Caulibugulones A−F, Novel Cytotoxic Isoquinoline Quinones and Iminoquinones from the Marine Bryozoan *Caulibugula intermis*

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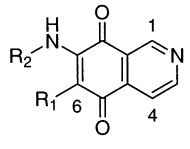
Received August 14, 2003

An extract of the marine bryozoan *Caulibugula intermis*, collected in the Indo-Pacific off Palau, produced a distinct pattern of differential cytotoxicity in the National Cancer Institute's 60 cell line antitumor screen. Bioactivity-directed fractionation of the extract provided six new compounds, caulibugulones A−F (**1**−**6**). The structures of these novel metabolites were determined by spectrochemical analyses including LC-MS, HRFABMS, 1-D and 2-D NMR experiments, and by comparison with related compounds. The structures of compounds **2** and **3** were confirmed by chemical interconversion. The isolated compounds exhibited IC_{50}'s of 0.03−1.67 μg/mL against murine tumor cells in an in vitro cytotoxicity assay.

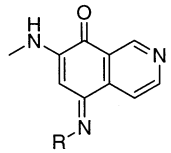
In our continuing search for novel cytotoxic metabolites from natural sources, the organic extract of the marine bryozoan *Caulibugula intermis* Harmer (Bugulidae) was selected for chemical evaluation based on the pattern of differential growth inhibition it exhibited in the U.S. National Cancer Institute (NCI)'s 60 cell line antitumor screen.[1,2] Several other members of the family Bugulidae, particularly bryozoans from the genus *Bugula*, have been the focus of extensive chemical studies. They have provided a large family of potent cytotoxic bryostatin macrolides,[3−15] a series of polypyrrole derivatives,[16−18] and several steroids and terpenoids.[19,20] There have not been any previous reports on the chemical constituents from *Caulibugula*. We report herein the isolation, characterization, and biological evaluation of four new isoquinoline quinones and two new isoquinoline iminoquinones from a collection of *C. intermis* made in Palau.

Results and Discussion

Solvent−solvent partitioning of the *C. intermis* extract concentrated the cytotoxic activity into the 9:1 methyl-*tert*-butyl ether/hexane-soluble fraction. Subsequent chromatography over Sephadex LH-20 followed by C_{18} HPLC led to the recovery of six related metabolites, which we have named caulibugulones A−F (**1**−**6**). The ^{1}H NMR spectra of compounds **1**−**6** each possessed a downfield singlet and a pair of doublets typical of the heteroaromatic ring of isoquinolines. The paucity of additional aromatic protons suggested a high degree of substitution for the second ring.



1 R_1 = H, R_2 = CH_3
2 R_1 = Br, R_2 = CH_3
3 R_1 = Cl, R_2 = CH_3
4 R_1 = H, R_2 = CH_2CH_2OH



5 R = H
6 R = CH_2CH_2OH

Caulibugulone A (**1**) was isolated in the highest yield as a dark red solid and gave a protonated molecule $[M + H]^+$ at *m/z* 189.0668 by HRFABMS, corresponding to a molecular formula of $C_{10}H_8N_2O_2$. The structure of **1** was deduced through a combination of 1-D ^{1}H and ^{13}C NMR experiments in combination with 2-D HMBC, HSQC, COSY, and ROESY experiments. The singlet at δ 9.16 (H-1) in the ^{1}H spectrum showed an HMBC correlation to C-3 (δ 155.5), while H-3 (δ 8.94) was *ortho*-coupled (J = 4.8 Hz) to a one-proton doublet at δ 7.94 (H-4), which suggested the presence of a 4,5-disubstituted pyridine moiety. An HMBC correlation from H-3 allowed assignment of C-4a, and HMBC correlations from H-1 and H-4 allowed assignment of C-8a. The ^{13}C spectrum indicated the presence of an additional two carbonyls and two sp^2 carbons, one bearing a proton at δ 5.77 and the other bearing an *N*-methyl substituent. One of the carbonyls (C-5, δ 181.1) was placed adjacent to C-4a and the other (C-8, δ 181.0) adjacent to C-8a by HMBC correlations from H-4 and H-1, respectively. An HMBC correlation from the proton at δ 5.77 to C-4a placed it on C-6. This in turn required the *N*-methyl group to be located at C-7. A three-bond HMBC correlation from the *N*-methyl protons at δ 2.92 to C-7, in addition to four-bond correlations to C-6 and C-8, confirmed the placement of the *N*-methyl group. Compound **1** is therefore 7-methylaminoisoquinoline-5,8-dione, which has not previously been reported in the chemical literature.

