Chemical investigation on Indonesian marine sponge *Mycale phyllophila*

Penelitian kandungan kimiawi spons *Mycale phyllophila* dari laut Indonesia

Triana Hertiani[1,2*)], Ru Angelie Edrada[1,3)], Rob W.M. van Soest [4)], Sudarsono[2)], Werner E.G., Müller [5)] and Peter Proksch [1,6)]

[1)] Institut für Pharmazeutische Biologie and Biotechnologie, Henrich-Heine Universität Düsseldorf, Universitätsstrasse 1 Geb. 26.23, 40225, Düsseldorf, Germany;

[2)] Pharmaceutical Biology Dept., Faculty of Pharmacy, Gadjah Mada University, Sekip Utara, 55281 Jogjakarta, Indonesia;

[3)] Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde, The John Arbuthnott Building, 27 Taylor Street, Glasgow G4 0NR, United Kingdom;

[4)] Institute for Systematics and Ecology, University of Amsterdam, P.O. Box 94766, 1090 GT Amsterdam, The Netherlands;

[5)] Institut für Physiologische Chemie und Pathobiochemie, Johannes-Guttenberg-Universität, Duesbergwerg 6, 55128 Mainz, Germany;

Abstract

Chemical investigation on marine sponge *Mycale phyllophila* collected from Bali, Indonesia has been performed. This study was aimed to isolate and to identify structures of the sponge secondary metabolites as well as to test their cytotoxic activity on mouse lymphoma cell line L5178Y.

The sponge extract was fractionated by liquid-liquid partition followed with a vacuum liquid chromatography method. Structure elucidation was performed on the basis of extensive spectroscopic analysis involving one and two dimensional NMR spectroscopy as well as mass spectrometry. Cytotoxicity was tested on mouse lymphoma cell line L5178Y by using the microculture tetrazolium (MTT) assay.

This study found a mixture of 5-pentadecyl-1*H*-pyrrole-2-carbaldehyde and (6'*E*)-5-(6'pentadecenyl)-1*H*-pyrrole-2-carbaldehyde as major constituents of the sponge extract. Those compounds were expected to be the active constituent to show growth inhibition of mouse lymphoma cell line (L5178Y) in vitro.

Key words : *Mycale phyllophila*, *cytotoxic agent,* NMR spectroscopy.

Abstrak

Telah dilakukan penelitian tentang komponen kimia spons laut *Mycale phyllophila* yang dikoleksi dari Bali, Indonesia. Penelitian ini bertujuan untuk mengisolasi dan mengidentifikasi struktur metabolit sekunder spons tersebut dan menguji aktivitas sitotoksisnya terhadap kultur sel limfoma tikus L5178Y.

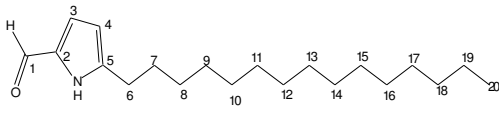
Ekstrak sponge difraksinasi menggunakan partisi cair-cair yang diteruskan dengan metode *vaccum liquid chromatography*. Elusidasi struktur dilakukan berdasarkan data spektroskopi resonansi magnetic inti 1D dan 2D serta spektrometri massa. Pengujian sitotoksisitas pada kultur sel limfoma tikus L5178Y dilakukan dengan menggunakan metode *microculture tetrazolium (MTT)*.

Penelitian ini mengungkap keberadaan 5-pentadesil-1*H*-pirol-2 karbaldehid dan (6'*E*)-5-(6'pentadesenil)-1*H*-pirol-2-karbaldehid sebagai kandungan utama ekstrak. Senyawa kimia tersebut diduga merupakan komponen aktif yang bertanggung jawab terhadap aktivitas penghambatan pertumbuhan sel limfoma tikus (L5178Y), *in vitro*.

