New Cytotoxic *N*-Methylated *β*-Carboline Alkaloids from the Marine Ascidian *Eudistoma gilboverde*

Mohammad A. Rashid,[†,‡] Kirk R. Gustafson,[§] and Michael R. Boyd*[,§]

Molecular Targets Drug Discovery Program, Center for Cancer Research, National Cancer Institute−Frederick, Building 1052, Room 121, Frederick, Maryland 21702-1201, and Intramural Research Support Program, SAIC−Frederick, NCI−Frederick, Frederick, Maryland 21702-1201

Received April 24, 2001

Bioassay-guided fractionation of an extract of the marine ascidian *Eudistoma gilboverde* provided three new *β*-carboline alkaloids identified as 2-methyleudistomin D (**1**), 2-methyleudistomin J (**2**), and 14-methyleudistomidin C (**3**). Six known metabolites, eudistomins C, D (**4**), E, J (**5**), K, and L, were also isolated and characterized. The structures of the new metabolites were elucidated by spectroscopic analyses and by comparison of their spectral data with related literature values. Of the three new compounds, 14-methyleudistomidin C (**3**) exhibited the most potent cytotoxic activity with IC_{50}'s of < 1.0 *μ*g/mL against four different human tumor cell lines.

Marine ascidians belonging to the genus *Eudistoma* (family Polycitoridae) have been the subject of extensive chemical and biological investigations. Numerous *β*-carboline alkaloids, including eudistomins A−T,[1−4] eudistomidins A−F,[5−7] eudistalbins A and B,[8] isoeudistomins,[9] and two new trypargine derivatives,[10] have been isolated from various *Eudistoma* species. Many of these compounds are reported to exhibit antiviral,[1−3] antimicrobial,[2] cytotoxic,[6,8,11] or calmodulin antagonistic[5] properties. The biosynthesis of complex *β*-carbolines is generally believed to involve the coupling of tryptophan with a second amino acid, and recent in vivo studies with *E. olivaceum* confirmed that tryptophan and proline are the primary precursors of eudistomin I.[12] Our studies were initiated after an organic extract of *E. gilboverde* (Sluiter) exhibited cytotoxic activity in the U.S. National Cancer Institute (NCI)'s 60-cell line antitumor screen.[13,14] This species of *Eudistoma*, which was collected in the Siaes Tunnel, Palau, has not been the subject of previous reports in the chemical literature. Bioassay-guided fractionation of the cytotoxic extract provided 10 *β*-carboline alkaloids, including three new compounds, **1**−**3**.

The CH_2Cl_2−MeOH extract of *E. gilboverde* was subjected to solvent−solvent partitioning[15] followed by gel permeation on Sephadex LH-20, and the resulting fractions were screened for cytotoxic activity. Final C_{18} HPLC purification of the principal cytotoxic fractions obtained from the LH-20 column provided nine *β*-carboline alkaloids. Six of these were readily identified as eudistomins C, D (**4**), E, J (**5**), K, and L by comparison of their physical and spectral data with previously reported values.[1−3]

The molecular formula of compound **1**, established as $C_{12}H_{10}N_2O_3Br$ by HRFABMS measurements, required 8 unsaturation equivalents and the presence of a quaternary nitrogen atom. Its UV spectrum was characteristic of a *β*-carboline alkaloid,[16] and a broad IR absorption band from 3100 to 3400 cm^{-1} suggested the presence of hydroxyl and NH moieties. The ^1H and ^{13}C NMR data of **1** (DMSO-d_6) corresponded closely with those of eudistomin D (**4**),[2] and correlations observed in the HSQC and HMBC experiments allowed unambiguous assignment of all the resonances in **1** (Table 1). A pair of 1H doublets (J = 6.5 Hz) at δ 8.58 and 8.96 were assigned to H-3 and H-4, while a broad signal at δ 9.38 (1H) was attributed to H-1. A second set of 1H doublets (J = 9 Hz) at δ 7.53 and 7.72 were assigned to H-7 and H-8, respectively. A three-proton singlet at δ 4.49 (δ_C 47.6) was characteristic of a quaternary *N*-Me group, and two deuterium-exchangeable proton signals at δ 10.45 and 12.99 were also apparent. HMBC correlations from the δ 10.45 proton to C-5 (δ 101.9), C-6 (δ 149.5), and C-7 (δ 122.4) established this resonance as OH-6, while an HMBC correlation between C-8a (δ 138.8) and the δ 12.99 proton revealed this signal as NH-9. The substitution of a methyl group on the nitrogen at position 2 of a *β*-carbolinium ring system in **1** was supported by NOE interactions observed between the *N*-methyl protons and both H-1 and H-3, and by HMBC correlations from H-1 and H-3 to the *N*-methyl carbon resonance. We were thus able to assign the structure of **1** as 2-methyleudistomin D.

