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Bioorganic & Medicinal Chemistry Letters 17 (2007) 4387–4391
Bioorganic & Medicinal Chemistry Letters

# Antifungal activity in triterpene glycosides from the sea cucumber *Actinopyga lecanora*☆

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Received 25 September 2006; revised 22 November 2006; accepted 12 December 2006
Available online 21 December 2006

**Abstract**—Bioassay-guided fractionation of methanol extract of sea cucumber *Actinopyga lecanora* led to the isolation of a new triterpene glycoside (**1**), along with two known glycosides holothurin B (**3**) and holothurin A (**4**). The structure has been elucidated on the basis of extensive 2D NMR spectroscopic analysis. The saponin (**3**) showed in vitro antifungal activity against all the twenty fungal test isolates including ATCC strain and was found to be most effective against *Trychophyton mentagrophytes* and *Sporothrix schenckii*, MIC range of 1.56 μg/ml.


In recent years, there has been a rise in the frequency, intensity and divergence of fungal infections in humans ranging from the superficial, such as dermatophytosis and onychomycoses, to deeply invasive and disseminated, like candidiasis and aspergillosis.[1] The urgency of development of new antifungal agents rose owing to therapeutic limitations of the current antifungal compounds, drug-related toxicity, hazardous drug–drug interactions and nonoptimal pharmacokinetics. All these reasons make present antifungals inadequate in treatments for immunocompromised patients, such as HIV-infected, transplant recipients and patients with cancer.[2,3] As a part of our screening programme in search for bioactive molecules from marine organisms of Indian Ocean,[4] we have investigated the sea cucumber *Actinopyga lecanora* for antifungal activity.

A preliminary biological screening of the methanol extract of freshly collected *Actinopyga lecanora* showed promising in vitro antifungal activity which was found to be localized in *n*-butanol soluble fraction only. The *n*-butanol soluble fraction on repeated column chromatographic purification led to the isolation of three pure compounds, **1**–**4** (Fig. 2). We report herein the isolation and characterization of a novel triterpene glycoside (**1**), along with two known triterpene glycosides holothurin B (**3**) and holothurin A (**4**) and in vitro antifungal activity of holothurin B (**3**).

The animal material (10 Kg) was chopped into small pieces, filled in glass percolators and extracted with methanol, affording extract (60.0 g). Crude methanol extract (45.0 g) was fractionated into ethyl acetate (10.5 g) and *n*-butanol (20.8 g) soluble fractions. Since activity was localized in *n*-butanol soluble fraction, therefore the *n*-butanol fraction was subjected to column chromatography over flash silica gel (230–400 mesh), eluting with a gradient of EtOAc saturated with $H_2O$/MeOH (99:1–70:30) to give fractions F-6 to F-12. Flash column chromatography of F-7 using a gradient elution of $CHCl_3$/MeOH (1:0–92:08) afforded semipurified compound **1** (200 mg) which was further purified by prep. TLC on precoated silica gel plates (20 × 20 cm, Merck) and developed in $CHCl_3$/MeOH (90:10) solvent system to afford pure compound **1** (80 mg). Similar purification of F-8 by flash CC with a gradient elution of $CHCl_3$/MeOH/$H_2O$ (90:9.5:0.5–88:11.25:0.75) afforded a pure compound **3** (400 mg). Similarly, compound **4** (50 mg) was obtained by repeated column chromatography of fraction F-11 by gradient elution with $CHCl_3$/MeOH/$H_2O$ (90:9.5:0.5–80:19.0:1.0).

Compound **1** had a molecular formula of $C_{37}H_{58}O_9$ as determined by the positive FAB mass spectrum

*Keywords*: Sea cucumber; *Actinopyga lecanora*; Triterpene glycosides; 2D NMR; Antifungal activity.
☆ CDRI communication No. 6685.
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doi:10.1016/j.bmcl.2006.12.052

