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Structural relationship among steroids from Sulawesi Tenggara’s sponge Clathria sp. and their radical scavenger activity

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Abstract. Four steroids were isolated and identified from the acetone extract of Clathria sp., namely clathruhoate or 3β-(butiryloxymethyl)-A-nor-5α-cholestan (1), 3β-(acetoxymethyl)-A-nor-5α-cholestan (2), 3β-(hydroxymethyl)-A-nor-5α-cholestan (3), and 3β-(hydroxymethyl)-A-nor-5α-cholest-15-ene (4). Isolation was carried out using chromatography techniques including Thin Layer Chromatography (TLC), vacuum liquid chromatography (VLC) and radial chromatography (RC) with silica gel as adsorbent and mixture of solvents as eluent. The Structure of isolated compounds was determined by spectroscopic methods i.e. FTIR, 1H and 13C NMR and also by comparing the spectroscopic data with similar data from references. The compounds isolated were evaluated for biological activity against a radical agent (DPPH radical). The relationship of the steroids showed that compound 3 is an oxidation product of compound 4. Clathruhoate (1) is formed by esterification of compound 3 with buthanoic acid, and compound 2 is produced by esterification of compound 3 with acetic acid. The radical scavenger potential of the methanol extract and steroids from Clathria sp. can be summarized as follows: methanol extracts > compound 4 > compound 3 > compound 2 > compound 1.

1. Introduction
Sulawesi Tenggara is one of the archipelagic provinces in Indonesia, with 561 islands and a territory which is about 75% ocean [1]. Consequently, the marine natural resources in this province are very abundant, one of which is a sponge [2,3]. Clathria is a genus of the family Microcionidae which comprises about 544 species [4], one of which grows in Sulawesi Tenggara. Some species of Clathria produce various secondary metabolites such as alkaloids [5–11], anthraquinones [12], and terpenoids [6,13–18]. Some of the compounds identified have interesting types of biological activity including antibacterial [10,16,17,19], anti-HIV [13], cytotoxicity towards cancer cell lines [8,9,17], anti-inflammatory [14], and antiplasmodial [20].

Clathria sp. Can be found in the sea of Sulawesi Tenggara, including the waters around the Maritime Education Centre “Bintang Samudra” in Kendari, the capital of Sulawesi Tenggara. Chemical studies of the species have isolated and identified four steroid compounds, namely clathruhoate or 3β-(butiryloxymethyl)-A-nor-5α-cholestan (1) 3β-(hydroxymethyl)-A-nor-5α-cholest-15-ene (2), 3β-(hydroxymethyl)-A-nor-5α-cholestan (3), and 3β-(acetoxymethyl)-A-nor-5α-cholestan
This paper reports the results of a study on the radical scavenger potential and the structural relationship among those steroids derived from Clathria sp.

2. Material and Methods

2.1. Sample collection, materials and equipment

Samples of Clathria sp. (Figure 1) were collected by hand using SCUBA (Self Contained Underwater Breathing Apparatus) from the reef slope area (70° slope) of the Bintang Samudra Marine Education Park, Sulawesi Tenggara, Indonesia, at depth of 2–10 m, in January 2017. The chemicals and materials used in this study included methanol, ethylacetate, n-hexane, chloroform, aquades, acetone, thin layer chromatography plate: Kieselgel 60 F254 p.a, silica gel 60 G (Merck®), cerium sulphate (CeSO4) (Merck®), DPPH (2,2-diphenyl-1-picrylhydrazyl).

Spectroscopy instruments were used. Ultraviolet (UV) spectra were obtained by using Cary Varian 100 concentration. Infrared (IR) spectra were acquired from Perkin-Elmer Spectrum One FT-IR Spectrophotometer. Spectrum 1H NMR, 13C NMR and NMR 2D (2-Dimension) were obtained using a JEOL ECP 500 spectrometer, operated at 500 MHz (1H) and 125 MHz (13C).

2.2. Extraction and Isolation

The sponge (in the form of dried powder, 4 kg) was extracted at room temperature with MeOH (3 x 8 L, for 24 h each time) and filtered. The methanol extract was concentrated using a vacuum rotary evaporator at low/reduced pressure, giving a brownish-yellow gum (300 g). An aliquot of this extract (30 g) was fractionated using vacuum liquid chromatography (VLC) (13 x 5 cm) with silica gel as adsorbent and gradient elution using n-hexane/EtOAc (9:1, 8:2, 7:3, 5:5, v/v) as eluent to produce five main fractions (F1-F5).

