Isolation and Molecular Structure Elucidation of Active Compound Produced by Marine Actinomycetes Isolate A32

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Abstract

The continuation of new antibiotics exploration becomes an important research program in the world for pharmaceutical and agricultural applications. Marine filamentous bacteria such as actinomycetes have been widely used as an important biological tool to generate a variety of new secondary metabolites, such as antibiotic. The aim of this study was to obtain identified active compound and determine its antimicrobial activity. Isolation, identification, and antimicrobial activity assay of active compound produced by marine actinomycetes isolate A32 had been conducted. Production of active compound using isolate actinomycetes A32 was conducted involving glucose, yeast, peptone medium. The fermentation was carried out at 30 ºC for 5 days. The broth of supernatant was extracted using ethyl acetate. Purification of active compound used chromatography column and eluted stepwise with the chloroform and methanol solvents. Antimicrobial activity was monitored using agar disc diffusion, and microbial test was conducted by analyzing the samples diameter of clear zone towards *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 66923, and *Candida albican* BIOMCC 00122. Results of isolation and purification of active compound produced by actinomycetes isolate A32 show that this compound has a molecular weight of 503.1 g/mol with molecular formula $C_{26}H_{37}N_3O_7$. Furthermore, this compound was suspected as Madumycin II after analysis of spectrum using ^{1}HNMR and COSY. The antimicrobial activity assay confirms that this active compound inhibited the growth of *Staphylococcus aureus* ATCC 25923 and *Candida albican* BIOMCC 00122.

Keywords: marine actinomycetes A32, Madumycin II, antimicrobial activity

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Introduction

Actinomycetes (order of Actinomytales) are the most widely distributed group of gram positive-bacteria in nature, which primarily inhabit the soil (Goodfellow & Williams, 1983). Approximately 70% of antibiotics in the world were originated from actinomycetes, mostly from *Streptomyces* and *Micromonospora* (Berdy, 2005; Goodfellow *et al.,*1988). Previously, researcher more focuses to explore the terrestrial actinomycetes. Currently, new antibiotic has been founded from marine actinomycetes (Fiedler *et al.,* 2005; Ghanem *et al.,* 2000; Lam, 2006)

Although the exploitation of marine actinomycetes as a source for discovery of novel secondary metabolites is at early stage, numerous novel metabolites have been isolated in past few years. For example, abyssomicin C is novel polycyclic polyketide antibiotic produced by a marine *Verrucosispora* strain (Riegdlinger *et al.,* 2004). Abyssomicin C possesses potent activity against Gram-positive bacteria, including clinical isolates of multiple-resistant. Another examples of newly developed metabolites are diazepinimicin and salinosporamide A. Diazepinomicin is a unique farnesylated dibenzodiazepinone produced by a *Micromonospora* strain (Charan *et al.,* 2004), which is having antibacterial, anti-inflammatory and antitumor activities. Salinosporamide A is a novel blactone-g-lactam isolated from a fermentation broth of a new obligate marine *actinomycetes,* namely *Salinispora tropical* (Feling *et al.,* 2003).

Indonesian archipelago covers more than 3.1 million km^2 of sea. It has a high level of biodiversity in microorganisms, plants, and

animals. Nevertheless this potential has not been exploited. Current exploration of actinomycetes in Indonesia is still limited to terrestrial actinomycetes. The objectives of this research were to isolate, purify, and identify the active compound produced by marine actinomycetes isolate A32.

Materials and Methods

Isolation of Actinomycetes. Actinomycetes A32 was isolated from sediment of marine site in Banten West Java. Sediment samples were processed in laboratory immediately after collection. The samples were suspended in sterilized water and serial dilutions of each sample were made. The samples were individually treated with acid and heat-shock. Acid treatment was conducted by acidifying the samples to pH 2 for 2 hours. Heat-shock treatment was conducted by heating the samples at 60 °C for 60 minutes (Pisano *et al*., 1986). The acid and heat-shock treated samples were inoculated onto starch agar medium (1 % w/v starch, 0.4 % w/v yeast extract, 0.2 % w/v peptone, natural seawater, and 1.6% w/v agar) and incubated for 4-8 weeks at room temperature. One hundred gram per mL of nalidixic acid, and 5 g/mL of rifampicin were added to reduce the number of unicellular bacteria (Pisano *et al*., 1986). The antifungal agent cycloheximide (100 g/mL) and 25 g/mL of nystatin were added to all isolation media. Actinomycetes colonies were recognized by the presence of branching, vegetative filaments and the formation of tough, leathery colonies that adhered to the agar surface. Morphologically diverse Actinomycetes were repeatedly transferred to the same media until pure cultures were obtained.