HRFABMS of compound **2** established its molecular formula as $C_{12}H_{10}N_2O_3Br$, which was isomeric with that of 2-methyleudistomin D (**1**). The primary difference observed in the ^1H NMR spectral data of **2** was that signals assigned to the *ortho*-coupled H-7 and H-8 protons in **1** were replaced by a pair of *para*-oriented protons (δ 7.87 and 8.02) in compound **2**. This indicated that carbons 5 and 8 were protonated and that the hydroxyl and bromine substituents were located on carbons 6 and 7. In DMSO-d_6, NOE interactions were observed between H-5 and the OH resonance (δ 10.53), as well as between H-8 and NH-9 (δ 12.67). This placed the hydroxyl group on C-6 and required that the bromine was substituted on C-7. This same pattern of substituents occurs in eudistomin J (**5**), and indeed the NMR spectral data recorded for **2** corresponded closely with the NMR data reported for **5**.[2] Placement of a quaternary *N*-methyl group at N-2 in compound **2** was supported by NOE interactions and HMBC correlations between the methyl group (δ_H 4.44, δ_C 47.5) and both H-1 (δ 9.28) and H-3 (δ 8.54). Thus, compound **2** was characterized as 2-methyleudistomin J.

HRFABMS of compound **3** provided a $[M + H]^+$ ion at *m*/*z* 380.0439, which established its molecular formula as $C_{16}H_{18}N_3OBrS$. This differed from the molecular formula

* To whom correspondence should be addressed. Tel: 301-846-5391. Fax: 301-846-6919. E-mail: boyd@dtpax2.ncifcrf.gov.
† SAIC−Frederick.
‡ On leave from the Department of Pharmacy, University of Dhaka, Dhaka-1000, Banglasdesh.
§ Molecular Targets Drug Discovery Program, NCI−Frederick.

10.1021/np010214+ CCC: $20.00
Published on Web 10/19/2001

Table 1. NMR Spectral Data for Compounds **1**−**3**

	1[a]		**2**[a]		**3**[b]	
pos.	δ^{13}C	δ^{1}H mult *J* (Hz)	δ^{13}C	δ^{1}H mult *J* (Hz)	δ^{13}C	δ^{1}H mult *J* (Hz)
1	130.7	9.38 s	130.6	9.28 s	137.0	
2-Me	47.6	4.49 s	47.5	4.44 s		
3	132.3	8.58 d (6.5)	132.7	8.54 d (6.5)	138.4	8.47 d (5.5)
4	117.9	8.96 d (6.5)	117.7	8.73 d (6.5)	118.6	8.71 d (5.5)
4a	130.6		130.8		131.4	
4b	118.8		119.5		121.9	
5	101.9		107.0	7.87 s	104.3	
6	149.5	10.45 s	149.2	10.53 s	149.9	10.03 s[c]
7	122.4	7.53 d (9.0)	117.6		120.2	7.28 d (8.5)
8	113.1	7.72 d (9.0)	116.9	8.02 s	112.7	7.49 d (8.5)
8a	138.8		138.2		137.8	
9		12.99 s		12.67 s		12.23 s[c]
9a	135.1		135.1		137.7	
10					66.6	5.08 dd (8.0, 5.5)
11					35.1	3.46 dd (14.0, 8.0)
						3.47 dd (14.0, 5.5)
13					16.1	1.82 s
14-Me_2					42.3	2.93 s

[a] Data acquired in DMSO-d_6. [b] Data acquired in CD_3OD. [c] Assigned from spectrum acquired in DMSO-d_6.

of eudistomidin C (**6**) only by the addition of CH_2, and the ^{1}H and ^{13}C NMR data obtained with compound **3** were quite similar to those reported for **6**.[6] However, the H-10 methine (δ 4.68), H_2-11 methylene (δ 3.16), and H_3-15 methyl (δ 2.50) resonances in **6** were shifted downfield in **3**, where they appeared at δ 5.08, 3.46, and 3.47 (1H each), and 2.93, respectively. In addition, the ^{1}H NMR signal at δ 2.93 integrated for six protons and displayed both HSQC and HMBC correlations to δ_C 42.3, which revealed the presence of a *gem*-dimethyl group in **3**. These spectral changes indicated that the proton on N-14 in eudistomidin C (**6**) was replaced by a methyl group in **3**. HMBC data confirmed the substituted β-carboline ring system and the presence of two methyl substituents on N-14 in **3**. The structure of compound **3** was therefore identified as the 14-methyl analogue of eudistomidin C. The absolute stereochemistry at C-10 in **3** was tentatively assigned as *S*, based on analogy with compound **6**, since the optical rotations of **3** (α_D +12.9°) and **6** (α_D +15.6°)[6] had the same sign and were close in magnitude.