Fraction F1 (2 g) was fractionated using a silica gel radial chromatography (RC), eluted isocratically with n-hexane/EtOAc (9.5:0.5, v/v) to yield nine subfractions (F11-F19). Combined subfractions F11-F13 (1.36 g) were further fractionated by silica gel radial chromatography (RC) with elution by n-hexane/CHC13 (9.5:0.5, v/v) to yield compound 1 (30 mg). Purification of subfraction F14 (0.14 g) using silica gel RC with n-hexane/CH2Cl2 (6:4, v/v) as a mobile phase produced compound 2 (23 mg).

Fraction F2 (10 g) was subjected to a silica gel vacuum liquid chromatography (VLC) (10 x 5 cm), eluted with n-hexane/EtOAc (9:1-0:10, v/v), yielding eight subfractions (F21-F28). Subfraction F24 (0.8 g) was purified using silica gel RC with n-hexane/EtOAc/acetone (7:1:0.5, v/v) as mobile phase to produce compound 3 (200 mg). Purification of subfraction F26 (1.1 g) by silica gel RC with elution of n-hexane/EtOAc/acetone (7:1:0.5, v/v) yielded compound 4 (120 mg).

2.3. Compound structure

The structure of isolated compounds was determined using spectroscopic methods including Fourier Transform Infrared Spectroscopy (FTIR), Infrared Spectroscopy (1H) and Nuclear Magnetic Resonance Spectroscopy (13C NMR) as well as by comparing the spectroscopic data with similar data from references.

2.4. Radical Scavenger Activity

The potency of isolated compounds as radical scavengers was evaluated against the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. The inhibition of DPPH radicals was determined qualitatively and quantitatively. The qualitative analysis was conducted using TLC autographic spray. The procedures of the TLC autographic assay were as follows. After developing and drying, TLC plates (samples ranging from 0.1–100 μg) were sprayed with 0.2% (2mg/mL) of DPPH solution in methanol. The plates were observed for 30 minutes after being sprayed. Active compounds appeared as yellow spots with a purple background.
The Bios method was adapted for quantitative analysis. One mL of DPPH (500µM, 0.2 mg/mL) in methanol was mixed with the same volume of the tested compounds at various concentrations. The mixture was kept in the dark for 30 minutes. The absorbance of each mixture was measured at 517 nm using a UV spectrophotometer after which the absorbance of the blank was measured. The concentration of the sample at which the absorbance at 517 nm decreased to a half of its initial value was used as the IC50 value of the compound tested. IC50 values were expressed in µg/mL. The analysis was performed in triplicate for the standard and for each compound [22].

3. Results and Discussion
Four steroid compounds have been isolated and identified from the methanol extract of Clathria sp. namely clathruhoate or 3β-(butyryloxymethyl)-A-nor-5α-cholestan (1), 3β-(acetoxymethyl)-A-nor-5α-cholestan (2), 3β-(hydroxymethyl)-A-nor-5α-cholestan (3), and 3β-(hydroxymethyl)-A-nor-5α-cholest-15-ene (4) [21]. All compound structures were inferred by interpreting spectroscopic data and comparing the data with references. Compound 1 is a novel compound; the spectroscopic data have been interpreted including Infrared, 1D and 2D NMR and mass spectroscopy.

- **clathruhoate or 3β-(butyryloxymethyl)-A-nor-5α-cholestan (1)**: white solid; ATR-IR (v_{max}, cm^{-1}): 2922, 2853, 1734, 1464, 1379, 1173, 968, 723; 1H NMR (CDCl₃, 500 MHz, δ/ppm, J/Hz): 1.08 (1H, m), 1.61 (1H, m, H-1), 1.44 (1H, m), 1.94 (1H, m, H-2), 2.31 (1H, m, H-3), 3.94 (1H, dd, J=10.27, 6.85, H-4a), 4.09 (1H, dd, J=10.27, 6.85, H-4b), 1.44 (1H, m, H-5), 1.04 (1H, m, H-6a), 1.55 (1H, m, H-6b), 0.83 (1H, m, H-7a), 1.74 (1H, m, H-7b), 1.36 (1H, m, H-8), 0.73 (1H, m, H-9), 1.31 (2H, m, H-11), 1.14 (2H, m, H-12), 0.99 (1H, m, H-14), 1.14 (1H, m, H-15a), 1.34 (1H, m, H-15b), 1.26 (2H, m, H-16), 1.00 (1H, m, H-17), 0.66 (3H, s, H-18), 0.78 (3H, s, H-19), 1.29 (1H, m, H-20), 0.91 (3H, m, H-21), 0.99 (2H, m, H-22), 1.33 (1H, m, H-23a), 1.44 (1H, m, H-23b), 1.14 (1H, m, H-24a), 1.95 (1H, m, H-24b), 1.24 (1H, m, H-25a), 1.81 (1H, m, H-25b), 0.86 (3H, m, H-26), 0.86 (3H, m, H-27), 2.27 (2H, t, J=7.83, H-2'), 1.63 (2H, m, H-3') and 0.89 (3H, m, H-4'). 13C NMR (CDCl₃, 125 MHz, ppm): 39.0 (C-1), 27.3 (C-2), 38.5 (C-3), 68.3 (C-4), 52.4 (C-5), 24.4 (C-6), 32.7 (C-7), 35.8 (C-8), 55.4 (C-9), 44.1 (C-10), 22.8 (C-11), 39.5 (C-12), 43.1 (C-13), 56.3 (C-14), 23.8 (C-15), 29.3 (C-16), 56.2 (C-17), 12.2 (C-18), 14.5 (C-19), 35.5 (C-20), 18.7 (C-21), 36.2 (C-22), 23.2 (C-23), 39.9 (C-24), 28.2 (C-25), 22.7 (C-26), 22.6 (C-27), 174.1 (C-1'), 34.5 (C-2'), 22.5 (C-3') and 14.1 (C-4'); HRMS-ESI: m/z 459.4112 [M + H]+ (calcd for C₂₅H₃₅O₂): 459.4197.

