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Symplocin A, a Linear Peptide from the Bahamian Cyanobacterium *Symploca* sp. Configurational Analysis of *N,N*-Dimethylamino Acids by Chiral-Phase HPLC of Naphthacyl Esters

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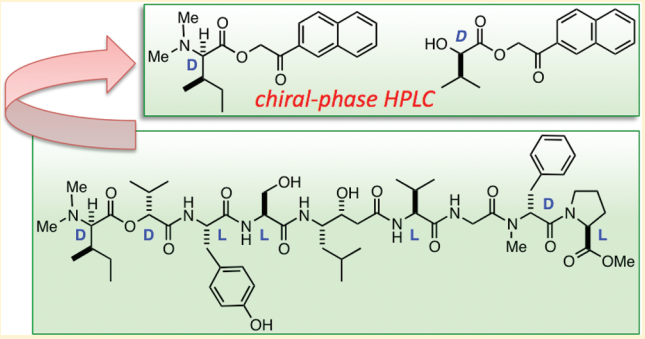
Supporting Information

ABSTRACT: The absolute stereostructures of the components of symplocin A (**3**), a new *N,N*-dimethyl-terminated peptide from the Bahamian cyanobacterium *Symploca* sp., were assigned from spectroscopic analysis, including MS, 2D NMR, and Marfey's analysis. The complete absolute configuration of symplocin A, including the unexpected D-configurations of the terminal *N,N*-dimethylisoleucine and valic acid residues, was assigned by chiral-phase HPLC of the corresponding 2-naphthacyl esters, a highly sensitive, complementary strategy for assignment of N-blocked peptide residues where Marfey's method is ineffectual or other methods fall short. Symplocin A exhibited potent activity as an inhibitor of cathepsin E (IC_{50} 300 pM).



Linear peptides terminated with *N,N*-dimethyl amino acid residues (DMAAs) are a minority family of natural products among the larger array of cyclic peptides reported from cyanobacteria.[1] For example, the exceedingly potent anticancer compounds dolastatin-15[2] and -10 (**1**)[3]—which has completed phase I clinical trials[4]—were initially found in the sea hare *Dolabella auricularia* from the Indian Ocean and, later, in the cyanobacterium *Symploca* sp.[1b] collected in Palau. Grassystatins A and B (**2a,b**),[1c] two DMAA-terminated peptides from *Lyngbya* cf. *confervoides*, and grassystatin C (**2c**) exhibit selective inhibition of the protease cathepsin E. Gallinamide A,[1a] from a Panamanian *Schizothrix* sp., is moderately active against the malarial parasite, *Plasmodium falciparum*, and the cytotoxic belamide A,[1d] from a Panamanian *Symploca* sp., shows tubulin-destabilizing activity.

While analyses of many highly modified α-amino acids have been achieved[5] conveniently by application of Marfey's analysis,[3] the configuration of DMAA-containing peptides is complicated by lack of reactivity: the hindered N-terminal amino acid is inert in standard Marfey's analysis and Edman degradation. A similar problem is encountered with analysis of 2-hydroxy acids that occasionally replace α-amino acid residues in DMAA-modified peptides or other cyclic peptides arising from nonribosomal polypeptide synthetase (NRPS) pathways.[6] Here, we report a new peptide, symplocin A (**3**), from a mixed cyanobacterial assemblage containing *Symploca* sp. collected in the Bahamas and its complete stereoassignment aided by a new protocol for DMAA and other nonstandard residues by analysis of their corresponding 2-naphthacyl esters. The sensitivity of the latter protocol is comparable to Marfey's method and well-suited to DMAA and 2-hydroxy acids, and may find application to other N-terminal blocked peptides.

RESULTS AND DISCUSSION

The molecular formula for **3**, $C_{56}H_{86}N_8O_{14}$, was established from HRESIMS data (m/z 1094.6257, $[M + H]^+$). The high N-content of **3** together with the presence of several C═O groups (HMBC) was suggestive of a peptide; this was confirmed by identification of peptide residue ^1H and ^{13}C spin systems through analysis of COSY, TOCSY, HSQC, and HMBC spectra. Amino acid (aa) analysis of the hydrolysate of **3** (6 M HCl, 110°) revealed the presence of the residues glycine (Gly), proline (Pro), serine (Ser), tyrosine (Tyr), and valine (Val). The ^1H NMR spectrum of **3** (CD_3CN, Table 1) showed broad N−H doublets coupled to α-CH protons of amino acid residues, the partial sequence of which was established by HMBC (Figure 1). A downfield AA′BB′ system (δ 6.73, d, J = 8.3 Hz; 7.09, d, J = 8.3 Hz) was assigned to the Tyr residue and supported by HMBC C−H correlations (Table 1). The remaining aromatic proton signals were suggestive of a phenylalanine (Phe) residue; however, Phe was not detected in standard analysis for standard proteinogenic amino acids. An HSQC spectrum showed the presence of eight C-substituted methyl groups, indicating hydrophobic amino acid residues. In addition, three upfield *N*-Me signals (δ 2.92, s; 2.83, s, 6H, two overlapped Me) appeared in the ^1H NMR spectrum of **3**. The HMBC spectrum