β-Carboline alkaloids with a quaternary methylated nitrogen are known from a number of different terrestrial plant genera;[17−22] however, they have rarely been isolated from marine organisms. To the best of our knowledge, the only *N*-methyl β-carbolinium derivatives described from marine sources were recently reported from the soft coral *Lignopsis spongiosum*[23] and the ascidian *Pseudodistoma* sp.[24] Compounds **1**−**3** were evaluated for cytotoxic properties in a 2-day in vitro assay. The IC_{50} values (μg/mL) for these compounds against LOX (melanoma), OVCAR-3 (ovarian), COLO-205 (colon), and MOLT-4 (leukemia) human tumor cell lines are listed below with the individual cell line identifiers: 2-methyleudistomin D (**1**), LOX (15.0), OVCAR-3 (20.0), COLO-205 (19.1), MOLT-4 (16.6); 2-methyleudistomin J (**2**), LOX (15.1), OVCAR-3 (20.0), COLO-205 (15.1), MOLT-4 (17.5); 14-methyleudistomidin C (**3**), LOX (0.41), OVCAR-3 (0.98), COLO-205 (0.42), MOLT-4 (0.57).

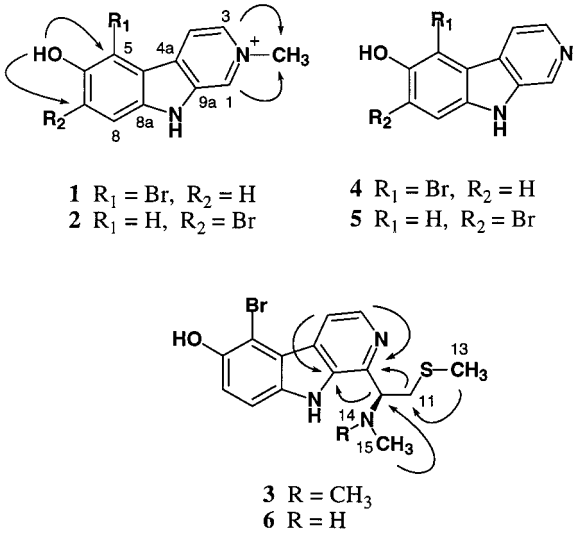

Figure 1. Arrows indicate some key HMBC correlations in **1**−**3**.

Experimental Section

General Experimental Procedures. HPLC was performed on a Varian-Rainin system employing a Dynamax C_{18} column (1 × 25 cm or 0.46 × 25 cm), using a flow rate of 3 or 0.6 mL/min and UV detection at 220 nm. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Ultraviolet (UV) and infrared (IR) spectra were obtained on a Beckman DU-640 and Perkin-Elmer 1600 FTIR spectrometer, respectively. The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded in CD_3OD and/or DMSO-d_6 on a Varian INOVA 500 spectrometer, and the chemical shifts are reported in ppm relative to the residual nondeuterated solvent signals. Inverse-detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{CH}$ = 140 Hz) and HMBC (optimized for $^nJ_{CH}$ = 8.5 and 3.5 Hz) pulse sequences. High-resolution mass spectra were acquired on a JEOL SX102 mass spectrometer.

Animal Material. Samples of the ascidian *E. gilboverde* were collected from Sias Tunnel, Palau, by the Coral Reef Research Foundation and subsequently identified by F. Monniot. A voucher specimen (voucher # 0CDN5058) for this collection is maintained at the Smithsonian Institution, Washington, D.C.