Caulibugulones B (**2**) and C (**3**) provided ^{1}H NMR spectra that lacked an H-6 singlet, but otherwise were very similar to the spectrum of **1**. Compound **2** gave a protonated molecule $[M + H]^+$ at *m/z* 223.0286 by HRFABMS corresponding to a molecular formula of $C_{10}H_7N_2O_2Cl$ and had an isotope pattern consistent with the presence of one chlorine atom. Compound **3** showed an isotopic distribution indicative of a single bromine atom and gave a protonated molecule $[M + H]^+$ at *m/z* 266.9764 that established a molecular formula of $C_{10}H_7N_2O_2Br$. The MS data together with the NMR data clearly indicated that H-6 had been replaced by a chlorine atom in **2** and a bromine atom in **3**. This was confirmed by conversion of **1** to either **2**, or **3** upon treatment with *N*-chlorosuccinimide (NCS), or *N*-bromosuccinimide (NBS), respectively. Bromination with NBS occurred rapidly in dioxane at room temperature and went to completion, as evidenced by HPLC and LCMS. Treatment of **1** with NCS under the conditions described above provided only a 38% yield, but subsequent reaction in

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10.1021/np030378l
Published on Web 01/06/2004

Table 1. ^{1}H and ^{13}C NMR Spectral Data for Caulibugulones A−E (**1**−**6**)[a]

	1[b]			**2**[c]		**3**[c]		**4**[d]		**5**[d]		**6**[d]	
no.	δ_H mult (*J*, Hz)	δ_C	HMBC	δ_H mult (*J*, Hz)	δ_C	δ_H mult (*J*, Hz)	δ_C	δ_H mult (*J*, Hz)	δ_C	δ_H mult (*J*, Hz)	δ_C	δ_H mult (*J*, Hz)	δ_C
1	9.16 s	147.4	C-3, C-4a, C-8, C-8a	9.35 s	148.1	9.36 s	148.4	9.17 s	148.3	9.30 s	149.8	9.33 s	149.8
3	8.94 d (4.8)	155.5	C-1, C-4, C-4a	9.01 d (4.8)	156.1	9.00 d (5.0)	156.1	8.97 d (5.0)	156.9	9.09 d (5.0)	157.4	9.09 d (5.0)	157.3
4	7.94 d (4.8)	120.1	C-3, C-4a, C-5, C-8a	7.98 d (4.8)	118.8	7.98 d (5.0)	119.2	7.81 d (5.0)	119.3	8.01 d (5.0)	118.8	8.45 d (5.0)	118.8
4a		141.0			138.4		138.2		140.1		136.5		137.2
5		181.1			174.8		173.4		181.5		162.1		159.0
6	5.77 s	100.7	C-4a, C-7, C-8		f		f	5.84 s	101.0	6.35 s	93.5	6.10 s	90.2
7		150.7			146.4		148.4		149.6		151.7		151.5
8		181.0			180.5		180.1		182.2		179.3		179.3
8a		125.8			124.4		123.8		125.6		124.3		123.9
7-NH	6.07[e] bs			8.29 bs		8.19 bs		6.32[e] bs					
7-NCH$_3$	2.92 d (5.1)[e]	29.3	C-6, C-7, C-8	3.38 d (5.5)	32.5	3.40 d (5.5)	33.0			3.08 s	29.6	3.12 s	29.8
N*CH*$_2$CH$_2$OH								3.37 dt (5.0, 5.5)[e]	45.2			3.88 t (4.5)	49.1
NCH$_2$*CH*$_2$OH								3.71 t (5.5)	59.7			3.93 t (4.5)	59.1

[a] Spectra recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C. [b] Spectra recorded in CD_3OD−$CDCl_3$ (1:1) and referenced to residual solvent (δ_H 3.30, δ_C 49.0). [c] Spectra recorded in pyridine-d_5 and referenced to residual solvent (δ_H 8.71, δ_C 123.5). [d] Spectra recorded in CD_3CN and referenced to residual solvent (δ_H 1.93, δ_C 118.2). [e] Recorded in $CDCl_3$. [f] Not observed.

