New Amino Acid Derivatives from the Marine Ascidian *Leptoclinides dubius*

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From the cytotoxic extracts of the marine ascidian *Leptoclinides dubius*, *N*-(*p*-hydroxybenzoyl)-L-arginine (**1**), *N*-(1*H*-indolyl-3-carbonyl)-D-arginine (**2**), and *N*-(6-bromo-1*H*-indolyl-3-carbonyl)-R, where R is L-Arg (**4**), L-His (**5**), and the very rare amino acid L-enduracididine (**3**), have been isolated and identified by spectroscopic data, hydrolysis, and comparison with authentic samples.

Marine ascidians have been shown to be a very rich source of unique and biologically active secondary metabolites that have attracted the interest of both chemists and pharmacologists.[1] A major group of those metabolites are nitrogen-containing compounds, particularly aromatic heterocycles.[2] As part of our ongoing investigation on bioactive compounds from marine organisms, we describe in this paper the isolation of simple peptides **1**−**5** (Chart 1) from the polar cytotoxic extract (100% inhibition of KB cells at 10 μg/mL and 80% inhibition of P-388 cells at 10 μg/mL) of the ascidian *Leptoclinides dubius*[3] (Sluiter, 1909) (order Enterogona, family Didemnidae), collected in New Caledonia.

The methanolic extracts of *L. dubius* (340 g dry wt) were sequentially submitted to solvent partition between aqueous MeOH and hexanes, then CH_2Cl_2, and then *n*-BuOH. The *n*-BuOH-soluble material was first chromatographed on Amberlite XAD-2 and then on Sephadex LH-20 and finally subjected to reversed-phase HPLC to afford pure **1** (11 mg), **2** (3 mg), **3** (5 mg), **4** (15 mg), and **5** (5 mg).

Compound **1** was obtained as a pale yellow solid. The UV spectra indicated the presence of a phenol chromophore with bands at λ_{max} 210 nm and 256 nm that shifted to longer wavelenghts on addition of base [MeOH/NaOH, λ_{max} 216 nm and 294 nm]. Its (+)-FABMS (in glycerol) showed the molecular ion at 295 ($[M + H]^+$), confirmed by the ion at *m*/*z* 317 ($[M + Na]^+$), which was obtained when the (+)-FABMS was taken in glycerol + NaCl. These ions are consistent with the molecular formula $C_{13}H_{18}N_4O_4$ for **1**, requiring seven sites of unsaturation. For its part, HREIMS showed the highest mass ion at *m*/*z* = 234.0768 ($[M - CH_6N_3]^+$; $C_{12}H_{12}NO_4$, Δ = 0.2 ppm) in accordance with the facile β cleavage of the guanidine moiety and the proposed structure.

The ^{1}H NMR spectrum (D_2O; 500 MHz) of **1** contained the resonances of an AA'BB' system at δ 6.68, d (H4/H6) and δ 7.46, d (H3/H7), due to a *p*-hydroxyphenyl fragment. The ^{13}C and DEPT NMR spectra showed 13 resonances that were correlated with the corresponding protons by standard 2D NMR experiments (COSY, HMQC, and HMBC). Thus, the carbon resonance at 55.2 ppm and its proton at 4.13 ppm (dd, H10) indicated the presence of an amino acid moiety, and that at δ 156.9 (s, C15) was in good agreement with the expected value for the terminal guanidine group. Acid hydrolysis of **1** (6 N HCl/120 °C/12 h) afforded *p*-hydroxybenzoic acid and L-Arg [CD (+) ($c = 1.49 \times 10^{-3}$ M, HCl 2 N), λ_{max} = 215 nm ($\Delta\epsilon$ = 0.06)] whose stereochemistry was determined by Marfey's method.[4]