Extraction and Isolation. The frozen samples (57.8 g) were first extracted with H_2O to give a separate aqueous extract, and the remaining solids were then percolated with CH_2Cl_2−MeOH (1:1) followed by MeOH (100%). The combined organic solvent extracts were evaporated in vacuo to provide a total of 3.6 g of material. A 2.0 g portion of the extract was subjected to a four-step solvent−solvent partitioning protocol[15] which concentrated the cytotoxic activity into the $CHCl_3$-soluble partition fraction (272 mg). This active fraction was separated by gel permeation chromatography on a Sephadex LH-20 column (2.5 × 100 cm) eluted with CH_2Cl_2−MeOH

(1:1). A total of 93 fractions (each 300 drops) were collected, and these were combined on the basis of the UV trace to give nine fractions. This further concentrated the activity into fractions 5 (17.0 mg) and 6 (5.0 mg). Purification of a 7.5 mg aliquot of fraction 5 by C_{18} HPLC using a linear gradient from 20% to 50% CH_3CN in H_2O (with 0.1% TFA) over 40 min yielded, in order of elution, 2-methyleudistomin D (**1**) (2.1 mg; t_R = 7.96 min), 2-methyleudistomin J (**2**) (1.2 mg; t_R = 10.32 min), eudistomin C salt (0.6 mg; t_R = 17.82 min), eudistomin E (0.6 mg; t_R = 21.62 min), and 14-methyleudistomidin C (**3**) (1.0 mg, t_R = 24.79 min). A similar purification of fraction 6 provided eudistomin D (0.5 mg; t_R = 11.42 min), eudistomin J (1.2 mg; t_R = 12.87 min), eudistomin C salt (0.5 mg; t_R = 19.77 min), and a mixture of eudistomins K and L (1.2 mg, t_R = 22.56 min). Repeated trituration of fraction 4 (27.5 mg) with CH_2Cl_2 and CH_3OH provided an additional 7.3 mg of virtually pure **1**.

2-Methyleudistomin D (1): yellow amorphous powder; UV (EtOH) λ_{max} (log ϵ) 214 (4.02), 236 (3.80), 268 (3.96), 303 sh (3.71), 314 (3.79), 416 (3.19) nm; IR (film) ν_{max} 3100−3400, 1670, 1490, 1320, 1194, 1130, 834, 807 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 276.9987 $[M]^+$ (calcd for $C_{12}H_{10}N_2O^{79}Br$, 276.9977).

2-Methyleudistomin J (2): yellow gum; UV (EtOH) λ_{max} (log ϵ) 212 (4.04), 236 (3.99), 271 (3.97), 307 sh (3.83), 321 (3.96), 412 (3.35) nm; IR (film) ν_{max} 3100−3400, 1680, 1493, 1460, 1327, 1202, 1137, 1028, 838, 800, 782 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HRFABMS *m/z* 276.9984 $[M]^+$ (calcd for $C_{12}H_{10}N_2O^{79}Br$, 276.9977).

14-Methyleudistomidin C (3): yellowish gum; $[\alpha]_D$ +12.9° (*c* 0.07, MeOH); UV (EtOH) λ_{max} (log ϵ) 216 (4.28), 234 (4.16), 252 (4.06), 287 sh (3.77), 298 (3.93), 369 (3.22) nm; IR (film) ν_{max} 3100−3400, 1677, 1565, 1429, 1317, 1283, 1255, 1208, 1181, 1130, 1025, 834, 794, 715, 1510, 810 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 12.23 (1H, bs, NH-9), 10.03 (1H, bs, OH-6), 8.57 (1H, d, J = 5.5 Hz, H-4), 8.46 (1H, d, J = 5.5 Hz, H-3), 7.55 (1H, d, J = 8.5 Hz, H-8), 7.34 (1H, d, J = 8.5 Hz, H-7), 5.31 (1H, dd, J = 8.0, 5.5 Hz, H-10), 3.40 (2H, m, H-11), 2.82 (6H, s, H-14 and H-15), 1.85 (3H, s, H-13); ^1H and ^{13}C NMR in CD_3OD, see Table 1; HRFABMS *m/z* 380.0439 $[M + H]^+$ (calcd for $C_{16}H_{19}N_3O^{79}BrS$, 380.0432).