methanol afforded nearly 100% conversion to the chloro derivative. Assignment of the ^{1}H and ^{13}C NMR resonances for **2** and **3** was done in a straightforward manner as described for **1**. However, a signal for C-6 could not be measured in the ^{13}C NMR spectrum of either **2** or **3**, regardless of the experimental conditions that were employed. Attempts to detect C-6 included increasing the delay time between each transient, decreasing the pulse width, and addition of the relaxation agent Cr(AcAc). The broad amino NH resonance in both **2** and **3** did not reveal any HMBC correlations, and appropriate four-bond heteronuclear correlations that could have allowed assignment of C-6 were never observed. While the inability to assign C-6 was frustrating, the chemical interconversion of **1** to either **2** or **3** allowed unambiguous assignment of the structures of caulibugulones B (**2**) and C (**3**). The difficulties encountered recording a signal for C-6 are likely due to the small sample size we obtained for both **2** and **3**, since complete ^{13}C assignments were made for structurally related synthetic compounds that were available in multimilligram quantities.[21]

Caulibugulone D (**4**) revealed a ^{1}H NMR spectrum that was similar to **1**, except that the *N*-methyl singlet of **1** was replaced by a pair of two-proton triplets at δ 3.32 and 3.71. The methylene triplets were mutually coupled as shown by COSY correlations. When the spectrum was acquired in $CDCl_3$, an additional COSY correlation was observed between the methylene protons at δ 3.32 and the NH proton at δ 6.32, which together with an HMBC correlation from these methylene protons to C-7 placed them on the C-7 nitrogen. Compound **4** gave a protonated molecule $[M + H]^+$ at *m*/*z* 219.0774 by HRFABMS corresponding to a molecular formula of $C_{11}H_{10}N_2O_3$. All that remained to be accounted for was an OH group, which was placed on the terminal methylene group based on its carbon chemical shift data (δ_C 59.7). Thus, the structure of caulibugulone D (**4**) was assigned as 7-(2-hydroxyethylamino)isoquinoline-5,8-dione.

The NMR spectra of caulibugulones E (**5**) and F (**6**) were also similar to those of **1**, with the major difference observed in their ^{13}C NMR spectra. One of the carbons that had been observed at ca. δ 180 in **1** was shifted upfield to ca. δ 160 in both **5** and **6**, which suggested that these compounds contained an imine functionality. The ^{1}H NMR data of **5**, together with the molecular formula of $C_{10}H_9N_3O$ determined by HRFABMS, indicated that this compound differed from **1** only by substitution of an NH for one of the quinone oxygens. The imine substituent in **5** was placed at C-5 on the basis of an HMBC correlation from H-4 to the upfield shifted carbon (δ 162.1). Caulibugulone F (**6**) was structurally related to **5** but had an additional $-CH_2$-CH_2OH group, as indicated by both NMR and MS data. Compound **6** was shown to possess an imino group at C-5 as in **5**, and an HMBC correlation from the methylene protons of the $-CH_2CH_2OH$ group to C-5 placed this substituent on the C-5 nitrogen. The geometry about the imine double bond was established by a ROESY correlation between the methylene protons at δ 3.88 and H-6.

A number of isoquinoline quinones, including the renierones and cribrostatins,[22−27] have been isolated as natural products from marine sponges. Similar compounds have also been isolated from microbial sources including cultures of *Streptomyces*[28−30] and *Calothrix*,[31] which suggests that the compounds derived from sponges may ultimately be of microbial origin as well. These isoquinoline quinones have been reported to exhibit antimicrobial and antitumor activities.[25−29] Quinoneimines are also well known from marine organisms, and the simplest of these are the pyrroloiminoquinones such as the isobatzellines,[32] makaluvamines,[33] and secobatzelline A.[34] Secobatzelline A was found to co-occur with its quinone analogue, secobatzelline B, which is similar to the co-occurrence of caulibugulone E (**5**) with its quinone counterpart **1**. To the best of our knowledge, caulibugulones E and F represent the first compounds with an isoquinoline iminoquinone skeleton to be isolated from a natural source. Compounds **1**−**6** were evaluated for cytotoxic activity against the murine IC-2^{WT} cell line[35] (Table 2). Compounds **1**−**3** displayed similar IC_{50}'s, indicating that halogen substitution at C-6 is not an important determinant of cytotoxicity. The iminoquinone **5** was approximately an order of magnitude more potent than the corresponding quinone **1**. Substitution of an ethyl alcohol at either the C-7 or C-5 nitrogen resulted in a 5−10-fold reduction in cytotoxicity. The caulibugulones

Table 2. IC_{50} of **1**−**6** against the Murine IC-2^{WT} Cell Line in an in Vitro Antiproliferative Assay

compound	IC_{50} (μg/mL)
1	0.34
2	0.22
3	0.28
4	1.67
5	0.03
6	0.10

are of similar potency to the cribrostatins and related isoquinoline quinones[22−24] and the iminoquinone isobatzellines[32] and secobatzelline A.[34]