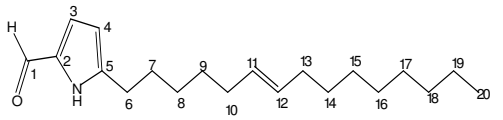
Kata kunci : *Mycale phyllophila*, senyawa sitotoksik, spektroskopi RMI.

Introduction

Marine sponges have been already proven to be a major producer of bioactive compounds from the sea, including those active as cytotoxic agents. As part of our research to explore marine sponges from different regions in Indonesia, several sponge extracts were screened for their cytotoxicity on mouse lymphoma cell line (L5178Y) by in vitro method. One active fraction detected was derived from *Mycale phyllophila*.



1



Sponges of the genus Mycale itself have been reported to be a rich source of a very chemically diverse group of bioactive natural compounds. The mycalamides (Perry *et al*., 1990); mycalolides (Fusetani *et al*., 1989); diterpenoid rotalins (Corriero *et al*., 1989); mycalisines (Kato *et al*., 1985); polybrominated C-15 acetogenins (Giordano *et al*., 1990) and brominated isocoumarins (Fusetani *et al*., 1991) are examples of different compounds isolated from this genus (Venkatesham *et al*., 2000). So far there were no reports on chemical constituents of *Mycale phyllophila*. Therefore it is interesting to do a chemical investigation of the active fraction to identify compounds which may be responsible for the activity.

Methodology

Sponge material

Sponge sample was collected from off shore Menjangan Island, Bali in October 2003 by means of SCUBA, and was directly preserved in ethanol after harvesting. A voucher specimen was deposited in the Zoological Museum in Amsterdam under reg. no. ZMAPOR18344.

Isolation method

The chemicals used in the detection and isolation methods were anisaldehyde (4-methoxybenzaldehyde), hydrochloric acid and concentrated sulphuric acid (all provided by Merck, Darmstadt, Germany); ammonium hydroxide, glacial acetic acid, (Fluka, Seelze, Germany); dimethylsulfoxide, trifluoroacetic acid 99 %, extra pure (Acros® organic, Geel, Belgium).

Solvents used for separation techniques were dichloromethane; ethyl acetate; *n*-hexane; methanol. These solvents were purchased from the Institute of Chemistry HHU Düsseldorf. They were distilled before using and special grade were used for spectroscopic measurements. Others solvent used were *n*-butanol and acetonitrile (Fluka, Seelze, Germany), and ethanol (technical grade).

Column chromatography was performed on silica gel (0.040-0.063 mm; Merck, Darmstadt, Germany). For HPLC analysis, samples were injected into an HPLC system equipped with a photodiode array detector (Dionex, München, Germany). Routine detection was at λ 235, 254, and 340 nm. The separation column (125 x 4 mm i.d.) was prefilled with Eurospher 100-C18, 5µm (Knauer, Berlin, Germany). Separation was achieved by applying a linear gradient from 90 % H_2O (pH 2.0) to 100 % MeOH over 40 min. TLC analysis was carried out using aluminium sheet precoated silica gel 60 F254 (Merck, Darmstadt, Germany).

Sponge tissue was separated from the supernatant (ethanol) and dried at room temperature. Dried sponge tissue (10.9 g) was ground and extracted exhaustively with methanol. After removing the solvent under reduced pressure, the resulting methanol extract was combined with the ethanol extract to obtain a total weight of 1.9 g. This crude extract was partitioned between ethyl acetate and water to obtain the ethyl acetate fraction (0.7 g). DAD-HPLC and LC/MS as well as TLC was used to guide the isolation procedure.

The ethyl acetate fraction was subjected to silica gel G60 vacuum liquid chromatography (VLC) by using mobile phase with increasing polarity from 100 % *n*-hexane to 100 % ethyl acetate followed by 100 % dichloromethane to 100 % MeOH, resulted 16 fractions. Fraction 3

(28 mg, 0.25 % of sponge dried weight) was later identified as a mixture of 1 and 2 at a ratio of 2:1.