($m/z$ = 647 $[M+H]^+$) and $^{13}C$ NMR data. The IR bands at 3400 (broad), 1759 (broad) and 1593 $cm^{-1}$ indicated the presence of hydroxyl, γ-lactone and olefinic groups. The $^1H$ NMR spectrum showed seven triterpene methyls ($\delta$ 0.91, 0.96, 1.10, 1.21, 1.37 and 1.57) and an acetoxy methyl ($\delta$ 2.17). The $^1H$, $^{13}C$ and DEPT spectra (Table 1) of **1** showed resonances for a 7(8) endocyclic double bond[5] [$\delta_C$ 120.1 (C-7) and 146.7 (C-8); $\delta_H$ 5.67 (1H br s)] and an acetoxy group [$\delta_C$ 170.8 and 21.4; $\delta_H$ 2.17 (3H, s)]. The position of double bond at C-7 was confirmed by HMBC[6] cross peaks (Table 1) at $\delta$ 5.67/51.3 (H-7/C-14), 5.67/23.4 (H-7/C-6) and 5.67/47.5 (H-7/C-9). The $^{13}C$ NMR spectrum (Table 1) featured two low field resonances at $\delta$ 179.9 and 170.8, assigned to γ-lactone carbonyl and acetoxy carbonyl carbons, respectively. The position of acetoxy group at C-16 was based on chemical shift[7,8] of the H-16 ($\delta$ 5.47) which showed correlation with signals at $\delta$ 2.15 (H-17), 1.78 (H-15α) and 1.59 (H-15β) in $^1H$-$^1H$ COSY spectrum and was confirmed by HMBC cross peaks at $\delta$ 5.47/170.8 (H-16/$CH_3CO$) and 5.47/83.3 (H-16/C-20). The relative stereochemistry of **1** was established by analysis of coupling constants and NOESY spectra (Fig. 1). The 16 β configuration of the acetoxy group was confirmed by NOESY cross peaks at $\delta$ 1.59/5.47 (H-15α /H-16α) and 2.15/5.47 (H-17α/H-16α) and from the coupling constant analysis for H-16 with the H-17α (7.6 Hz) and H-15α (7.6 Hz).[5] The presence of two doublets at $\delta$ 0.96 and 0.91 (each $J$ = 6.0 Hz) in the $^1H$ NMR spectrum of **1** and $^{13}C$ NMR resonances at $\delta$ 22.2 and 23.3 confirmed the presence of 16β-acetoxyholost-7-ene-3β-ol as the aglycon of glycoside **1**, identical to that of frondoside A from *Cucumaria frondosa*.[9]

Further $^1H$ NMR spectrum displayed one anomeric proton at $\delta$ 4.87 (1H, d, $J$ = 7.2 Hz) giving correlation with anomeric carbon atom at $\delta$ 107.8 in HMQC[10] spectrum, suggesting that this saponin contains one sugar unit. The anomeric proton was axial as judged from vicinal coupling constant of 7.2 Hz. The ring proton atoms of sugar unit were assigned starting from the readily identifiable anomeric proton, using $^1H$–$^1H$ COSY