- **3β-(acetoxymethyl)-A-nor-5α-cholestan (2)**: yellow crystal; ATR-IR (v_{max}, cm^{-1}): 2926, 2858, 1740, 1455, 1370, 1164, 969, 721; 1H NMR (CDCl₃, 500 MHz, δ/ppm, J/Hz): 4.09 (1H, dd, J=10.27, 7.15), 3.95 (1H, t, J=8.4), 2.33 (2H, m), 2.03 (3H, s), 0.86 (3H, m), 0.91 (3H, s), 0.86 (3H, m), 0.85 (3H, m); 13C NMR (CDCl₃, 125 MHz, ppm): 39.1 (C-1), 22.8 (C-2), 38.6 (C-3), 68.5 (C-4), 52.6 (C-5), 37.5 (C-6), 32.8 (C-7), 37.5 (C-8), 55.6 (C-9), 44.3 (C-10), 22.8 (C-11), 39.5 (C-12), 43.1 (C-13), 56.4 (C-14), 24.6 (C-15), 28.3 (C-16), 56.4 (C-17), 12.3 (C-18), 14.5 (C-19), 35.9 (C-20), 18.9 (C-21), 36.3 (C-22), 23.0 (C-23), 40.0 (C-24), 28.2 (C-25), 22.7 (C-26), 22.6 (C-27), 171.5 (C-1'), and 21.2 (C-2'); MW: 430.7061 (C₂₅H₃₅O₃). NMR data were identical to those given in [23].

- **3β-(hydroxymethyl)-A-nor-5α-cholestan (3)**: white solid; ATR-IR (v_{max}, cm^{-1}): 3357, 2915, 2820, 1454, 1378, 1276, 1196, 740; 1H NMR (CDCl₃, 500 MHz, δ/ppm, J/Hz): 3.67 (1H, dd, J=10.3, 6.3), 2.23 (1H, m), 1.93 (2H, m), 1.82 (1H, m), 0.91 (3H, m), 0.86 (3H, m), 0.84 (3H, m), 0.73 (3H, s), 0.64 (3H, s); 13C NMR (CDCl₃, 125 MHz, ppm): 39.1 (C-1), 22.9 (C-2), 39.7 (C-3), 67.2 (C-4), 52.7 (C-5), 37.4 (C-6), 32.9 (C-7), 35.7 (C-8), 55.6 (C-9), 44.3 (C-10), 23.4 (C-11), 36.3 (C-12), 43.1 (C-13), 56.5 (C-14), 24.6 (C-15), 28.3 (C-16), 56.4 (C-17), 12.3 (C-18), 14.7 (C-19), 29.8 (C-20), 18.9 (C-21), 35.9 (C-22), 24.6 (C-23), 40.0 (C-24), 28.2 (C-25), 22.7 (C-26), and 23.0 (C-27); MW: 388.6694 (C₂₂H₃₈O₅). NMR data were identical to those given in [23].
The compound structures of 2, 3 and 4 were determined based on comparison of Infrared, 1D NMR and mass spectroscopy data of isolates to references data. All compound structures are displayed at Figure 1.

