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EUDISTOMIN U AND ISOEUDISTOMIN U, NEW ALKALOIDS FROM THE CARRIBEAN ASCIDIAN *LISSOCLINUM FRAGILE*

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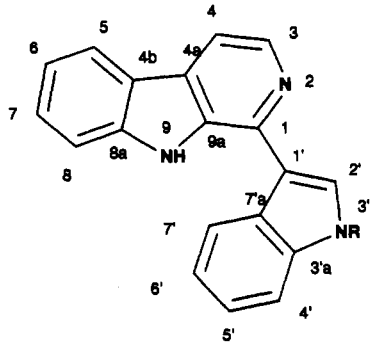
ABSTRACT.—Chemical investigations of the Caribbean ascidian *Lissoclinum fragile* (Didemnidae) have resulted in the isolation of new alkaloids which we have designated eudistomin U [**1**] and isoeudistomin U [**2**]. Their structures were determined by spectrometric and chemical means.

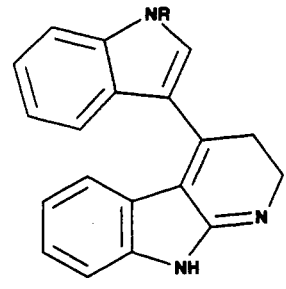
Marine ascidians are a very rich source of nitrogenous secondary metabolites, and particularly of alkaloids, with most of these occurring in the family Polycitoridae. In contrast, the number of marine alkaloids isolated from the family Didemnidae is rather small, namely, ascididemnin (1), the shermilamines (2), the lamellarins (3), the tridemnic acids (4), and diplamine (5). The varamines (6) possess a thiomethyl group and are the only alkaloids isolated from the genus *Lissoclinum*, which is known to contain cyclic peptides (7) and macrolides (8). In this paper, we report the structure of two new alkaloids isolated from the genus *Lissoclinum*, which were obtained using a simplified hplc system for the detection of compounds capable of binding to DNA (9) as a guide for chromatographic separations.

Brown and green color morphs of the colonial ascidian *L. fragile*, which is the host of a symbiotic alga (10), were collected near "Ilet Pigeon" (Cousteau's reservation) Guadeloupe, France, in June 1988, between −30 and −40 m depth. Solvent partition of the crude extract and extensive chromatographic purification, monitored by the aforementioned bioassay (9), allowed the separation of two yellow compounds which we have designated eudistomin U [**1**] and isoeudistomin U [**2**].

Accurate mass measurement (peak matching) of the highest ion at *m/z* 283.10945 in the eims of eudistomin U [**1**], provided the molecular formula $C_{19}H_{13}N_3$ (M^+, Δ=1.4 mmu), in agreement with ^{1}H- and ^{13}C-nmr data (see Table 1). The infrared spectrum ($CHCl_3$) established the presence of conjugated double bonds (ν max 3010 and 1610 cm^{-1}) and of OH and/or NH functionalities (ν max 3600–3400 cm^{-1}). The ^{1}H-nmr spectrum of eudistomin U displayed the best dispersion when measured in CD_2Cl_2 with 1 drop of CD_3OD.



1
1a



2
2a

TABLE 1. ^{1}H- (400 MHz) and ^{13}C[a]- (100 MHz) Nmr Data for **1**[b] and **2**[b].

Position	Compound									
	1					**2**				
	^{1}H			^{13}C		^{1}H			^{13}C	
	δ (ppm)	mult.	*J* (Hz)	δ (ppm)	$^nJ_{CH}$=10 Hz	δ (ppm)	mult.	*J* (Hz)	δ (ppm)	$^nJ_{CH}$=10 Hz
1				136.51	H-3					
2						3.90	t	11.7	42.30	H-3
3	8.30	d	6	128.00	H-4	3.20	t	11.7	20.35	H-2
4	8.05	d	6	114.50	H-3				132.06	H-2
4a				121.05	H-3/H-4				125.71	H-3
4b				122.43	H-5/H-6				138.31	H-6/H-8
5	8.20	d	8	123.48	H-7	7.65	d	7.2	119.07	H-7
6	7.35	br t	8, 7.3	121.47	H-8	7.41	br t	8, 7.2	124.24	H-8
7	7.60	br t	8, 7.3	131.00	H-5	7.39	br t	8, 7.2	124.96	H-5
8	7.61	d	8	114.87	H-6	7.81	d	7.2	114.48	H-6
8a				143.04	H-5/H-7				142.95	H-5/H-7
9a				132.40	H-4				158.63	H-2
1′				109.32					109.45	
2′	7.97	s		138.00		8.30	s		138.00	
3′a				137.79	H-5′/H-7′				141.10	H-5′/H-7′
4′	7.50	d	8	113.70	H-6′	7.55	d	7.3	113.78	H-6′
5′	7.30	br t	8, 7.2	124.00	H-7′	7.49	br t	8, 7.3	129.69	H-7′
6′	7.21	br t	8, 7.2	122.05	H-4′	7.25	br t	8, 7.3	122.69	H-4′
7′	7.80	d	8	120.32	H-5′	7.75	d	7.3	121.70	H-5′
7′a				126.16	H-4′/H-6′				124.90	H-4′/H-6′