**Table 1.** NMR data for compound **1** in pyridine-$d_5$[a]

| Position | $\delta$ C | $\delta$ H (m, $J$ in Hz) | HMBC correlation |
|---|---|---|---|
| 1 | 36.5 | 1.51 (2H, m) | |
| 2 | 67.2 | 1.95 (2H, m) | |
| 3 | 89.1 | 3.46 (1H, m) | C-1′, C-4, C-28, C-29 |
| 4 | 39.6 | | |
| 5 | 48.1 | 1.01 (1H, m) | |
| 6 | 23.4 | 2.01 (2H, m) | |
| 7 | 120.1 | 5.67 (1H, br s) | C-9, C-14, C-6 |
| 8 | 146.7 | | |
| 9 | 47.5 | 3.46 (1H, m) | |
| 10 | 35.7 | | |
| 11 | 23.0 | 1.87 (1H, m), 1.65 (1H, m) | |
| 12 | 30.3 | 1.91 (2H, m) | |
| 13 | 58.5 | | |
| 14 | 51.3 | | |
| 15 | 34.3 | 1.78 (1H, m), 1.59 (1H, dd, 12.0, 7.6) | |
| 16 | 68.4 | 5.47 (1H, ddd, 7.1, 7.6, 7.6) | $CH_3CO$, C-20 |
| 17 | 54.9 | 2.15 (1H, d, 7.6) | C-16, C-20, C-21 |
| 18 | 179.9 | | |
| 19 | 24.1 | 1.21 (3H, s) | C-9, C-10 |
| 20 | 83.3 | | |
| 21 | 27.1 | 1.57 (3H, s) | C-20, C-17, C-22 |
| 22 | 44.2 | 2.31 (1H, m), 1.81 (1H, m) | |
| 23 | 24.9 | 1.30 (2H, m) | |
| 24 | 45.4 | 1.20 (2H, m) | |
| 25 | 26.6 | 1.55 (1H, m) | |
| 26 | 22.2 | 0.96 (3H, d, 6.0) | C-24, C-25, C-27 |
| 27 | 23.3 | 0.91 (3H, d, 6.0) | C-24, C-25, C-26 |
| 28 | 29.0 | 1.37 (3H, s) | C-3, C-5, C-4, C-29 |
| 29 | 17.7 | 1.10 (3H, s) | C-3, C-5, C-4, C-28 |
| 30 | 31.1 | 1.10 (3H, s) | C-8, C-13, C-14, C-15 |
| $CH_3CO$ | 170.8 | | |
| $CH_3CO$ | 21.4 | 2.17 (3H, s) | $CH_3CO$ |
| Xyl. (1′ → C-3) | | | |
| 1′ | 107.8 | 4.87 (1H, d, 7.2) | C-3, C-5′, C-3′ |
| 2′ | 75.5 | 4.03 (1H, t, 7.8) | C-1′, C-3′, C-4′ |
| 3′ | 78.6 | 4.17 (1H, t, 8.4) | C-2′, C-4′ |
| 4′ | 71.2 | 4.23 (1H, m) | C-3′, C-5′ |
| 5′ | 67.2 | 3.80 (1H, dd, 11.1, 10.4) | C-1′, C-3′, C-4′ |
| | | 4.43 (1H, dd, 11.1, 4.8) | C-1′, C-3′, C-4′ |

[a] 300 MHz for $^1H$ NMR and 75 MHz for $^{13}C$ NMR.

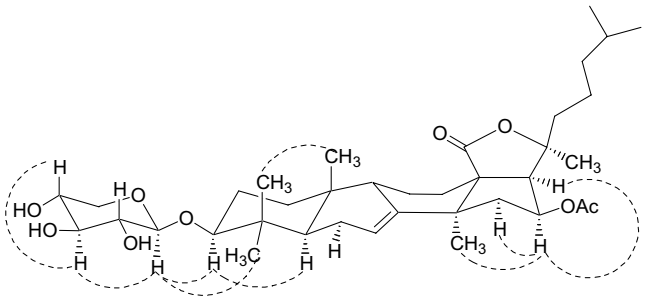

**Figure 1.** Selected NOESY correlations in compound **1**.

spectrum. After assignments of the protons, the $^{13}C$ NMR resonances were identified by direct $^{1}H$–$^{13}C$ correlation in HMQC spectrum and were further confirmed by HMBC spectrum. The sugar ring was assigned as β-D-xylopyranoside on the basis of large vicinal coupling constant ($J$ = 8–10 Hz) of C-5α proton.[11,12] The linkage of sugar unit to C-3 hydroxy of aglycon was determined by HMBC cross peaks at $\delta$ 4.87/89.1 (H-1′/C-3), 3.46/107.8 (H-3/C-1′) and further confirmed by NOESY cross peak at $\delta$ 4.87/3.46 (H-1′/H-3).

Acid hydrolysis[13] of **1** afforded β-D-xylopyranoside as carbohydrate component identified on the basis of Co-TLC with authentic sugar, while the aglycon part was identified as 16β-acetoxyholost-7,24-diene-3β-ol (**2**) by comparing NMR and mass data reported in the literature.[14] From the foregoing evidences, the structure of compound **1** was elucidated as 3-*O*-β-D-xylopyranosyl-16β-acetoxyholost-7-ene. This is the first report of naturally occurring holothurin containing one sugar unit.

Furthermore, two known compounds were characterized as holothurin A[15] (**4**) and holothurin B[16,17] (**3**), respectively, by comparing their spectroscopic data with those reported in the literature.