Parallel to the bioactivity assay, chemical investigation was done by using TLC, DAD HPLC and LC/MS analyses in order to predict the chemical constituents. DAD HPLC used in this study was as follows. Program used: Chromeleon version 6.3; pump: Dionex P580A LPG; detector: Dionex, Photodiode Arrray Detector UVD 340S; autosampler: ASI-100T; column Thermostat: STH 585; column: Knauer, 5.0 mmID; packing material 5 µm; Eurospher-100 C-18. Column was initially equilibrated isocratically with 10:9 [methanol: acidic-nanopure water (adjusted to pH 2 with phosphoric acid)] in 5 minutes then the solvent was gradually changed to 100 % methanol in 30 minutes which was continued to 40 minutes with 100 % methanol. Injection volume was 20 µL. Compounds having chromophores were detected by UV-Vis diode array detector at 240 nm, 254 nm, 280 nm and 365 nm.

LC/MS equipment used in this study was the Finnigan LCQ-DECA (mass spectrometer); Agilent 1100 series for HPLC system (pump, detector and autosampler): column Knauer, 125 mm L, 2 mm ID, prepacked with Eurospher-100 C18 (5 µm) and with integrated pre-column. As standard analytical program, column was initially equilibrated isocratically with 10:9 [acetonitrile: acidic-nanopure water (containing 1 % formic acid)] in 5 minutes then the solvent was gradually changed to acetonitrile 100 % in 30 minutes which was continued to 10 minutes with 100 % acetonitrile. HPLC was run on a Eurospher C-18 reversed phase column.

Structure elucidation

^{1}H and ^{13}C NMR spectra were recorded at 300 °K on Bruker DPX 300, ARX 400, 500 or AVANCE DMX 600 NMR spectrometers using deuterated methanol or DMSO-d_6 (Eurisotop, France) as solvents. All 1D and 2D spectra were obtained using the standard Bruker software. EIMS was measured on Mass spectrometer type Finnegan MAT 8200.