Liquid Culture and Extraction of Active Substance. An established slant of isolate was inoculated into a 250 mL flask containing 100 mL of vegetative medium (YEME medium) consisting of bacto peptone 5 g/L, yeast extract 3 g/L, malt extract 3 g/L, glucose 3 g/L, demineral water 25 mL, and sea water 75 mL. The pH value of the medium was adjusted to 7.6 before sterilization. The flask was incubated at 30 ºC for 2 days in an incubator-shaker. Fifty milliliter of the culture was transferred to 1,000 milliliter of the fermentation medium. Fermentation medium consisted of bacto peptone 15 g/L, yeast extract 3 g/L, Fe (III) citrate hydrate 0.3 g/L, demineralised water 250 mL/L, and sea water 750 mL/L (Nedialkova & Mariana, 2005). The pH value of the medium was adjusted to 7.6 before sterilization. The fermentation was carried out at 30 ºC for 5 days in incubator-shaker. The total volume of fermentation was 5 L, which is divided into 5 flasks. Extraction of active compound was conducted by centrifugation of culture broth at 14,000×g for 15 minutes. The broth supernatants were divided and extracted using ethyl acetate solvent. Supernatant and organic solvent were mixed thoroughly by shaking them in 2 L capacity separating funnel and allowed to stand for 30 minutes. Two layers were separated; the aqueous layer and the organic layer, which contained the solvent and the antimicrobial agent. The organic layer was concentrated by evaporation under vacuum to the least possible volume, after dehydration with anhydrous Na_2SO_4. The aqueous layer was re-extracted and the organic layer added above organic layer. The organic layer was a concentrated by repeated cycle of evaporation under vacuum. The dry extract of the supernatant was purified using silica gel column chromatography. A total of 0.33 g of dried extract was injected onto the column and then eluted stepwise with chloroform-methanol solvent system as follows: First the column was eluted with 100 % of 50 mL chloroform (Fraction 1). Then the process was repeated with reducing the chloroform by 10 % in each fraction while the methanol was increased by 10 % in each fraction, until the percentage of methanol was 100 %, and washing with 50 ml of 100 % methanol. The elution speed was 5 mL/min. Thirty fractions were collected (each of 20 mL) and then concentrated and dried for testing their antimicrobial activities. The active fraction (fraction number 11-20) were combined, dried and weighed. A total of 0.042 mg of the active fractions obtained from chromatography column was further purified by preparative HPLC.

Preparative HPLC. Purification by preparative HPLC was conducted using a Waters 2695 HPLC, photodiode array detector (PDA), and column puresil 5µ C18 4.6×150 mm. Ten thousand parts per million of 2 mL

active fraction solution in methanol was injected in preparative HPLC. The volume injected was 100 µL per injection under conditions of average pressure of 1,267 psi, and the flow rate was 1 mL/min where the mobile phase was 0-45 % methanol–water and time period was 25 minutes. (Kazakevich & Lobrutto, 2007). The active fractions obtained from preparative chromatography column were collected and dried. A total of 0,004 g of dried of pure active compound was identified using ESI-MS, ^{1}HNMR, and COSY.

Antimicrobial Activity Assay. Antimicrobial activity was monitored by agar diffusion paper-disc (6 mm) method. The discs were dripped with methanol solution of extract, dried and placed over the agar surface plates freshly inoculated with either *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 66923, and *Candida albicans* as test organisms. Suspensions of test organisms were adjusted to 10^{6} cfu/mL. The most potent isolates were noted for each test microorganism, based on the mean diameter of inhibition zones (Bonev *et al*., 2008).

Elucidation of Chemical Structure. Molecular weight and formula were determined using ESI-MS Spectrum ESI-LCMS Jeol JMS-SX102A, molecule structure elucidation of active compound were determined using ^{1}HNMR, and COSY (Varian UNITY INOVA 750).

Results and Discussion

Fermentation of actinomycetes isolate A32 was carried out for 5 days in yeast-peptone medium. Five liters of volume fermentation produced 5.01 g of dry biomass, and after methanol extraction as much as 1.25 g and 0.78 g of extract and supernatant extract (ethyl acetate phase) was collected, respectively. The concentrated supernatant was tested for its biological activity towards *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus Subtilis* ATCC 66923, and *Candida albican* BIOMCC 00122 as test organism. Antimicrobial activity of supernatant extract is shown in Table 1.

