Chemical Examination of the Sponge *Phycopsis* sp.

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Two new compounds 7α,8α-epoxy theonellin isothiocyanate (1) and 5α,8α-epidioxyergosta-6*Z*,22*Z*,25-trien-3β-ol (2) along with two known compounds, theonellin isothiocyanate (3) and theonellin formamide (4) have been isolated from the sponge *Phycopsis* sp. Compound 2 showed cytotoxic activity against HL-60 and U937 human cancer cell lines with IC_{50} values of 5.96±0.02 and 31.72±0.55 μg/ml, respectively.

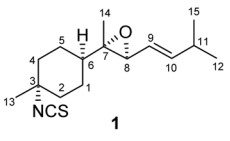
Key words 7α,8α-epoxy theonellin isothiocyanate; cytotoxic activity; *Phycopsis* sp.; 5α,8α-epidioxyergosta-6*Z*,22*Z*,25-trien-3β-ol

Several sesquiterpenoids, diterpenoids and steroids having rare functional groups like isocyano, isothiocyanate, formamides and endoperoxides have been isolated from sponges.[1—7] Interestingly, many of these nitrogenous functional group containing terpenes and 5α,8α-epidioxy sterols have been found to possess different biological activities.[8—11] Previously, phenolic derivatives[12] and 4-thiocyanatoneopupukeanane[13] were reported from the sponge species of *Phycopsis*.

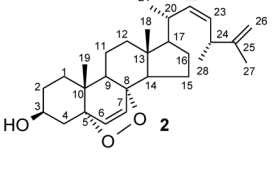
As a part of our ongoing research on the biologically active substances from marine organisms,[14] we have investigated the sponge *Phycopsis* sp. collected from Mandapam coast, India. Herein we report the isolation, and structure determination of the two new compounds; i) bisabolene type sesquiterpenoid, 7α,8α-epoxy theonellin isothiocyanate (**1**) and ii) 5α,8α-epidioxyergosta-6*Z*,22*Z*,25-trien-3β-ol (**2**), together with two known related sesquiterpenes, theonellin isothiocyanate (**3**)[15] and theonellin formamide (**4**)[15] from the sponge *Phycopsis* sp. Effect of **2** on cell proliferation of HL-60 (myeloid leukemia) and U937 (leukemic monocyte lymphoma) was investigated.

Results and Discussion

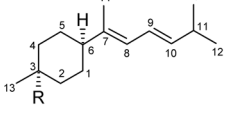
Freshly collected sponge specimens were placed in methanol at the site of collection until workup. The sponge specimens reextracted with dichloromethane : methanol (1 : 1, 3×1.5 l). The combined extract was concentrated under reduced pressure to a predominantly aqueous extract, which was extracted into ethyl acetate. The crude ethyl acetate extract was subjected to Sephadex LH-20 gel filtration chromatography followed by silica gel column chromatography to afford four pure compounds **1** to **4**.



1



2



3 R = NCS
4 R = NHCHO

Compounds **3** and **4** were readily identified as theonellin isothiocyanate (**3**)[15] and theonellin formamide (**4**),[15] respectively, by comparison of their spectral data with those reported in the literature and this is first report of isolation from the sponge *Phycopsis* sp.