Cytotoxicity assay

Cytotoxicity assay against L5178Y mouse lymphoma cells was determined by using

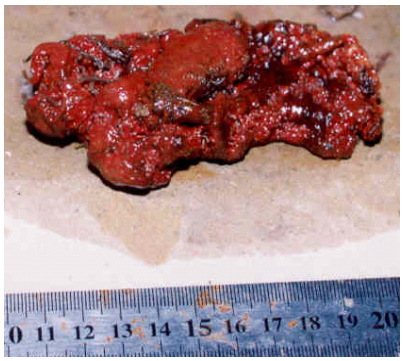


Fig. 1. *Mycale phyllophilla*

microculture tetrazolium (MTT) assay

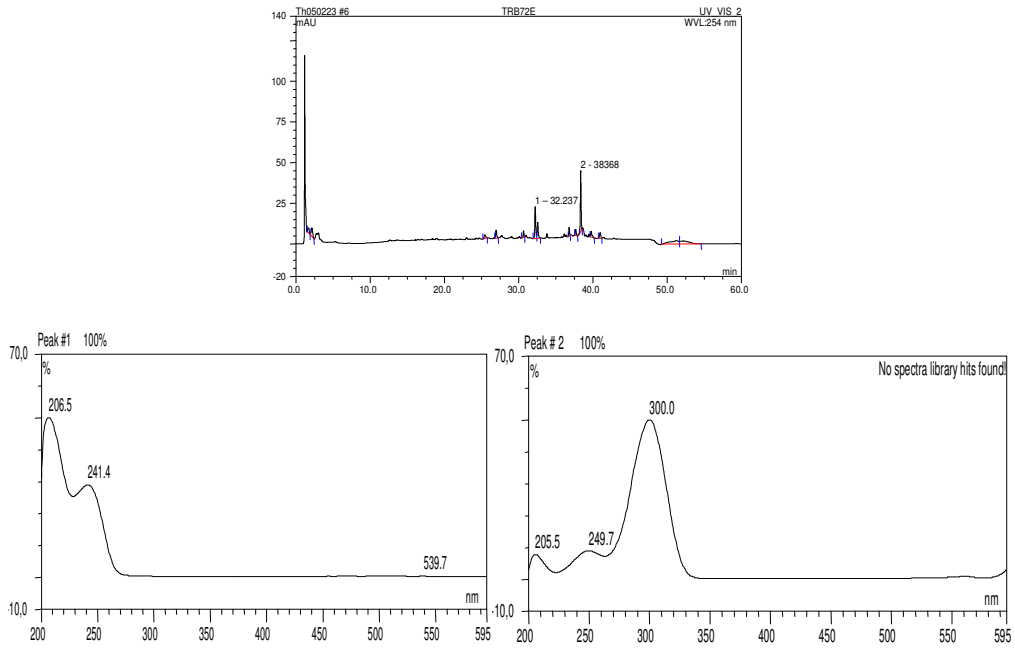


Fig. 2. DAD HPLC profile of *Mycale phylophilla* crude extract (above) and the UV absorption patterns of the major substances (below).

(Carmichael *et al*., 1987).

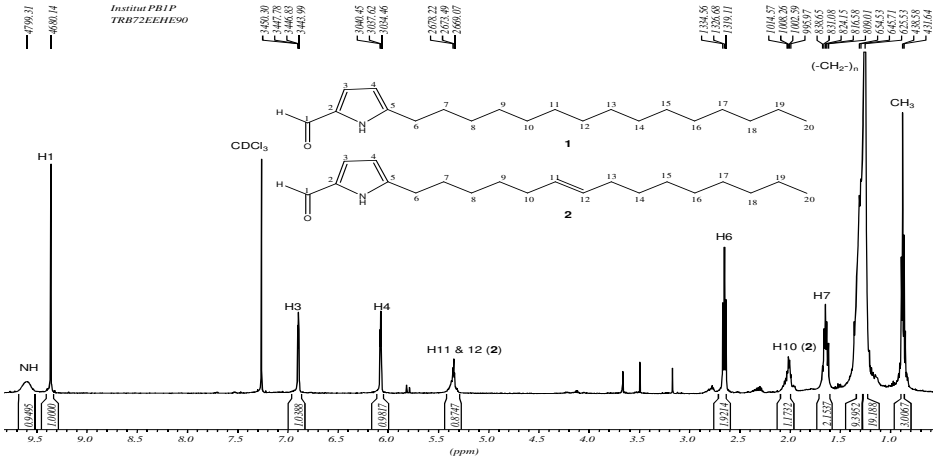


Fig. 3. ^{1}H-NMR spectrum of compounds 1 and 2 as a 2:1 mixture ($CDCl_3$, 500 MHz).