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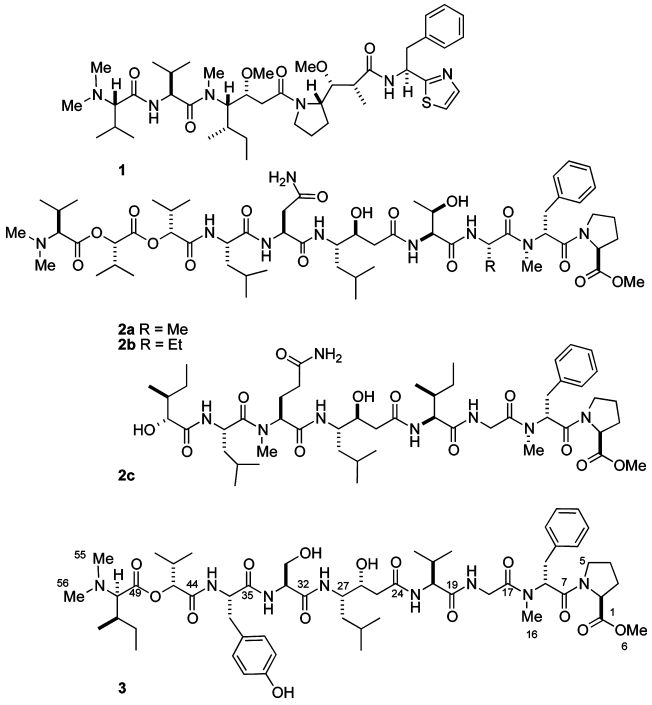

showed a correlation of the methyl group (δ 2.92, s) to a spin system consistent with *N*-MePhe.

A TOCSY experiment readily traced the α-CH proton signal (δ 3.94, d) to a spin network belong to isoleucine (Ile). Integration of the δ 2.83 singlet in the ^{1}H NMR spectrum showed the presence of two equivalent methyl groups; thus **3** is a linear peptide terminated with an *N*,*N*-dimethylisoleucine residue. An *O*-CH_3 group (δ_H 3.65 s) was linked to the terminal proline methyl ester (*O*-MePro) by HMBC correlations. The COSY and HMBC correlations from the downfield shifted CH (C-45 δ 81.2) were consistent with a 3-methyl-2-hydroxybutanoyl residue (2-valic acid). Finally, the remaining COSY and HMBC correlations of **3** revealed the presence of a γ-amino acid, statine (4-amino-3-hydroxy-6-methylheptanoic acid).

The configurations of the proteinogenic amino acid residues were determined, uneventfully, after hydrolysis of **3** (6 M HCl, 110 °C) and derivatization–HPLC analysis using Marfey's method.[7] The α-amino acid residues Tyr, Ser, Val, and *O*-MePro were found to be of the L-configuration, and the remaining residues were assigned either by Marfey's method (*N*-MePhe, statine) or an alternative O-derivatization (2-naphthacyl esters; see below) after preparation of amino acid standards as follows.

N-Me-L-Phe was prepared (Scheme 1) by methylation of *N*-Boc-L-phenylalanine ethyl ester (freshly prepared Ag_2O, CH_3I) to obtain *N*-Boc-*N*-methyl-L-phenylalanine ethyl ester followed by acid deprotection (3 M HCl, 80 °C).[8] Comparison of the L-Marfey's derivative of the peptide hydrolysate of **3** with that of standard *N*-MePhe (both L- and D-FDAA reagents; t_R = 33.35 and 44.65 min, respectively) revealed the presence of *N*-Me-D-Phe.

Two diastereomers of statine were prepared by modifications of published procedures from Schmidt[9] and Galeotti[10] (Scheme 1). (*S*)-*N*-Boc-Leu underwent intermolecular coupling–cyclization with Meldrum's acid (DCC, DMAP) to produce the corresponding pyrrolidin-2,4-dione,[11] which was reduced ($NaBH_4$) diastereoselectively to provide the 4-hydroxypyrrolidinone (4*S*,5*S*)-**4a** (pyrrole numbering) as a single diastereomer. Ring-opening of **4a** (NaOMe, MeOH) followed by deprotection (TFA) gave pure (3*S*,4*S*)-statine (**5a**). Alternatively, the (3*S*,4*R*)-diasteromer **5b** was obtained, along with **5a** (2:3, ^{1}H NMR), when borohydride reduction of the pyrrolidindione was carried out *after* removal of the *N*-Boc protecting group (TFA-CH_2Cl_2), followed by acid hydrolysis (6 M HCl, 110 °C, 2.5 h).[10,12] The diastereomeric ratio of the mixture of **5a**:**5b** was the same as that of the 4-hydroxypyrrolidinone from which it derived, suggesting that no epimerization had taken place at C-4.[13] HPLC-Marfey's analysis of **3**, as described above, revealed symplocin A contains the (3*R*,4*S*) diastereomer of statine.

Assignment of absolute configurations of the *N*,*N*-dimethylisoleucine and valic acid residues presented a larger obstacle: both are insufficiently nucleophilic to react with Marfey's reagents (FDAA[7] or FDLA[14]). As an alternative, α-bromoacetophenone has been used extensively for derivatization of carboxylic acids to their corresponding phenacyl esters.[15] We anticipated that free *N*,*N*-Ile and valic acid, present in the peptide hydrolysate of **3**, would undergo O-alkylation exclusively at their carboxy groups at pH = 6, conditions under which the OH group in α-hydroxy acids and α-*N*,*N*-dimethylamino groups should be protonated and unreactive. Subsequent assignment of the configuration of the ester products should be feasible under chiral-phase HPLC conditions.

