Eudistomins W and X, Two New β-Carbolines from the Micronesian Tunicate *Eudistoma* sp.

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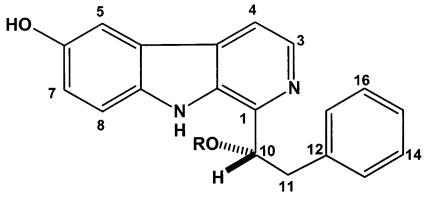
Chemical investigation of the Micronesian ascidian *Eudistoma* sp. afforded two new eudistomin congeners, which were designated eudistomins W (**1**) and X (**2**). The structures of the new compounds were unambiguously established on the basis of NMR spectroscopic (^{1}H, ^{13}C, COSY, ^{1}H detected direct, and long-range ^{13}C−^{1}H correlations) and mass spectrometric (EI and ESIMS) data. Compound **2** exhibited antibiotic activity toward *Bacillus subtilis, Staphyloccocus aureus,* and *Escherichia coli* and was also found to be fungicidal against *Candida albicans* in an agar diffusion assay. Compound **1** was selectively active against *C. albicans* but showed no antibacterial activity.

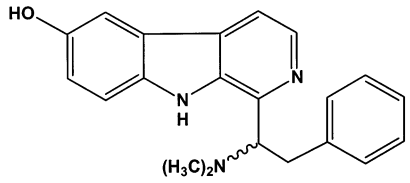
Marine ascidians of the genus *Eudistoma* are known to yield a plethora of novel and biologically active secondary metabolites.[1] They are a rich source of nitrogen-containing compounds which include eudistones,[2] segolines,[3] shermilamine,[3b] and rigidin[4] as well as the macrolides iejmalides.[5] A major group of *Eudistoma* constituents are indole alkaloids, as exemplified by the staurosporines,[6] trypargines,[7] woodinine,[8] eudistomins,[9] eudistalbins,[10] and eudistomidins.[11] β-Carboline congeners, structurally related to the eudistomins, have also been reported from other tunicate genera such as *Ritterella*,[12] *Pseudodistoma*,[13] and *Lissoclinum*.[14] The biosynthesis of these β-carbolines is generally believed to involve the coupling of a tryptophan unit with a second amino acid, as recently demonstrated in *E. olivaceum*.[15] β-Carbolines often exhibit pronounced biological activity, such as antimicrobial,[9a,16] cytotoxic,[17] and antiviral[9a,18] activity.

Our interest with this undescribed species of *Eudistoma* arose from the observation of a strong deterrent effect of its crude MeOH extract toward feeding by generalist reef fishes. This yellow colonial ascidian was first found on mangrove roots in areas of Chuuk, Micronesia. We also observed that despite its high abundance and lack of visible morphological defenses, only the flatworms *Pseudoceros indicus* and *Pseudoceros tristriatus* were observed to be feeding on the ascidian.[6c,d] Subsequent isolation of the major UV-active compounds afforded two new eudistomin congeners W (**1**) and X (**2**).

Sufficient quantities of the undescribed ascidian *Eudistoma* sp. were collected via snorkeling at depths of 1 to 2 m for extraction of their secondary metabolites. Samples were frozen immediately and freeze-dried prior to extraction. The β-carboline congeners **1** and **2** as well as several known nitrogenous secondary metabolites including 3,7-dimethylisoguanine[19] and 2-amino-6(1′,2′-dihydroxypropyl)-3-methyl-4-pterinone[20] besides the nucleosides thymidine and adenosine were isolated from the methanol-soluble extract. Eudistomins W (**1**) and X (**2**), which were present as the major metabolites, were separated from the aforementioned minor components by normal-phase chromatography on Lobar columns followed by reversed-phase semipreparative HPLC.



1: R = H
3: R = *S*-MPTA
4: R = *R*-MPTA



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High-resolution EIMS of eudistomin W (**1**) provided the molecular formula $C_{19}H_{16}N_2O_2$, which was in agreement with its ^{1}H and ^{13}C NMR data (Table 1). The base peak at *m*/*z* 213 for $C_{12}H_9N_2O_2$ was confirmed by HREIMS and corresponded to the loss of a benzyl unit. The ^{1}H data of compound **1** were comparable to those of eudistomins S and T,[9c,21] which pointed out the presence of both a carboline moiety and a phenyl function. The ^{1}H NMR spectral data of **1** revealed the presence of four spin systems as verified by COSY. Two doublet signals (J = 6.0 Hz) at δ 8.39 (H-4) and 8.10 (H-3) were indicative of the 2,3,4-trisubstituted pyridine moiety characteristic of the eudistomins.[9b] The second spin system constituted a 1,2,4-trisubstituted benzene ring, with signals at δ 7.64 (d, J = 2.2 Hz), 7.35 (dd, J = 8.8, 2.2 Hz), and 7.62 (d, J = 8.8 Hz) for H-5, H-7, and H-8, respectively. The spectral characteristics matched those of a 1,6-disubstituted β-carboline moiety present for example in eudistomin S.[9c] The multiplet sets of proton resonances at δ 6.98 and 7.12 integrating for two protons