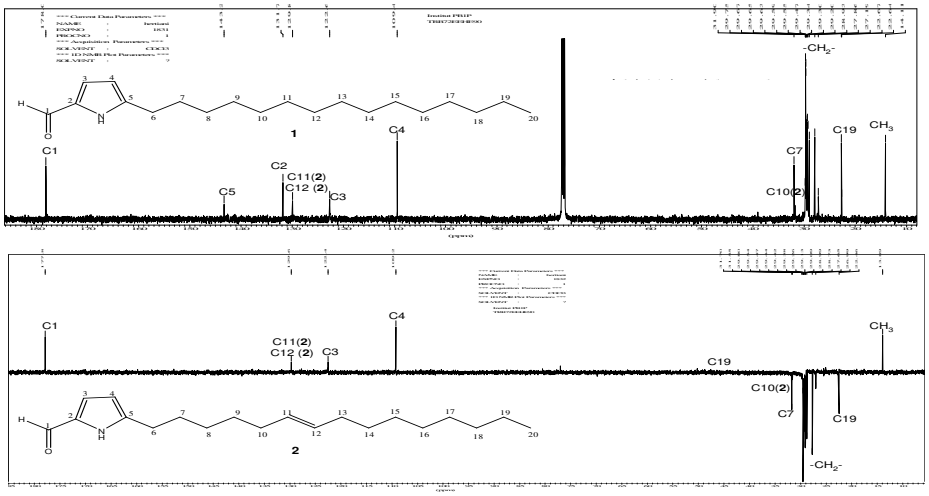


Fig. 4. ^{13}C - NMR (above) and DEPT (below) spectra of 1 and 2 as a 2:1 mixture ($CDCl_3$, 125 MHz).

Results and Discussion

DAD HPLC profile (Fig.2) showed that the crude extract (1 g) contained two major peaks. These peaks had delayed retention time and were eluted by 100 % MeOH showing that both were relatively non polar substances. The first peak was isolated and identified as a fatty acid derivative (not elucidated further) while the second peak was identified later as a mixture of 5-alkyl-2-pyrrole carbaldehyde derivatives (compounds 1 and 2).

Result of the cytotoxicity assay revealed that one of the VLC fractions yielded from solvent mixture of hexane-ethyl acetate 9:1, inhibited the growth of mouse lymphoma cell line (L5178Y) with IC_{50} 1.8 µg/mL. Further investigation on the fraction showed that it contained 1 and 2 in a 2:1 mixture, as the major constituents.

The optically inactive brown amorphous fraction was obtained at a yield of 28 mg (0.026 % of sponge dry weight). Its UV spectrum showed λ_{max} at 300.4 nm, which is a typical spectrum for pyrrole 2-

carbaldehyde (Stierle and Faulkner, 1980). EI-MS experiment revealed molecular ion peaks at m/z 305, 319, and 333, suggesting the presence of at least three 5-alkyl-2-pyrrole carbaldehydes which differ in the length of the alkyl chain.

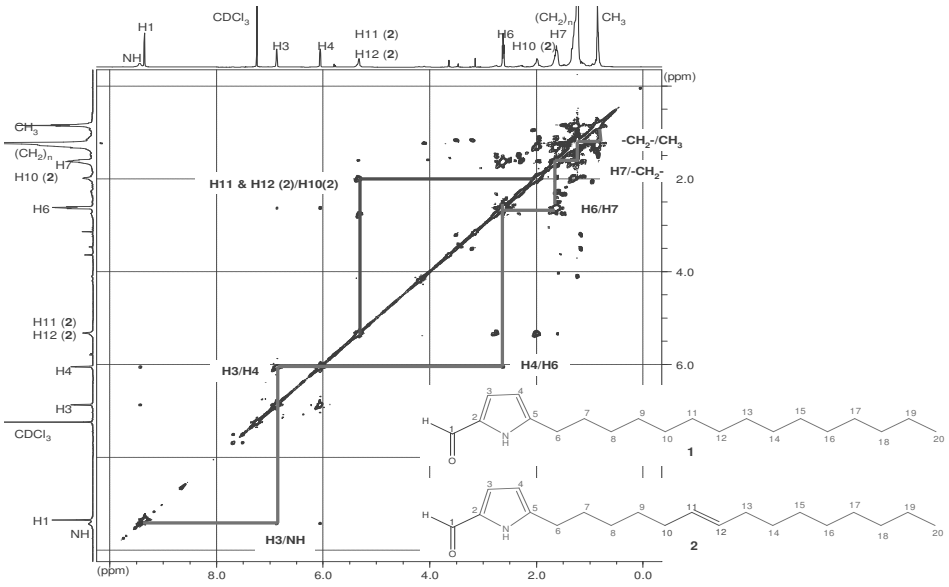


Fig. 5. ^{1}H-^{1}H COSY spectrum of 1 and 2 as a 2:1 mixture ($CDCl_3$).

