Cystodytins D-I, New Cytotoxic Tetracyclic Aromatic Alkaloids from the Okinawan Marine Tunicate Cystodytes dellechiajei

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CYSTODYTINS D–I, NEW CYTOTOXIC TETRACYCLIC AROMATIC ALKALOIDS FROM THE OKINAWAN MARINE TUNICATE *CYSTODYTES DELLECHIAJEI*

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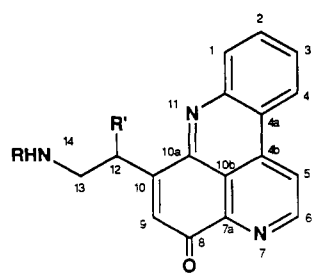
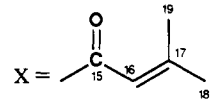
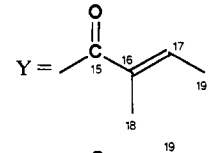
ABSTRACT.—Six new cytotoxic tetracyclic aromatic alkaloids, cystodytins D [**3a**], E [**3b**], F [**4a**], G [**4b**], H [**5a**], and I [**5b**] have been isolated from the Okinawan marine tunicate *Cystodytes dellechiajei* and their structures elucidated on the basis of spectroscopic data.

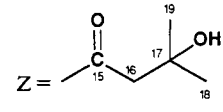
Recently a series of polycyclic aromatic alkaloids with structural similarities have been isolated from sponges, tunicates, or sea anemones and shown to exhibit interesting biological activities (1). We previously reported the isolation and structural elucidation of the tetracyclic aromatic alkaloids cystodytins A [**1a**], B [**1b**], and C [**2**], from the Okinawan marine tunicate *Cystodytes dellechiajei* (della Valle 1877) (family Didemnidae), and we showed that these alkaloids possess potent cytotoxic activity as well as powerful Ca^{2+}-releasing activity in the sarcoplasmic reticulum (2). In our continuing studies on bioactive substances from Okinawan marine organisms (3–7), we have further investigated extracts of the tunicate *C. dellechiajei* to obtain six new strongly cytotoxic tetracyclic aromatic alkaloids, named cystodytins D [**3a**], E [**3b**], F [**4a**], G [**4b**], H [**5a**], and I [**5b**], together with cystodytins A [**1a**] and B [**1b**]. In this paper we describe their isolation and structure elucidation.

The brown-colored compound tunicate *C. dellechiajei* was collected off the Kerama Islands, Okinawa, by scuba and kept frozen until used. The MeOH extract was subjected to separation by successive chromatographies on Diaion HP-20, Si gel, and Sephadex LH-20 columns to afford a 3.5:1 mixture of cystodytins D [**3a**] and E [**3b**], a









1a R = X, R′ = H
1b R = Y, R′ = H
2 R = Z, R′ = H
3a R = X, R′ = OH
3b R = Y, R′ = OH
4a R = X, R′ = OMe
4b R = Y, R′ = OMe
5a R = X, R′ = $OCO(CH_2)_7CH=CH(CH_2)_7CH_3$
5b R = Y, R′ = $OCO(CH_2)_7CH=CH(CH_2)_7CH_3$

TABLE 1. ^{1}H- and ^{13}C-nmr Chemical Shifts of Cystodytin D [**3a**] and Protons to Which Long-Range Correlations Were Observed in the HMBC Experiments.

Position	^{13}C	^{1}H	HMBC (^{1}H)
1	131.7 d	8.24 d	
2	131.9 d	7.94 dd	H-3, H-4
3	129.9 d	7.64 dd	H-1
4	123.0 d	8.49 d	H-3
4a	121.8 s		H-1, H-3
4b	137.1 s		
5	119.2 d	8.39 d	
6	150.1 d	9.10 d	
7a	144.9 s		
8	183.5 s		
9	131.2 d	7.20 s	
10	150.0 s		H-9
10a	146.7 s		H-9
10b	117.4 s		
11a	144.9 d		H-2, H-4
12	71.2 d	5.56 m	H-9
13	41.6 t	4.16 m	
		3.96 m	
14		6.07 m	
15	169.3 s		H-16
16	117.4 d	5.62 br s	H-18, H-19
17	153.4 s		H-18, H-19
18	27.3 q	1.64 br s	H-16, H-19
19	20.0 q	2.16 br s	H-16, H-18