Compound **1** obtained as colorless oil, $[\alpha]_D^{25}$ −36° (c=0.25, $CHCl_3$), *Rf*: 0.90 (hexane). Its electron ionization (EI) mass spectrum showed molecular ion peak at *m*/*z* 279 $[M]^+$ and the HR-EI-MS indicated molecular ion peak at *m*/*z* 279.1592 which corresponded to the molecular formula $C_{16}H_{25}ONS$, which required five degrees of unsaturation. The IR spectrum of **1** showed strong absorption at 2089 cm^{-1} indicated the presence of isothiocyanate functionality.[15] The ^{1}H-NMR spectrum of **1** (Table 1) showed signals for a 1,2 disubstituted double bond protons at δ 5.84 (1H, dd, J=14.4, 6.2 Hz) and 5.26 (1H, dd, J=14.4, 6.2 Hz), two quaternary methyls at δ 1.42 (3H, s) and 1.22 (3H, s), and an isopropyl at δ 1.00 (6H, d, J=6.8 Hz). Further, the ^{1}H-NMR spectrum of **1** displayed a signal at δ 3.16 (1H, d, J=6.2 Hz) attributable to an epoxy group. The ^{13}C-NMR spectrum of **1** (Table 1) showed signals for sixteen carbons, which include two double bond carbons at δ 144.67 and 121.69, two oxygenated carbons at δ 64.25 and 62.67, and a carbon at δ 139.4 due to isothiocyanate functional group.[15] The foregoing spectral data accounted for four degrees of unsaturation assuming the structure of the compound belongs to bisabolene type sesquiterpene, the fifth degree of unsaturation was assigned for epoxy group due to the signals at δ 64.25 and 62.67 in ^{13}C-NMR. The ^{1}H–^{1}H correlated spectroscopy (COSY) spectrum of compound **1** showed a linear correlations among the protons at C-8 through C-11; for example the signal at δ 3.16 (1H, d, J=6.2 Hz, H-8) showed correlation with a signal at δ 5.84 (1H, dd, J=14.4, 6.2 Hz, H-9) which in turn showed correlation with δ 5.26 (1H, dd, J=14.4, 6.2 Hz, H-10) and latter signal showed correlation with a methine proton at δ 2.30 (1H, m, H-11). It is evident from the literature survey and foregoing spectral data the structure of compound **1** was established as a new compound, 7,8-epoxy theonellin isothiocyanate, and further confirmed by ^{13}C-NMR and distortion-

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Compound **1**[a)]

Position[b)]	^{1}H	^{13}C
1	—	23.7 (CH_2)
2	—	38.4 (CH_2)
3	—	60.6 (C)
4	—	38.3 (CH_2)
5	—	24.4 (CH_2)
6	—	44.3 (CH)
7	—	64.25 (C)
8	3.16 (1H, d, J=6.2)	62.67 (CH)
9	5.84 (1H, dd, J=14.4, 6.2)	121.69 (CH)
10	5.26 (1H, dd, J=14.4, 6.2)	144.67 (CH)
11	2.30 (1H, m)	31.1 (CH)
12	1.00 (3H, d, J=6.8)	22.0 (CH_3)
13	1.42 (3H, br s)	24.5 (CH_3)
14	1.22 (3H, s)	14.2 (CH_3)
15	1.00 (3H, d, J=6.8)	22.0 (CH_3)
NCS	—	139.4

a) Measured in $CDCl_3$, ^{13}C; 75 MHz, ^{1}H; 300 MHz, δ ppm, J in Hz. *b*) Assignments were made based on previous literature.[15)]

less enhancement by polarization transfer (DEPT) spectral data. The relative stereochemistry of the epoxy group was determined by molecular model calculations using PCM6 package. From molecular mechanics calculations, the coupling constants between 8α-H and Δ^9-H and 8β-H and Δ^9-H were calculated as 3.24 Hz and 6.98 Hz, respectively, from energy-minimized molecular structures. The experimentally observed coupling constant (6.2 Hz) is close to the calculated value, between 8β-H and Δ^9-H. Hence, the structure of the compound was determined as 7α,8α-epoxy theonellin isothiocyanate (**1**).