The four stereoisomers of *N*,*N*-dimethylisoleucine (**6a–d**) were prepared by separate reductive alkylation (CH_2O, H_2, Pd–C)[16] of stereoisomers of Ile. For reasons of sensitivity, we chose α-bromo-2-acetonaphthone (**7**) to prepare naphthacyl esters. The latter possesses two advantages over phenacyl esters: a stronger chromophore (λ_{max} 248 nm, $\log_{10}$ ε 3.94[13]) that is inherently fluorescent. Compounds **6a–d** were separately converted (Scheme 2) into the corresponding 2-naphthacyl esters **8a–d**, which were readily visualized and collected from preparative TLC plates under long-wavelength UV irradiation. Optimized conditions for chiral-phase HPLC separation of the four esters (Chiralcel OD-H, 2:98 *i*-PrOH + 0.1% TFA–hexane, 0.5 mL/min) gave baseline separations of **8a–d** (t_R = 28.60, 29.91, 27.42, 25.22, respectively). Derivatization of the acid hydrolysate of **3** and HPLC under identical conditions gave a compound that co-eluted with **8b**; therefore, **3** contains *N*,*N*-Me_2-D-Ile.

The absolute configuration of valic acid was determined in a similar manner. Samples of L- and D-valic acid (**9a**,**b**), prepared by diazotization–hydrolysis[17] of L- and D-Val (Scheme 2), were converted to the corresponding 2-naphthacyl esters **10a**,**b** under conditions similar to those described above. The two enantiomers were separated with baseline resolution on chiral-phase HPLC (t_R = 30.20; 18.16, respectively). The EtOAc-soluble material, obtained by two-phase extraction of the acid hydrolysate of **3**, was derivatized in the same manner. Chiral-phase HPLC of this material showed only the presence of *O*-(2-naphthacyl) ester of valic acid (D-**10b**) that co-eluted with an authentic standard. Therefore, D-valic acid is present in **3**, and the complete configuration of the peptide is revealed.

Peptide **3** is most similar to grassystatins A and B (**2a**,**b**); however, important differences in the structures of **3** and **2a**,**b** are apparent. Compounds **2a**,**b** contain two units of *S* valic acid, whereas **3** has an *R* valic acid residue. Hydrolysis of statine-like residues is sometimes accompanied by epimerization at C-3. We find that under conditions of acid hydrolysis of **3** the statine residue undergoes only partial epimerization at C-3, unlike that of grassypeptin A (**2a**), or the homologated-Phe γ-amino acid residue in stictamides A–C,[18] or the isostatine residue in didemnin B.[19] In contrast, the C-4 stereocenter (pyrrole numbering) of **4a**

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Table 1. ^{1}H (600 MHz) and ^{13}C NMR (125 MHz) for 3 (CD_3CN)

amino acid	no.	δ_C, mult.[a]	δ_H, mult (J in Hz)	COSY	HMBC[b]	ROESY
O-Me-Pro	1	173.4, C				
	2	59.1, CH	4.26, dd (2.3, 5.9)	3a, 3b	1, 3, 4	3a, 3b
	3a	29.5, CH_2	1.77, m	2, 3b	1, 2, 4, 5	2, 3b, 5
	3b		2.12, m	2, 3a	1, 4, 5	2, 3a
	4a	25.7, CH_2	1.85, m	3b, 5	2, 3, 5	
	4b		1.78, m	3a, 3b, 5	2, 5	3a, 3b, 5
	5	47.4, CH_2	3.31, m	4a, 4b	3, 4	3a
	6	52.5, CH_2	3.65, s		1	
N-MePhe	7	168.7, C				
	8	57.5, CH	5.42, t (7.1)	9a, 9b	7, 9, 10, 16	5, 11
	9a	35.3, CH_2	2.75, m	8, 9b	8, 10, 11	7, 8, 9b, 11
	9b		3.18, m	8, 9a	7, 8, 10, 11	8, 9b, 11
	10	138.7, C				
	11/15	130.2, CH	7.23, m		9, 10, 13	8, 9a, 9b, 16
	12/14	129.0, CH	7.23, m		10, 13	8, 9a, 9b, 16
	13	127.2, CH	7.18, m		12/14	
	16	30.6, CH_3	2.92, s		8, 17	
Gly	17	169.5, C				
	18a	41.5, CH_2	3.94, m	NH	17, 19	16
	18b		4.02, m	NH	17, 19	16
	NH		7.45, m			18b
Val	19	172.5, C				
	20	59.9, CH	4.12[c]	NH, 21	21, 22, 23	21, 22, 25, 28a
	21	30.9, CH	2.08, m	20, 23	20, 22, 23	20, 23
	22	18.4, CH_3	0.91, s	21	20, 21	21
	23	22.56, CH_3	0.86, s	21	20, 21	21
	NH		7.36, m	20		21, 25, 30
Sta	24	172.7, C				
	25a	41.1, CH_2	2.36, m	26	24, 26, 27	26,28, NH(Val), NH
	25b		2.42, m	26	24, 26, 27	
	26	71.4, CH	3.92, m		[c]	25
	27	52.5, CH	3.83, m		[c]	
	28a	39.7, CH_2	1.29, m		[c]	27, 28b
	28b		1.49, m		[c]	28a
	29	25.4, CH	1.57, m	30	28, 30, 31	31
	30	23.6, CH_3	0.88, s	29		
	31	22.4, CH_3	0.85, s	29		
	NH		7.04[b]			27
Ser	32	171.8, C				
	33	56.9, CH	4.31, m	34, NH	34	34, NH
	34	62.6, CH_2	3.77, m	33	32	
Tyr	35	172.5, C				
	36	56.1, CH	4.58, m	37a, 37b, NH	37, 38, 32	37a,b, NH
	37a	36.9, CH_2	2.81, m	36, 37b	35, 39/43	37b
	37b		3.14, m	36, 37a	35,36, 39/43	36, 37a, 39/43
	38	129.0, C				
	39/43	131.0, CH	7.09, d (8.3)	40/42	37,38, 40/42, 41	37a,b, 36
	40/42	115.9, CH	6.72, d (8.4)	39/43	38, 41, 40/42	
	41	156.6, C				
	NH		7.94, m		44	36, 37a, 46, 39/43
Valic acid	44					
	45	81.2, CH	4.87, d (4.1)	46	46, 47, 48	46, 47, 48, NH (Tyr)
	46	31.1, CH	2.01, m	45, 47, 48	45, 47, 48	45, NH (Tyr)
	47	16.8, CH_3	0.67, d (6.8)	46	45, 46, 48	46, 45
	48	18.9, CH_3	0.84[d]	46	45, 46, 47	46
N,*N*-Me_2-Ile	49	168.0, C				
	50	71.8, CH_3	3.94, d(4.5)	51	51, 52, 53	51, 52, 53
	51	34.1, CH	2.15, m	50, 52	49, 50, 52, 53, 54	52
	52	14.0, CH_3	0.95, s	51	50, 51, 53	
	53a	27.5, CH_2	1.32, m		50, 51, 52, 54	50, 51
	53b		1.42, m	53a		50, 53a