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Table 1. ^{13}C NMR, ^{1}H NMR, and HMBC Data for Eudistomins W (**1**) and X (**2**) in CD_3OD

	1			**2**		
	δ_C	δ_H (mult, *J* in Hz)	HMBC (H to C)	δ_C	δ_H (mult, *J* in Hz)	HMBC
1	143.5 s			137.5 s		
3	128.1 d	8.10 d (6.0)	C-1, C-4, C-4a	139.7 d	8.46 d (5.4)	C-1, C-4, C-4a, C-4b,[a,b] C-9a[b]
4	117.0 d	8.39 d (6.0)	C-1,[a,b] C-4b, C-9a	117.4 d	8.02 d (5.4)	C-3, C-4a, C-4b, C-9a
4a	134.7 s			133.6 s		
4b	121.9 s			123.9 s		
5	106.7 d	7.64 d (2.2)	C-6, C-7, C-8a,[b] C-4b[b]	107.8 d	7.50 d (2.5)	C-4a, C-6, C-7, C-8a, C-8[b]
6	154.2 s	7.35 dd (8.8, 2.2)		153.7 d	7.13 dd (8.8, 2.5)	
7	124.2 d	7.62 d (8.8)		121.3 d	7.34 d (8.8)	C-5, C-6, C-8, C-8a
8	114.7 d			113.8 d		C-4a,[a] C-4b, C-6, C-7, C-8a, C-5[b]
8a	140.1 s		C-4b, C-6, C-8a[b]	138.1 s		
9a	133.2 s			139.5 s		
10	70.2 d	5.77 t (5.8)	C-9a,[b] C-12	69.1 d	5.06 dd (5.0, 10.0)	C-9a, C-11, C-12, N(CH_3)$_2$
11	43.5 t	3.40[a]	C-1, C-12, C-13/17, C-10	38.9 t	3.65 m	C-1, C-10, C-13, C-17
12	136.9 s			137.3 s		
13/17	130.7 d	6.98−7.12 m	C-15, C-12[b]	130.8 d	6.95−7.01 m	C-11[c]
14/16	129.3 d	6.98−7.12 m	C-12[c]	129.7 d	6.95−7.01 m	C-12[c]
15	128.0 d	6.98−7.12 m	c	128.0 d	6.95−7.01 m	
16	129.3 d			129.7 d		
17	130.7 d			130.8 d		
N(CH_3)$_2$				43.7 q	3.00 bs	C-10, N(CH_3)$_2$

[a] Signal partially obscured by solvent peak. [b] Weak correlation. [c] Correlations in the aromatic region were not discernible due to signal overlap.

and three protons, respectively, were assigned to a monosubstituted phenyl moiety. The remaining proton signal was a triplet at δ 5.77 (J = 5.8 Hz), indicating the presence of a hydroxyl methine coupled to a methylene which was found to be partially obscured by the solvent signal but was readily identified from 2D NMR spectra.

The carbon assignments and multiplicities for compound **1** were confirmed both by HMQC and HMBC experiments (Table 1). The high-field signals for the quaternary sp^2 carbons (C-4a and C-4b) and for the protonated carbon at C-8 were consistent with the β-shielding effect of a nitrogen atom in a β-carboline system[22] as well as with those of its 1,6-disubstituted congeners.[9b,23] The position of the hydroxyl function was confirmed through HMBC correlations and by comparison of its ^{1}H NMR and ^{13}C data with both 6-hydroxy- (e.g., eudistomins M and Q, manzamine Y, and hyrtiomanzamine)[9b,23] and 7-hydroxy-substituted β-carbolines (e.g., harmol and arenarine C).[22,24] The NMR data of eudistomin W matched those of eudistomins M and Q,[9b] manzamine Y, and hyrtiomanzamine,[23] which favored the substitution at C-6.