Compound **2** also contained arginine as the amino acid component, but its aromatic chromophore had UV bands at λ_{max} 222, 250, and 280 nm, typical of an indole system. The (+)-FABMS showed the molecular ion at *m*/*z* 318 ($[M + H]^+$; molecular formula $C_{15}H_{19}N_5O_3$), that together with the ^{1}H NMR spectral data suggested structure **2**, with an amide bond between the indole carboxylic acid and the Arg group. Similar analyses were carried out with compound **4**, which showed a molecular mass 78 amu higher than **2** in the (+)-FABMS. It also showed a typical cluster due to the presence of one bromine atom, and this atom was located at C6 on the basis of 2D NMR data.[5,6] Acid hydrolysis of **2** afforded the expected 1*H*-indole-3-carboxylic acid and D-Arg [CD (−) ($c = 2.87 \times 10^{-3}$ M, HCl 2 N), λ_{max} = 207.5 nm ($\Delta\epsilon$ = −0.09)], while hydrolysis of **4** produced 6-bromo-1*H*-indole-3-carboxylic acid and L-Arg [CD (+) ($c = 2.42 \times 10^{-3}$ M, HCl 2 N), λ_{max} = 213.0 nm ($\Delta\epsilon$ = 0.63)].

Spectroscopic data and hydrolysis indicated that compounds **3** and **5** contained the 6-bromo-1*H*-indole-3-carboxylic acid moiety and that the amino acid component of **5** was L-His. For its part, comparison of the ^{13}C and DEPT NMR data of **3** with those of **4** showed differences mainly at C12, C13 (a CH_2 in **4**; a CH in **3**), and C14. All these signals were shifted to lower field in **3**, suggesting that C13 was bonded to a nitrogen atom. These and other significant data from ^{1}H NMR and FABMS indicated that the amino acid of **3** was a cyclic derivative of Arg, with the guanidine moiety forming a partially saturated imidazole ring. In addition, the amino acid isolated by hydrolysis of **3** showed a positive CD band indicative of an L configuration at C11 and a positive optical rotation.[7]

Two natural amino acids with a partially saturated imidazole structure were reported 30 years ago and their absolute stereochemistry determined by X-ray diffraction and ORD. These amino acids were named enduracididine [**6**; L-series; (2*S*,4*R*)] and its diastereoisomer alloenduracididine [D-series; (2*R*,4*R*)] isolated from the fungal peptide antibiotic enduracidin.[8] Enduracididine has since been found as a component of

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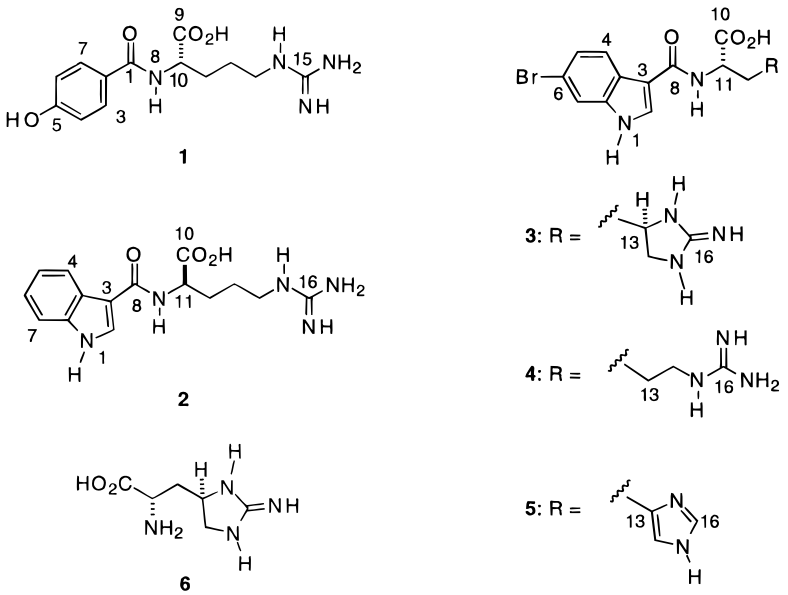
Chart 1



the seeds of a leguminoseae.[9] Comparison with the reported data for those isomers indicates that the amino acid component of **3** is enduracididine, and therefore the absolute stereochemistry of **3** is (11*S*,13*R*). This is the first time the very rare amino acid enduracididine has been found in a marine organism, albeit in very small amounts.

In summary, *L. dubius* is the source, as other ascidians, of amino acid-derived metabolites.[2] In this particular case, they are modified dipeptides where one of the amino acid constituents has lost two carbon atoms and the amino group.