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Table 1. continued

amino acid	no.	δ_C, mult.[a]	δ_H, mult (J in Hz)	COSY	HMBC[b]	ROESY
	54	12.0, CH_3	0.96, s	53a,b	51, 52	
	55/56	43.0, CH_3	2.83, s	50		

[a]Determined from edited HSQC. [b]$^nJ_{CH}$ = 8 Hz. [c]Significant overlap with isoleucine. [d]Overlapped

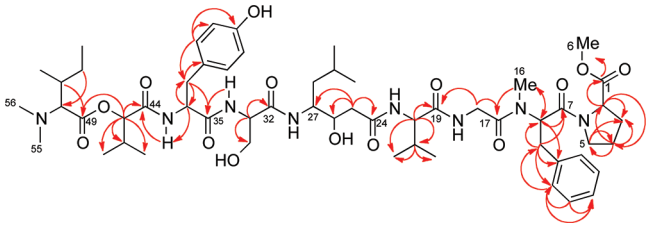
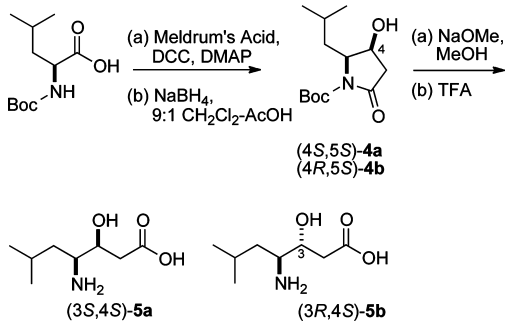


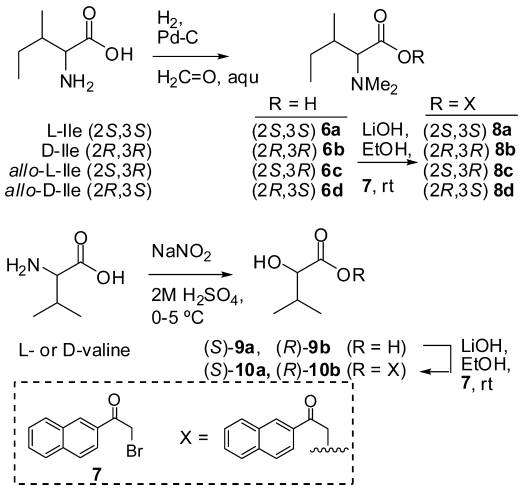
Figure 1. HMBC correlations in compound **3**.

Scheme 1. Synthesis of Diastereomeric Statine Standards, (3*S*,4*S*)-5a and (3*R*,4*S*)-5b[a]



[a]See refs 9 and 10.

Scheme 2. Synthesis of 2-Naphthacyl Esters of *N,N*-Dimethylisoleucine Stereoisomers and (*S*)- and (*R*)-Valic Acid



(Scheme 1) does not epimerize under similar conditions. Consequently, the observed ratio of **5a**:**5b** in the hydrolysate of **3** suggests the major diastereomer retains the configuration (3*S*,4*S*) of the intact peptide.

The configurational analysis of DMAAs has, in the past, relied on total synthesis (e.g., the need for total synthesis of **1** and its diastereomers and systematic spectroscopic comparisons[20]) or methods of lower sensitivity[21] based on chiral-phase HPLC (penicillamine-bonded phase, elution with Cu^{2+}, CH_3CN(aq)). The 2-naphthacyl ester approach provides an independent, highly sensitive tool for configurational analysis of DMAAs. Our finding that the α-CH carbon in **1** in the terminal *N,N*-Me_2-Ile has the D-configuration refutes an earlier assumption, "the precedent that all marine natural products containing an *N,N*-dimethyl terminal amino acid residue possess the L configuration at this center",[1a] and offers a more satisfying experimental solution for independent verification of DMAA configuration.

