

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 metabolite compound which can be used as antibiotic and medicine. Terpenoid compound derivatives have been successfully isolated from sponge *Callyspongia pseudoreticulata* 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 (^1H , ^{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*. *Demospongiae* 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 *Xestospongia 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 material 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 pseudoreticulata* 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°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 R_f value of compound 3 TLC can be seen in Table 1.

Table 1. TLC Chromatogram data for compound 3 and its R_f 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 °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 ¹³C NMR signals at δ_c 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 sp³ group consisting of 1 quaternary carbon atom δ_c 32.12 ppm, 1 methyl carbon atom δ_c 14.31 ppm, and 26 methyl carbon

atoms (Table 2). The ¹³C 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 δ_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, ^1H 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

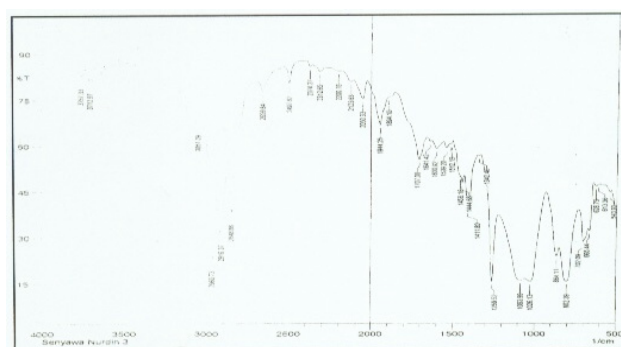


Figure 1. IR spectra of compound 3

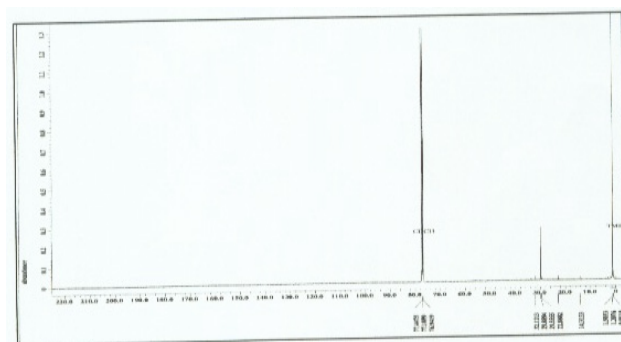
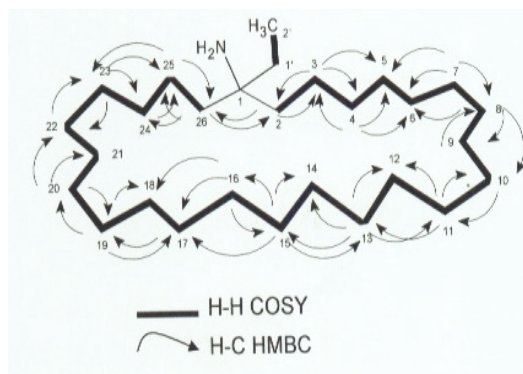
Figure 2. ^{13}C NMR spectra (compound 3)

Figure 3. Structure of compound 3

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

No.	δ_{C} 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°C melting point.