Experimental Section

General Experimental Procedures. Optical rotations were measured in MeOH using a JASCO DIP-370 polarimeter with a sodium lamp operating at 598 nm. The UV spectra were recorded on a Hewlett-Packard 8452A spectrometer. The IR spectra on a MINAC Prospect FTI spectrometer. CD data were obtained in 2 N HCl on a JASCO J-720 spectropolarimeter. The NMR spectra were recorded on a Bruker AMX-500 at 500.13 MHz for ^{1}H and 125.77 for ^{13}C. The two-dimensional ^{1}H−^{13}C heteronuclear shift correlation (HMQC) spectra (512 × 2K) for directly bonded protons and carbons were obtained by accumulating 48 scans per t_1, with a relaxation delay of 1 s and J_{CH} = 130 Hz. The two-dimensional ^{1}H−^{13}C heteronuclear shift correlation (HMBC) spectrum (1024 × 2K) was obtained by accumulating 128 scans per t_1; the relaxation delay was 1.5 s, and the value of J_{CH} selected was 9 Hz. The data were zero-filled to 1024 in F_1 and subjected to a QSINE transformation.

HPLC was performed with a Waters Model 590 apparatus equipped with an R401 differential refractometer and a 440 UV detector working at 254 nm. Semipreparative HPLC was carried out using 300 × 7.8 mm μBondapak NH_2 and μBondapak C_{18} reversed-phase columns and a 500 × 7.8 mm Partisil M9 10/50 ODS-3 reversed-phase column; for analytical HPLC a 330 × 3.9 mm μBondapak C_{18} column was used. Fast atom bombardment mass (FABMS) spectra were obtained on a Kratos MS50 mass spectrometer in glycerol matrix with NaCl as additive for positive ion mode. HREIMS were obtained on a Kratos MS50 spectrometer and LREIMS on a Hewlett-Packard 5988A mass spectrometer operating at 70 eV.

Animal Material. Speciments of the ascidian *L. dubius* (voucher ref. UA-284) were collected by hand using SCUBA (−5 to −10 m) in Woodin Canal, New Caledonia and stored at −5°C. They were identified by Mrs. F. Monniot of the Muséum National d'Histoire Naturelle (Paris, France).[3]

Extraction and Isolation. The freeze-dried ascidians (340 g) were homogeneized in methanol (six times) and the solvent evaporated to dryness under reduced pressure. The extract was partitioned between 10% aqueous MeOH (200 mL) and hexane (2 × 200 mL). The aqueous portion was made 40% MeOH and extracted sequentially with CH_2Cl_2 (3 × 200 mL) and *n*-BuOH (5 × 200 mL). The *n*-BuOH portion was passed through an Amberlite XAD-2 column, eluting first with water (3 L) to retain the organic compounds that were then eluted with MeOH (4 L). The methanol eluates were concentrated under reduced pressure to give 1.2 g of material, which was chromatographed on Sephadex LH-20 (flow rate 50 mL/h) with MeOH/H_2O (2:1) as eluant and TLC monitoring (silicagel, 6:4:1 $CHCl_3$/MeOH/H_2O) giving seven main fractions. Fractions 4 and 6 contained compounds **1**−**5**. The fraction four (127 mg) was submitted to HPLC on a 300 × 7.8 mm μBondapak NH_2 column, eluting with MeOH, to afford pure compound **1** (11 mg, retention time 22 min, flow rate 2 mL/min). Identical separation conditions applied to fraction 6 (214 mg) afforded 5 mg of pure compound **5** (retention time 30 min, flow rate 3 mL/min).

The rest of the compounds were also isolated from fraction 6, using different HPLC conditions as follows: HPLC on a 300 × 7.8 mm μBondapak C_{18} column, eluting with 30:70 MeOH/H_2O, afforded 3 mg of pure compound **2** (retention time 21 min, flow rate 1.5 mL/min) and HPLC on a 500 × 7.8 mm Partisil M9 10/50 ODS-3 column eluting with 45:55 MeOH/10^{-2} M NH_4-AcO gave pure compounds **4** (15 mg, retention time 46 min, flow rate 2 mL/min) and **3** (5 mg, retention time 48 min, flow rate 2 mL/min).

