table 1.

Desulfurization of Antatollamide A (1) – Desthio-antatollamide A (4).

A suspension of Raney Ni (Raney® 2800 approx. 50 μL, 50% w/v H_2O) was added to a solution of **1** (0.5 mg, 0.4 μmol) in MeOH (1.5 mL). The solution was purged with N_2 (5 min), then stirred at 65 °C for 3 h while monitoring the disappearance of **1** (LC-MS). The mixture was cooled to room temperature, and passed onto an equilibrated SPE cartridge (C_{18}) and eluted with MeOH to deliver desthio-antatollamide A (**4**, 0.4 mg, 84%) as colorless solid that was used for acid hydrolysis (see below). An analytical sample of **4** was obtained by purification of an aliquot of crude compound (100 μg) by HPLC, using the same conditions for purification of **1** and **2**, and quantitated by QSCS (40 μg).[xviii]. Colorless solid; ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) 8.24 (d, *J* = 7.2 Hz, 1H), 8.22 (d, *J* = 8.4 Hz, 1H), 7.55 (d, *J* = 7.2 Hz, 1H), 7.25 (m, 2H), 7.19 (m, 1H), 7.13 (m, 2H), 4.50 (m, 2H), 4.42 (t, *J* = 7.2 Hz, 1H), 4.37 (t, *J* = 8.4 Hz, 1H), 4.20 (m, 2H), 3.81 (m, 1H), 3.79 (t, *J* = 6.3 Hz, 1H), 3.53 (q, *J* = 8.4 Hz, 1H), 3.20 (m, 1H), 3.10 (dd, *J* = 13.8, 6.0 Hz, 1H), 2.96 (dd, *J* = 13.2, 9.6 Hz, 1H), 2.89 (t, *J* = 9.6 Hz, 1H), 2.25 (m, 1H), 2.10 (m, 1H), 2.03 (m, 1H), 1.96 (m, 1H), 1.81 (m, 3H, 1.62 (m, 1H), 1.56 (m, 1H), 1.46 (m, 1H), 1.24 (m 2× 1H), 1.03 (d, *J* = 6.6 Hz, 3H), 0.93 (d, *J* = 7.2 Hz, 3H), 0.88 (d, *J* = 7.2 Hz, 3H), 0.86 (m, 6H).; HRMS (ESI-TOF) *m/z*: $[M+Na]^+$ Calcd for $C_{33}H_{48}N_6O_6Na$ 647.3528; Found 647.3528.

Acid Hydrolysis of Desthio-antatollamide A (4).

Compound **4** (0.4 mg, 0.640 μmol) was dissolved in 6 M HCl (500 μL) and heated at 110 °C with stirring for 12 h. The solution was cooled to 23 °C and extracted with EtOAc (3 × 500 μL). The combined organic layers were dried under a stream of N_2 and derivatized with (5-fluoro-2,4-dinitrophenyl)-N^{α}-L-tryptophanamide (FDTA, **8**, see below) to yield the DTA-amino acid derivatives for LCMS analysis.

Synthesis of 2-((5-fluoro-2,4-dinitrophenyl)-*N*$^{\alpha}$-L-tryptophanamide (FDTA, 8).

Thionyl chloride (3.6 mL, 49.7 mmol) was slowly added to a suspension of L-tryptophan (1.00 g, 4.90 mmol) in anhydrous MeOH (50 mL) at 0 °C. The solution was heated at reflux for 20 h, cooled, and the solvent removed under reduced pressure. The crude residue was dissolved in EtOAc, and the solution washed with saturated Na_2CO_3 followed by brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to yield methyl ester **6** (1.41 g, 81 %) as a pale yellow solid, identical by ^{1}H NMR and ESI-MS with literature values.[xix] Concentrated aqueous ammonia (15 mL, 0.222 mol) was added dropwise to a solution of **6** (1.41 g, 6.47 mmol) in anhydrous MeOH (2 mL) at 0 °C, then the mixture warmed to room temperature with stirring until no **6** remained (TLC, ninhydrin). Removal of the solvent gave the primary amide **7** as an off-white solid (quantitative), identical by ^{1}H NMR and ESI-MS with literature values.[xx]