3.5:1 mixture of cystodytins F [**4a**] and G [**4b**], and a 3.5:1 mixture of cystodytins H [**5a**] and I [**5b**] in 0.0004, 0.001, and 0.001% yield, respectively, based on the wet wt of the tunicate. It was difficult to separate each mixture (**3a** and **3b**, **4a** and **4b**, and **5a** and **5b**), since the two components of each pair had the same retention times on hplc under several solvent systems.

The fabms of the mixture of **3a** and **3b** showed the quasi-molecular ion of the reduced form $[M+2+H]^+$, which is often observed for quinones and related compounds (8), at *m/z* 376. The molecular formula for this mixture was established to be $C_{22}H_{19}N_3O_3$ by hrfabms [*m/z* 376.1669, $C_{22}H_{22}N_3O_3$, Δ −0.8 mmu, $(M+2+H)^+$]. The uv spectrum showed absorptions at 222 (ϵ 37000), 268 (30000), and 380 (11000) nm, indicative of aromatic ring systems. The ir band observed at 3400 cm^{-1} was attributable to an OH and/or NH group. An intense ir absorption at 1650 cm^{-1} and ^{13}C signals at δ 183.5 and 169.3 suggested the presence of a conjugated ketone and an amide carbonyl group. In the ^{1}H nmr spectrum of the mixture in $CDCl_3$, the signals for **3a** were accompanied by those for **3b** in a ratio of 3.5:1. Signals due to six aromatic and two olefinic protons, one oxymethine proton, two protons attached on an sp^3 methylene, and six protons on two methyl groups were observed for each of **3a** and **3b**. The ^{13}C-nmr data of **3a**, including data from a DEPT experiment, showed the presence of two methyls, one sp^3 methylene, eight sp^2 methines, one oxygenated sp^3 methine, eight sp^2 quaternary carbons other than a conjugated ketone and an amide carbonyl, thus accounting for all carbons of **3a**. The HMQC (^{1}H detected heteronuclear multiple quantum coherence) experiment (9) provided assignments for all protonated carbons (Table 1). The double quantum filtered homonuclear-correlated (DQF COSY) spectrum (10) of the mixture revealed the proton connectivities for four adjoining benzenoid ring protons (H-1–H-4) and two vicinally coupled ($J=6$ Hz) sp^2 protons,

which were assigned to α and β protons on the pyridine ring from their chemical shifts (δ 8.39 and 9.10, H-5 and H-6, respectively). An oxymethine proton at δ 5.56 was coupled with methylene protons at δ 4.16 and 3.96, which were in turn coupled to an exchangeable NH proton (δ 6.07). The olefinic proton at δ 6.52 showed allylic coupling to two methyl protons at δ 2.16 and 1.64. Further information on the skeletal framework of **3a** was obtained by ^{1}H-^{13}C long-range couplings observed through the HMBC (^{1}H detected heteronuclear multiple bond correlation) experiment (11). The presence of a disubstituted benzene ring (C-1–C-4, C-4a, and C-11a) was confirmed by the HMBC cross peaks for H-1/C-4a (δ 121.3), H-3/C-4a, H-2/C-11a (δ 145.0), and H-4/C-11a. The isolated aromatic proton at δ 7.20 (H-9) showed connectivities to the carbon signals at δ 150.0, 146.7, and 71.2, which were attributed to C-10, C-10a, and C-12. Thus the aliphatic side chain starting from C-12 was suggested to be attached at C-10. The presence of a senecioyl amide unit was clarified by the HMBC cross-peaks for H-16/C-15, H-16/C-18, H-16/C-19, H_3-18/C-6, H_3-18/C-17, H_3-18/C-19, H_3-19/C-16, H_3-19/C-17, and H_3-19/C-18. From all of the observations described above cystodytin D [**3a**] was deduced to possess the same tetracyclic aromatic chromophore as cystodytin A [**1a**]. The ^{13}C chemical shifts for the aromatic ring portion of cystodytin D [**3a**] corresponded exactly to those of cystodytin A [**1a**] (2). The molecular formula of cystodytin D [**3a**], $C_{22}H_{19}N_3O_3$, implied that **3a** possesses one more oxygen atom than **1a**. Cystodytin A [**1a**] comprises two sp^3 methylenes (C-12 and C-13) in the side chain, while cystodytin D [**3a**] was suggested to have a hydroxyl group at C-12 position from the ^{1}H- and ^{13}C-nmr data (vide supra). The structure of cystodytin D was, therefore, concluded to be **3a**.