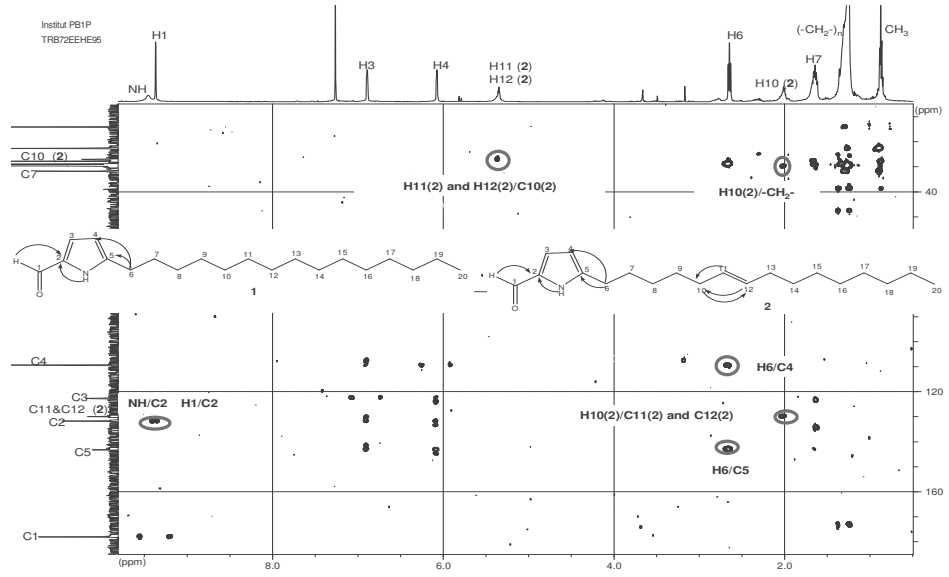


Fig. 6. ^{1}H–^{13}C HMBC spectrum of 1 and 2 as a 2:1 mixture ($CDCl_3$).

^{1}H-NMR spectrum (Fig.3) suggested a presence of a 5-alkyl-2-pyrrole-carboxaldehyde derivative. This proposed structure was exhibited by signals of aldehyde proton at δ_H 9.38 (1H, s); protons of the pyrrole ring at δ_H 6.87 (1H, dd, J = 3.2, 2.8 Hz) and at δ_H 6.05 (1H, dd, J = 3.2, 2.8 Hz) which coupled to a NH signal at δ_H 9.41; a deshielded methylene unit signal at δ_H 2.62 (2H, t, J = 7.0 Hz); and a signal for a primary methyl group at δ_H 0.86 (3H, t, J = 6.6 Hz). As a typical $J_{3,4}$ pyrrole

protons was detected as 3.2 Hz (Jones and Bean, 1977), the pyrrole ring of the compounds might be substituted at positions 2 and 5. This proposed sub structure was confirmed in the 2D-NMR analyses.

Table I. NMR data of 1 and 2, recorded in $CDCl_3$, at 500 MHz (^1H) and 125 MHz (^{13}C)

Atom No.	1			2		
	^1H		^{13}C	^1H		^{13}C
	δ	**Multiplicity, *J* in Hz**	**δ, DEPT**	**δ**	**Multiplicity, *J* in Hz**	**δ, DEPT**
NH	9.41	br s	-	9.41	br s	-
HC=O	9.38	s	178.1, CH	9.38	s	178.1, CH
3	6.87	dd, 3.2, 2.8	122.6, CH	6.87	dd, 3.2, 2.8	122.6, CH
4	6.05	dd, 3.2, 2.8	109.4, CH	6.05	dd, 3.2, 2.8	109.4, CH
11 & 12	-	-	-	5.30	m	129.9, CH
6	2.62	t, 7.8	22.6, CH_2	2.62	t, 7.8	22.6, CH_2
10	-	-	-	1.99	m	31.8, CH_2
7	1.62	m	31.9, CH_2	1.62	m	31.9, CH_2
$(-CH_2-)_n$	1.40-1.25	br s	29.6, CH_2 29.3, CH_2 29.2, CH_2 28.9, CH_2 27.9, CH_2	1.40 - 1.25	br s	29.6, CH_2 29.3, CH_2 29.2, CH_2 28.9, CH_2 27.9, CH_2

