

Note

Secondary metabolites from the Gorgonian *Echinomuraceae splendens* (Thomson & Simson)

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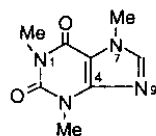
Two xanthine derivatives, caffeine (1, 3, 7-trimethylxanthine) **1** and its 2-*O*-methyl analog (Δ^1 , O^2 -methyl theobromine) **2**, *N*-methyl-pyrazole-5-carboxylic acid **3** and a tetrahydroxy sterol, 1 β , 3 β , 5 α , 6 β -tetrahydroxycholestane **4** have been isolated from the methanol extract of the gorgonian *Echinomuraceae splendens* (Thomson & Simson). These compounds have been purified on silica gel and Sephadex LH-20 columns and their structures elucidated by spectroscopic studies. This is the first report of the occurrence of compounds **2** and **3** in nature. Caffeine exhibits mild antifouling activity against marine fouling organisms while its *O*-methyl analog **2** is inactive.

Continuing our search for promising bioactive molecules from marine organisms, we examined the gorgonian *Echinomuraceae splendens* collected from Karwar region, India. The crude MeOH extract of this animal had mild antifouling properties.

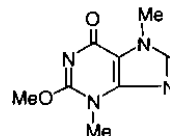
Results and Discussion

The gorgonian coral *E. splendens* was collected by SCUBA diving from Karwar region (-10 M). They were immediately preserved in MeOH and transported to the laboratory for further processing. After two weeks the solvent was drained off and concentrated *in vacuo* to yield the crude extract (10 g). The above extract was suspended in aq. MeOH and successively extracted with hexane (2 g) and chloroform (2 g). Bioassay studies indicated the chloroform fraction to be active against antifouling diatoms. This was purified over silica gel and sephadex LH 20 columns to yield compounds **1-4** in pure form.

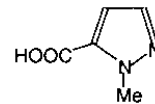
The compound **1** (50 mg) had absorptions at 1701



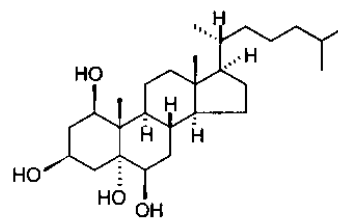
1



2



3



4

and 1659 cm^{-1} in the IR and 272 nm in UV-Vis spectra, indicating the presence of carbonyl group and multiple unsaturations. EIMS revealed its molecular weight to be 194. The ^1H NMR spectrum indicated the presence of three *N*-methyls and one vinyl proton singlet while its ^{13}C NMR and DEPT spectra revealed the presence of 8 carbon signals between 155.3 and 27.8 ppm. These data were in agreement with those of caffeine, as confirmed by comparison with spectral data of authentic sample.

Compound **2** (45 mg) had strong absorption at 1650 and 1550 cm^{-1} in the IR and at 265 nm in the UV-Vis absorption spectra. Its ^1H NMR spectrum had signals at δ 7.46 (1H, s), 3.96(3H, s), 3.93(3H, s) & 3.51(3H, s) while its ^{13}C NMR and DEPT spectra indicated presence of 8 signals: δ 162.3(s), 154.9(s), 148.8(s), 141.0(d), 112.8(s), 56.3(q), 33.5(q) & 30.2(q). FABMS revealed its molecular weight to be 194, the same as that of caffeine **1**.

The above results suggested that compound **2** is an isomer of caffeine **1**. The presence of vinylic proton signals in these two molecules at the same place, *viz.*, δ 7.46 suggested that the rings containing these protons have the same chemical environment. The major difference in the structures of these two molecules were that caffeine **1** has three *N*-methyls while compound **2** has two *N*-methyls and one *O*-methyl group. Comparison of the proton and carbon chemical shifts of the methyl groups also revealed that the *N*-1 methyl signal of caffeine **1** (δ 3.24 & 27.8) has been replaced by an OMe group in the latter. This OMe signal now appears at δ 3.96 and