Symplocin A (**3**) lacked significant cytotoxicity against cultured tumor cells (HCT-116) but exhibited potent activity as an inhibitor of the protease enzyme cathepsin E[22] (IC_{50} 300 pM), comparable to that of pepstatin.[23] This is consistent with the findings by Luesch and co-workers, who reported inhibition of cathepsin E by grassystatins A and B (IC_{50} 886 and 354 pM, respectively).[1c]

In conclusion, a new peptide, symplocin A (**3**), from *Symploca* sp. has been characterized and shown to contain the *N,N*-Me_2-D-Ile terminal amino acid residue and an acetate-homologated γ-amino acid, (3*S*,4*S*)-statine. A new method to assign the absolute configurations of *N,N*-dimethylamino acids and 2-hydroxy acids was deployed, along with Marfey's analysis, to secure the complete stereostructure of **3**.

EXPERIMENTAL SECTION

General Experimental Procedures. General procedures are described elsewhere.[24]

Microbial Material. A mixed cyanobacterial assemblage (10−10−039) was collected at San Salvador Island, Bahamas, in July 2010, at a depth of 25 m, and frozen until required. The following characteristics of the major filamentous components (>70%) were observed by comparative microscopy: presence of sheath, cell dimensions: width ~7.5 μm, length ~5 μm, shallow constrictions with a single trichome, lack of calyptra, which are characteristic of *Symploca* sp.[25] On the basis of the general appearance of the type sample slide, approximately 75−80% of the type sample appears to be *Symploca*, and the remaining sample is made up of diatoms, other cyanobacteria resembling the genus *Lyngbya* spp. A voucher specimen is archived at UC San Diego, Department of Chemistry and Biochemistry.

Extraction and Isolation. A sample of a cyanobacterial assemblage (116 g wet wt) was extracted with MeOH (2 × 900 mL over 8 h). The concentrated extract was partitioned between EtOAc (3 × 700 mL) and H_2O (300 mL), and the organic layer concentrated under reduced pressure to give a green solid (130.0 mg). The EtOAc extract (121.5 mg) was subjected to Sephadex LH-20 chromatography eluting with 100% MeOH to give 45 fractions. Fractions 12−14 (22.9 mg) were combined, dried under reduced pressure, and subjected to semipreparative reversed-phase HPLC (C_{18}, 2 mL/min, gradient, 40:60 to 100:0 (CH_3CN + 0.1% TFA(aq))−(H_2O + 0.1% TFA(aq)) over 40 min) to give **3** (3.1 mg).

3: colorless, amorphous solid; $[\alpha]^{22.5}_D$ +16.0 (*c* 2.18, MeOH); FTIR (ATR) ν 3311, 2972, 1745, 1671, 1518, 1447, 1204, 1138 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 1095.6330 $[M + H]^+$ (calcd for $C_{56}H_{86}N_8O_{14}$, 1095.6336).

N,N-Dimethyl-L-isoleucine (ref 26). A mixture of L-isoleucine (0.020 g in 0.8 mL water), formaldehyde (0.049 mL, 37% aq), and 10% Pd−C (20 mg, 0.188 mmol) was stirred vigorously overnight under 1 atm of H_2. After venting, the mixture was heated to reflux and filtered hot (×2), and the filtrate concentrated under reduced pressure to afford *N,N*-dimethyl-L-isoleucine[26] as a colorless solid: ^1H NMR (300 MHz, D_2O)

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δ 3.39 (1H, d, J = 10.2 Hz), 3.30 (1H, s), 2.87 (6H, s), 2.01 (1H, m), 1.41 (1H, m), 1.03 (3H, d, J = 7.3 Hz), 0.89 (3H, t, J = 7.4 Hz).

N-Boc-L-Phenylalanine Ethyl Ester. To a solution of L-phenylalanine ethyl ester (1.0 g in 10 mL CH_2Cl_2) was added di-*tert*-butyl dicarbonate (1.31 g, 5.57 mmol) followed by dry, distilled diisopropylethylamine (10 mL, 7.42 mmol). The mixture was stirred at room temperature (rt; 0.5 h), extracted (3 × 25 mL H_2O), dried over $MgSO_4$, and concentrated under reduced pressure to yield 1.0 g of *N*-Boc-L-phenylalanine ethyl ester: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.12−7.33 (m, 5H), 4.97 (1H, m), 4.56 (1H, m), 4.15 (3H, q, J = 7.1 Hz), 3.07 (1H, br s), 1.52 (3H, s), 1.41 (9H s), 1.22 (3H, t, J = 7.2 Hz).