Table 1. Antimicrobial activity of supernatant extract

Sample concentration	Diameter of clear zone (mm)			
	S. aureus ATCC 25923	*B. subtilis* ATCC 66923	*E. coli* ATCC 25922	*C. albican* BIOMCC 00122
(100µg/disc)	30	-	-	32
(10µg/disc)	25	-	-	24
(1µg/disc)	10	-	-	20
Control (Rifampicin 1µg/disc)	21	15	15	-

Table 1 shows antimicrobial activity of supernatant extract towards 2 microorganisms, *Staphylococcus aureus* ATCC 25923 and *Candida albican* BIOMCC 00122. Compare to control (rifampicin), this extract has less inhibition activity due to 1 µg/disc of extract form 10mm diameter of clear zone, while control could form 21 mm diameter of clear zone. However, the tested extract was not pure or single compound.

A total of 0.33 g of dried extract was injected onto column chromatography for further purification. The active fraction of chromatography column numbers 10-20 were combined and dried. A total of 0.007 g of the active fraction of chromatography column were obtained. The bioassay test of column chromatography fraction is shown in Figure 1.

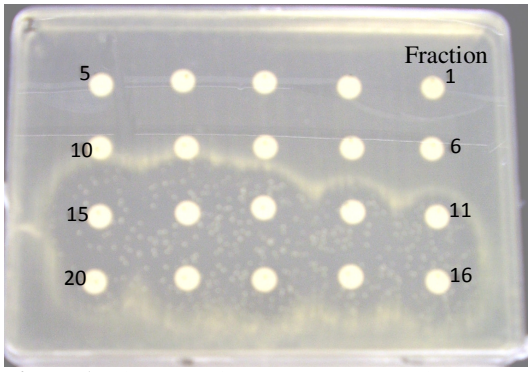


Figure 1. Bioassay of chromatography column fraction against bacterial test *S. aureus* ATCC 25923 as a microbial test.

A total of 0.007 g of chromatography column active fraction were purified using preparative HPLC to obtain a pure compound. A total of 0.003 g of a pure compound was obtained. The color of a pure compound was white powder. Bioassay test of pure compound against bacterial test *S. aureus* ATCC 25923 and *C. albican* BIOMCC 00122 is presented in Figure 2. Structure elucidation of pure active compound was determined using ESI-MS, H^1NMR, and COSY. ESI-MS spectra were obtained on Jeol JMS-SX102A. ESI-MS spectrum result shows that this active compound has molecular weight of 503.1 g/mol with molecular formula $C_{26}H_{37}N_3O_7$. This chemical characteristics are indicated by ESI-MS at m/z 504.1 $(M+H)^+$ as shown on Figure 3.

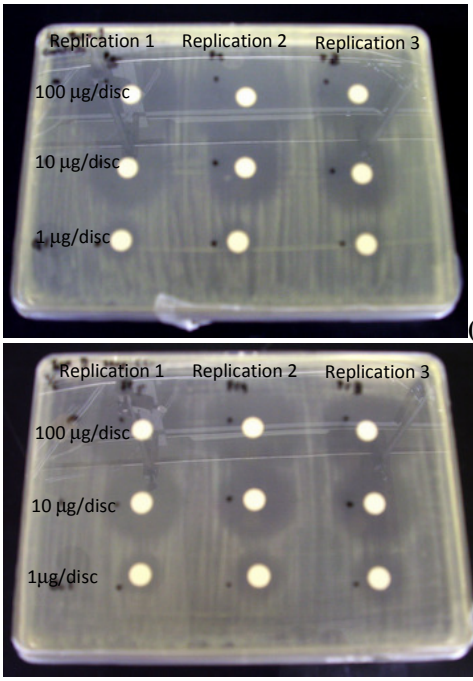


Figure 2. Bioassay of single active compound against bacterial test *S. aureus* ATCC 25923 (A) and *C. albican* BIOMCC 00122 (B).