Compound **8** was prepared according to a variation of the original protocol reported by Marfey.9 Anhydrous $MgSO_4$ (1.25 g) was added to a stirred solution of **7** dissolved in NaOH (0.5 mL, 0.5 mmol) and acetone (10 mL). The solution was filtered, 1,5-difluoro-2,4-dinitrobenzene (0.10 g, 0.51 mmol) was added and the solution stirred for 0.5 h at room temperature. Standard workup gave **8** (0.122 g, 63 %); recrystallization from aqueous acetone delivered pure **8** as bright orange rosettes, m.p. 220 – 222 °C °C; $[\alpha]_D$ −230 (*c* 1.0, acetone); UV (MeOH) λ_{max} 220 nm (ε $\log_{10}$ 4.57), 252 (4.04), 266 (4.07), 339 (4.12); FTIR (ATR, ZnSe plate) ν 3416, 3330, 3196, 3114, 2925, 2253, 1682, 1630, 1579, 1542, 1520, 1457, 1421, 1367, 1329, 1287, 1126, 1099, 1050, 922, 834, 742, 707 cm^{-1}. ^{1}H NMR (600 MHz, DMSO-d_6, J_{HF} = ^{1}H-^{19}F coupling): δ 10.93 (bs, 1H), 8.94 (d, *J* = 7.8 Hz, 1H), 8.79 (d, J_{HF} = 8.4 Hz, 1H), 7.85 (bs, 1H), 7.57 (d, *J* = 7.8 Hz, 1H), 7.53 (bs, 1H), 7.30 (d, *J* = 8.4 Hz, 1H), 7.18 (s, 1H), 7.04 (t, *J* = 7.5 Hz, 1H), 6.94 (t, *J* = 7.5 Hz, 1H), 6.69 (d, J_{HF} = 14.4 Hz, 1H), 4.67 (m, 1H), 3.38 (dd, *J* = 14.4, 4.8 Hz, 1H), 3.27 (dd, *J* = 14.4, 7.2 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6, J_{CF} = ^{13}C-^{19}F coupling): δ 171.5 Cq, 158.7, Cq (J_{CF} = 265 Hz), 148.3 Cq (J_{CF} = 14.0 Hz), 136.2 Cq, 127.3 CH, 127.2 Cq, 127.1 Cq (br), 125.0 Cq (J_{CF} = 9.5 Hz), 124.6 CH, 121.2 CH, 118.6 CH, 118.4 CH, 111.4 CH, 108.4 Cq, 102.1 CH (J_{CF} = 27.4 Hz), 56.8 CH, 28.4 CH_2; HRMS (ESI-TOF) *m/z*: $[M+Na]^+$ Calcd for $C_{17}H_{14}N_5O_5FNa$ 410.0871; Found 410.0878.

Absolute Configuration of the Amino Acids of Desthio-antatollamide A (4) and Antatollamide A (1).

The acid hydrolysate obtained from **4** (see above) was treated with FDTA (**8**, 2 mg, 5 μmol) in acetone (200 μL) and 1M $NaHCO_3$ (50 μL, 50 μmol), and the mixture heated at 85 °C, with stirring, for 30 minutes. After cooling, the mixture was neutralized with 1M HCl (50 μL, 50 μmol) and concentrated under a stream of N_2. The residue was dissolved in H_2O (100 μL) and centrifuged to remove insoluble material before analysis by LCMS (Hypersil Gold, C_{18} column, 50 × 2.1 mm, 1.9 μm, linear gradient, initial conditions 85:15 H_2O-0.1% HCOOH-CH_3CN to 55:45 over 25 minutes, flow rate = 0.50 mL·min^{-1}). The retention times (t_R, min) for the standard amino acids DTA-amino acid derivatives were as follows: L-Ala (13.00), D-Ala (15.52), L-Pro (13.51), D-Pro (15.21), L-Val (16.24), D-Val (19.76), L-Ile and L-*allo*-Ile (co-elution, 18.30), D-Ile and D-*allo*-Ile (co-elution, 21.98), L-Phe (18.72), and D-Phe (21.53). LCMS analysis of DTA derivatives obtained from **4** showed the presence