***N*-Methyl-L-Phenylalanine Hydrochloride.** A mixture of *N*-Boc-phenylalanine ethyl ester (0.4 g, 1.4 mmol), freshly prepared Ag_2O (1.28 g, 5.5 mmol), and CH_3I (1.54 mL, 11.0 mmol) in DMF (15 mL) was stirred overnight at rt. The mixture was diluted with H_2O (300 mL) and extracted (200 mL of $CHCl_3$, 3×), and the combined organic extracts were dried over $MgSO_4$ and concentrated. The product was purified by flash chromatography (silica, 1:10 acetone−hexane, flow rate: 25 mL/min) to obtain *N*-Boc-*N*-methyl-L-phenylalanine ethyl ester[27] (0.236 g, 56%). A solution of *N*-Boc-*N*-methyl-L-phenylalanine ethyl ester (1 mg) in MeOH (0.25 mL) was treated with HCl (3 M HCl(aq), 0.25 mL). The reaction mixture was stirred for 2 h at rt and extracted with EtOAc (3 × 1 mL). The combined organic extracts were concentrated under a stream of N_2 to give pure *N*-methyl-L-phenylalanine hydrochloride. ^{1}H NMR data were in agreement with literature values.[27]

*(4S,5S)-4-Hydroxypyrrolidin-2-one (**4a**).* A solution of *N*-Boc-L-leucine (0.5 g, 2.16 mmol) in CH_2Cl_2 (5 mL) was cooled to 0 °C and treated with Meldrum's acid (0.809 g, 2.16 mmol), DMAP (0.369 g, 3.2 mmol), and freshly triturated DCC (0.5 g, 2.51 mmol). The solution was warmed to rt, left to stir for 3 h, and poured into cold EtOAc (50 mL). The solution was then filtered, and the filtrate was washed with cold, aqueous $NaHSO_4$ (8 mL, 5% w/v), followed by brine (8 mL). After concentration under reduced pressure, the resulting solid was redissolved in EtOAc (20 mL) and heated at reflux (0.5 h). After removal of volatiles, the residue was purified by flash chromatography (silica, gradient: 1:9 to 1:1 EtOAc−hexane). Fractions identified by TLC were then combined and concentrated to yield the known pyrrolidine-2,4-dione[9] (0.42 g, 42%), which was used directly in the next step. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.40 (1H, m), 3.28 (1H, d, J = 18.9 Hz), 3.16 (1H, d, J = 18.9 Hz), 1.87−2.01 (1H, m), 1.56 (9H, s), 1.1−1.4 (1H, m), 0.95 (6H, d, J = 6 Hz), in agreement with literature values.[9]

A solution of the above-described pyrrolidine-2,4-dione (0.186 g, 0.71 mmol) in CH_2Cl_2−AcOH (3.5 mL, 9:1) was cooled to 0 °C and treated with $NaBH_4$ (0.055 g, 1.4 mmol) in two portions. The mixture was stirred at 0 °C for 30 min, concentrated, and redissolved in EtOAc (7 mL). The organic solution was washed with 5% aqueous $NaHCO_3$ (3 × 3 mL). The crude product was then purified by flash chromatography (silica, gradient 3:1 EtOAc−hexane to EtOAc) to yield known 4-hydroxypyrrolidinone **4a**[9] (55.5 mg, 30% yield): ^{1}H NMR (400 MHz, DMSO) δ 5.29 (1H, d, 9.66), 4.30 (1H, m), 4.00 (1H, m), 2.45 (2H, dq, J = 16.8, 6.9), 1.72 (2H, m), 1.44 (9H, s), 0.90 (6H, m), in agreement with literature values.[9]

*(4R,5S)-4-Hydroxypyrrolidin-2-one (**4b**).* To a suspension of pyrrolidine-2,4-dione (0.128 g, 0.78 mmol) in CH_2Cl_2 (1 mL) was added TFA (1 mL), and the the mixture was stirred for 20 min. The solution was concentrated under a stream of N_2 to provide crude product (0.121 g), which was dissolved in CH_2Cl_2−AcOH (9:1, 4 mL). $NaBH_4$ (60 mg, 1.56 mmol) was added at 0 °C in two portions, and the mixture was allowed to warm to rt over 45 min with stirring. The organic solution was washed with 5% $NaHCO_3$(aq) (3 × 5 mL), and the organic phase dried over $MgSO_4$, concentrated, and purified by flash chromatography (silica, 9:1 CH_2Cl_2−MeOH) to yield a mixture of **4a**:**4b** (62:38, 38.0 mg, 30%).

*(3S,4S)-Statine TFA Salt (**5a**).* A mixture of **4a** (55.5 mg in 1 mL MeOH) and NaOMe (0.5 mL, 0.44 M) was stirred for 1 h, concentrated under reduced pressure, and taken up in EtOAc (10 mL). The EtOAc solution was then washed with H_2O (5 mL) and brine (5 mL). Acidification of the aqueous layer (1 M HCl, 0.5 mL) yielded pure (3*S*,4*S*)-*N*-Boc statine[9] (21.4 mg, 38%) with data consistent with literature values. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.02 (1H, m), 3.61 (1H, m), 2.55 (2H, d, J = 3.0 Hz), 1.43 (9H, s), 0.91 (6H, m). A sample of the latter compound (1.0 mg, 0.36 μmole) was stirred in CH_2Cl_2−TFA (1:1, 0.5 mL) at rt for 2 h to yield, after removal of volatiles, (3*S*,4*S*)-statine TFA salt (**5a**, quant): ^{1}H NMR (400 MHz, D_2O) δ 4.10 (1H, m), 3.31 (1H, m), 2.66 (2H, m), 1.68 (1H, m), 1.50 (2H, m), 0.91 (6H, m).[9]

*(3R,4S)-Statine TFA Salt (**5b**).* *S*-*N*-Boc pyrrolidin-2,4-one was subject to deprotection (TFA), followed by $NaBH_4$ reduction[10] and hydrolysis (6 M HCl, 110 °C, 2.5 h) to give a mixture of diastereomers **5a**:**5b** (62:38). LRMS *m*/*z* 176.4 $[M + H]^+$ (calcd 176.2).

