Structural Elucidation of Secondary Metabolites in Sponge (*Callyspongia pseudoreticulata*) with N-Hexane Extract

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Abstract: Sponge is one of the marine species that has a high bioactivity and contains secondary metabobolite compound which can be used as antibiotic and medicine. Terpenoid compound derivatives have been successfully isolated from sponge *Callyspongia pseudoretilulata* by a method in which the n-hexane extract weighing 11.28 g was fractionated using vacuum column chromatography with a non-polar eluent, n-hexane. The polarity of this eluent was improved by ethyl acetic resulting in fairly polar eluent, methanol. A total of 27 fractions were obtained and one of the compounds from n-hexane extract was structurally elucidated by NMR spectroscopy and two dimensions of homonuclear and heteronuclear (^{1}H, ^{13}C, DEPT, H-H COSY, HMQC, and HMBC). The identified compound was a hydrocarbon (1-ethyl cyclo hex cosana-1-amin) that indicated LC50 of 60.58 against *A. salina* and has a great potential as antitumor or anticancer.

Keywords: Callyspongia pseudoreticulata; n-hexane

1. Introduction

Sponge is a rich source of terpenoid, peptide, polyketida, alkaloid, steroid, and other compounds. A study on cytotoxic secondary metabolites from sponge (1986-1991) suggested that the compounds had potential as antitumor (Schemitz *et al.,* 1993). Sponges can be found alive and distributed in various waters in islands and continents (Soest *et al*., 1994) including in South Sulawesi water (Voogd and Soest, 2002). Sponges have various different species, numbering more than 10,000 species in four classes of *Hexatinellidae*, *Calcarea*, *Demospongiae*, and *Sclerospongiae*. Demospongia class has the largest distribution (Barnes *et al.,* 1989).

In the last decade, exploratory researches on sponge in South Sulawesi waters have been developed. In the waters there are many sponge types whose secondary metabolites are very potential as antiviral, antibacterial, and antifungal (Razak and Ridhay, 2004). This is in accordance to a study by Rusli (2005), who had successfully isolated and identified several secondary metabolites from various sponge derived from Samalona islands water, Makassar, and found to be bioactive against microbes. Other study on sponge *Xentospongia aschmorica* identified four manzamine A compounds, which were previously studied by many due to its potential as anticancer and its ability to inhibit malarial parasites (Sakai *et al.,* 1992).

The prevalent and commonly found sponges in Makassar waters are *Callyspongia* sp, and they are considered candidates for new pharmaceutical mate rial discoveries. This species included *Callyspongia pseudoreticulata.* This sponge had been studied in an attempt to develop the prospect and benefit of secondary metabolite compounds, but still limited to the identification of their compound group (suriani, 2006).

According to Amir and Budianto (1996), the sponge *Callyspongia pseudoreticulata* is a commonly found sponge in Indonesia waters. This sponge is an oceanic biota that contains many secondary metabolite compounds. Isolates from this sponge have been identified containing some secondary metabolite compound groups such as terpenoid, alkaloid, and steroid.

This study was aimed to find out the toxicity of fractionated n-hexane extract of sponge (*Callyspongia pseudoreticulata*) against shrimp fries of *Artemua saliana.* This test was a secondary test with positive correlation to primary test of P388 cancerous cell and usually used as preliminary step in determining bioactive properties of an organic sample component. When the sample turns out to have bioactivity, further study is performed to elucidate the structure and tested for its benefit as pharmaceutical material (Anderson *et al.,* 1991).

2. Materials and Methods

2.1 Sample Preparation and Extraction

A survey was conducted before collecting the *Callyspongia pseudoreticulate* sample to determine the samples that met the pre-determined classification. Samples were collected from Samalona islands locations with 6 m depth. The collected samples were then transferred to laboratory and reconfirmed for its classification. The samples were cleaned immediately by distilled water and then air-dried for 1 x 24 hours. After that, the samples were crushed with blender and then macerated with methanol for 2 x 24 hours four times. The macerates of methanol was evaporated in a low-pressure evaporator operated at 40^{o}C temperature to obtain concentrated macerates. The macerate was then extracted subsequently with n-hexane, chloroform, and ethylase. The extraction was analyzed with C-NMR and H-NMR spectroscopy. C-NMR give the number of C atoms and H-NMR give the number of proton or hydrogen of the sample. .