of L-Pro (13.57), D-Ala (15.47), L-Ile or L-*allo*-Ile (18.26), L-Phe (18.77), and L-Val (16.22). By correlation, **1** has the same absolute configuration.

As L-Ile and L-*allo*-Ile DTA derivatives co-eluted under the above conditions, they were resolved under a different combination of solid phase, solvent gradient and buffer (Agilent Zorbax SB-Aq column, 4.6 × 250 mm, 5 μm, step gradient, initial conditions 30%−40% CH_3CN-20 mM NH_4OAc-0.1% TFA, 40 min; 40%−50% CH_3CN-20 mM NH_4OAc-0.1% TFA, 5 min. Flow rate = 0.70 mL·min^{-1}). The retention times for the standard amino acid DTA derivatives were as follows: L-Ile (t_R = 34.41 min), and L-*allo*-Ile (t_R = 33.38 min). The HPLC analysis of the DTA derivatives obtained from **4**, co-injected with L-Ile and L-*allo*-Ile, established the presence of L-Ile.

Cultured CLL Cytotoxicity Assay.

Primary chronic lymphocytic leukemia (CLL) cells were seeded in RPMI 1640 with 10% of FBS (Fetal Bovine Serum) in 96-well plates (10^6 cells/well/100 μL) and treated with compounds at determined concentrations (0–10^4 nM). Cells were harvested after 48 h, washed and stained with PI (Propidium Iodide) and DioC6 (30 min, 37 °C). After the incubation time, the cells were washed and run in a flow cytometer (FACS Calibur) with detection of fluorescence in channels 1 (DioC6) and 3 (PI). Cells that were positive by DioC6 were considered alive while cells that were positive by PI were considered dead.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS.

We thank J. MacMillan for assistance with collection of *D. molle*, Y. Su for HRMS data, A. Mrse and B. Duggan for assistance with NMR measurements, and J. Castro and J. Vélez Luján (Moores Cancer Center) for cytotoxicity data. We are grateful to the Federated States of Micronesia for permission to collect in territorial waters. The 500 MHz NMR and the HPLC TOFMS spectrometers were purchased with funding from the NSF (Chemical Research Instrument Fund, CHE0741968) and the NIH Shared Instrument Grant (S10RR025636) programs, respectively. This research was supported by the NIH (AI100776).

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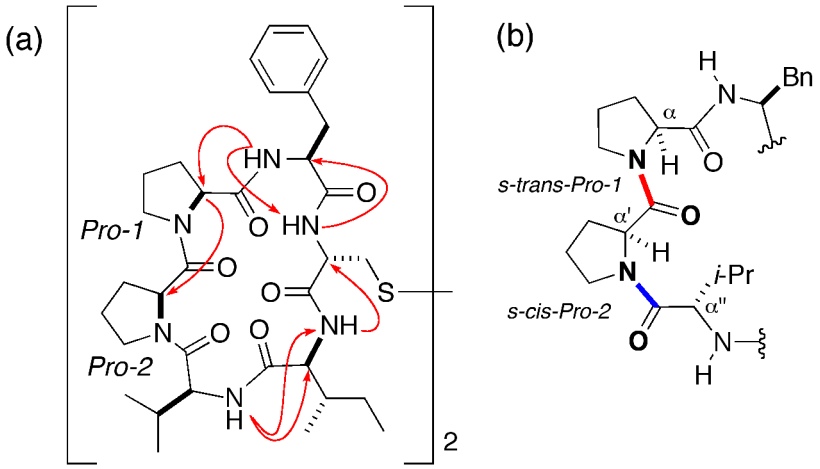


Figure 1.