The crude methanol extract of *A. lecanora* showed significant antifungal activity, against *C. albicans* (MIC, 62.5 μg/ml), *C. neoformans* (MIC, 125 μg/ml), *S. schenckii* (MIC, 62.5 μg/ml), *T. mentagrophytes* (MIC, 125 μg/ml) and *A. fumigatus* (MIC, 31.2 μg/ml). On further fractionation the activity was localized in *n*-butanol soluble fraction only (Table 2). Comparing the antifungal activity of holothurin B (**3**) with fluconazole, it was observed that holothurin B was more active than fluconazole in inhibiting the growth of *Trychophyton mentagrophytes* (Table 3). On the other hand, holothurin B (**3**) showed almost comparable MIC to fluconazole against *Sporothrix schenckii* and *Aspergillus fumigatus*. Other two compounds failed to show significant activity.

The fungal strains were grown on Sabroaud dextrose agar. After the incubation, fungal growth were suspended in normal saline and maintained at $1.0–5.0 \times 10^3$ cfu/ml. The activity of test samples was determined by the NCCLS[18,19] method for fungus using RPMI-1640 media buffered with MOPS (3-[*N*-morpholino]propane sulfonic acid) (Sigma Chemical Co.). The 96-well tissue culture plates were used for twofold serial dilution. The proper growth control, drug control and blank were adjusted

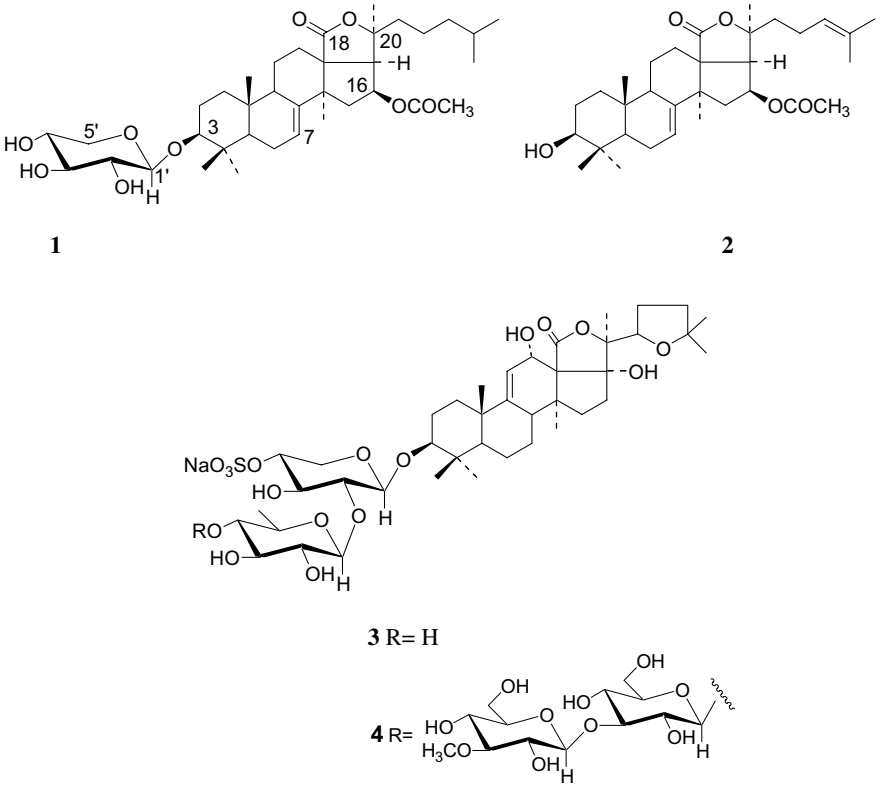

**Figure 2.** Isolated compounds.

**Table 2.** In vitro antifungal activity of methanol extract, ethyl acetate, *n*-butanol fractions and compound **1** and compound **4** of *Actinopyga lecanora*

| Code no. | Minimum inhibitory concn (MIC) in μg/ml against | | | | | |
|---|---|---|---|---|---|---|
| | Fungi | | | | | |
| | Ca | Cn | Ss | Tm | Af | Cp |
| Methanol extract[a] | — | 62.5 | 250 | 250 | 125 | — |
| Ethyl acetate fraction[a] | 500 | 250 | 250 | 250 | 500 | — |
| *n*-Butanol[a] | 125 | 62.5 | 62.5 | 125 | 125 | 125 |
| Compound **1**[b] | 50.0 | 25.0 | 25.0 | 25.0 | 50.0 | 50.0 |
| Compound **4**[b] | 50.0 | 25.0 | 25.0 | 25.0 | 50.0 | — |
| Fluconazole | 0.5 | 1.0 | 1.0 | 2.0 | 2.0 | 1.0 |

Ca, *Candida albicans*; Tm, *Trichophyton mentagrophytes*; Cn, *Cryptococcus neoformans*; Af, *Aspergillus fumigatus*; Ss, *Sporothrix schenckii*; Cp, *Candida parapsilosis* (ATCC-22019).