^{13}C-NMR spectrum (Fig.4) confirmed the aldehyde function by a presence of a low field sp^2 resonance at δ_C 178.1. The pyrrole ring carbons were shown by resonances at δ_C 143.2 (C); δ_C 131.8 (C); δ_C 122.5 (CH) and 109.2 (CH). The remaining ^{13}C NMR signals were assigned to the *n*-alkyl side chains (Stierle and Faulkner, 1980).

Beside the 5-alkyl-2-pyrrole carboxaldehyde signals, co-occurrence of a (6'*E*)-5-(6' penta decenyl)-1*H*-pyrrole-2-carbaldehyde deri-vative in the fraction was detected as shown by two olefinic proton signals overlapped at δ_H 5.30 (1H, t, J = 7.5 Hz) and a signal of the adjacent methylene unit at δ_H 2.0 (1H, m). ^{13}C NMR resonances at δ_C 129.9 (CH) and at δ_C 31.9 (CH_2) supported the structure. Considering that 2 showed integration signals as half of 1 suggested that 1 and 2 were present in a 2:1 mixture.

The olefinic bond position of 2 in the long chain was determined based on its mass spectrum fragmentation. A fragment ion at *m/z* 150 and *m/z* 204 resulted from an allylic cleavage of the side chain indicated that the olefinic bond was separated from the pyrrole ring by five methylene group (Stierle and Faulkner, 1980). Furthermore, geometry of the double bond was assigned as *E* based on chemical shifts of the allylic methylenes resonances at ^{13}C-NMR which appeared at δ_C 31.9 (CH_2) and 31.6 (CH_2) (Stierle and Faulkner, 1980).

^1H–^1H COSY spectrum exhibited two different spin systems (Fig. 5). The first spin system correlated the alkylene side chain to the pyrrole protons, while the second one correlated the olefinic protons to the rest of the alkylene side chain and to the pyrrole ring. HMBC data also secured this argument as shown by cross peaks correlated the olefinic protons to the methylene unit adjacent to it and then to the rest of the alkylene chain (Fig.6).

5-Alkyl 2-pyrrole carboxaldehyde deri-vates were first reported from *Laxosuberites* sp. (Stierle and Faulkner, 1980) and later, it was reported to be isolated together with 2 from *Mycale tenuispiculata* (Venkatesham *et al*., 2000). Their congeners, the mycalazols, a 5-alkyl-2-hydroxymethylpyrroles derivatives, as well as mycalazals were previously reported by Ortega and collaborators (1997, 2004) to exhibit cytotoxicity activity against several cell line e.g., P388 and SCHABEL mice lymphoma, A 549 human lung carcinoma, HT29 human colon carcinoma, MEL28 human melanoma cell lines and LNcaP, while several pyrrole-2-carbaldehydes possessing hydrocarbon side chains of different length and/or number of unsaturations at C-5 was reported to exhibit anti diabetic activities (Reddy and Dhananjaya, 2000).

Conclusion

This research revealed 1 and 2 as the major metabolites of sponge *Mycale phillophyla*. Fraction containing both compounds in 2:1 mixture exhibited mouse lymphoma cell line (L5178Y) growth inhibition with IC_{50} 1.8 μg/mL. Therefore, besides providing information about the chemical profile of *Mycale phillophyla* which has never been reported before, this research has also again proven marine environment as an interesting source for promising anti cancer agents. Further research should be conducted in the future in order to find the above compounds mechanism of action.