2.2 Secondary Compounds Isolation

The n-hexane extract weighting 11.28 gram was fractionated using vacuum column chromatography with a non-polar eluent,

n-hexane. The polarity of this eluent was improved by ethyl acetic resulting in fairly polar eluent; methanol and 27 fractions were obtained. The fractions were then analyzed by thin layer chromatography with eluent chloroform 100% and six primary fractions were obtained (Figure 1). Each of the primary fractions were further fractionated and then were crystallized and recrystallized to obtain first, second, and third compound. Compound 3 was a component in n-hexane extract. Thin-layer chromatography tests for third compound using three different eluent systems consistently revealed single spot, that the compound was considered as a pure compound. Combination of eluent and Rf value of compound 3 TLC can be seen in Table 1.

Table 1. TLC Chromatogram data for compound 3 and its Rf value

Eluent	R_f
n-hexane 100%	0.75
Chloroform : n-hexane = 1 : 9	0.95
Ethyl acetic : n-hexane = 1 : 9	0.97

3. Results and Discussion

The crystals obtained were solid white with melting point of 79-80 ^{o}C. Liebermann Burchard test was negative for terpenoid or steroid. In the IR spectra of the compound 3 (Figure 1), the peak of 2,960 and 2,916 cm^{-1} represented -CH (*str*) of -CH2 and -CH3 groups. The peak at 1,444 cm^{-1} represented -CH (*bending*). The peak at 3,712 and 3,757 cm^{-1} represented N-H (*str*) of NH2 group and 1083 cm^{-1} represented peak of C-N (*str*) (Figure 1).

NMR spectra data of compound 3 revealed 6 ^{13}C NMR signals at δc 32.12; 29.95; 29.89; 29.56; and 14.31 (Figure 2 and Table 2). The DEPT NMR 135 spectra data indicated 5 signals (Table 2). These signals represented 28 carbon atoms and interpreted as sp^3 group consisting of 1 quaternary carbon atom dc 32.12 ppm, 1 methyl carbon atom dc 14.31 ppm, and 26 methyl carbon atoms (Table 2). The ^{13}C NMR spectra data suggested a structure as described in Figure 3.

1H NMR spectra data (Table 3) is in accordance to the suggested compound 3 structure (Figure 3). Proton shift at d_H 0.85 ppm (3H, t, 7,5 Hz) represents H-2' and 1.20 ppm (2H, q, 7,5 Hz) represents H-1'. This structure became more apparent from H-H COSY spectra data. H-1' has a H-H COSY correlation to H-2' and H-3 to H2 and H-4, and H-4 to H-3 and H-5. H-H COSY correlation as a whole was indicated in Table 3. The next spectra data supporting the compound 3 structure was the H-C HMBC correlation. In Figure 3, the proton H-3 correlated to carbon atoms C-2 and C-4 in short distance and to C-5 in long distance. Whereas the proton H-25 correlated to C-24 and 26 in short distance and to C-23 in long distance.

Table 2. ^{13}C NMR, DEPT NMR, ^{1}H NMR spectra data and H Integration of Compound 3.

No.	δc NMR (ppm)	Group	δH NMR (ppm) (multiplicity, J)	H integration (one)
1	32.12	-	-	-
2	29.95	CH_2	1.65 2H (m)	2
3	29.89	CH_2	1.20 (m)	50
4	29.56	CH_2	1.20 (m)	2
5	22.89	CH_2	1.20 (m)	2
6	14.31	CH_3	0.85 3H (t =7.5 Hz)	3