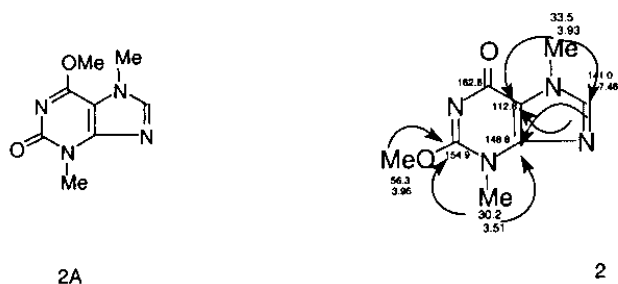


Figure 1

56.3 in ^1H and ^{13}C NMR spectra respectively. Two possible structures, consistent with these results are shown in **Figure 1**. The UV absorption of this compound does not show the extended conjugation predicted by **2a**, thereby establishing the correct structure as **2**. This is also confirmed by HMBC studies (**Figure 1**).

Compound **3** (20 mg) had absorption at 3500-3100, 1710, 1650, 1598 and 1508 cm^{-1} in the IR and 259 nm in UV spectra, indicating the presence of carboxylic acid and aromaticity. Its molecular weight was determined to be 126 by FABMS, suggesting an even number of nitrogen or none. The ^1H NMR spectrum was quite simple, with two doublets as part of an AB quartet at δ 7.2 and 5.7 (1H each, $J=7.58$ Hz) and an *N*-methyl signal at δ 3.30. The ^{13}C NMR and DEPT spectra revealed the presence of five carbons: δ 164.4(s), 152.2(s), 139.1(d), 100.6(d) and 26.7(q). Comparison of the above values with those of pyrazole and imidazole derivatives indicated the molecule to be *N*-methyl-pyrazole-5-carboxylic acid¹. The proximity of the carboxylic acid group to the *N*-methyl moiety was evident from the upfield shift of methyl signal (δ 3.30 and 26.7) as compared to those in *N*-methyl-pyrazole (δ 3.80² and 38.1³).

The structure was further confirmed by comparison of the ^{13}C NMR spectral data of compound **3** with that of *N,N*-dimethyl derivative of pyrazole-3(5)-carboxylic acid prepared by treating a suspension of pyrazole-3(5)-carboxylic acid in chloroform with an ethereal solution of diazomethane. The ^1H NMR spectrum of the reaction mixture indicated >90% *N,N*-dimethylation as compared to *N,O*-dimethylation. The ^{13}C NMR spectrum of the same had 4 distinct *N*-Me signals at δ 35.9, 35.6, 26.8 and 26.6. The former two are from the *N,N*-dimethyl pyrazole-3-carboxylate zwitterion while the latter two must be from the corresponding pyrazole-5-carboxylate derivative. In the latter isomer, the methyl groups are vicinal to the carboxyl group.

Compound **4** (10 mg) had absorption peaks at 3450, 2980, 2725, 1466 and 1380 cm^{-1} in the IR

spectrum and only end absorption in UV, indicative of terpenoid / sterol structure. The molecular ion was not clear in FABMS with highest ion observed at m/z 419 $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$, followed by fragment ions at m/z 401, 383, and 365, indicating successive losses of elements of water. The ^1H NMR spectrum had three proton signals in the region 3-4 ppm, while the ^{13}C NMR and DEPT spectra revealed the presence of four oxygenated carbons: δ 76.5(s), 76.0(d), 73.1(d) and 64.6(d). These results indicated the molecule to be a tetrahydroxy cholestane. Among these, the carbon signals at δ 76.5, 76.0 and 64.6 could be easily assigned to 3β , 5α , 6β -trihydroxycholestane system^{4,5}. The fourth hydroxyl group must be at C-1 considering the large downfield shift of 19-H in pyridine- d_5 to δ 1.90 as compared to δ 1.02 in CDCl_3 -DMSO(d_6). The large coupling of protons at δ 3.96 (3-H) and 3.89 (1-H, 11.4 Hz) indicated them to be diaxial, leaving the hydroxyl groups to have β stereochemistry (diequatorial). Similarly, the smaller coupling of 6-H indicated this proton to be equatorial and the hydroxyl group to be axial (β). From the above results, the structure of this compound was finalized as 1β , 3β , 5α , 6β -tetrahydroxycholestane **4**. This was further confirmed by comparing the NMR spectral data with those of 1β , 3β , 5α , 6β -tetrahydroxycholestane derivatives^{6,7}. The ^{13}C NMR chemical shifts of this molecule in both pyridine- d_5 and CDCl_3 +DMSO- d_6 are reported here for the first time.