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were obtained on NaCl disks in a Perkin-Elmer Spectrum 2000 FT-IR spectrometer. NMR spectra were acquired on a Varian Unity INOVA spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C and referenced to the residual nondeuterated solvent. Positive-ion, fast-atom bombardment mass spectra (FABMS) were obtained on either a JEOL SX102 or VG 7070E-HF double-focusing mass spectrometer. A sample matrix of either 5:1 dithiothreitol and dithioeriythritol (magic bullet) or 3-nitrobenzyl alcohol (NBA) was employed. Electrospray mass spectra were obtained on a Hewlett-Packard HP1100 integrated LC-MS system. HPLC was performed on a Waters 600E system with a Waters 990 DAD, or a Varian ProStar 215 system with ProStar 320 UV/vis detector.

Animal Material. Samples of the marine bryozoan *Caulibugula intermis* were collected by P. L. Colin (Coral Reef Research Foundation) at a depth of 33 m in the south Pacific off Palau. Animal material was frozen shortly after collection and maintained frozen prior to extraction. A voucher specimen (0CDN 1079) for this collection is maintained at the Smithsonian Institution, Washington, D.C.

Isolation and Identification of Compounds 1−6. The frozen animal material was ground into a coarse powder (227.7 g) and extracted with H_2O to yield 25.92 g of crude aqueous extract after lyophilization. The marc was then extracted with CH_2Cl_2−MeOH (1:1) followed by MeOH (100%) to yield 5.14 g of combined crude organic extract. A 1.78 g portion of the crude organic extract was dissolved in 9:1 MeOH−H_2O and partitioned with hexane (3 × 200 mL). H_2O (120 mL) was added to the MeOH/H_2O layer, which was then extracted (3 × 200 mL) with 9:1 MTBE−hexane. The residual organic solvents were removed from the aqueous layer by rotary evaporation under reduced pressure, and the aqueous layer was extracted with 3 × 200 mL of EtOAc. The MTBE fraction (413 mg) was dissolved in 2:5:1 hexane−CH_2Cl_2−MeOH and chromatographed by gel permeation chromatography on Sephadex LH-20 (2 × 125 cm) eluting with hexane−CH_2Cl_2−MeOH (2:5:1) monitoring at 254 nm. The main cytotoxic fraction (23.0 mg) was further purified by C_{18} HPLC (Dynamax, 1 × 25 cm, 8 μm) employing a linear gradient from 0 to 100% MeCN in H_2O (+0.1% TFA) over 30 min (3 mL/min) with detection at 220 nm, yielding compounds **1** (4.6 mg, t_R = 16.8 min), **2** (0.2 mg, t_R = 19.7 min), and **3** (0.5 mg, t_R = 20.2 min). The subsequent LH-20 fraction (10.4 mg) which also showed cytotoxicity was further purified by C_{18} HPLC as above, yielding compound **4** (0.8 mg, t_R = 14.7 min) and **5**/**6** (0.6 mg, t_R = 13.9 min) as a 1:1 mixture. Workup of the remaining crude organic extract provided additional amounts of **1** (7.1 mg), **2** (2.0 mg), **3** (0.8 mg), **4** (0.8 mg), and **5**/**6** (0.3 mg). Caulibugulones A−F were also recovered from an aqueous extract of *C. intermis*. A 5.01 g sample of aqueous extract was redissolved in H_2O and chromatographed over C_4 using a MeOH−H_2O step gradient. The fraction eluting with 2:1 H_2O−MeOH (144.2 mg) was partitioned between CH_2Cl_2 and H_2O. The organic layer (22.0 mg) was chromatographed on C_{18} as above to yield **1** (16.6 mg), **4** (5.3 mg), and **5**/**6** (3.4 mg), along with trace amounts of **2** and **3**. Compounds **5** and **6** were separated by C_{18} HPLC employing a linear gradient from 0 to 10% MeCN in H_2O (+0.1% TFA) over 20 min followed by isocractic elution at 10% MeCN (3 mL/min) with detection at 220 nm, yielding compounds **5** (0.8 mg, t_R = 29.1 min) and **6** (2.5 mg, t_R = 29.8 min) as their TFA salts.