[a]A relaxation delay (4 sec) was used during ^{13}C-nmr spectroscopy to improve the signals of quaternary carbons having longer relaxation times. Identification was based on DEPT, QUAT (selective quaternary carbon observation with ^{1}H decoupling), HMQC and HMBC experiments.

[b]Nmr data for [**1**] in CD_2Cl_2+1 drop of CD_3OD; nmr data for **2** in CD_2Cl_2+1 drop of trifluoroacetic acid-*d*.

Single frequency decoupling and double-quantum filtered COSY nmr verified three spin-coupled networks. Two spin systems, H-5, -6, -7, -8 and H-4′, -5′, -6′, -7′ (see Table 1), constituted signals of disubstituted benzenes. The remaining proton signals were two mutually coupled one-proton signals at H-3 and H-4 (δ 8.05, d and 8.30, d; J=6 Hz) and an olefinic singlet at H-2′ (δ 7.97, 1H). Furthermore, broad exchangeable protons (δ 9.5, bs, 2H) were detected in the ^{1}H-nmr spectrum of eudistomin U in CD_2Cl_2. Due to the limited amount of available compound, the ^{13}C-nmr spectrum of **1** was assigned on the basis of reverse 2D nmr (optimized in an HMQC experiment for $^1J_{CH}$=160 Hz, and in HMBC experiments for $^3J_{CH}$=10 Hz for dihedral angles=180°, and $^3J_{CH}$=5 and 3 Hz for dihedral angles=0°) (11). The high-field signals for the quaternary sp^2 carbons (C-4a, C-4b, C-1′, and C-7′a) and for the protonated carbons C-8 and C-4′, at δ 114.87 and 113.70, respectively, were consistent with the β-shielding effect of nitrogen functionalities. The chemical shift values observed during the nmr studies were found to be very close to those reported for a β-carboline moiety (12–14) and for an indole ring (14–16). A large number of mono-, di-, and tri-substituted β-carbolines have already been extracted (12–14) from ascidians (named eudistomins A–T), so this new derivative was called eudistomin U [**1**].

Only one nuclear Overhauser effect was observed by using the nOe difference nmr spectrometric technique. Irradiation of H-5 produced a marked enhancement (6%) for H-4. In DMSO-d_6, the ^{1}H nmr spectrum of **1** showed broad singlets at δ 11.6 for NH-3′, at δ 11.2 for NH-9 and a doublet at δ 8.10 with J=2.1 Hz for H-2′. The monoacetylated derivative **1a** with (M^+) at *m/z* 326 and $(M-42)^+$ at *m/z* 284 in the cims, was produced by acetylation of **1** (excess Ac_2O in pyridine). The ^{1}H-nmr spectrum of **1** in CD_2Cl_2+1 drop of CD_3OD, confirmed the presence of one

acetyl functionality (δ CH_3=2.75) inducing a deshielded effect [δ 7.95 (H-4′) and 9.50 (H-2′)] on two aromatic protons. This result indicated that the acetyl group was located on the indole ring. The ir spectrum of **1a** showed absorptions of a residual NH (ν max 3550 cm^{-1}) and of amide functionalities (ν max 1655 cm^{-1}). A color change from yellow to pink was correlated with a bathochromic shift in the uv spectrum upon acidification (17) of **1a**, indicating a free base. These results gave considerable support to the proposed structure [**1**] for eudistomin U.

Isoeudistomin U [**2**] was obtained as a yellow foam. The molecular formula $C_{19}H_{15}N_3$ was determined from the molecular ion at *m/z* 285.12578 (M^+, Δ=0.7 mmu) in the eims (peak matching) and positive parent ion at 286 (M^++H) in the fabms, in conjunction with ^{1}H- and ^{13}C-nmr data (Table 1). However, the cims, fabms, and eims were always found to contain significant ions at *m/z* 283 (or *m/z* 284, M^++H) corresponding to M^+−2H (peak matching 283.11005, Δ=0.8 mmu) and *m/z* 257 (or 258, M^++H) for $M^+-C_2H_4$ (peak matching 257.09474, Δ=0.5 mmu). The ir and uv spectra of **2** were very similar to those of **1**.