Compound **2** was obtained as colorless crystalline solid, mp 114—116 °C, $[\alpha]_D^{25}$ +19.0° (c=0.25, $CHCl_3$), *Rf*: 0.50 (hexane : ethyl acetate, 3 : 2). Its ESI mass spectrum showed molecular ion peak at *m/z* 449 $[M+Na]^+$ and the HR-EI-MS indicated molecular ion peak at *m/z* 449.3011 which corresponded to the molecular formula $C_{28}H_{42}O_3Na$, which required eight degrees of unsaturation. The IR spectrum showed strong absorption (IR ν_{max}) at 3440 cm^{-1} indicated the presence of hydroxyl group. The ^{1}H-NMR spectrum (Table 2) of **2** contained signals for two 1,2 disubstituted olefinic protons at δ 6.50 (1H, d, J=8.8 Hz), 6.24 (1H, d, J=8.8 Hz), 5.26 (1H, dd, J=5.8, 6.2 Hz) and 5.23 (1H, dd, J=5.8, 6.2 Hz), and methylene protons at δ 4.70 (2H, br s). Further, the ^{1}H-NMR spectrum of **2** showed signals for a methine proton at δ 3.94 (1H, m), five methyl groups at δ 1.67 (3H, s), 1.08 (3H, d, J=7.3 Hz), 1.00 (3H, d, J=7.3 Hz), 0.88 (3H, s) and 0.82 (3H, s). The following spectral data is of typical sterols and the signals at δ 3.94 (1H, m), δ 6.50 (1H, d, J=8.8 Hz) and 6.24 (1H, d, J=8.8 Hz) indicated the compound **2** belongs to 3β hydroxy 5α,8α, epidioxy Δ^6 steroids.[16)] The ^{13}C-NMR spectrum of **2** (Table 2) showed signals for twenty eight carbons, which include six double bond carbons at δ 149.6, 135.41, 135.28, 131.85, 130.64 and 108.82, three oxygen attached carbons at δ 82.14, 79.37 and 66.36, and five methyls at δ 20.58 (2C), 18.78, 18.12 and 12.81. From the foregoing spectral data and a literature survey revealed that the ^{1}H- and ^{13}C-NMR spectra of **2** are identical with that of 5α,8α-epidioxyergosta-6*Z*,22*E*,25-trien-3β-ol, isolated from the sponge *Axinyssa* sp.,[17)] except with a substantial difference in the coupling constants $^{H}J_{22}$ of the double bond at Δ^{22}. The reported coupling constants for Δ^{22} were δ 5.26 (1H, dd, J=6.3, 15.2 Hz) and 5.23 (1H, dd, J=7.2, 15.2 Hz) due to *E*-configuration of the double bond. However, in the ^{1}H-NMR spectrum of **2**, the coupling constants for Δ^{22} were observed at δ 5.26 (1H, dd, J=5.8, 6.3 Hz) and 5.23 (1H, dd, J=5.8, 6.2 Hz), which indicated the Δ^{22} double bond stereochemistry is in *Z*-configuration. From the foregoing spectral data and a literature survey revealed that the structure of compound **2** was determined as new compound, 5α,8α-epidioxyergosta-6*Z*,22*Z*,25-trien-3β-ol.

Table 2. ^{1}H- and ^{13}C- NMR Spectral Data of Compound **2**[a)]

Position[b)]	^{1}H	^{13}C
1	—	34.6 (CH_2)
2	—	30.6 (CH_2)
3	3.94 (1H, m)	66.36 (CH)
4	—	36.8 (CH_2)
5	—	82.14 (C)
6	6.24 (1H, d, J=8.8)	135.28 (CH)
7	6.50 (1H, d, J=8.8)	130.64 (CH)
8	—	79.37 (C)
9	—	51 (CH)
10	—	36.8 (C)
11	—	20.5 (CH_2)
12	—	39.3 (CH_2)
13	—	43.5 (C)
14	—	51.6 (CH)
15	—	23.3 (CH_2)
16	—	28.5 (CH_2)
17	—	56.1 (CH)
18	0.82 (3H, s)	12.81 (CH_3)
19	0.88 (3H, s)	18.12 (CH_3)
20	—	39.5 (CH)
21	1.00 (3H, d, J=7.3)	20.58 (CH_3)
22	5.23 (1H, dd, J=5.8, 6.2)	135.41 (CH)
23	5.26 (1H, dd, J=5.8, 6.2)	131.85 (CH)
24	2.71 (1H, m)	44.5 (CH)
25	—	149.6 (C)
26	4.70 (2H, br s)	108.82 (CH_2)
27	1.67 (3H, s)	20.58 (CH_3)
28	1.08 (1H, d, J=7.3)	18.78 (CH_3)

a) Measured in $CDCl_3$, ^{13}C; 75 MHz, ^{1}H; 300 MHz, δ ppm, J in Hz. *b*) Assignments were made based on previous literature.[17)]

Table 3. Cytotoxic Activity of 5α,8α-Epidioxyergosta-6*Z*,22*Z*,25-trien-3β-ol (**2**)

Compound	Cytotoxic activity[a)]	
	HL-60 IC_{50}±S.E. (μg/ml)[b)]	U937 IC_{50}±S.E. (μg/ml)[b)]
2	5.96±0.02	31.72±0.55
Etoposide	1.08±0.12	10.56±0.7

a) Cytotoxic activity: Exponentially growing cells were treated with different concentrations of compounds for 48 h and cell growth inhibition was analyzed through MTT assay. *b*) IC_{50} is defined as the concentration, which results in a 50% decrease in cell number as compared with that of the control cultures in the absence of an inhibitor. The values represent the mean±S.E. of three individual observations.