Table 1. ^{1}H NMR Data of **1**−**5**

	1[a] δ_H, mult, *J* (Hz)	**2**[b] δ_H, mult, *J* (Hz)	**3**[a] δ_H, mult, *J* (Hz)	**4**[a] δ_H, mult, *J* (Hz)	**5**[a] δ_H, mult, *J* (Hz)
H2		7.81, s	8.04, s	7.99, s	7.96, s
H3	7.46, d, 8.7				7.55, d, 1.2
H4	6.68, d, 8.7	7.82, dd, 8.8, 3.4	8.06, d, 7.7	7.90, d, 8.5	7.90, d, 8.5
H5		7.10, m	7.28, dd, 7.6, 1.7	7.20, dd, 8.5, 1.5	7.20, dd, 8.5, 1.2
H6	6.68, d, 8.7	7.10, m			
H7	7.46, d, 8.7	7.39, dd, 6.0, 3.0	7.66, d, 1.7	7.38, d, 1.5	7.55, d, 1.2
H10	4.13, dd, 8.3, 5.0				
H11	*a* = 1.58, m	4.27, dd, 7.7, 4.9	4.61, m	4.50, dd, 8.7, 4.3	4.70, dd, 7.5, 5.1
	b = 1.70, m				
H12	1.42, q	*a* = 1.66, m	*a* = 2.20, m	*a* = 1.80, m	*a* = 3.2, dd, 15.0, 5.1
		b = 1.78, m	*b* = 2.30, m	*b* = 1.90, m	*b* = 3.4, dd, 15.0, 5.1
H13	2.90, t, 6.9	1.52, q	4.27, m	1.64, q	
H14		3.00, t, 6.9	*a* = 3.56, t, 9.6	*a* = 3.10, m	7.20, s
			b = 3.94, t, 9.5	*b* = 3.20, m	
H16					8.50, s

[a] In CD_3OD. [b] In D_2O.

Table 2. ^{13}C NMR Data of **1**−**5**[a]

	δ_C (**1**)[b]	δ_C (**2**)[c]	δ_C (**3**)[b]	δ_C (**4**)[b]	δ_C (**5**)[b]
C1	169.9				
C2	125.4	128.4	130.8	130.1	130.5
C3	129.6	110.5	112.3	111.8	112.1
C3a		137.6	139.3	138.6	133.0
C4	115.2	123.0	123.3	122.9	123.1
C5	159.4	121.1	125.6	124.9	125.4
C6	115.2	120.9	117.2	116.7	117.0
C7	129.6	113.5	116.2	115.6	116.0
C7a		128.2	126.2	125.9	126.4
C8		168.4	167.9	167.4	167.3
C9	178.9				
C10	55.2	180.5	179.0	180.1	176.8
C11	29.0,	56.5	55.3	55.5	54.9
C12	24.7	31.6	40.5	31.0	29.9
C13	40.8	27.0	55.3	26.3	139.1
C14		42.2	50.1	42.1	118.5
C15	156.9				
C16		160.0	161.5	158.9	134.5

[a] Assignments based on DEPT experiments. [b] In CD_3OD. [c] In D_2O.

Compound 1, $C_{13}H_{18}N_4O_4$: $[\alpha]^{20}_D$ +19.5° (*c* 2.05 , MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (3.46), 256 (3.49) nm; IR (dry film) ν max 3200−3600 (OH), 1630 (C═N, guanidine), 1614 (C═O, amide), 1576 (C═O, carboxylic acid), 1264 (OH, phenol moeity) cm^{-1}; ^{1}H NMR see Table 1; ^{13}C NMR see Table 2; FABMS (positive ion mode) *m*/*z* 295 (100), 121 (14), 93 (11); EIMS (70 eV) *m*/*z* 234 (9), 137 (5), 121 (100), 93 (16); HREIMS 234.0768 ($C_{12}H_{12}NO_4$, Δ = 0.2 ppm).

Compound 2, $C_{15}H_{19}N_5O_3$: $[\alpha]^{20}_D$ −133.3° (*c* 0.15 , MeOH); UV (MeOH) λ_{max} (log ϵ) 214 (3.65), 246 (3.13), 282 (3.11) 286 (3.10) nm; IR (dry film) ν max 1651 (C═N, guanidine), 1623 (C═O, amide), 1592 (C═C), 1539 (C═O, carboxylic acid) cm^{-1}; ^{1}H NMR see Table 1; FABMS (positive ion mode) *m*/*z* 318 (22), 259 (19), 115 (100); EIMS (70 eV) *m*/*z* 257 (19), 160 (11), 145 (12), 144 (100), 116 (15); HREIMS 258.1212 ($C_{14}H_{14}N_2O_3$, Δ = 0.3 ppm).