Acknowledgements

We wish to thank Mr. Juswono and Mr. Susilo Hadi (Gadjah Mada University, Indonesia) for collecting the sponge sample. This report is part of PhD. thesis of T. Hertiani at the University of Düsseldorf, Germany, 2007 (Supervisor: Prof. Dr. P. Proksch). A DAAD scholarship granted to T. Hertiani is gratefully acknowledged.

References

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D., and Mitchell, J.B., 1987, Evaluation of Tetrazolium-based semi automated colorimetric assay: Assessment of radio sensitivity, *Cancer Res*., 47, 943-946.

Corriero, G., Madaio, A., Mayol, L., Piccialli, V., and Sica, D., 1989, Rotalin A and B, two novel diterpene metabolites from the encrusting sponge *Mycale rotalis*, *Tetrahedron*, 45, 277-288.

Fusetani, N., Sugawara, T., Matsunaga, S., and Hirota, H., 1991, Bioactive marine metabolites. Part 35. Cytotoxic metabolites of the marine sponge *Mycale adhaerens* Lambe, *J. Org. Chem*., 56 (16), 4971-4974.

Fusetani, N., Yasumuro, K., Matsunaga, S., and Hashimoto, K., 1989, Mycalolides A-C, hybrid macrolides of ulapualides and halichondramide, from a sponge of the genus mycale, *Tetrahedron Lett*., 30(21), 2809-2812.

Giordano, F., Mayol, L., Notaro, G., Piccialli, V., and Sica, D., 1990, Structure and absolute configuration of two new polybrominated C16 acetogenins from the sponge *Mycale rotalis*, *J. Chem. Soc. Chem. Commun*., 22, 1559-1561.

Jones, R.A., and Bean, G.P., 1977, *The chemistry of pyrroles*, Academic Press, New York.

Kato,Y., Fusetani, N., Matsunaga, S., and Hashimoto, K., 1985, Bioactive marine metabolites IX. Mycalisines A and B, novel nucleosides which inhibit cell division of

fertilized starfish eggs, from the marine sponge *Mycale* sp. *Tetrahedron Lett*., 26(29), 3483-3486.

Ortega, Maria J.; Zubia, Eva; Sánchez, M. Carmen; Salvá, Javier; and Carballo, J. Luis, 2004, Structure and cytotoxicity of new metabolites from the sponge Mycale Cecilia, *Tetrahedron*, 60(11), 2517 – 2524.

Ortega, M.J., Zubia, E., Carballo, J.L., and Salvá, 1997, New cytotoxic metabolites from the sponge *Mycale micracanthoxea*, *Tetrahedron*, 53(1), 331 – 340.

Perry, N.B., Blunt, W.J., Munro, M.H.G., and Thompson, A.M., 1990, Antiviral and antitumor agents from a New Zealand sponge, *Mycale* sp. 2. Structures and conformations of mycalamides A and B, *J. Org. Chem*., 55(1), 223-227.

Reddy, G. Bala Show; and Dhananjaya, N., 2000, Chemical investigations of *Mycale mytilorum* and a study on toxicity and anti diabetic activity of 5-octadecylpyrrole-2-carboxaldehyde, *Bioorg. Med. Chem*., 8(1), 27–36.

Stierle, D.B., and Faulkner, D.J., 1980, Metabolites of the marine sponge *Laxosuberites* sp., *J. Org. Chem*., 45(24), 4980-4982.

Venkatesham, U., Rao, M.R., and Venkateswarlu, Y., 2000, New 5-alkylpyrrole-2-carboxaldehyde derivatives from the sponge *Mycale tenuisiculata*, *J. Nat. Prod*., 63(9) 1318-1320.

* Korespondensi: Dr. Triana Hertiani, S.Si., M.Si., Apt.
Bagian Biologi Farmasi,Fakultas Farmasi UGM
Jl. Sekip Utara Yogyakarta,55281
Email: hadna3ana@yahoo.com