Cytotoxicity The cytotoxic activity of compound **2** was tested against two human cancer cell lines HL-60 and U937 by measuring the number of live cells after 48 h of treatment (MTT assay).[18)] It exhibited potent antiproliferative activity and inhibited cell growth on HL-60 and U937 cells, with cytotoxic potency (IC_{50} values) of 5.96 and 31.72 μg/ml, re-

spectively. As such based on the IC_{50} values, compound **2** showed more toxic effect on HL-60 and was found to be 5.32 fold greater potency than U937. However, the respective activities were comparatively 5.5 to 3.0 fold less than the standard drug, etoposide (Table 3).

Experimental

Melting points were obtained on a Mel-Temp apparatus and are uncorrected and IR spectra were recorded on Shimadzu-240 and Perkin-Elmer 240-C instruments, respectively. The ^{1}H- and ^{13}C-NMR was recorded on 300 MHz (Avance), 200 MHz (Gemini) instruments using TMS as internal standard. Chemical shifts are reported in δ (ppm) and coupling constants (J) are expressed in hertz. The MS were recorded on a VG Auto Spec-M instrument.

Collection The sponge *Phycopsis* sp. (IIC-611) was collected from the Mandapam coast in the Gulf of Mannar, Tamilnadu, India, during November 2003 by skin diving at a depth of 20 feet.

Identification The sponge was identified by Dr. P. A. Thomas, Emeritus Scientist, Central Marine Fisheries Research Institute, Vizhinjam, Thiruvnanthapuram, India. The voucher specimen is on deposit at the National Institute of Oceanography, Goa, India with the registration IIC-611.

Extraction and Isolation The freshly collected sponge material was cut into pieces and soaked in methanol at the site of collection until workup. The initial aqueous methanol extract was removed from the sponge and then the sponge material was reextracted with dichloromethane : methanol (1 : 1, 3×1.5 l) by percolation at room temperature. The combined extract including methanol extract was concentrated under reduced pressure to obtain predominantly an aqueous suspension and was extracted into ethyl acetate. The concentrated crude ethyl acetate extract (25 g) was subjected to gel filtration chromatography on Sephadex LH-20 (1 : 1 dichloromethane : methanol) (120 cm×4 cm) followed by silica gel (100—200 mesh) column (50 cm×2.5 cm) chromatography eluting with hexane through hexane–ethyl acetate mixtures to ethyl acetate as eluent by increasing polarities afforded 7α,8α-epoxy theonellin isothiocyanate (**1**, 20 mg), 5α,8α-epidioxyergosta-6*Z*,22*Z*,25-trien-3β-ol (**2**, 74 mg), theonellin isothiocyanate (**3**, 64 mg), theonellin formamide (**4**, 95 mg).

7α,8α-Epoxy Theonellin Isothiocyanate [(2*S*,3*S*)-2-((1*R*,4*S*)-4-Isothiocyanato-4-methylcyclohexyl)-2-methyl-3-((*E*)-3-methylbut-1-enyl)oxirane, **1**]: Colorless oil, $[\alpha]_D^{25}$ −36° (c=0.25, $CHCl_3$), *Rf*: 0.90 (hexane), UV (MeOH): 238 nm (ε 20200), IR (KBr): 2938, 2869, 2089, 1709 and 1132 cm^{-1}, For ^{1}H- and ^{13}C-NMR see Table 1, EI-MS *m*/*z*: 279 $[M]^+$ HR-EI-MS *m*/*z*: 279.1592 (Calcd for $C_{16}H_{25}ONS$, 279.1651).