Caffeine **1**, a known terrestrial plant product is not common in marine organisms. The only other report of its occurrence in a marine organism is from another gorgonian *Echinogorgia pseudossapo*⁸. Compounds **2** and **3** are reported for the first time from a natural source. This is also the first report of occurrence of compound **4** from a gorgonian. Earlier it has been reported from a soft coral *Sarcophyton glaucum* as a mixture of both C_{27} and C_{28} sterols⁷. Among these, caffeine exhibited mild activity against marine fouling organisms while, its isomer **2** was devoid of this property. The details of these studies would be published elsewhere.

Experimental Section

General

IR spectra were recorded on Perkin Elmer FTIR Model 1640, UV spectra on a Hewlett Packard 8450 A UV-Vis spectrophotometer, ^1H and ^{13}C NMR spectra on Varian XL-400 (400 MHz) and XL-300 (300 MHz) spectrometers (chemical shifts in δ , ppm)

using TMS as an internal standard and FABMS and EIMS on a VG-70-SE instrument with glycerol and thioglycerol used as matrix for the former.

Animal material

The gorgonian coral was collected from Karwar (Karnataka), in west coast of India, during spring 1992 by SCUBA diving (-10M). The samples were immediately soaked in MeOH and transported to the laboratory for further processing. The animal was identified to be *Echinomuraceae splendens* (Thomson & Simpson) and a voucher specimen is deposited at the Biology Museum, National Institute of Oceanography, Dona Paula, Goa.

Extraction and isolation of compounds

The animals (2 kg) were collected from Karwar by SCUBA diving, preserved in MeOH and transported to the lab. After two weeks, the solution was decanted, filtered and concentrated *in vacuo* to a semi-liquid form. This was resuspended in 90% aq. MeOH and sequentially extracted with hexane (2 g) and CHCl₃ (2 g). The hexane fraction consisted of a mixture of fatty acids and several monohydroxy sterols and was not processed further. The CHCl₃ fraction upon chromatography over silica gel (CHCl₃-MeOH gradient) and Sephadex LH-20 (CHCl₃-MeOH, 1:1) yielded compounds **1-4**.

Caffeine 1: IR(KBr): 3112, 2954, 1701, 1659, 1548, 1484, 1457, 1402, 1359, 1285, 1238, 1189, 1026, 974, 861, 745 and 610 cm⁻¹; UV-Vis (MeOH): 272 (16 600) nm; ¹H NMR (CDCl₃): δ 7.46 (1H, s, 6-H), 3.86(3H, s, N₇-Me), 3.42(3H, s, N₃-Me), and 3.24(3H, s, N₁-Me); ¹³C NMR (CDCl₃ + DMSO-*d*₆): 155.3(s), 151.7(s), 148.5(s), 141.6(d), 107.5(s), 33.4(q), 29.6(q), and 27.8(q); EIMS *m/z*(%): 195([M+H]⁺, 100), 194(18.6), 181(9), 171(7.3), 141(9.8), 138(5.7).