The ^{1}H-nmr spectrum of **2** displayed the best dispersion when run in CD_2Cl_2 with two drops of trifluoroacetic acid-*d*. Single-frequency and double-quantum filtered COSY verified two spin-coupled networks of disubstituted benzenes, H-5, -6, -7, -8 and H-4′, -5′, -6′, -7′ (see Table 1). The remaining proton signals were two mutually coupled methylenes H-2 and H-3 (δ 3.9, t, 2H and δ 3.2, t, 2H with J=11.7 Hz) and an olefinic singlet H-2′ (δ 8.30, 1H). Two supplementary broad exchangeable protons (10.5 ppm) were observed for **2** in CD_2Cl_2. The ^{13}C-nmr spectrum of **2** (Table 1) was assigned mainly on the basis of reverse heteronuclear correlation spectroscopy, HMQC and HMBC, respectively optimized for observing 160 and 10 Hz $J_{C\text{-}H}$ couplings.

In the ^{1}H-nmr spectrum of the monoacetylated derivative **2a**, produced by acetylation of **2**, two aromatic protons were found at 8.60 and 8.25 ppm in agreement with a deshielding effect by the acetyl group (δ CH_3=2.72). The monoacetylated derivative **2a** showed a molecular ion at *m/z* 326 (M^+−2H) and a fragment $(M^+-2H-42)^+$ at *m/z* 284 in the cims, probably corresponding to more stable ions during mass spectrometric fragmentation, since the ^{1}H-nmr spectrum of **2a** did not show a supplementary unsaturation when compared to those of **2**. Selective spin decouplings readily assigned the whole set of aromatic protons, six of them having similar chemical shifts in **2** and **2a**. From the uv spectra of **2a**, bathochromic shifts were observed upon acidification. The ^{1}H-nmr spectrum of **2** in DMSO-d_6 showed broad singlets at δ 11.6 for NH-3′ and at δ 10.5 for NH-9, and a doublet at δ 8.39 with J=2.1 Hz for H-2′. The main structural variation between compounds **1** and **2** was based on observed differences occurring in the ^{13}C-nmr spectrum of **2**. The carbon C-9a (δ 158.63) was located between two nitrogen atoms like in a pyrrolo [2,3-d] pyrimidine (18) or α-carboline ring (δ 152.2) (14), a deshielded value corresponding to the lack of a β-shielding effect due to the nitrogen atom N-2 in the β-carboline ring. With such a structure, the other deshielded carbon value (δ 138.31 for C-4b) was in agreement with those found for indole alkaloids possessing an unsaturated double bond [e.g., pandoline (19)] or unsaturated functionalities [rhyncophylline derivatives (20)] in an α orientation from the NH group. Therefore, a dihydro α-carboline moiety was proposed for **2** to account for these features. This proposal was confirmed by the HMBC couplings between C-9a (δ 158.63)/H-2 (δ 3.90) and C-4a (δ 125.71)/H-3 (δ 3.20). The β-carboline ring possibility was thus eliminated as these HMBC couplings could not be obtained with such a structure {cf. the C-H couplings in eudistomin U [**1**] be-

tween C-9a (δ 132.40)/H-4 (δ 8.30) and C-4a (δ 121.05)/H-4 (δ 8.30) and H-3 (δ 8.05)}. Moreover, this structure was supported by a conspicuous nOe, observed by using nOe difference nmr technique, wherein irradiation of H-2′ produced a marked enhancement for H-3 (12%), a strong nOe that implied a close proximity of the respective ring residues. This result did not fit well with the aforementioned result obtained on the β-carboline moiety of eudistomin U (i.e., an nOe between H-5 and H-4) but was in agreement with the proposed dihydro α-carboline structure, placing the indole ring as shown in **2**. This was also confirmed by an observed nOe enhancement (8%) of H-5 (δ 7.65) when H-7′ (δ 7.75) was irradiated. The α-carboline skeleton—a quite rare moiety in natural product chemistry—has already been described from a solitary tunicate (14).