Compound 3. $C_{15}H_{16}N_5O_3Br$, $[\alpha]^{20}_D$ +16.5° (*c* 0.85 , MeOH); UV (MeOH) λ_{max} (log ϵ) 222 (2.98), 250 (2.63), 282 (2.39) nm; IR (dry film) ν max 1682 (C═N, guanidine), 1616 (C═O, amide), 1592 (C═C), 1535 (C═O, carboxylic acid) cm^{-1}; ^{1}H NMR see Table 1; ^{13}C NMR: see Table 2; FABMS (positive ion mode) *m*/*z* 396(42), 394 (40), 224 (12), 222 (11), 115 (100); EIMS (70 eV) *m*/*z* 337 (12), 335 (12), 240 (16), 238 (17), 224 (97), 222 (100), 197 (10), 195 (11), 143 (15), 115 (21), 114 (40); HREIMS 335.0032 ($C_{14}H_{12}N_2O_3{}^{79}Br$, Δ = 0.2 ppm).

Compound 4, $C_{15}H_{18}N_5O_3Br$. $[\alpha]^{20}_D$ +3.6° (*c* 0.55 , MeOH); UV (MeOH) λ_{max} (log ϵ) 222 (3.78), 250 (3.43), 280 (3.18) nm; IR (dry film) ν max 1670 (C═N, guanidine), 1623 (C═O, amide), 1592 (C═C), 1576 (C═O, carboxylic acid) cm^{-1}; ^{1}H NMR see Table 1; ^{13}C NMR see Table 2; FABMS (positive ion mode) *m*/*z* 398 (55), 396 (53), 224 (23), 222 (22), 144 (10), 115 (100); EIMS (70 eV) *m*/*z* 337 (16), 335 (16), 240 (23), 238 (24), 224 (97), 222 (100), 197 (25), 195 (27), 115 (30), 114 (40); HREIMS 335.0029 ($C_{14}H_{12}N_2O_3{}^{79}Br$, Δ = 0.3 ppm).

Compound 5, $C_{15}H_{13}N_4O_3Br$. $[\alpha]^{20}_D$ +26.6° (*c* 0.55 , MeOH); UV (MeOH) λ_{max} (log ϵ) 222 (3.53), 250 (3.17), 282 (2.92) nm; IR (dry film) ν max 3400 (N-H), 1595 (C═O, amide), 1538 (C═O, carboxylic acid) cm^{-1}; ^{1}H NMR: see Table 1; ^{13}C NMR see Table 2; FABMS (positive ion mode) *m*/*z* 379 (17), 377 (16), 224 (9), 222 (8), 115 (100); EIMS (70 eV) *m*/*z* 241 (48), 240 (28), 239 (41), 224 (96), 222 (100), 197 (97),196 (35), 195 (99), 194 (20), 143 (21), 116(64), 115 (39), 82(48), 81(42); HREIMS 376.0169 ($C_{15}H_{13}N_4O_3{}^{79}Br$, Δ = 0.3 ppm).

Acid Hydrolysis of 1−5. A solution of the compound (2−4 mg) in 6 N HCl (1 mL) was heated at 120 °C overnight in a stoppered reaction vial. The solution was then evaporated under reduced pressure and the residue partitioned between water (3 mL) and $CHCl_3$ (3 × 2 mL). The combined organic extracts were washed with water and evaporated to dryness under reduced pressure to give *p*-hydroxybenzoic acid from **1**, 1*H*-indole-3-carboxylic acid from **2**, and 6-bromo-1*H*-indole-3-carboxylic acid from **3**−**5**, identified by MS and comparison with authentic samples.

The aqueous layer was lyophilized and the residue derivatized with Marfey's reagent and compared by HPLC on 330 × 3.9 mm μBondapak C_{18} column, with the corresponding derivatives of L- and D-Arg and L- and D-His. In this way, L-Arg was identified in **1** and **4**, D-Arg in **2,** and L-His in **5.** Hydrolysis of **3** gave enduracididine (**6**), identified by comparison of its spectroscopic properties (NMR, CD, α) with reported data.[9]

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