Antatollamide A (a) Summary of NOESY correlations observed for **1** (DMSO-d_6, 600 MHz, t_m = 500 mS) (b) 3D approximation of the *s-trans*-Pro-1–*s-cis*-Pro-2 element (amide bonds highlighted in color).

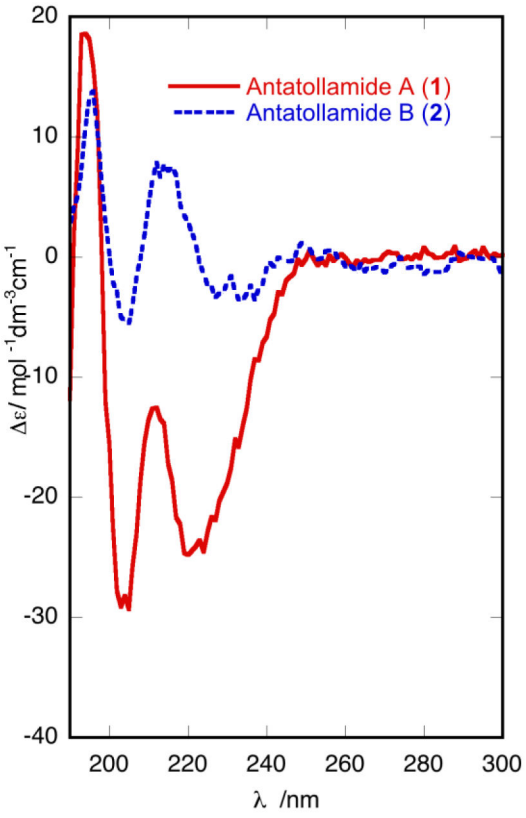
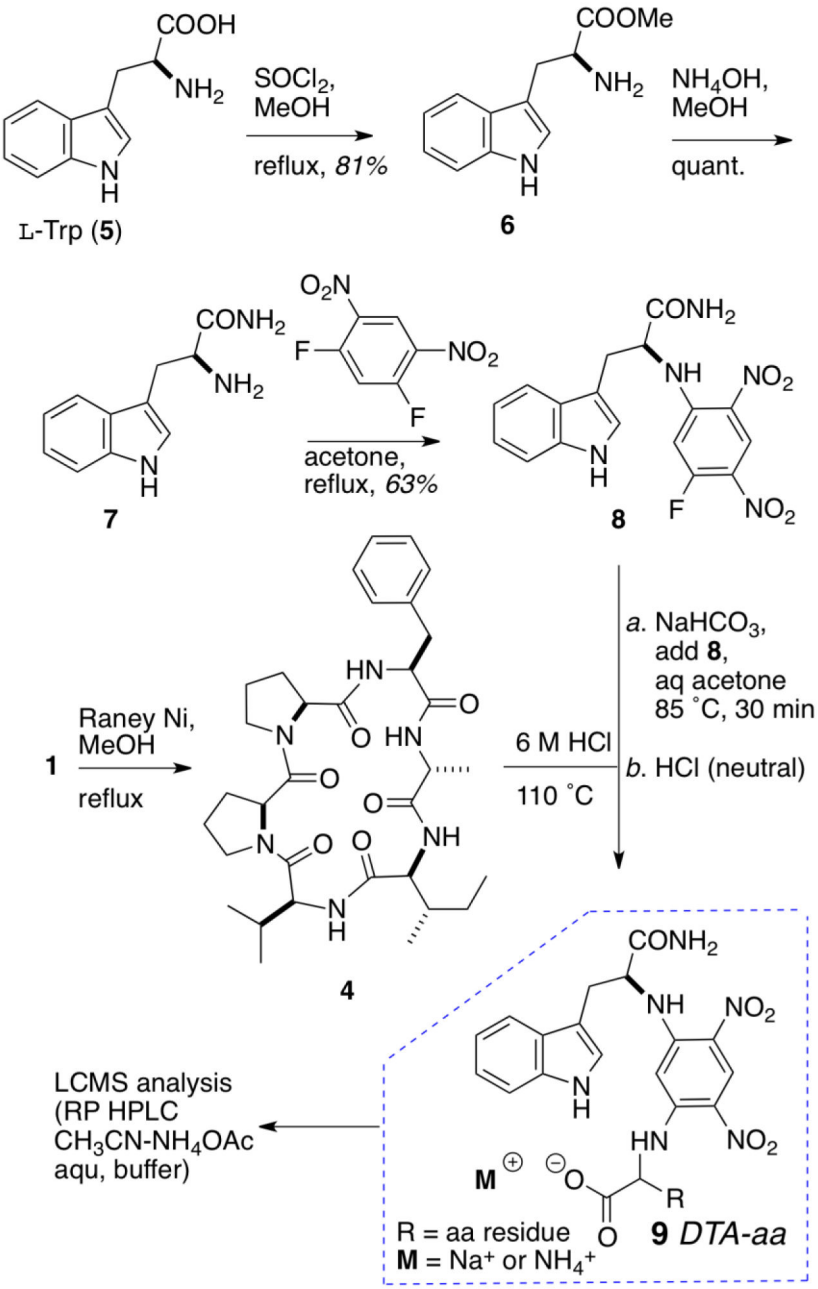