The presence of a hydroxyl methine adjacent to a methylene was also compatible with the ^{13}C resonances in the aliphatic region at δ 70.2 (d, C-10) and 43.5 (t, C-11), respectively. The attachment of the hydroxylated phenethyl function at C-1 was verified by $^{13}C-^{1}H$ long-range cross-peaks from the hydroxyl methine proton (H-10) at δ 5.77 to C-9a (δ 133.2) and C-12 (δ 136.9) of the phenyl function. Additional evidence for the proposed structure was obtained from cross-peaks observed between the proton signal for H-11 (partially hidden under the solvent peak) with C-1 (δ 143.5) and C-12 (δ 136.9). The ^{1}H and ^{13}C chemical shifts for positions 1 and 10 in eudistomin W (**1**) were comparable to that reported for picrasidine Y, which carries a similar hydroxylated alkyl substituent at C-1.[25] Thus, eudistomin W (**1**) differed from eudistomin T[9c] by reduction of the keto group at C-10 in the latter to a hydroxyl function and by an additional hydroxyl group at C-6. The unusual upfield shift of C-3 at (δ 128.1) and the downfield shift for C-7 (δ 124.2) indicated the protonation effect of the nitrogen in a pyridine system.[26] A similar phenomenon was also observed with the ^{13}C NMR data of eudistomin U.[14] The absolute configuration of the hydroxyl function at C-10 was determined as *S* by analysis of the ^{1}H NMR data of the (*S*)- and (*R*)-MPTA ester derivatives **3** and **4** through the modified Mosher's method.[27]

Eudistomin X (**2**) had the molecular formula $C_{21}H_{21}N_3O$, as determined by high-resolution EIMS. As in the case of eudistomin X (**2**), the base peak at *m*/*z* 240 was due to a loss of a benzyl moiety, and its molecular formula $C_{14}H_{14}N_3O$ was consequently confirmed by HREIMS. Inspection of the ^{1}H NMR and COSY spectra of eudistomin X (**2**) revealed that the proton spin systems were comparable to those of eudistomin W (**1**). The presence of a similar 1,6-disubstituted β-carboline unit in **2** was verified by the HMBC correlation data (Table 1). However, changes were observed for H-10, which was shifted upfield by 0.7 ppm, while the methylene multiplet of H_2-11 was shifted downfield by 0.25 ppm. In comparison to eudistomin W (**1**), significant differences were also observed in the ^{13}C chemical shifts of eudistomin X (**2**) (C-1 − 6.0 ppm, C-3 + 11.6 ppm, C-9a + 6.3 ppm, and C-11− 4.6 ppm), which were indicative of a change of substituent at C-10 and a lack of protonation of the nitrogen in the pyridine system. The ^{1}H and ^{13}C NMR resonances for positions 1, 3, 4, 9a, 10, and 11 were comparable to those of the *N*-methylated analogues, eudistomidin C[11b] and its 14-methyl congener.[28] The appearance of a broad singlet at δ 3.00 integrating for six protons revealed the presence of a *N*,*N*-dimethyl function as in 14-methyl eudistomidin C.[28] The position of the *N*,*N*-dimethyl function was confirmed a $^{13}C-^{1}H$ long-range correlation between the methyl carbons at δ_C 43.7 and the methine multiplet of H-10 at δ_H 5.07 and vice versa. Thus, eudistomin X (**2**) differed from 14-methyl eudistomidin C[28] by debromination at C-5 and by the presence of a phenyl function at C-11 instead of a methylthio substituent. Eudistomidin C and its 14-methyl congener were reported as dextrorotatory (α_D +15.6° and +12.9°, respectively) and were both assigned the *S* configuration.[11b,28] The stereochemistry of eudistomidin C was determined by chiral synthesis of the enantiomeric 10(*R*)-6-*O*-methyleudistomidin C, which was consequently observed to be levorotatory (α_D −13.8).[11b] Although eudistomin X was also levorotatory (α_D −7.0°), the stereochemistry at C-10 could not be determined due to differences in substituents which were not comparable to those of eudistomidin C and its 14-methyl congener.

In comparison to previously described eudistomin congeners, eudistomin W (**1**) exhibited the same carbon skeleton as eudistomin T,[9c] but differed from the latter by reduction of the keto group at C-10 to a hydroxyl function and by an additional hydroxyl group at C-6. Similar β-carboline congeners with hydroxylated alkyl substituents at C-1 have been previously isolated from the Chinese medicinal plants *Arenaria kansuensis*[24] (family Caryophyllaceae) and *Picrasma quassioides*[25] (family Simaroubaceae) as well as the marine bryozoan *Costaticella hastata*.[29] The *N*,*N*-dimethyl function at C-10 in eudistomin X (**2**) was already observed in 14-methyl eudistomidin C,[28] but the latter carried a methylthio substituent at C-11 instead of the phenyl group present in **2**.