(S)-Valic Acid. To an ice-cold solution of L-valine (100 mg, 0.854 mmol) in 2 M H_2SO_4 (0.97 mL) was added $NaNO_2$ (116 mg) in H_2O (0.155 mL) over 4 h, and the mixture allowed to warm to rt with stirring over 6 h. The reaction mixture was saturated with NaCl and extracted with EtOAc (10 × 20 mL), and the combined organics were dried over $MgSO_4$. Removal of the volatiles under reduced pressure afforded (*S*)-valic acid (**9a**) as a viscous oil (0.053 g, 53%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.16 (1H, d, J = 3.4 Hz), 2.16 (1H, m), 1.07 (3H, d, J = 6.9), 0.93 (3H, d, J = 6.9 Hz).[28]

(*S*)-Valic Acid 2-Naphthacyl Ester (10a). To a solution of (*S*)-valic acid (**9a**, 20 mg, 0.17 mmol in 0.50 mL of EtOAc) was added α-bromo-2-acetonaphthone (0.042 g, 0.17 mmol) in EtOAc (2.5 mL) followed by Et_3N (23 μL). The solution was stirred at rt for 3 h followed by extraction with H_2O (3 × 2 mL). The combined organic layers were washed, sequentially, with 10% citric acid (1 mL), 7% $NaHCO_3$ (1 mL), and brine (1 mL), and the volatiles were removed under reduced pressure. The residue was dissolved in EtOAc and separated by preparative TLC (1:1 hexane−EtOAc), and the middle-eluting product band was extracted from silica gel (2 mL of EtOAc) to yield L-valic acid 2-naphthacyl ester (**10a**, 5.0 mg): colorless solid; $[\alpha]^{26}_D$ +5.0 (*c* 1.01, EtOAc); UV (MeOH), λ_{max} (log ε) 248 (4.34), 283 (3.65), 338 (2.95) nm; FTIR (ATR): ν 3349, 2921, 2851, 1741, 1695 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (1H, s), 7.94 (5H, m), 7.63 (2H, m), 5.69 (1H, d, J = 21.6 Hz), 5.52 (1H, d, J = 21.6 Hz, 5.18 (1H, d, J = 5.8 Hz), 2.53 (1H, m), 1.20 (6H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191 (C), 168 (C), 136 (C), 133 (C), 131 (C), 129.9 (CH), 129.8 (CH), 129.3 (CH), 129.2 (CH), 128 (CH), 127 (CH), 123 (CH), 84.1 (CH), 67.0 (CH_2), 30.0 (CH), 19.0 (CH_3), 17.4 (CH_3).; HRESIMS *m*/*z* 309.1098 $[M + Na]^+$ (calcd for $C_{17}H_{18}O_4Na$, 309.1097).

(±)-D,L-**10a**,**b** was prepared from (±)-valic acid in a similar manner. See below for chiral-phase HPLC retention times.

***N*,*N*-Dimethyl-L-isoleucine 2-Naphthacyl Ester (8a).** To a solution of *N*,*N*-dimethyl-L-isoleucine (**6a**, 1.18 mg, 11.4 μmol) in EtOH (200 μL) was added $NaHCO_3$ (3 mg, 23 μmol) followed by α-bromo-2-acetonaphthone (**7**, 4 mg, 11.4 μmol). The mixture was stirred at rt overnight, dried down, and separated by preparative TLC (9:1 hexane−EtOAc). The band corresponding to product was extracted with CH_2Cl_2 to yield *N*,*N*-dimethyl-L-isoleucine naphthacyl ester (**8a**, 0.8 mg, 40%): $[\alpha]^{23}_D$ +12.2 (*c* 0.35, CH_3CN); UV (CH_3CN) λ_{max} 248 (log ε 4.45), 283 (3.73), 338 (3.00) nm; FTIR (ATR) ν 2920, 2851, 2346, 1680, 1466 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (1H, s), 7.93 (4H, m), 7.60 (2H, m), 5.53 (2H, s), 3.06 (2H, d, J = 10.1), 2.41 (6H, s), 1.93 (1H, m), 1.72 (1H, m), 1.21 (2H, m), 0.99 (3H, d, J = 6.6), 0.93 (3H, t, J = 7.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192 (C), 171 (C), 136 (C), 132 (C), 131 (C), 130 (CH), 129 (CH), 128 (CH), 127 (CH), 123 (CH), 72.5 (CH), 65.6 (CH_2), 41.6 (CH), 33.6 (CH), 25.3 (CH_2), 15.8 (CH_3), 10.7 (CH_3); HRESIMS *m*/*z* 328.1909 $[M + H]^+$ (calcd for $C_{20}H_{26}NO_3$, 328.1907).

Chiral-Phase HPLC Analysis of *N*,*N*-Dimethyl-L-isoleucine 2-Naphthacyl Esters 8a−d. Stereoisomerically pure and mixed *N*,*N*-Me_2 isoleucines [*allo*-L-Ile, **6c**, 1:1 mixture of L-Ile, **6a**, and *allo*-D-Ile (**6d**, Sigma), and a 1:1:1:1 mixture of **6a**−**d** (Sigma)] were converted to the corresponding naphthacyl esters **8a**−**d**, as described above for pure *S*-**6a**, and analyzed by chiral-phase HPLC (Chiralcel OD-H, 250 × 4.60, 0.5 mL.min^{-1}, 2:98 *i*-PrOH + 0.1% TFA−hexane) and gave the following retention times: t_R = 28.60, 29.91, 27.42, 25.23 min, respectively.

