CLAVULAZINE, A NEW MARINE PYRAZINE CONGENER FROM THE OKINAWAN SOFT CORAL *CLAVULARIA VIRIDIS*

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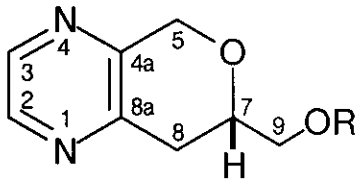
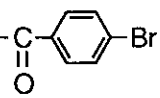
Abstract — A new marine pyrazine congener, clavulazine (**2**), was obtained from the polar portion of the MeOH extract of the Okinawan soft coral *Clavularia viridis* as its acetate (**1**). The structure of **2** was determined on the basis of spectral analysis and chemical conversion. The absolute configuration of **2** was unambigously determined by X-Ray crystallographic analysis on the *p*-bromobenzoate (**3**).

The Okinawan soft coral, *Clavularia viridis* Quoy and Gaimard (class Anthozoa, subclass Octocorallia, order Stolonifera), contains numerous structurally unique antitumor prostanoids[1] and cytotoxic steroids.[2] In recent study on *C. viridis*, new prostanoid lactones,[3] prostanoid carboxylate salts[4] and steroids[5] were isolated. To find bioactive compounds in the polar portion of the MeOH extract of *C. viridis*, a new pyrazine congener, clavulazine (**2**), was obtained. This paper describes the isolation and structure determination of **2**.

Specimens of *C. viridis* (wet wt 17.1 Kg), collected from the coral reef of Ishigaki Island (Okinawa Prefecture, Japan) in December 1995, were immersed in MeOH, the extract of which was partitioned between AcOEt and H_2O and the aqueous layer was successively partitioned with BuOH to afford AcOEt-, BuOH- and H_2O-soluble portions. A part (15.0 g) of the BuOH soluble portion (39.6 g) was chromatographed on a silanised silica gel column that had been eluted successively with H_2O, H_2O-MeOH [3:1, 1:1 and then 1:3] and MeOH to obtain 13 fractions. The 5th and 6th fractions (363 mg) were separated by reversed phase flash column and reverse phase medium pressure liquid chromatography (MPLC) to give the crude fraction of **2** (66 mg) that contained an unseparable impurity. After confirming the absence of acetyl methyl proton signals of this fraction on the basis of its ^{1}H NMR spectrum, the fraction (30 mg) was treated with acetic anhydride in pyridine at room temperature and the acetylated product thus obtained was purified by normal and reverse phase chromatography to afford compound **1** (6.4 mg). Hydrolysis of **1** with 0.5 *M* K_2CO_3 *aqueous solution in* MeOH gave the natural product (**2**),[6] whose structure was determined using its acetate (**1**).



1; R = Ac
2; R = H
3; R = 

The molecular formula, $C_{10}H_{12}N_2O_3$, of **1** was derived based on elemental analysis and HREIMS. ^1H and ^{13}C NMR data for **1** are shown in Table 1. All 10 carbons appeared in the ^{13}C NMR spectrum and DEPT indicated the presence of a methyl, three sp^3 methylenes, sp^3 methine, two sp^2 methines and three sp^2 quaternary carbons. In addition to an acetyl group [IR ν_{max} 1736, 1248 cm^{-1}, δ_H 2.14 (3H, s), δ_C 20.8 (CH_3), 170.4 (C)], the presence of 2,3-disubstituted pyrazine ring was demonstrated by UV [λ_{max} (log ε) 309 (3.01), 277 (3.86), 272 (3.87)], ^1H NMR [δ_H 8.41 (1H, br d, J = 2.5 Hz), 8.38 (1H, br d, J = 2.5 Hz)][7] and ^{13}C NMR [δ_C 142.3 (CH), 143.0 (CH), 148.9 (C), 150.3 (C)] spectra. Partial structure of -CH_2-CH(O)-CH_2-O- was indicated by ^1H-^1H COSY measurement. All correlations between ^{13}C and ^1H signals were confirmed by analysis of the ^{13}C-^1H COSY spectrum, and then ^{13}C-^1H long range correlations (two and three bonds) were found on the COLOC spectrum of **1** as shown in Figure 1. The correlations provide connectivities between quaternary aromatic carbon on the pyrazine ring at C-8a [δ_C 148.9] and aromatic proton at H-2 [δ_H 8.41 (1H, br d, J = 2.5 Hz)], C-8a and methylene protons at H-8 [δ_H 3.05 (1H, dd, J = 11.3, 17.1 Hz), 2.95 (1H, dd, J = 3.1, 17.1 Hz)], and another quaternary aromatic carbon at C-4a [δ_C 150.3] and H-3 [δ_H 8.38 (1H, br d, J = 2.5 Hz)] and H-5 [δ_H 4.96 (1H, d, J = 16.0 Hz), 4.85 (1H, dd, J = 1.3, 16.0 Hz)]. From the COLOC spectrum, it was also evident that the