Due to the known antibiotic activity of β-carboline alkaloids, the two new congeners were subjected to an antimicrobial agar diffusion assay. Eudistomin X (**2**) exhibited antibacterial activity which showed zones of inhibitions toward *B. subtilis* (17 and 18 mm), *S. aureus* (11 and 12 mm), and *E. coli* (15 and 20 mm) at loading doses of 5 and 10 μg per disk, respectively. Compound **2** was also found to be fungicidal against *C. albicans*, which displayed zones of inhibitions of 17 and 18 mm at similar loading doses. On the contrary, eudistomin W (**1**) was found to be selectively active toward *C. albicans*, giving a zone of inhibition of 13 mm at 10 μg.

Experimental Section

General Experimental Procedures. Optical rotation was recorded on a Perkin-Elmer Model 341 LC polarimeter. 1D and 2D NMR spectra (chemical shifts in ppm) were recorded on Bruker AMX 300, ARX 400, or DRX 500 NMR spectrometers using standard Bruker software. Mass spectra (ESIMS) were recorded on a Finnigan MAT TSQ-7000 triple-stage quadrupole mass spectrometer. The temperature of the heated capillary (20 V) was 200 °C, and the electrospray capillary voltage was set to 3.5 kV. Nitrogen served as both sheath (70 psi) and auxiliary gas; argon served as collision gas. High-resolution EIMS were recorded on a Finnigan MAT 8430 and on an Intectra AMD 402. For HPLC analysis, samples were injected into a HPLC system equipped with a photodiode-array detector (Gynkotek, München, Germany). Routine detection was at 254 and 292 nm. The separation column (125 × 4 mm, i.d.) was prefilled with Eurospher 100-C_{18}, 5 μm (Knauer, Berlin, Germany). Separation was achieved by applying a linear gradient from 100% H_2O (with 0.2% TFA) to 100% MeOH over 40 min. Semipreparative HPLC was conducted on a Merck Hitachi LaChrome L-7100 pump and Merck Hitachi LaChrome L-7400 UV detector. Chromatograms were recorded on a Merck Hitachi D-2000 Chromato-Integrator. Separation columns (300 × 8 mm, i.d.) were prefilled with Eurospher 100-C_{18}, 7 μm (Knauer, Berlin, Germany). Compounds were purified by applying a linear gradient from 100% H_2O (with 0.2% TFA) to 100% MeOH over 40 min. TLC was performed on precoated TLC plates with Si gel 60 F_{254} and Si gel RP-18 F_{254} (Merck, Darmstadt, Germany). Compounds were detected by UV absorbance or by fluorescence at 254 and 366 nm. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements.

Animal Material. *Eudistoma* sp. (family Polycitoridae) is a colonial ascidian that closely resembles *Eudistoma viride* Tokioka, 1955[30] redescribed by Monniot and Monniot in 2001. Contrary to *E. viride* the new species show well-delimited yellow spots at the top of the zooids. The zooids were yellow and slightly transparent. Several zooids were converged and sitting on small 1 cm short branches which were attached to the mangrove roots. A voucher specimen is kept under MNHN A3 EUD 242 at the Museum National d'Histoire Naturelle, Paris, France.

Extraction and Isolation. The freeze-dried samples of the ascidian *Eudistoma* sp. (11 g) were extracted exhaustively with MeOH. The total MeOH extract was evaporated under reduced pressure to give a residue (2 g), which was then chromatographed on a silica gel column [elution with MeOH/CH_2Cl_2/NH_3 (30:67:3)], yielding 12 fractions. Eudistomin W (**1**) was isolated from fraction 12, which was then subjected to isocratic reversed-phase chromatography (Lobar Merck size B 310-25, LiChroprep RP18, 40−63 μm) using MeOH/H_2O (45:55) with 0.2% TFA as eluent. Further purification was achieved by semipreparative HPLC [MeOH/H_2O (0.2% TFA) with a gradient of 0−100% MeOH in 40 min)], yielding 11.0 mg of **1** (0.1%). Eudistomin X (**2**) was isolated from fraction 6, which was purified by elution over silica gel utilizing MeOH/CH_2Cl_2 (30:70 with 2% NH_3). The yield was 31.9 mg (0.29%).