Figure 2.
ECD Spectra of antatollamides A (**1**) and B (**2**) (CH_3CN, 23 °C)



Scheme 1.

Preparation of L-FDTA (**8**) and Application in Configurational Analysis of **1**.

Table 1.

NMR data for 1 and 2 (600 MHz, DMSO-d_6)

		1			2		
	Position	δ_C [a]		δ_H [b], mult (*J*, Hz)	δ_C [a]		δ_H [b], mult (*J*, Hz)
Pro-1	C=O	170.3			169.9		
	α	58.7	CH	4.24, t (7.5)	58.7	CH	4.27, m
	β	28.3	CH_2	1.62, m	31.5	CH_2	1.30, m
				2.29, m			1.89, m
	γ	25.0	CH_2	1.83, m	22.0	CH_2	1.53, m
				1.98, m			1.59, m
	δ	47.5	CH_2	3.54, q (8.4)	46.7	CH_2	3.20, m
				3.79, m			3.27, m
Pro-2	C=O	170.6	C		170.2	C	
	α	60.5	CH	4.50, m	58.7	CH	4.16. dd (8.1, 5.1)
	β	30.6	CH_2	1.82, m	28.9	CH_2	1.66, m
				2.12, dd (12, 6.0)			2.19, m
	γ	21.4	CH_2	0.94, m	24.7	CH_2	1.82, m
				1.59, m			1.90, m
	δ	45.9	CH_2	2.90, t (9.9)	47.6	CH_2	3.49, m
				3.20, m			3.76, m
Val	C=O	169.67	C		171.2	C	
	α	54.8	CH	4.39, t (8.4)	56.0	CH	4.28, m
	β	30.3	CH	2.02, m	29.7	CH	1.91, m
	γ	18.0	CH_3	0.86, d (6.6)	19.0	CH_3	0.81, m
		19.2	CH_3	0.94, d (6.6)	19.5	CH_3	0.83, m
	NH			7.04, d (9.0)			7.08, d (9.6)
Ile	C=O	170.2	C		169.8	C	
	α	59.7	CH	3.84, t (6.6)	60.9	CH	3.70, t (6.3)
	β	35.2	CH	1.85, m	35.4	CH	1.76, m
	β-Me	15.6	CH_3	0.89, d (7.2)	15.9	CH_3	0.88, d (7.2)
	δ	25.0	CH_2	1.25, m	25.7	CH_2	1.23, m
				1.47, m			1.49, m
	δ	11.3	CH_3	0.85, t (7.2)	11.6	CH_3	0.84, m
	NH			8.43, d (6.6)			8.22, d (5.4)
Cys	C=O	169.71	C		170.9	C	
	α	54.8	CH	4.46, m	54.6	CH	4.57, m
	β	24.8	CH_2	2.48, m [c]	26.2	CH_2	2.69, m
							2.82, m
	NH			7.69, d (7.8)			7.26, m
Phe	C=O	170.4	C		171.6	C	