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References

1. Achmad, S. A. (2004). Empat Puluh Tahun dalam Kimia Organik Bahan Alam Tumbuh-tumbuhan Tropika Indonesia, Rekoleksi dan Prospek. Bulletin of the Indonesian Society of Natural Product Chemistry. 4(2): 50-54.
2. Amir, I. and Budianto, A. (1996). Mengenal Spons Laut (Demospongia) Secara Umum. *Oseana*. LIPI Jakarta. 21(2): 15-30.
3. Anderson, J. E. Goetz, C. M., McLaughlin. (1991). A. Blind Comparison of Simple Bench-top Bioassay and Human Tumor Cell Cytotoxicities as Antitumor Prescreen. *Journal Phytochemical Analysis*, 2: 107-111.
4. Barnes, R, Calon, P., and Olive, P. (1989). *The Invertebrata*, Blackkywell Scientific Pub. Oxford, London, Endinburgh, Boston, Melbourne, Five Pub: 49-53.
5. Endrada, R. U., Proksch, P., Wray, W., Witte, L., Mller, W. E. G., and Soest, R. W. M. van. (1996). Four New Bioactive Manzamine-Type Alkaloid from the Philippine Marine Sponge *Xestospongia aschmoica*. *Journal of Natural Product*. 59:1056-1060.
6. Ireland, C., Roll, D., Molnki, T., Mc Kee, T. and Swersey, J. (1988). *Uniqeness of the Marine Chemical Environment, Catagories of Marine Natural Products from Invertebrates in Biomedical Importance of Marine Organism*, Fautin, D.G., (ed), California Academy of Sciences, San Fransisco, 45-47.
7. Plisson, F. (2014). Callyspongisines A–D: bromopyrrole alkaloids from an Australian marine sponge, *Callyspongia* sp. *Org. Biomol. Chem.* 1579-1584
8. Rahmaniar. (1992). Suatu Pengantar Oseana. *Toxin Marine*. 62(1): 1-11.
9. Razak, A. R. and Ridhay, A. (2004). *Penepisan Senyawa Anti Mikroba dari Beberapa Jenis Bunga Karang (Porifera) secara Kromtorafi Lapis Tipis Bioatografi*. Laporan Hasil Penelitian Nomor 89/P2IPT/DPPM/P10/III/2004. Kerjasama Departemen Pendidikan nasional dan Fakultas Keguruan dan Ilmu Pendidikan Universitas Tadulako (*in Indonesian*).
10. Rusli, (2005). *Isolasi dan Identifikasi Senyawa Aktif Antimikroba Beberapa Spons dari Perairan Pulau Samalona*. Tesis tidak diterbitkan. Makassar: Program Pascasarjana Universitas Hasanuddin.
11. Sakai, R., Higa, T., and Jefoord, C. (1992). Manzamin A, A Novel Antitumor Alkaloid from a Sponge. *J. Am. Chem. Soc.*, 11(1):8925-8927.
12. San, T. W. (1993). Toxicity Testing Using The Brine Shrimp, *Artemia salina*, *Bioactive Natural Products, Detection, Isolation and Structural Determination*, Chi Press, Inc., London.

13. Satari, R. (2003). *Produk Bahan Alam Laut sebagai Lead Compound untuk Farmasi dan Pertanian*. Makalah disajikan dalam Seminar Sehari Prospektif Baru dalam Drug Discovery, Makassar, 26 Oktober 2003 (*in Indonesian*)
14. Schemitz, F. J., Bowden, B. F., and Toth, S. I. (1993). *Antitumour and Cytotoxic Compounds from Marine Organism, Pharmaceutical and Bioactive Natural Products*. New York: Plenum Press.
15. Soest, R. W. M. van. (1989). The Indonesian Sponges Fauna, A Status Report Netherlands, *Journal of Sea Research*, 23: 223-230.
16. Soest, R. W. M. van., Kempen, T. M. G. van., and Breakman, J. C. (1994). *Sponges in Time and Space: Biology, Chemistry, Paleontology*. A.A Balkema/Rotterdam.
17. Suriani. (2006). *Isolasi, Karakterisasi, dan Uji Bioaktivitas Metabolit Sekunder Spon Callyspongia sp.* Tesis tidak diterbitkan. Makassar: Program Pascasarjana Universitas Hasanuddin (*in Indonesian*).
18. Usman, H. (2005). *Isolasi, Karakterisasi, dan Uji Bioaktivitas Metabolit Sekunder dari Tumbuhan Cryptocarya costata*. Disertasi tidak diterbitkan. Makassar: Program Pascasarjana Universitas Hasanuddin. (*in Indonesian*).
19. Voodg, N. J. de, and Soest, R. W. M. van. (2002). *Indonesian Sponges of Genus Petrosia Vormaeer*, Amsterdam, University of Amsterdam.
20. Wiryowidagdo, S. and Moka. W. (1995). *Identifikasi dan Eksplorasi organisme laut sebagai Sumber Bahan Obat Baru di Kepulauan Supermonde*. (*in Indonesian*).