High-resolution ^{1}HNMR spectrum were obtained on a Varian UNITY INOVA 750 (750 MHz) with tetramethylsilane (TMS) as internal standard in DMSO and give following data: δ_H: The proton appearing as a singlet at chemical shift δ 8.497 (a) is a proton ring Oxazoles, The doublet at δ 7.354 (c) was assigned to the amide proton of the alanine moiety, the same thing proton bonded to the amide shown in chemical shift δ 8.044 (b). Duplet proton peak at a chemical shift δ 3.879 (l) is methylene proton adjacent to the amide nitrogen. The higher chemical shift (downfield) occurs due to the nitrogen atom nearby. Proton peaks adjacent to the hydroxyl group shown in the chemical shift δ 3.811 (m). Proton multiplet peaks adjacent to two methyl groups are shown in chemical shift peak at δ1.895 (o). Chromatogram of ^{1}HNMR spectrums is shown on Figure 4 and comparison between ^{1}HNMR spectrums and those reported by Chamberlin and Chen (1976) is shown in Table 2.

Table 2. ^{1}H NMR spectrum at 750MHz in DMSO of Madumycin II and comparison with ^{1}H NMR spectrum at 270 MHz in DMSO by Chamberlin and Chen (1976).

Proton	Madumycin II		Reference (Chamberlin & Chen, 1976)	
	δ(ppm)	Multiple	δ(ppm)	Multiple
H-c	7.354	d	7.42	d
H-j	4.576	dq	4.75	dq
H-q	1.366	d	1.44	d
H-i	4.730	dd	4.75	dd
H-o	1.895	m	1.85	m
H-t	0.840	d	0.91	d
H-s	0.946	d	0.91	d
H-n	2.754	m	2.78	m
H-r	1.040	d	1.08	d
H-d	6.528	dd	6.60	dd
H-f	5.855	bd	5.85	bd
H-b	8.044	t	8.16	t
H-l	3.879	ddd	3.65	ddd
H-v	5.571	ddd	5.85	ddd
H-e	6.015	d	6.16	d
H-u	1.75	bs	1.73	bs
H-h	5.301	bd	5.48	bd
H-x	3.654	dt	3.75	dt
H-m	1.650	m	2.30	m
H-w	3.811	m	3,20	m
H-k	2.912	2dd	2.90	2dd
H-a	8.497	s	8.08	s

Identification of spins of proton which are coupled to each other was determined using COSY. COSY spectrum of pure active compounds produced by isolate A32 is shown in Figure 5.

Figure 5 shows that the proton (s) δ 0.946 is located adjacent to the proton (o) δ 1.895, and protons (o) is also adjacent to the proton (t) δ 0.840, proton (r) δ1.040 adjacent to the proton (n) δ 2.754, protons (j) δ 4.574 adjacent

to the proton (q) δ 1.366, proton (k) δ2.912 adjacent to the proton (w) δ 3.811, proton (w) δ 3.811 adjacent to the proton (m) δ 1.65, proton (i) δ4.730 adjacent to the proton (o) δ 1.895, proton (h) δ5.301 adjacent to the proton (m) δ 1.65, proton (d) δ 6.528 adjacent to the proton (n) δ 2,754, proton (v) δ5.572 adjacent to the proton (i) δ 3.879, proton (c) δ 7.314 adjacent to the proton (j) δ 4.576, proton (e) δ 6.015 adjacent to the proton (v) δ 5.571, and proton (d) δ 6.528 adjacent to the proton (f) δ 5.866.