Cytotoxicity Evaluations. DMSO solutions of chromatography fractions and aliquots of the purified compounds were assayed for cytotoxic properties in a 2-day in vitro assay, experimental details of which have been reported previously.[25]

Acknowledgment. The authors wish to thank D. Newman (NPB) for coordinating collections, L. Bell Colin (CRRF) for collection and sample information, T. McCloud for extractions, L. Pannell for mass spectral measurements, and T. Johnson for cytotoxicity evaluations. This project was supported by NCI contract No. NO1-CO-56000. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References and Notes

(1) Rinehart, K. L., Jr.; Kobayashi, J.; Harbour, G. C.; Hughes, R. G., Jr.; Mizask, S. A.; Scahill, T. A. *J. Am. Chem. Soc.* **1984**, *106*, 1524−1526.
(2) Kobayashi, J.; Harbour, G. C.; Gilmore, J.; Rinehart Jr., K. L. *J. Am. Chem. Soc.* **1984**, *106*, 1526−1528.
(3) Rinehart, K. L., Jr.; Kobayashi, J.; Harbour, G. C.; Gilmore, J.; Mascal, M.; Holt, T. G.; Shield, L. S.; Lafargue, F. *J. Am. Chem. Soc.* **1987**, *109*, 3378−3387.
(4) Kinzer, K. F.; Cardellina, J. H., II. *Tetrahedron Lett.* **1987**, *28*, 925−926.
(5) Kobayashi, J.; Nakamura, H.; Ohizumi, Y.; Hirata, Y. *Tetrahedron Lett.* **1986**, *27*, 1191−1194.
(6) Kobayashi, J.; Cheng, J.; Ohta, T.; Nozoe, S.; Ohizumi, Y.; Sasaki, T. *J. Org. Chem.* **1990**, *55*, 3666−3670.
(7) Murata, O.; Shigemori, H.; Ishibashi, K. S.; Hayashi, K.; Kobayashi, J. *Tetrahedron Lett.* **1991**, *32*, 3539−3542.
(8) Adesanya, S. A.; Chbani, M.; Païs, M.; Debitus, C. *J. Nat. Prod.* **1992**, *55*, 525−527.
(9) Kang, H.; Fenical. W. *Nat. Prod. Lett.* **1996**, *9*, 7−12.
(10) Wagoner, R. M. V.; Jompa, J.; Tahir, A.; Ireland, C. M. *J. Nat. Prod.* **1999**, *62*, 794−797.
(11) Blunt, J. W.; Lake, B. J.; Munro, M. H. G. *Tetrahedron Lett.* **1987**, *28*, 1825−1826.
(12) Shen, G. Q.; Baker, B. J. *Tetrahedron Lett.* **1994**, *35*, 1141−1144.
(13) Boyd, M. R. In *Current Therapy in Oncology*; Niederhuber, J. E., Ed.; B. C. Decker, Inc.: Philadelphia, 1993; pp 11−22.
(14) Boyd, M. R.; Paull, K. D. *Drug Dev. Res.* **1995**, *34*, 91−109.
(15) Van Wagenen, B. C.; Cardellina, J. H., II; Lidert, Z. C.; Swithenbank, C. *J. Org. Chem.* **1993**, *58*, 335−337.
(16) Scott, A. I. *Interpretation of the Ultraviolet Spectra of Natural Products*; Valeriote, F. A., Corbett, T., Baker, L., Eds.; Pergamon Press: New York, 1964; p 176.
(17) Bannarjee, P. K.; Ghosal, S. *Aust. J. Chem.* **1969**, *22*, 275−277.
(18) Ghosal, S.; Banerjee, P. K.; Banerjee, S. K. *Phytochemistry* **1970**, *9*. 429−433.
(19) Ghosal, S.; Banerjee, S. K.; Bhattacharya, S. K.; Sanyal, A. K. *Planta Med.* **1972**, *21*, 398−409.
(20) Ghosal, S.; Bhattacharya, S. K. *Planta Med.* **1972**, *22*, 434−440.
(21) Jiang-Nam, P.; Xiao-Zheng, F.; Qi-Tai, Z.; Xiao-Tiam, L. *Phytochemistry* **1997**, *46*, 1119−1122.
(22) Baechli, E.; Vamvacas, C.; Schmid, H.; Karrer, P. *Helv. Chim. Acta* **1957**, *40*, 1167−1187.
(23) Carbrera, G. M.; Seldes, A. M. *J. Nat. Prod.* **1999**, *62*, 759−760.
(24) Rashid, M. A.; Gustafson, K. R.; Cartner, L. K.; Pannell, L. K.; Boyd, M. R. *Tetrahedron* **2001**, *57*, 5751−5755.
(25) Bokesch, H. R.; Blunt, J. W.; Westergaard, C. K.; Cardellina, J. H., II; Johnson, T. R.; Michael, J. A.; McKee, T. C.; Hollingshed, M. G.; Boyd, M. R. *J. Nat. Prod.* **1999**, *62*, 633−635.

NP010214+