The detailed analysis of the ^{1}H-nmr data of the mixture of **3a** and **3b** allowed us to assign the structure of the minor component, cystodytin E [**3b**]. Structural differences from cystodytin D [**3a**] were found only in the C-16–C-19 moiety. Two methyl signals [δ 1.62 (s, H_3-18) and 1.74 (d, $J = 7.0$ Hz, H_3-19)] were coupled to an olefinic proton at δ 6.48 (H-17) in the DQF COSY spectrum. The coupling pattern of H_3-19 and H-17 implied the presence of an α,β-dimethylacryloyl (tigloyl) group for the C-16–C-19 moiety, which is also contained in cystodytin B [**1b**]. Thus the structure of cystodytin E was deduced to be **3b**.

The eims of the mixture of cystodytins F and G showed a molecular ion peak of reduced form $[M + 2]^+$ at *m/z* 389. The molecular formula, $C_{23}H_{21}N_3O$, of the mixture was established by hreims (*m/z* 389.1749, $C_{23}H_{23}N_3O_3$, Δ +0.9 mmu, $[M + 2]^+$). This molecular formula implied that the mixture has an additional CH_2 unit as compared with the mixture of **3a** and **3b**. The ^{1}H-nmr spectrum of cystodytin F [**4a**] was quite similar to that of **3a**, except for the presence of a methoxy signal at δ 3.50 (3H, s) and a low-field chemical shift of an oxymethine signal (δ 5.40, H-12), which was coupled to methylene signals (δ 3.99 and 3.82, H_2-13) in the ^{1}H-^{1}H COSY spectrum. The ^{13}C-nmr spectrum of **4a** showed a methoxy carbon signal (δ 57.6), and the oxymethine carbon resonated at a lower field (δ 76.7, C-12) than that of **3a** (δ 71.2), indicating that the methoxy group was attached to this carbon. Thus the structure of cystodytin F [**4a**] was deduced to be 12-*O*-methylcystodytin D. The ^{1}H- and ^{13}C-nmr spectra revealed that the minor component, cystodytin G [**4b**], possesses an α,β-dimethylacryloyl (tigloyl) group [δ_H 6.24 (1H, m, H-17), 1.78 (3H, br s, H_3-18), and 1.72 (3H, d, $J = 6.8$ Hz); δ_C 12.5 (q, C-18) and 13.9 (q, C-19)]. Thus cystodytin G [**4b**] proved to be 12-*O*-methylcystodytin E.

The ^{1}H-nmr spectra of the mixture of cystodytins H and I showed signals due to an unsaturated fatty acid moiety [δ 5.35 (2H, m), 2.83 (2H, m), 2.50 (2H, t), 2.04 (2H, m), 1.25 (22H, br s), and 0.88 (3H, t)] in addition to signals commonly observed for other cystodytins. The fabms of cystodytins H and I showed a quasi-molecular ion peak