[a] (Crude extract, fraction) = Maximum tested concn 500 μg/ml.

[b] (Pure compound) = Maximum tested concn 50 μg/ml.

**Table 3.** In vitro antifungal activity of compound **3** against twenty different fungi

| Fungal strains | MIC (μg/ml) |
|---|---|
| *Aspergillus terreus* IMI-235704 | 25 |
| *Penicillium funiculoram* MTCC-287 | 12.5 |
| *Penicillium ochrochloron* MTCC-517 | 12.5 |
| *Trichophyton rubrum* MTCC-296 | 3.12 |
| *Candida albicans* ATCC-14053 | 12.5 |
| *Candida albicans* ATCC-60193 | 12.5 |
| *Candida albicans* ATCC-66027 | 25 |
| *Cryptococcus neoformans* ATCC-6603 | 3.12 |
| *Candida albicans* ATCC-10231 | 25 |
| *Candida krusei* ATCC-6258 | 12.5 |
| *Candida albicans* (CDRI) | 3.12 |
| *Candida albicans* MTCC-183 | 25 |
| *Candida albicans* MTCC-227 | 12.5 |
| *Fetobasidella neoformans* MTCC-1346 | 6.25 |
| *Aspergillus flavus* (CDRI) | 12.5 |
| *Cryptococcus neoformans* (CDRI) | 3.12 |
| *Sporothrix schenckii* (CDRI) | 1.56 |
| *Trichophyton mentagrophytes* (CDRI) | 1.56 |
| *Aspergillus fumigatus* (CDRI) | 3.12 |
| *Candida parapsilosis* ATCC-22019 | 6.25 |

IMI, International Mycological Institute; MTCC, Microbial Type Culture Collection (India); ATCC, American Type Culture Collection; CDRI, Central Drug Research Institute (India).

onto the plate. Test samples were dissolved in DMSO (at a concentration of 10 mg/ml for crude extract and 1 mg/ml for pure compounds) and 20 μl of this was added to the 96-well tissue culture plate having 180 μl RPMI-1640 so that the maximum concentration of the compound became 500 μg/ml (for 10 mg/ml) and 50 μg/ml (1 mg/ml). From here the solution was serially diluted resulting in the half of the concentration of test compounds and then inocula were added and kept in incubator. Microtiter plates were incubated at 35 °C in a moist dark chamber and MICs were recorded spectrophotometrically.

In conclusion, results obtained from the present study suggest that marine natural products isolated from the sea cucumber (*Actinopyga lecanora*) continue to be an interesting source of antifungal compounds. Holothurin B could be a lead molecule in the development of a potent drug for the treatment of fungal infections. Further studies are still warranted to establish the toxicity data of holothurin B and its in vivo activity in the animal model.

## Acknowledgments

We are thankful to the Director, Central Drug Research Institute, Lucknow, India, for providing necessary research facilities as well as his keen interest in marine natural products. We are grateful to the Department of Ocean Development, Government of India, for providing financial assistance. We are also thankful to Mr. N. P. Mishra and Mr. H. R. Mishra for technical assistance and SAIF for spectral data.

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13. Acid hydrolysis of compound **1**: The solution of compound **1** in 2.0 N HCl in 80% v/v of ethanol (5 ml) was refluxed for 3 h. After this, water was added to the reaction mixture and ethanol was evaporated, it was again refluxed for one more hour. Reaction mixture was then extracted with chloroform; Organic layer was identified as 16β-acetoxyholost-7,24-diene-3β-ol (**2**) by comparing NMR and mass data. The aqueous phase was neutralized with Amberlite IR 410 $CO_3^{2-}$ resin, filtered and concen-

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