Table 1 ^{13}C and ^1H NMR spectral data[a] for compound **1**

position	^{13}C [b]	^1H [c]
2	142.3 (CH)	8.41 (1H, br d, J = 2.5 Hz)
3	143.0 (CH)	8.38 (1H, br d, J = 2.5 Hz)
4a	150.3 (C)	
5	68.9 (CH_2)	4.96 (1H, d, J = 16.0 Hz) 4.85 (1H, dd, J = 1.3, 16.0 Hz)[d]
7	72.9 (CH)	4.11 (1H, dddd, J = 3.1, 3.3, 6.8, 11.3 Hz)
8	33.0 (CH_2)	3.05 (1H, dd, J = 11.3, 17.1 Hz) 2.95 (1H, dd, J = 3.1, 17.1 Hz)
8a	148.9 (C)	
9	65.9 (CH_2)	4.34 (1H, dd, J = 3.3, 12.0 Hz) 4.26 (1H, dd, J = 6.8, 12.0 Hz)
$\underline{CH_3}$CO-	20.8 (CH_3)	2.14 (3H, s)
$CH_3\underline{C}$O-	170.4 (C)	

[a] δ ppm [b] 100 MHz, $CDCl_3$ [c] 400 MHz, $CDCl_3$, [d] The signal changed to a doublet (J = 16.0 Hz) by irradiation at δ 8.38 (br d, J = 2.5 Hz, H-3) ppm.

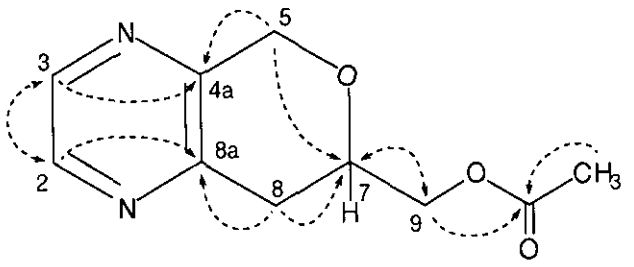


Figure 1 ^{13}C-^{1}H long range correlations observed on COLOC spectrum for **1**

sp^3 methine carbon at C-7 [δ_C 72.9] was correlated to H-5, H-8 and H-9 [δ_H 4.34 (1H, dd, J = 3.3, 12.0 Hz), 4.26 (1H, dd, J = 6.8, 12.0 Hz)] and an acetyl carbonyl carbon [δ_C 170.4] with H-9. Acetate 1 is thus shown to have a planer structure as shown in Figure 1 and thus to be that of the natural product, **2**.

The absolute stereochemistry of the chiral center at C-7 was unambiguously determined based on X-Ray crystallographic analysis of *p*-bromobenzoate (**3**). By Cu K-α radiation at room temperature, 1591 reflections were observed. The structure of **3** was found by direct methods using the SIR 92 (Giacovazzo, 1994)[8] program, which was refined by full matrix least-squares techniques to R = 0.038. The computer-generated perspective drawing in Figure 2 indicates *S* configuration at C-7. It thus follows that the structure of clavulazine (**2**) is (7*S*)-7,8-dihydro-5*H*-pyrano[3,4-*b*]pyrazin-7-ylmethanol.