at *m/z* 640 $[M+2+H]^+$ and an intense peak at *m/z* 358, which corresponded to a fragment ion generated by loss of the fatty acid moiety $[M+2+H-C_{18}H_{34}O_2]^+$. In the ^{1}H-nmr spectrum the signal due to the oxymethine proton on C-12 was observed in the lower field [δ 6.85 (1H, t, J = 3.4 Hz, H-12)] than that of cystodytin E [**2a**], indicating that a fatty acid ester group is attached on C-12 position. Acid hydrolysis of cystodytins H and I with 5% hydrogen chloride in MeOH afforded an unsaturated fatty acid (eims *m/z* 282 $[M]^+$) and cystodytins F [**4a**] and G [**4b**] (hreims *m/z* 389.1725, Δ −1.4 mmu, $[M+2]^+$). The fatty acid was identified as octadecenoic acid by gc-ms analysis of its methyl ester. Thus the structures of cystodytins H [**5a**] and I [**5b**] were deduced to be 12-*O*-octadecenoylcystodytins D and E, respectively.

Cystodytins D–I are considered to be biogenetically related to cystodytins A [**1a**] and B [**1b**], whose origin and biosynthetic pathway remain to be resolved. Compounds **3a** and **3b**, **4a** and **4b**, and **5a** and **5b** were cytotoxic, exhibiting IC_{50} values of 1.1 (**3a** and **3b**), 0.068 (**4a** and **4b**), and 0.080 (**5a** and **5b**) μg/ml against murine lymphoma L1210 cells, and values of 1.4 (**3a** and **3b**), 0.078 (**4a** and **4b**), and 0.092 (**5a** and **5b**) μg/ml against human epidermoid carcinoma KB cells in vitro.

EXPERIMENTAL

GENERAL EXPERIMENTAL METHODS.—Ir and uv spectra were measured on a JASCO A-120 spectrophotometer and a Shimadzu uv-220 spectrometer, respectively. ^{1}H- and ^{13}C-nmr spectra were recorded on Bruker AM-500, JEOL EX-400, and GX-270 spectrometers in $CDCl_3$. The 7.26 ppm resonance of residual $CHCl_3$ and 77.0 ppm of $CDCl_3$ were used as internal references for ^{1}H and ^{13}C nmr, respectively. Eims and hreims spectra were obtained on a JEOL DX-303 spectrometer operating at 70 eV. Fabms and hrfabms spectra were obtained on a JEOL HX-110 spectrometer using glycerol as a matrix.

ISOLATION.—The brown-colored tunicate *C. dellechiajei* was collected off the Kerama Islands, Okinawa, by scuba, and a voucher specimen was deposited at Faculty of Pharmaceutical Sciences, Hokkaido University. The tunicate (250 g, wet wt) was extracted with MeOH (500 ml × 2). After evaporation under reduced pressure, the residue (5.89 g), which was dissolved in MeOH-H_2O (5:95), was adsorbed on a Diaion HP-20 column (Mitsubishi Kasei, 200 g) and eluted with an MeOH/H_2O system. The fraction (250.2 mg) eluted with MeOH (100%) was subjected to a Si gel column (Wako gel C-300, Wako Pure Chemical, 10 × 250 mm) with MeOH-$CHCl_3$ (1.5:98.5↦3:97). The fraction (200–270 ml) eluting by MeOH-$CHCl_3$ (1.5:98.5) was purified by a Sephadex LH-20 column (Pharmacia Fine Chemical, 10 × 950 mm) with $CHCl_3$-MeOH (1:1) to give a mixture of cystodytins F [**4a**] and G [**4b**] (0.001%, wet wt) and a mixture of cystodytins H [**5a**] and I [**5b**] (0.001%). The fraction (280–390 ml) of the Si gel column eluted with MeOH-$CHCl_3$ (3:97) was rechromatographed on a Sephadex LH-20 column (10 × 950 mm) eluting with $CHCl_3$-MeOH (1:1) to give a mixture of known cystodytins A [**1a**] and B [**1b**] (0.006%), which was identified by ^{1}H-nmr and eims spectral data as well as direct comparison with the authentic sample by tlc examination, and a mixture of cystodytins D [**3a**] and E [**3b**] (0.0004%). None of the three mixtures could be separated by using hplc columns (Silica gel: Senshu-pack® silica, Senshu Science, with 3% MeOH/$CHCl_3$; ODS: Develosil® ODS, Nomura Chemical, or YMC-pack® ODS, Yamamura Chemical, with 60% MeOH), because the two components of each pair had the same retention times with low recovery.