Alkaloids **1** and **2** were found to be capable of binding to DNA in a chromatographic purification procedure (9), but their detailed biological profiles have not been determined as yet. However, the compounds showed a strong antibacterial activity against *Agrobacterium tumefaciens*, employing this crown-gall bioassay (21,22) to estimate antineoplastic potencies. Antibacterial activities, however, were not detected against marine strains. Compounds **1** and **2** did not show cytotoxic effects against CEM human leukemic lymphoblasts. The presence of eudistomin derivatives in an ascidian of the family Didemnidae is surprising since they are usually found only in the family Polycitoridae. However, eudistomin K (23) has also been isolated from the family Polyclinidae. These features could be related to a possible microbial origin of such molecules, especially for ascidians that harbor prokaryotic unicellular symbionts.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Uv and Ft-ir spectra were recorded on Perkin-Elmer model spectrophotometers. ^{1}H- and ^{13}C-nmr spectra (respectively at 400 MHz and 100 MHz) were recorded on a JEOL-EX400 instrument. Mass spectra were obtained on a VG ZAB HS instrument. Standard parameters were used for 2D nmr experiments.

EXTRACTION AND ISOLATION.—The colonial ascidian (700 g dry wt) was collected in June 1988 near "Ilet Pigeon," (Cousteau's reservation) Guadeloupe, France, at between −30 and −40 m depth. A voucher specimen has been deposited in the Observatoire Océanologique, Banyuls sur Mer, France. The material was preserved in EtOH, then ground and extracted with $CHCl_3$-MeOH (1:1). After filtration and evaporation, the crude extract was partitioned between H_2O and successively hexane, Et_2O, $CHCl_2$ and BuOH. After evaporation, the BuOH-soluble material was dissolved in acidic H_2O and extracted with CH_2Cl_2. The organic-soluble material was evaporated to yield 2 g of a crude organic extract. Chromatographic separations were done on a Si gel column with mixtures of CH_2Cl_2/MeOH. The novel isoeudistomin U (**2**, 8 mg, 0.0012% dry wt) and eudistomin U (**1**, 5 mg, 0.0007% dry wt) were eluted with CH_2Cl_2-MeOH (90:10), purified by a reversed-phase open column (RP-8, MeOH/H_2O, 1:1) and by repeated hplc on a C_{18} reversed-phase column with a 75:25 solvent mixture of MeOH-H_2O.

EUDISTOMIN U [**1**].—Yellow foam; uv λ max (MeOH) (ε) 220 (8000), 240 (3500), 252 (10500), 270 (7700), 278 (7210), 340 (9020), 380 (13000), 470 (5120) nm; ir ν max ($CHCl_3$) 3600, 3520, 3010, 2981, 1610, 1405, 1290, 1255, 1221, 890 cm^{-1}; hreims (peak matching) found *m/z* 283.10945 (M^+), $C_{19}H_{13}N_3$ requires 283.11087; eims *m/z* 283 (100) M^+, 128 (37), 127 (19), 85 (36), 84 (22); cims *m/z* 284 (M^++H, 100) 213 (51), 133 (82); ^{1}H- and ^{13}C-nmr spectra, see Table 1; ^{1}H nmr (CD_2Cl_2) δ 9.5 (2H, br s), 8.46 (1H, d, *J*=5.6 Hz, H-3), 8.41 (1H, br s, H-2′), 8.38 (1H, d, *J*=5.6 Hz, H-4), 8.35 (1H, d, *J*=8 Hz, H-5), 7.82 (1H, d, *J*=8 Hz, H-4′), 7.78 (1H, d, *J*=8 Hz, H-8), 7.64 (1H, d, *J*=8 Hz, H-7′), 7.43 (1H, br t, *J*=7.3 and 8 Hz, H-6), 7.42 (1H, br t, *J*=7.3 and 8 Hz, H-7), 7.32 (1H, br t, *J*=7.3 and 8 Hz, H-6′), 7.24 (1H, br t, *J*=7.3 and 8 Hz, H-5′); selected values from ^{1}H nmr in DMSO-d_6 δ 11.6 (1H, br s, NH-3′), 11.2 (1H, s, NH-9), 8.10 (1H, d, *J*=2.1 Hz, CH-2′).