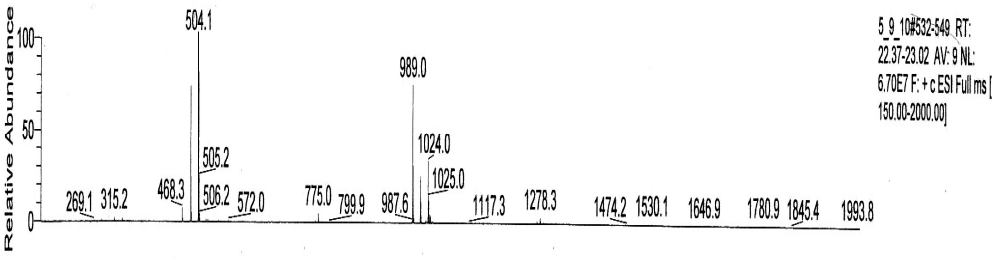


Figure 3. Spectrum ESI-MS m/z 504.1 $(M+H)^+$ of active compound

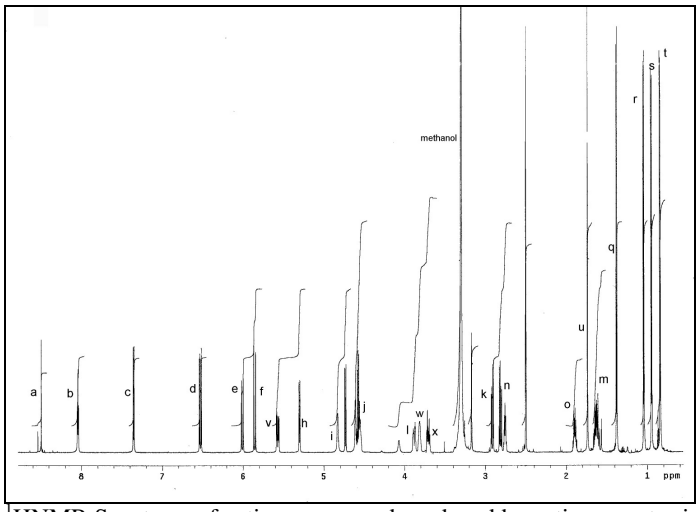


Figure 4. ^{1}HNMR Spectrum of active compound produced by actinomycetes isolate A32

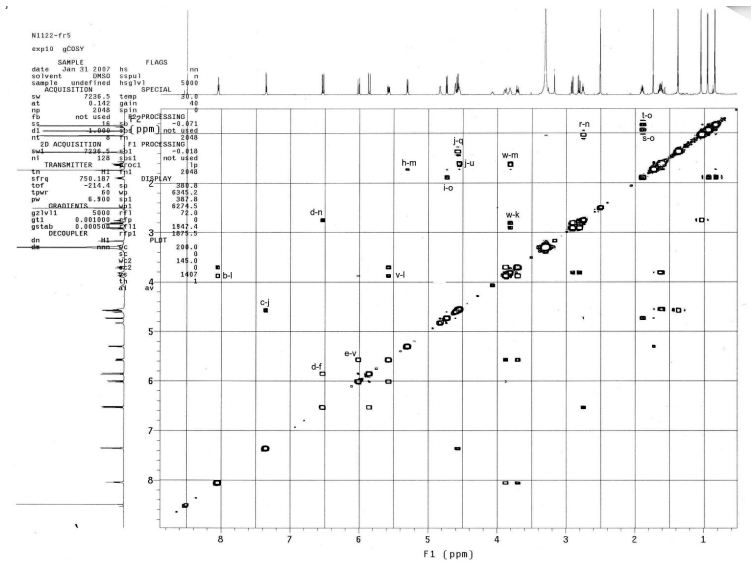
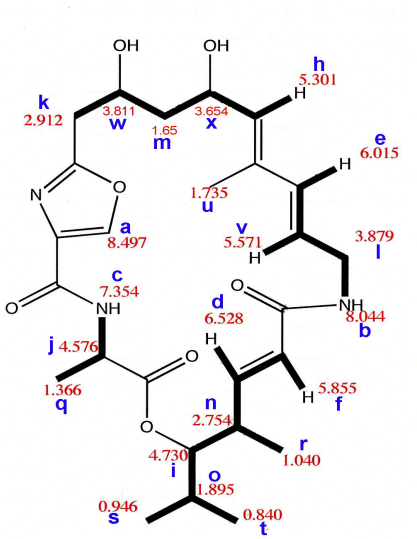


Figure 5. COSY spectrum of pure active compound produced by actinomycetes isolate A32

Results of interpretation ^{1}HNMR and COSY spectra show that the active compound produced by isolates A32 is Madumycin II, molecular formula $C_{26}H_{37}N_3O_7$. The molecular structure prediction of this compound is presented in Figure 6. This compound included in the group macrolactone virginiamycin (Cocito, 1979).



Explanation : number that presented show chemical shift of proton

Figure 6. Molecule structure prediction of active compound (Madumycin II) produced by actinomycetes isolate A32

Madumycin II had been discovered earlier by Chamberlin and Chen (1976). However, Madumycin II was produced by *Actinoplanes philippinensis* belonging to the phylum of Actinobacteria (Actinomycetes). Madumycin II also called A2315A, belong to the virginiamycin family of antibiotic (Martinelli *et al.,* 1978).

Conclusion

Results of this study can be concluded as follows; Marine actinomycetes isolate A32 was able to inhibit the growth of *Staphylococcus aureus* ATCC 25923 and *Candida albican* BIOMCC 00122. The results of isolation and purification of active compound produced by actinomycetes isolate A32 show that this compound has a molecular weight of 503.1 g/mol with molecular formula $C_{26}H_{37}N_3O_7$. Analysis of spectrum using ^{1}HNMR and COSY, this compound was suspected as Madumycin II.

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