Eudistomin W (1): brown amorphous powder; $[\alpha]^{20}_D$ −23.0° (*c* 0.24, MeOH); UV (MeOH) λ_{max} 208, 234, 266, 308, 405; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 305 $[M + H]^+$; EIMS *m*/*z* 304 $[M]^+$ (11), 285 (23), 257 (23), 236 (34), 213 (100), 194 (14), 183 (12), 152 (16), 137 (21), 123 (23), 111 (32), 97 (59), 83 (64); HREIMS *m*/*z* 304.1225 $[M]^+$ (calcd for $C_{19}H_{16}N_2O_2$, 304.1212), *m*/*z* 213.0659 $[M - C_7H_7]^+$ (calcd for $C_{12}H_9N_2O_2$, 213.0664).

Eudistomin X (2): brown amorphous powder; $[\alpha]^{20}_D$ −7.0° (*c* 0.49, MeOH); UV (MeOH) λ_{max} 201, 234, 251, 298, 373; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 332 $[M + H]^+$; EIMS *m*/*z* 331 $[M]^+$ (2), 288 (43), 240 (100), 226 (34), 211 (14), 199 (24), 184 (8), 155 (5), 111 (6), 91 (10), 83 (9); HREIMS *m*/*z* 331.1621 $[M]^+$ (calcd for $C_{21}H_{21}N_3O$, 331.1685), *m*/*z* 240.1129 $[M - C_7H_7]^+$ (calcd for $C_{14}H_{14}N_3O$, 240.1137).

Preparation of (*S*)- and (*R*)-MPTA Ester Derivatives of Eudistomin W (1). To 1.0 mg of **1** in 0.5 mg of CH_2Cl_2 were added the following reagents sequentially: 100 μL of pyridine, 0.5 mg of 4-(dimethylamino)pyridine, and 10 mg of *R*(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride. The mixture was heated at 50 °C for 4 h under N_2 and then passed through a disposable pipet packed with silica gel and eluted with 10 mL of *i*-PrOH/CH_2Cl_2 (20:80). The solvent was then evaporated under vacuum to obtain the respective *S*-MPTA ester. The same procedure was adopted for preparation of the *R*-MPTA ester using *S*(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride. ^{1}H NMR (CD_3OD, 500 MHz) for *S*-MPTA ester (**3**): δ 8.258 (1H, d, 6.0 Hz, H-4), 7.888 (1H, d, 6.0 Hz, H-3), 7.507 (3H, m, H-14/15/16), 7.183 (2H, m, H-13/17), 7.145 (1H, d, 3.0 Hz, H-5), 7.134 (1H, d, 7.5 Hz, H-8), 6.797 (1H, dd, 3.0/7.5 Hz, H-7), 5.479 (1H, bs, H-10), 3.383 (1H, bd, 11.6 Hz, H-11A), 3.328 (1H, bd, 11.6, H-11B Hz). ^{1}H NMR (CD_3OD, 500 MHz) for *R*-MPTA ester (**4**): δ 8.257 (1H, d, 6.0 Hz, H-4), 7.887 (1H, d, 6.0 Hz, H-3), 7.514 (3H, m, H-14/15/16), 7.191 (2H, m, H-13/17), 7.143 (1H, d, 3.0 Hz, H-5), 7.132 (1H, d, 7.5 Hz, H-8), 6.795 (1H, dd, 3.0/7.5 Hz, H-7), 5.479 (1H, bs, H-10), 3.381 (1H, bd, 11.6 Hz, H-11A), 3.330 (1H, bd, 11.6, H-11B Hz). $\Delta\delta = \delta_S - \delta_R$: H-3 (+0.002), H-4 (+0.001), H-5 (+0.002), H-7 (+0.002), H-8 (+0.002), H-10 (+0.001), H-11A (+0.002), H-11B (−0.002), H-13/17 (−0.008), H-14/15/16 (−0.007).

Agar Diffusion Assay. Susceptibility disks (5 mm in diameter) were impregnated with 5 and 10 μg of the isolated compounds and placed on LB agar plates inoculated with the test bacteria *B. subtilis* 168, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922. The plates were observed for zones of inhibitition after 24 h of incubation at 37 °C. Fungicidal activity was assayed using *C. albicans* as test organism which was inoculated on YPD agar plates, and zones of inhibitition were recorded after 24 h of incubation at 27 °C.

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