5α,8α-Epidioxyergosta-6*Z*,22*Z*,25-trien-3β-ol (**2**): Colorless crystalline solid, mp 114—116 °C, $[\alpha]_D^{25}$ +19° (c=0.25, $CHCl_3$), *Rf*: 0.50 (hexane : ethyl acetate, 3 : 2), IR (KBr): 3432, 2949, 2863, 1640, 1449, 1373, 1040, 927 and 884 cm^{-1}, For ^{1}H- and ^{13}C-NMR, see Table 2, EI-MS *m*/*z*: 449 $[M+Na]^+$ HR-EI-MS *m*/*z*: 449.3011 $[M+Na]^+$ (Calcd for $C_{28}H_{42}O_3Na$, 449.3026).

Theonellin Isothiocyanate [(1*R*,4*R*)-1-Isothiocyanato-1-methyl-4-((2*E*,4*E*)-6-methylhepta-2,4-dien-2-yl)cyclohexane, **3**]: Colorless oil, $[\alpha]_D^{25}$ +21° (c=0.25, $CHCl_3$), *Rf*: 0.95 (hexane), UV (MeOH): 238 nm (ε 30200), IR (KBr): 2938, 2100 cm^{-1}, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.21 (1H, ddq, J=15.4, 10.7, 1.5 Hz), 5.81 (1H, d, J=10.7 Hz), 5.60 (1H, dd, J=15.6, 7.5 Hz), 2.35 (1H, m), 1.72 (3H, s), 1.42 (3H, s) and 1.02 (6H, d, J=6.8 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 140.4, 138.6, 129.9, 123.7, 123.3, 60.7, 44.9, 38.4 (2C), 31.3, 26.8 (2C), 24.8, 22.4 (2C), 15.1, EI-MS *m*/*z*: 263 $[M]^+$ ($C_{16}H_{25}NS$).

Theonellin Formamide (*N*-((1*R*,4*R*)-1-Methyl-4-((2*E*,4*E*)-6-methylhepta-2,4-dien-2-yl)cyclohexyl)formamide, **4**): Colorless oil, $[\alpha]_D^{25}$ +18° (c=0.25, $CHCl_3$), *Rf*: 0.50 (hexane : ethyl acetate, 7 : 3), IR (KBr): 3280 and 1685 cm^{-1}, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 8.24 (1/2H, d, J=12.0 Hz), 7.98 (1/2H, d, J=1.7 Hz), 7.18 (1H, br s), 6.14 (1H, dd, J=15.4, 10.8 Hz), 5.75 (1H, d, J=10.8 Hz), 5.50 (1H, dd, J=15.1, 6.6 Hz), 2.34 (1H, m) 1.70 (3H, br s), 1.42, 1.38 (3/2H, s) and 1.00 (6H, d, J=6.8 Hz).

Cell Lines The cell lines HL-60 (human myeloid leukemia) and U937 (human leukemic monocyte lymphoma) were obtained from National Centre for Cellular Sciences (NCCS), Pune, India. Both HL-60 and U937 cells were cultured in RPMI 1640 media supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin and were maintained in culture at 37 °C in an atmosphere of 5% CO_2.

Bioassay Cell proliferation or viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay.[18] Cells were seeded in each well containing 100 μl medium at a final density of 2×10^4 cells/well, in flat-bottom 96 well plate at identical conditions. After overnight incubation, the cells were treated with different concentrations of test compounds (1.25—100 μg/ml) or dimethyl sulfoxide (DMSO) (carrier solvent) in a final volume of 200 μl with three replicates each. After 48 h, 10 μl of MTT (5 mg/ml) was added to each well and the plate was incubated at 37 °C in the dark for 4 h. The formazan crystals were solubilized in DMSO (100 μl/well) and the reduction of MTT was quantified by absorbance at 570 nm in a spectrophotometer (Spectra MAX Plus; Molecular Devices; supported by SOFTmax PRO-3.0). Effects of the test compounds on cell viability were calculated using cells treated with DMSO as control. The data were subjected to linear regression analysis and the regression lines were plotted for the best straight line fit. The IC_{50} (inhibition of cell viability) concentrations were calculated using the respective regression equation.

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