Δ¹, O²-Methyl theobromine (2-O-Methyl analog of caffeine) 2: IR(KBr): 1650, 1550, 1490, 1460, 1410, 1370, 1230, 1200, 1170, 1120, 1050, 1010, 890, 780, 750, 740, and 620 cm⁻¹; UV-Vis (MeOH): 265 (8555) nm; ¹H NMR (CDCl₃): δ 7.46(1H, s, 6-H), 3.96 (3H, s, O-Me), 3.93 (3H, s, N₇-Me), 3.51(3H, s, N₃-Me); ¹³C NMR (CDCl₃ + DMSO-*d*₆): 162.3(s, C-6), 154.9(s, C-2), 148.8(s, C-4), 141(d, C-8), 112.8(s, C-5), 56.3(q, OMe), 33.5(q, N₇-Me), 30.2(q, N₃-Me); FABMS *m/z*: 411 [2M+Na]⁺, 389[2M+H]⁺, 217[M+Na]⁺, 195 [2M+H]⁺; EIMS *m/z*(%): 194(M⁺, 100), 193(74), 180(18.4), 179(16.2), 165(8.1), 164(5.7), 137 (13.9), 136(30.6) 109(64.5) and 82(43.5).

N-Methyl-pyrazole-3-carboxylic acid 3: IR(KBr): 3500-3100, 1710, 1650, 1593, 1508, 1380, 1240, 1200 and 1070 cm⁻¹; UV-Vis (MeOH): 259(6490); ¹H NMR(CDCl₃+DMSO-*d*₆): δ 7.2(1H, d, *J*=7.6Hz), 5.7(1H, d, *J*=7.6Hz), 3.30 (3H, s, *N*-Me); ¹³C NMR (CDCl₃ + DMSO-*d*₆): 164.4(s), 152.2(s), 139.1(d), 100.6(d) and 26.7 (q, *N*-Me); FABMS *m/z*: 253[2M+H]⁺ 127[M+H]⁺.

1β, 3β, 5α, 6β-Tetrahydroxycholestane 4: [α]_D²⁰=-14.9(1.28 mg/mL, MeOH); IR (KBr):3450. 2980, 2725, 1470, 1380, 1220, 1050, 1013 and 965 cm⁻¹; ¹H NMR (CDCl₃): δ 3.96 (1H, pentet, 3α-H), 3.89 (1H, dd, *J*=11.4, 4.2 Hz, 1α-H), 3.38(1H, brs, 6α-H), 1.07(3H, s, 19-H), 0.85(3H, d, *J*=5.8Hz), 0.82(6H, d, *J*=6.5Hz), 0.62(3H, s, 18-H); ¹H NMR (Py-*d*₅): δ 4.96(1H, pentet), 4.91 (1H, dd, *J*=11.4 and 4.2 Hz, 1α-H), 4.2(1H, brs, 6α-H), 1.9 (3H, s, 19-H), 0.95(3H, d, *J*=5.8Hz, 21-H), 0.88(6H, d, *J*=6.5 Hz, 26 and 27-H), 0.80 (3H, s, 18-H); ¹³C NMR (CDCl₃+CD₃OD)*: 73.2(73.8, d, C-1), 40.0(44.1, t, C-2), 64.6(65.4, d, C-3), 41.1(43.2, t, C-4), 76.5(77.1, s, C-5), 76.0(78.0, d, C-6), 34.1(35.7, t, C-7), 30.6(31.9, d, C-8), 46.0(47.2, d, C-9), 43.6(44.9, s, C-10), 24.4(25.0, t, C-11), 40.1(41.4, t, C-12), 40.3(41.4, t, C-13), 55.9(56.9, d, C-14), 23.9(24.9, t, C-15), 27.9(28.5, t, C-16), 56.4(56.9, d, C-17), 11.9(12.5, q, C-18), 9.1(10.7, q, C-19), 35.7(36.2, d, C-20), 18.5(18.9, q, C-21), 36.1(36.5, t, C-22), 23.8(24.2, t, C-23), 39.4(39.7, t, C-24), 27.9(28.2, d, C-25), 22.7(22.9, q, C-26) and 22.4(22.7, q, C-27); FABMS *m/z*: 419 [M-H₂O+H]⁺, 401, 383 and 365.

* The values obtained using pyridine-*d*₅ as solvent are given in parentheses

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