CYSTODYTINS D [**3a**] AND E [**3b**].—Brown-colored amorphous solids; $[\alpha]^{30}D$ −160° (c = 0.3, $CHCl_3$); uv (MeOH) λ max 214 (ε 37000), 274 (30000), 384 nm (11000); ir (KBr) ν max 3400 (br), 2930, 2850, 1650 (br) cm^{-1}. ^{1}H and ^{13}C nmr for **3a** see Table 1. ^{1}H nmr for **3b** δ 6.36 (1H, m, H-14), 6.48 (1H, m, H-17), 1.82 (3H, br s, H_3-18), 1.73 (3H, dd, J = 6.9 and 1.1 Hz, H_3-19). Fabms *m/z* $[M+2+H]^+$ 376, $[M-H_2O+2+H]^+$ 358; hrfabms *m/z* $[M+2+H]^+$ 376.1669 (calcd for $C_{22}H_{22}N_3O_3$, 376.1661).

CYSTODYTINS F [**4a**] AND G [**4b**].—Yellow-colored amorphous solids; $[\alpha]^{30}D$ −133° (c = 0.3, $CHCl_3$); uv (EtOH) λ max 225 (ε 30000), 272 (23000), 380 nm (11000); ir (film) ν max 3330, 2940, 2850, 1660, 1590, 1175, 1110 cm^{-1}. ^{1}H nmr ($CDCl_3$) for **4a** δ 9.25 (1H, d, J = 5.4 Hz, H-6), 8.59 (1H, dd, J = 1.8 and 8.1 Hz, H-4), 8.58 (1H, d, J = 5.4 Hz, H-5), 7.92 (1H, dt, J = 1.0 and 7.3 Hz, H-2), 7.82 (1H, dt, J = 1.0 and 8.3 Hz, H-3), 7.12 (1H, s, H-9), 5.86 (1H, m, H-14), 5.57 (1H, br s, H-16), 5.40 (1H, t, J = 4.9 Hz, H-12), 3.99 (1H, ddd, J = 3.9, 5.4, 14.0 Hz, H_a-13), 3.82 (1H, ddd, J = 5.4, 6.4, 14.0 Hz, H_b-13), 3.50 (3H, s, OMe), 2.00 (3H, d, J = 1.0 Hz, H-18), 1.81 (3H, d, J = 2.0 Hz, H-19). ^{1}H nmr ($CDCl_3$) for **4b** δ 6.37 (1H, dd, J = 1.5 and 6.8 Hz, H-14), 6.24 (1H, t, J = 4.9 Hz, H-17), 3.52 (3H, s OMe), 1.78 (3H, t, J = 1.2 Hz, H-18), 1.72 (3H, dd, J = 1.2 and 6.8 Hz, H-19). ^{13}C

nmr ($CDCl_3$) for **4a** δ 183.8 (s, C-8), 167.0 (s, C-15), 151.2 (d, C-6), 150.8 (s, C-17), 150.1 (d, C-6), 149.7 (s, C-10a), 146.8 (s, C-7a), 145.4 (s, C-11a), 137.4 (s, C-4b), 132.2 (d, C-2), 131.7 (d, C-9), 130.1 (d, C-1), 129.9 (d, C-3), 122.0 (d, C-4a), 119.5 (d, C-5), 118.4 (d, C-16), 118.2 (s, C-10b), 76.72 (d, C-12), 57.75 (q, OMe), 43.25 (t, C-13), 27.09 (q, C-18), 19.77 (q, C-19). ^{13}C nmr ($CDCl_3$) for **4b** δ 130.8 (d, C-17), 57.82 (q, OMe), 13.92 (q, C-19), 12.46 (q, C-18). Eims *m/z* $[M+2]^+$ 389, 357, 328, 302, 277, 262, 247; hreims *m/z* $[M+2]^+$ 389.1749 (calcd for $C_{23}H_{23}O_3N_3$, 389.1740).