The dihydropyranopyrazine ring system of **2** is quite rare in natural products and only palythazine[9] and isopalythazine[9] have been reported as related compounds. The bioactivity and biogenesis of these compounds are presently being studied.

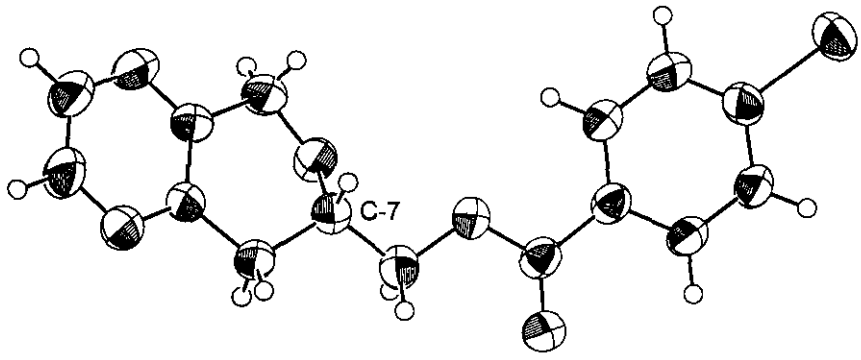


Figure 2 Computer-generated perspective drawing of **3**

EXPERIMENTAL

General Experimental Procedures. No correction was made for melting points. Melting points and optical rotation were measured using a YAZAWA BY-1 micro melting point apparatus and a JASCO DIP-370 automatic polarimeter, respectively. IR spectra were recorded with a Perkin-Elmer FT-IR 1600

spectrophotometer and UV spectra with a JASCO V-520 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM-400 spectrometer (^{1}H; 400 MHz, ^{13}C; 100 MHz) in $CDCl_3$ Two dimensional NMR spectra were obtained using a JEOL JNM-A-500 spectrometer (^{1}H; 500 MHz, ^{13}C; 125 MHz). ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, and COLOC were measured based on standard JEOL pulse sequences. Chemical shifts were given on a δ (ppm) scale with $CHCl_3$ (^{1}H; 7.26 ppm, ^{13}C; 77.0 ppm) as the internal standard (s, singlet; d, doublet; t, triplet; br, broad). MS were taken with a Micromass Auto Spec spectrometer. Column chromatography was carried out on a silica gel (Merck Silica gel 60, 70-230 mesh) and silanized silica gel (Merck Silica gel 60 silanized, 70-230 mesh) for normal and reverse phase separation, respectively. Reverse phase flash column chromatography was conducted on YMC-GEL ODS-A 120-230/70. Medium pressure liquid chromatography (MPLC) was carried out with KHLC-201-43 (Kusano) apparatus using a CIG prepack column (silica gel, CPS-HS-221-05, for the normal phase and ODS silica gel, CPO-HS-221-20, for the reverse phase). HPLC was conducted with a YMC-Pack ODS-AM column (ODS silica gel, SH-343-5AM, reverse phase).