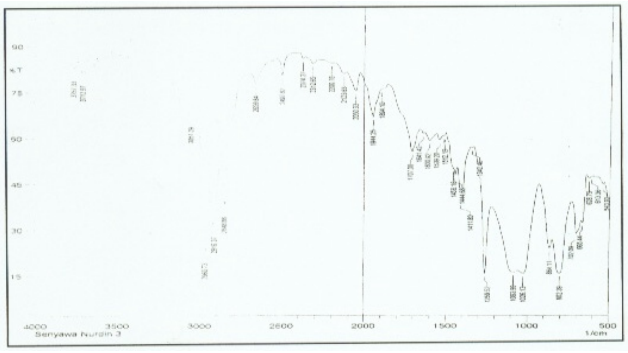

Figure 1. IR spectra of compound 3

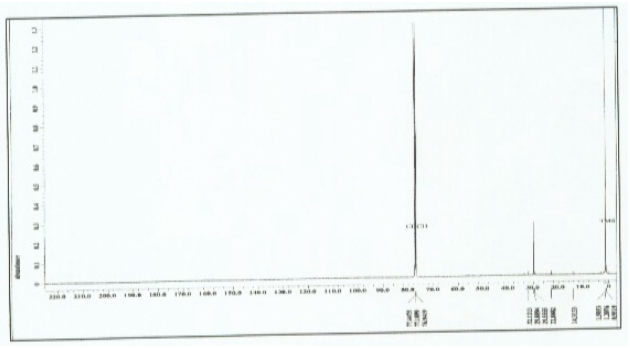

Figure 2. 13C NMR spectra (compound 3)

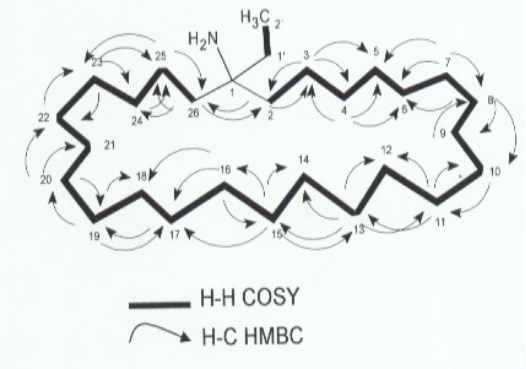

Figure 3. Structure of compound 3

Table 3. ^{13}C NMR, ^{1}H NMR, H-H COSY and H-C HMBC data of Compound 3

No.	δc NMR (ppm)	δ_H NMR (ppm) (multiplicity, *J*)	H-H COSY	H-C HMBS
1	32,12	-		
2	29,95	1,65, 2H (m)	3	3,4,26
3	29,88	1,20, 2H (m)	2,4	2,4,5
4	29,88	1,20, 2H (m)	3,5	2,3,5,6
5	29,88	1,20, 2H (m)	4,6	3,4,6,7
6	29,88	1,20, 2H (m)	5,7	4,5,7,8
7	29,88	1,20, 2H (m)	6,8	5,6,8,9
8	29,88	1,20, 2H (m)	7,9	6,7,9,10
9	29,88	1,20, 2H (m)	8,10	7,8,10,11
10	29,88	1,20, 2H (m)	9,11	8,9,11,12
11	29,88	1,20, 2H (m)	10,12	9,10,12,13
12	29,88	1,20, 2H (m)	11,13	10,11,13,14
13	29,88	1,20, 2H (m)	12,14	11,12,14,15
14	29,88	1,20, 2H (m)	13,15	12,13,15,16
15	29,88	1,20, 2H (m)	14,16	13,14,16,17
16	29,88	1,20, 2H (m)	15,17	14,15,17,18
17	29,88	1,20, 2H (m)	16,18	15,16,18,19
18	29,88	1,20, 2H (m)	17,19	16,17,19,20
19	29,88	1,20, 2H (m)	18,20	17,18,20,21
20	29,88	1,20, 2H (m)	19,21	18,19,21,22
21	29,88	1,20, 2H (m)	20,22	19,20,22,23
22	29,88	1,20, 2H (m)	21,23	20,21,23,24
23	29,88	1,20, 2H (m)	22,24	21,22,24,25
24	29,88	1,20, 2H (m)	23,25	22,23,25,26
25	29,88	1,20, 2H (m)	24,26	23,24,26
26	29,88	1,20, 2H (m)	25	24,25,2
1’	22,89	1,20, 2H (q, 7, 5 Hz)	2’	2’
2’	14,31	0,85, 3H (t, 7, 5 Hz)	1’	1’

4. Conclusion

One of the secondary compounds found in sponge *Callyspongia pseudoreticulata* with n-hexane extract was a hydrocarbon compound (1-ethyl cyclo hex cosana-1-amin). This isolated compound for the first time from sponge *Callyspongia pseudoreticulata* indicated LC50 of 60.58 against *A. salina* and has a great potential as antitumor or anticancer. It is a white crystal with 79-80^{o}C melting point.

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