CYSTODYTINS H [**5a**] AND I [**5b**].—Yellow-colored amorphous solids; $[\alpha]^{30}D$ −29.1° (*c* = 0.3, $CHCl_3$); uv (EtOH) λ max 225 (ε 37000), 273 (25000), 382 nm (10000); ir (neat) ν max 3320, 2910, 2840, 1730, 1650, 1590, 1540, 1250, 1190 cm^{-1}; ^1H nmr ($CDCl_3$) δ 9.24 (1H, d, *J* = 5.4 Hz, H-6), 8.58 (1H, dd, *J* = 1.8 and 8.1 Hz, H-4), 8.57 (1H, d, *J* = 5.4 Hz, H-5), 8.32 (1H, br d, *J* = 8.3 Hz, H-1), 7.93 (1H, dt, *J* = 1.5 and 7.8 Hz, H-2), 7.83 (1H, br t, *J* = 7.3 Hz, H-4), 6.99 (1H, s, H-9), 6.85 (1H, t, *J* = 3.4 Hz, H-12), 5.71 (1H, m, H-14), 5.53 (1H, br s, H-16), 5.35 (2H, m, -*CH* = *CH*-), 4.14 (1H, m, H_a-13), 3.94 (1H, m, H_b-13), 2.83 (2H, m, -CH = CH-CH_2), 2.50 (2H, t, *J* = 6.3 Hz, H-2′), 2.04 (2H, m, -CH = CH-CH_2), 1.97 (3H, d, *J* = 1.5 Hz, H-19), 1.79 (3H, d, *J* = 1.5 Hz, H-18), 1.70 (2H, m, CH_2), 1.25 (22H, m, CH_2), 0.88 (3H, t, *J* = 6.3 Hz, H-18); fabms *m/z* $[M+2+H]^+$ 640, 374 $[M+2-C_{18}H_{33}O_2+H]^+$ 358, 274, 263, 259.

METHANOLYSIS OF CYSTODYTINS H [**5a**] AND I [**5b**].—The mixture of compounds **5a** and **5b** (0.9 mg) was treated with 5% HCl in MeOH (1 ml) under reflux for 6 h. To the reaction mixture H_2O (5 ml) was added, and the mixture was partitioned with hexane (10 ml × 3). The aqueous layer was made alkaline with 1 N $NaHCO_3$ and extracted with $CHCl_3$ (10 ml × 3). The hexane-soluble fraction was evaporated under reduced pressure to give a colorless oil (0.2 mg). This oil was treated with CH_2N_2, and the product was identified as methyl octadecenoate by gc-ms analysis. The $CHCl_3$ layer was subjected to a Si gel column (Wako gel C-300, 5 × 75 mm) with MeOH-$CHCl_3$ (3:97) to give compounds **4a** and **4b** (0.5 mg), which were identified by tlc, ^1H nmr, eims, and hreims (*m/z* 389.1725, Δ −1.4 mmu, $[M+2]^+$).

GC-MS ANALYSIS OF FATTY ACID METHYL ESTERS.—Gc-ms was carried out by using a silicone SE-30 glass column (3 mm × 2 m, GL Science; injection temperature 250°; initial oven temperature 140°; program rate 4°/min; carrier gas He; detection FID) and a DX-300 mass spectrometer operating in the ei mode (70 eV, scan range between 1 and 715 with repetition times of 2 sec). Retention times (min) of peaks due to standard fatty acid methyl esters: methyl tetradecanoate (6.3 min), methyl 9-hexadecenoate (10.4 min), methyl hexadecanoate (11.2 min), and methyl 9-octadecenoate (15.1 min). The retention time of the methyl ester of the methanolysis product of the mixture of **5a** and **5b** was 15.1 min.

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