Extraction and Isolation. The soft coral, *Clavularia viridis* Quoy and Gaimard, was collected from the coral reef of Ishigaki Island (Okinawa Prefecture, Japan) in December, 1995 at a depth of 1-2 m. A voucher specimen (No. SC-95-1) is presently on deposit at this laboratory, Tokyo University of Pharmacy and Life Science (Tokyo, Japan). Wet specimens (17.1 kg) were extracted three times with MeOH (19 L, each) at rt for 2 d. After filtration, the MeOH solution was concentrated under reduced pressure to give extract (total, 644 g) which was then partitioned between H_2O and AcOEt. The AcOEt layer was concentrated under reduced pressure to give AcOEt-soluble portion (123.6 g) and the remaining aqueous layer was extracted with BuOH. The BuOH and aqueous solutions were concentrated under reduced pressure to give BuOH- and H_2O-soluble portions (39.6 g and 379 g, respectively).
A part (15.0 g) of the BuOH soluble portion was chromatographed on a silanised silica gel column (150 g). Stepwise elution with H_2O (600 mL), H_2O-MeOH (3:1, 1:1 and 1:3, each 600 mL), and MeOH (900 mL) gave 13 fractions. The 5th and 6th fractions (363 mg) [eluted with H_2O-MeOH (1:1)] were subjected to reverse phase flash column chromatography [H_2O-MeOH (8:2 and 7:3) as eluents] and reverse phase medium pressure liquid chromatography (MPLC) [H_2O-MeCN (85:15) as the eluent] to gave a crude pyrazine congener fraction (66 mg).
To the solution of a part (30 mg) of the crude pyrazine congener fraction in pyridine (2 mL), acetic anhydride (0.5 mL) was added and the mixture allowed to stand for 4 h. The reaction mixture was then concentrated under reduced pressure. The residue was subjected to reverse and normal phase MPLC [H_2O-MeCN (7:3) and hexane-2-propanol (1:1 and 1:3) as the eluent, respectively] to gave acetylated clavulazine (**1**) (6.4 mg).

Compound (1). Colorless needles (recrystallized from hexane), mp 95.0-96.0 °C; $[\alpha]_D$ -88.6° (*c* 0.19, $CHCl_3$); EIMS (relative intensity) *m/z* 208 (M^+, 11), 149 (45), 148 (57), 107 (91), 43 (100); HREIMS *m/z* 208.0847 (M^+, $C_{10}H_{12}N_2O_3$ requires 208.0848); Anal. Calcd for $C_{10}H_{12}N_2O_3$: C, 57.69; H, 5.81; N, 13.45. Found: C, 57.59; H, 5.98; N, 13.37. UV (EtOH) λ_{max} nm (log ε) 309.0 (3.01), 277 (3.86),

272 (3.87); IR ν_{max} cm^{-1} (KBr) 1736, 1404, 1248, 1161, 1099, 1068, 1049; ^1H and ^{13}C NMR data of **1** are given in Table 1.

Conversion of 1 to 2. To a solution of **1** (4.4 mg) in MeOH (0.2 mL), 0.5 M K_2CO_3 aqueous solution (0.07 mL) was added, followed by standing for 2 h. To the reaction mixture, saturated NH_4Cl solution (0.2 mL) was added and the mixture was concentrated under reduced pressure. The residue was dissolved in H_2O and the solution was passed through a silanised silica gel (5 g) short column. After elution with H_2O to wash out inorganic salts, elution with MeOH gave a crude product. Reverse phase MPLC [H_2O-MeOH (3:1) as the eluent] purification of the crude product afforded clavulazine (**2**) (2.5 mg, 79 %).

Clavulazine (2). Colorless needles (recrystallized from hexane-AcOEt), mp 101.0-102.0 °C; $[\alpha]_D$ -99.4° (*c* 0.17, $CHCl_3$); EIMS (relative intensity) *m/z* 166 (M^+, 37), 148 (M^+-H_2O, 17), 135 (87), 107(100); HREIMS *m/z* 166.0746 (M^+, $C_8H_{10}N_2O_3$ requires 166.0742); UV (EtOH) λ_{max} nm (log ε) 310 (3.06), 277 (3.89), 272 (3.89); IR ν_{max} cm^{-1} (KBr) 3301, 1400, 1160, 1104, 1062, 1039; ^1H NMR ($CDCl_3$, 400 MHz) δ ppm 8.41 (1H, br d, *J* = 2.4 Hz, H-2), 8.37 (1H, br d, *J* = 2.4 Hz, H-3), 4.97 (1H, d, *J* = 16.2 Hz, H-5), 4.85 (1H, dd, *J* = 1.1, 16.2 Hz, H-5), 3.99 (1H, tdd, *J* = 3.1, 6.8, 11.3 Hz, H-7), 3.87 (1H, ddd, *J* = 3.1, 7.5, 11.8 Hz, changed to dd, *J* = 3.1, 11.8 Hz by addition of D_2O, H-9), 3.75 (1H, ddd, *J* = 5.0, 6.8, 11.8 Hz, changed to dd, *J* = 6.8, 11.8 Hz by addition of D_2O, H-9), 3.06 (1H, dd, *J* = 11.3, 17.1 Hz, H-8), 2.88 (1H, dd, *J* = 3.1, 17.1 Hz, H-8), 2.17 (1H, dd, *J* = 5.0, 7.5 Hz, disappeared by addition of D_2O, HO-); ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 150.5(C, C-4a), 149.5 (C, C-8a), 143.0 (CH, C-3), 142.2 (CH, C-2), 75.5 (CH, C-7), 68.9 (CH_2, C-5), 65.2 (CH_2, C-9), 32.6(CH_2, C-8).