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Hydrolysis of Symplocin A. Symplocin A (**3**, 200 μg) was subjected to acid hydrolysis (6 N HCl, 120 °C, 20 h), the mixture was cooled, and a portion was extracted with EtOAc. The organic layer was dried and used in the preparation of **10a** (see below), and the remainder of the aqueous hydrolysate was dried under a stream of nitrogen and high vacuum before conversion to Marfey's derivatives or 2-naphthacyl esters as described below.

*Preparation of 2-Naphthacyl Esters from the Peptide Hydrolysate of **3**.* An aliquot of the peptide hydrolysate of **3** (~100 μg in 200 μL of EtOAc) of the ethyl acetate-soluble fraction was treated with α-bromo-2-acetonaphthone (200 μg) followed by triethylamine (~10 μL). The reaction was stirred overnight at rt followed by isolation by preparative TLC (1:1 EtOAc–hexane). The extracted band was identified as product by LRMS (*m/z* 287.5 $[M + H]^+$; calcd 287.1) as valic acid 2-naphthacyl ester (**10**). The product was concentrated under a stream of N_2 and redissolved (1 mg/mL, 1:1 hexane–*i*-PrOH) and analyzed by chiral-phase HPLC (see below).

An aliquot of the total hydrolysate (2.0 mg) was dissolved in EtOH (250 μL) and adjusted to pH = 6 (LiOH(aq)). To the reaction mixture was added α-bromo-2-acetonaphthone (4.2 mg). The reaction mixture was left to stir at rt overnight. The reaction mixture was then dried under a stream of N_2, redissolved in CH_2Cl_2 (0.5 mL), and separated by preparative TLC (1:9 EtOAc–hexane). The product band containing **8** (LRMS, *m/z* 328.52 $[M + H]^+$; calcd 328.18) was concentrated under a stream of N_2, reconstituted (1 mg/mL, 1:1 hexane–*i*-PrOH), and analyzed by chiral-phase HPLC (Chiracel OD-H, 250 × 4.6 mm, 0.5 mL·min^{-1}, 2:98 *i*-PrOH + 0.1% TFA–hexane) along with standard naphthacyl esters **8a–d** prepared as described above. The natural product-derived *N,N*-dimethylisoleucine 2-naphthacyl ester was identified as (2*R*,3*R*)-**8b** (t_R = 28.75) and confirmed by co-injection with standard **8b**.

Chiral-Phase HPLC Analysis of 2-Naphthacyl Valic Acid Esters. An aliquot of the EtOAc-soluble hydrolysate of peptide **3** (~100 μg) was derivatized with α-bromo-2-acetonaphthone (see above) and compared with the standard 2-naphthacyl esters (**10a**,**b**) (prepared from (±)-valic acid **9a**,**b**, see above) by chiral-phase HPLC (Chiracel OD-H, 250 × 4.60, 0.5 mL·min^{-1}, 25:75 *i*-PrOH–hexane), giving the following respective retention times: t_R = 18.17, 30.14, and 18.16 min. The identity of the natural product-derived *S*-**10b** and the synthetic standard was confirmed by co-injection.

*Marfey's Analysis of **3**.* Aliquots (~100 μg) of hydrolyzed symplocin A (**3**) were dissolved in 1 M $NaHCO_3$(aq) (400 μL) and treated, separately, with L-FDAA or D-FDAA (50 μL, 2 mg/mL in acetone). The mixture was stirred at 80 °C for 30 min followed by the addition of HCl (2 M, 200 μL) and dilution with CH_3CN (100 μL). The freshly prepared Marfey's derivatives of the peptide hydrolysate and standard amino acids were analyzed under two sets of conditions. Condition A (RP HPLC, Agilent Eclipse XDB-C_{18}, 1.0 mL/min, gradient elution with 1:4 to 1:1 CH_3CN–H_2O + 0.1% HCOOH over 45 min, monitoring at 340 nm) was used to assign Ser, Tyr, and *N*-MePhe. Condition B (RP HPLC, Agilent Eclipse XDB-C_{18}, 1.0 mL/min, gradient elution with 1:9 to 100% CH_3CN–H_2O + 0.1% formic acid over 45 min, monitoring at 340 nm) was used to assign Pro and Val. The retention times (min) of authentic L-FDAA derivatives were L-Ser (22.39), D-Ser (23.6), L-Pro (31.25), D-Pro (33.0), L-Tyr (26.08), D-Tyr (30.79), L-Val (33.42), D-Val (37.84), L-*N*-MePhe (33.35), and D-*N*-MePhe (44.46). The Marfey's derivatives of hydrolyzed **3** gave retention times of 22.00, 31.92, 27.37, 33.21, and 43.71 min, corresponding to L-Ser, L-Pro, L-Try, L-Val, and D-*N*-MePhe, respectively, which were confirmed by co-injections.

■ ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C NMR and 2D NMR spectra of **3** and ^1H,^{13}C NMR of naphthacyl esters **8a** and **10a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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