![Figure 1. Structure and structural relationship of four steroids isolated from the methanol extract of the sponge Clathria sp.](image)

The four steroids that were successfully isolated and identified from Clathria sp. had a different basic structure compared to steroids from plants. Ring 1 (R1) steroids from plants are cyclohexane whereas the Clathria sp. steroids are cyclopentane. Furthermore, four steroids from Clathria sp. can be distinguished based on the presence of a cycloalkene ring. For three compounds (1, 2, 3), the difference in the compound structure lies in the substituent bond to C-4. Compound 1 binds to the buthanoic group, compound 2 binds with the ethanoic group and compound 3 binds to the hydroxyl group. In another compound, 3β-(hydroxymethyl)-A-nor-5α-choleste-15-ene, the basic structure of the compound has an alkene group on C-15 and C-16. This is quite interesting in terms of chemistry, because the four compounds come from one organism. The structural relationship among compounds 1 to 4 can be explained as follows. Compound 3 is the result of hydrogenation reaction on compound 4. Furthermore compound 3 can undergo an esterification reaction with buthanoic acid to produce

- 3β-(hydroxymethyl)-A-nor-5α-cholest-15-ene (4): white solid; ATR-IR ($\nu_{\text{max}}$, cm$^{-1}$): 3334, 2918, 2853, 1508, 1454, 1376, 1265, 1035, 977; H NMR (CDCl$_3$, 500 MHz, δ/ppm, J/Hz): 5.15 (1H, m), 5.13 (1H, m), 3.71 (1H, dd, J=10.45, 6.50), 3.47 (1H, t, J=9.72), 2.22 (1H, m), 1.95 (2H, m), 0.90 (3H, m), 0.89 (3H, m), 0.85 (3H, m), 0.74 (3H, s), 0.65 (3H, s); C NMR (CDCl$_3$, 125 MHz, ppm): 52.7 (C-3), 37.4 (C-5), 32.9 (C-6), 32.9 (C-7), 35.7 (C-8), 55.6 (C-9), 44.3 (C-10), 23.4 (C-11), 36.3 (C-12), 43.1 (C-13), 56.5(C-14), 130.1 (C-15), 131.5 (C-16), 56.4 (C-17), 12.3 (C-18), 14.7 (C-19), 29.8 (C-20), 18.9 (C-21), 35.9 (C-22), 24.6 (C-23), 40.0 (C-24), 28.2 (C-25), 22.7 (C-26), and 23.0 (C-27); MW: 386.6535 (C$_{27}$H$_{46}$O). NMR data were identical to those given in [24].
compound 1, and compound 3 can also react with acetic acid to form compound 2. These reactions can occur with other compounds so that the compounds in the sponge *Clathria* sp. can be more diverse.

The differences in the chemical structure of the steroids can affect their biological activity. The type of biological activity which was evaluated was the ability to act as a radical scavenger. The results of the radical scavenger test are shown in Table 1.

**Table 1. Radical scavenger activity of methanol extract and compounds isolated from *Clathria* sp.**

| Sample (s) | Methanol extract | Compound 1 | Compound 2 | Compound 3 | Compound 4 | Ascorbic Acid |
|------------|------------------|------------|------------|------------|------------|--------------|
| IC₅₀ (g/mL) | 218.6 ± 2.4      | 376.2 ± 2.8| 366.4 ± 2.7| 312.2 ± 2.2| 288.4 ± 2.6| 25.2 ± 0.8   |

The data in Table 1 indicates that, in general, the test samples were less active as radical scavengers than the standard (ascorbic acid). Furthermore, the four isolated compounds were less active than the raw unfractionated methanol extract of *Clathria* sp. This was thought to be caused by the presence of four hydroxyl groups on ascorbic acid, thus facilitating the release of hydrogen radicals and neutralizing DPPH radicals, whereas only two are conjugated with carbonyl groups [25]. The methanol extract of *Clathria* sp. is more active as a radical scavenger than the isolated compounds indicating that there are further present compounds in the methanol extract which have radical scavenger potential but have not yet been isolated and identified.

Compound 4 had the highest activity as a radical scavenger among the isolated compounds, due to the presence of an alkene group on C-15/C-16. This group can facilitate the formation of radicals which neutralize DPPH radicals [26]. Clathruhoate or 3β-(butyryloxymethyl)-A-nor-5α-cholestane (1) is an ester compound, which can act as a driver of oxidation reactions rather than as an antioxidant; therefore, this compound had the lowest ability to neutralize DPPH radicals. In addition, the presence of a longer alkane chain in compound 1 compared to compound 2 could cause compound 1 to be less active in neutralizing DPPH radicals.

4. Conclusion

Four steroids from the methanol extract of *Clathria* sp. have been isolated and described. The structural relationship between the compounds showed that compound 3 is an oxidation product of compound 4. Clathruhoate (1) is formed by esterification of compound 3 with buthanoic acid, and compound 2 is produced by esterification of compound 3 with acetic acid. The order of radical scavenger potency of the methanol extracts and steroids from *Clathria* sp. was: methanol extracts > compound 4 > compound 3 > compound 2 > compound 1.

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