Conversion of 2 to 3. To a solution of **2** (3.1 mg) in pyridine (0.2 mL), *p*-bromobenzoyl chloride (20 mg) was added and the mixture stood for 4 h. To the reaction mixture, toluene (1 mL) was added and the solvent was evaporated under reduced pressure. The residue was dissolved in AcOEt (1 mL) and the solution was passed through a normal phase silica gel (5 g) short column [hexane-AcOEt (1:1) as the eluent] to give a crude product. The product was purified by normal phase MPLC [hexane-AcOEt (1:1) as the eluent] to give **3** (5.2 mg, 87 %).

Compound (3). Colorless needles (recrystallized from hexane-AcOEt), mp 106.5-107.0 °C; $[\alpha]_D$ -58.2° (*c* 0.16, $CHCl_3$), EIMS (relative intensity) *m/z* 350 (M^+, 5), 348 (M^+, 5), 185 (25), 183 (25), 148 (100), 107 (87); HREIMS *m/z* 348.0110 (M^+, $C_{15}H_{13}N_2O_3Br$ requires 348.0110); UV (EtOH) λ_{max} nm (log ε) 311 (3.01), 277 (shoulder, 3.92), 271 (3.95), 246 (4.26); IR ν_{max} cm^{-1} (KBr) 1719, 1592, 1404, 1289, 1101, 1072; ^1H NMR ($CDCl_3$, 400 MHz) δ ppm 8.43 (1H, br d, *J* = 2.3 Hz), 8.39 (1H, br d, *J* = 2.3 Hz), 7.94 (2H, d, *J* = 8.5 Hz), 7.60 (2H, d, *J* = 8.5 Hz), 4.99 (1H, d, *J* = 16.3 Hz), 4.87 (1H, dd, *J* = 0.8, 16.3 Hz), 4.57 (1H, dd, *J* = 4.0, 11.8 Hz), 4.52 (1H, dd, *J* = 6.0, 11.8 Hz), 4.25 (1H, dddd, *J* = 3.6, 4.0, 6.0, 10.8 Hz), 3.14 (1H, dd, *J* = 10.8, 17.1 Hz), 3.04 (1H, dd, *J* = 3.6, 17.1 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ ppm 165.67 (C), 150.40 (C), 148.93 (C), 143.03 (CH), 142.37

(CH), 131.80 (CH × 2), 131.28 (CH × 2), 128.63 (C), 128.42 (C), 72.94 (CH), 68.90 (CH_2), 66.50 (CH_2), 33.21 (CH_2).

X-Ray crystallographic analysis of 3. A single crystal of **3** was obtained by recrystallization from hexane-AcOEt. **3**: orthorhombic, $P2_12_12_1$, a = 14.806 (4) Å, b = 23.944 (7) Å, c = 4.132 (2) Å, V = 1464.8 (8) $Å^3$, Z = 4, D_x = 1.583 gcm^{-3}, crystal size 0.5 × 1.0 × 1.0 mm^3. Diffraction data were obtained using a Mac Sciences MXC18 diffractometer at room temperature with Cu K-α (λ = 1.54178 Å) radiation. Total reflections measured was 1591. The structure of **3** was determined by direct methods using SIR 92 (Giacovazzo, 1994)[8] program with refinement by full matrix least-squares techniques to R = 0.038.

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