Acetylation of **1**.—Standard procedures were used. Purification was performed with reversed-phase hplc (12% MeOH/H_2O) to obtain 1 mg of pure monoacetylated derivative [**1a**]; uv λ max (MeOH) (ε) 220 (7980), 240 (3480), 268 (5840), 289 (5120), 303 (4700), 349 (3100), 364 (3050) nm; uv in MeOH+1 drop of 1N HCl λ max (ε) 258 (8570), 268 (7390), 303 (5340), 325 (4360), 374 (3078), 393 (2930) nm; ir ν max ($CHCl_3$) 3550, 3050, 2997, 2988, 1655, 1598, 1455,

1248, 1205, 897 cm^{-1}; cims *m/z* 326 (M^++H, 100), 284 (M^++H−42, 91); ^1H nmr of [**1a**] in CD_2Cl_2+1 drop of CD_3OD δ 9.50 (1H, s, H-2′), 8.30 (1H, d, *J*=6 Hz, H-3), 8.21 (1H, d, *J*=8 Hz, H-5), 8.05 (1H, d, *J*=6 Hz, H-4), 7.95 (1H, d, *J*=8 Hz, H-4′), 7.80 (1H, d, *J*=8 Hz, H-7′), 7.62 (1H, br t, *J*=8 and 7.3 Hz, H-8), 7.60 (1H, br t, *J*=8 and 7.3 Hz, H-7), 7.35 (1H, br t, *J*=8 and 7.3 Hz, H-6), 7.31 (1H, br t, *J*=8 and 7.2 Hz, H-5′), 7.20 (1H, br t, *J*=8 and 7.2 Hz, H-6′), 2.75 (3H, s, Ac).

ISOEUDISTOMIN U [**2**].—Yellow foam; uv λ max (MeOH) (ε) 250 (10230), 270 (7700), 278 (7150), 340 (9160), 380 (12990), 470 (5140) nm; ir ν max ($CHCl_3$) 3600, 3515, 3046, 2987, 1605, 1422, 1282, 1245, 1200, 895 cm^{-1}; hreims (peak matching) found *m/z* 285.12578 (M^+), $C_{19}H_{15}N_3$ requires 285.12651, 283.11005 (M^+−2H), $C_{19}H_{13}N_3$ requires 283.11087, 257.09474 ($M^+-C_2H_4$), $C_{17}H_{11}N_3$ requires 257.09523; eims *m/z* 285 (M^+, 100), 283 (90), 257 (30), 128 (37), 127 (19), 85 (36), 84 (22); fabms *m/z* 286 (M^++H, 100) 258 (48), 214 (51), 133 (82); ^1H- and ^{13}C-nmr spectra, see Table 2; ^1H nmr (CD_2Cl_2) δ 10.5 (2H, br s), 8.60 (1H, s, H-2′), 8.00 (1H, d, *J*=8 Hz, H-8), 7.90 (1H, d, *J*=8 Hz, H-4′), 7.79 (1H, d, *J*=8 Hz, H-7′), 7.71 (1H, d, *J*=8 Hz, H-5), 7.60 (1H, br t, *J*=7.3 and 8 Hz, H-5′), 7.50–7.35 (3H, H-7+H-6′+H-6), 3.82 (2H, t, *J*=12 Hz, H-2), 3.28 (2H, t, *J*=12 Hz, H-3); selected values from ^1H nmr in DMSO-d_6 δ 11.6 (1H, br s, NH-3′), 10.5 (1H, s, NH-9), 8.39 (1H′, d, *J*=2.1 Hz, CH-2′).

Acetylation of **2**.—Standard procedures were used. Purification was performed with reversed-phase hplc (12% H_2O in MeOH) to obtain 1 mg of pure monoacetylated derivative [**2a**]; uv λ max (MeOH) (ε) 250 (15970), 290 (9330), 340 (7960), 360 (6925), 400 (2196), 460 (594) nm; uv λ max (MeOH+1 drop of 1N HCl) (ε) 270 (8412), 287 (4452), 313 (7269), 328 (6888), 355 (5233), 393 (3110) nm; ir ν max ($CHCl_3$) 3545, 3050, 2997, 2987, 1655, 1600, 1452, 1245, 1200, 897 cm^{-1}; cims *m/z* 326 (M^++H, 100), 284 (M^++H−42, 19); ^1H nmr (CD_2Cl_2+1 drop of CF_3COOD) δ 8.60 (1H, s, H-2′), 8.25 (1H, d, *J*=8 Hz, H-4′), 7.82 (1H, s, H-8), 7.75 (1H, d, *J*=8 Hz, H-7′), 7.55 (1H, d, *J*=8 Hz, H-5), 7.50 (1H, t, *J*=7.3 and 8 Hz, H-5′), 7.41 (1H, t, *J*=7.2 and 8 Hz, H-6), 7.39 (1H, t, *J*=7.2 and 8 Hz, H-7), 7.25 (1H, t, *J*=7.3 and 8 Hz, H-6′), 3.55 (2H, t, *J*=12 Hz, H-2), 3.30 (2H, t, *J*=12 Hz, H-3), 2.72 (3H, s, Ac).

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