	1			2		
Position	**δ_C [a]**		**δ_H [b], mult (*J*, Hz)**	**δ_C [a]**		**δ_H [b], mult (*J*, Hz)**
α	55.0	CH	4.53, m	55.0	CH	4.42, m
β	38.0	CH_2	3.00, dd (9.9, 3.6)	37.8	CH_2	2.89 dd (13.8, 10.8)
			3.12, dd (13.2, 6.0)			3.22, m
γ	137.6	C		138.1	C	
δ	129.2	CH	7.15, d (7.2)	129.6	CH	7.18, m
ε	128.3	CH	7.25, m	128.6	CH	7.27, m
ζ	126.4	C	7.19, m	126.8	C	7.19, m
NH			8.15, d (7.8)			8.46, d (8.4)

[a] 125 MHz Multiplicity determined from HSQC spectra.

[b] 600 MHz.

[c] 2H integration

Table 2.

Comparative UHPLC Retention times (t_R, min) and Orders of Elution of L-DTA and L-DAA (Marfey's) Derivatives of Selected L- and D- Amino Acids (aa)

		t_R, min •DTA					t_R, min •DAA			
aa		**L-**	**D-**	**Δt[b]**	**α[c]**		**L-**	**D-**	**Δt[b]**	**α[c]**
Ala	L→D	13.08	15.52	2.44	1.19	L→D	5.20	7.38	2.18	1.42
Ser	L→D	10.43	10.93	0.50	1.05	L→D	3.02	3.02	0.00	1.00
Asn	[d]	9.73	9.73	0.00	1.00	[d]	2.72	2.72	0.00	1.00
Cit[e]	D→L	10.93	11.31	–0.38	1.03	D→L	4.15	4.15	0.00	1.00
N-Me-Ala	L→D	19.40	19.95	0.55	1.03	[d]	8.39	8.39	0.00	1.00
N-Me-Asp	D→L	11.67	10.51	–1.16	1.11	D→L	3.49	4.29	0.80	1.23
iso-Ser[f]	L→D	16.67	17.04	0.37	1.02	[d]	3.82	3.82	0.00	1.00
Ile	L→D	18.40	22.06	3.66	1.20	L→D	12.67	16.18	3.51	1.28
allo-Ile	L→D	18.32	22.01	3.69	1.20					
Ile[g]	L→D	34.41	–	–	–					
allo-Ile[g]	L→D	33.38	–	–	–					

[a] RP C_{18} column, 50 × 2.1 mm (1.9 μm) and a flow rate = 0.50 mL.min^{-1}. Elution was completed with a linear gradient of 15% – 45% CH_3CN/H_2O–0.1% HCOOH for 25 minutes, followed by 100% CH_3CN for 2 minutes and re-equilibration with 15% CH_3CN-H_2O-0.1% HCOOH for 3 minutes before the next measurement; void time, $t_V°$= 0.53 min.

[b] $\Delta t = t_R$ (D-aa) – t_R (L-aa).

[c] $\alpha = [t_R(B) - t_V°)]/[t_R(A) - t_V°]$, where A, B are the faster and slower eluting components, respectively.

[d] Coelution.

[e] Citrulline

[f] 3-Amino-2-hydroxypropanoic acid.

[g] Agilent Zorbax SB-Aq column, 4.6 × 250 mm (5 μm), stepped gradient, initial conditions 30%–40% CH_3CN-20 mM NH_4OAc-0.1% TFA, 40 min; 40% – 50% CH_3CN-20 mM NH_4OAc-0.1% TFA, flow rate = 0.7 mL.min^{-1}.