Caulibugulone A (1): dark red solid; UV (MeOH) λ_{max} (log ϵ) 237 (4.24), 268 (4.10), 451 (3.61) nm; IR (film, NaCl) ν_{max} 3271, 1683, 1597, 1583, 1554, 1500, 1422, 1363, 1338, 1181, 1079, 1036, 844, 812, 756 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS (magic bullet), *m*/*z* 189.0668 [M + H]$^+$, calcd for $C_{10}H_9N_2O_2$, 189.0664 (Δ +0.4 mmu).

Caulibugulone B (2): dark red solid; UV (MeOH) λ_{max} (log ϵ) 251 (4.03), 274 (3.97), 475 (3.36) nm; IR (film, NaCl) ν_{max} 3281, 1694, 1593, 1557, 1515, 1414, 1336, 1267, 1229, 1194, 1117, 969, 882, 745 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS (magic bullet), *m*/*z* 223.0286 [M + H]$^+$, calcd for $C_{10}H_8N_2O_2Cl$, 223.0274 (Δ +1.2 mmu).

Caulibugulone C (3): dark red solid; UV (MeOH) λ_{max} (log ϵ) 254 (4.00), 272 (3.97), 474 (3.49) nm; IR (film, NaCl) ν_{max} 3276, 1689, 1591, 1552, 1513, 1412, 1314, 1262, 1192, 1137, 1036, 868, 802, 743 cm^{-1}; ^{1}H NMR and ^{13}C data, see Table 1; HRFABMS (magic bullet), *m*/*z* 266.9764 [M + H]$^+$, calcd for $C_{10}H_8N_2O_2Br$, 266.9769 (Δ −0.5 mmu).

Caulibugulone D (4): dark orange solid; UV (MeOH) λ_{max} (log ϵ) 273 (3.95), 450 (3.35) nm; IR (film, NaCl) ν_{max} 3368, 1682, 1601, 1557, 1520, 1435, 1365, 1329, 1277, 1206, 1135, 835, 798 cm^{-1}; ^{1}H NMR and ^{13}C data, see Table 1; HRFABMS (NBA), *m*/*z* 219.0774 [M + H]$^+$, calcd for $C_{11}H_{11}N_2O_3$, 219.0770 (Δ +0.4 mmu).

Caulibugulone E (5): dark orange solid (TFA salt); UV (MeOH) λ_{max} (log ϵ) 213 (3.71), 245 (3.75), 441 (3.09) nm; IR (film, NaCl) ν_{max} 3000, 1673, 1594, 1506, 1415, 1339, 1184, 1136, 835, 797 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS (NBA), *m*/*z* 188.0831 [M + H]$^+$, calcd for $C_{10}H_{10}N_3O$, 188.0824 (Δ +0.7 mmu).

Caulibugulone F (6): dark orange solid (TFA salt); UV (MeOH) λ_{max} (log ϵ) 215 (3.79), 245 (3.75), 440 (3.16) nm; IR (film, NaCl) ν_{max} 3233, 1681, 1600, 1564, 1512, 1423, 1338, 1203, 1132, 838, 800, 722 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS (NBA), *m*/*z* 232.1067 [M + H]$^+$, calcd for $C_{12}H_{14}N_3O_2$, 232.1086 (Δ −1.9 mmu).

Halogenation of 1.[36,37] Compound **1** (3.0 mg, 0.016 mmol) was dissolved in 400 μL of dioxane to which 100 μL of dioxane containing 2.85 mg (0.016 mmol) of *N*-bromosuccinimide (NBS) was added. The reaction mixture was stirred 7 h at room temperature and then dried under N_2. The resulting mixture was purified by C_{18} HPLC as described above to provide 3.0 mg of **3**. No starting material (**1**) was detected by HPLC. An analogous procedure was used to provide 1.8 mg of **2** by treatment with *N*-chlorosuccinimide (NCS) substituting methanol for dioxane.

Cytotoxicity Assay. Chromatography fractions were made up in DMSO and assayed in an in vitro cytotoxicity assay against LOX and OVCAR-3 human tumor cell lines to direct purification. Pure **1**−**6** were made up in MeOH and tested using the murine IC-2^{WT} cell line[35] to provide a relative comparison of cytotoxicity. Experimental details of the two-day antiproliferation assay employed are published elsewhere.[38]

Acknowledgment. We thank D. Newman for coordinating collections, P. L. Colin (CRRF) for sample collections, T. McCloud for sample extractions, T. Johnson, J. Wilson, and N. Oku for cytotoxicity evaluations, G. Chmurny for helpful NMR discussions, and L. Pannell for selected HRFABMS.

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NP030378L