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An Antibacterial Compound Isolated from Sponge-associated bacteria *Rhodobacteracea bacterium*

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Information about antimicrobial compound derived from *Rhodobacteraceae bacterium* is not reported yet. The goal of this study was to get chemical structure of substance that play an important role inhibits pathogenic bacteria growth. The investigation of antibacterial compounds derived from ethyl acetate extract of *Rhodobacteracea bacterium* from *Aaptos* sp., was carried out and gave rise a new antibiotic lead compound. Ethyl acetate extract was run on normal phase open column and reverse phase high performance liquid chromatographies. High Resolution LC-Mass-Mass, 1D and 2D NMR were used to structural analysis. In this study, reported that *Rhodobacteraceae bacterium* that was isolated from sponge *Aaptos* sp., contained a new antibacterial compound N-benzyl-2-methoxy-N-(2-[4-nonylphenoxy]ethanamine. This compound was moderately inhibit against phatogenic *Vibrio eltor*, *Bacillus subtilis* and *Staphylococcus aureus*.

Key words: *Rhodobacteraceae bacterium*, N-benzyl-2-methoxy-N-[2-(4-nonylphenoxy)] ethanamine, antibacterial

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INTRODUCTION

Secondary metabolite derived from microorganisms associated surface of marine invertebrate is a new target for finding drug lead compound (Armstrong *et al*., 2001; Penesyan *et al*., 2010, 2011). Sponges especially from tropical area have diverse bioactive metabolites (De Rosa *et al*., 2003). Sponges was the most place where the microorganisms harbored, more than 40% of sponge body contained microorganisms (Friedrich *et al*., 1999). Several bioactive compounds derived from sponge have structural similarities to metabolites of microbial origin, suggesting that microbial is the true or involve to the metabolite biosynthesis (Proksch *et al*., 2002). *Aaptos* sp., is the most producer of antibacterial compounds such as aaptamine and it's analog. Characterization of their potential antibiotic producer of associate microorganisms reveal to *Halomonas aquamarina*, α *Proteobacterium* and *Pseudoalteromonas luteviolacea* (Radjasa *et al*., 2007).

Rhodobacteraceae bacterium is marine α proteobacteria that reveal to potent antibacterial metabolite (Murniasih *et al*., 2013). This data would be incomplete if the compounds that play an important role to the biologically active were unknown. Separation using organic chemical technique and spectroscophycal analysis was conducted to identify the antibacterial compound containing in *Rhodobacteraceae bacterium*.

Information about antimicrobial compound derived from *Rhodobacteraceae bacterium* is not reported yet. The goal of this study was to get chemical structure of substance that play an important role inhibits pathogenic bacteria growth.

MATERIALS AND METHODS

Bacterial cultivation: *Rhodobacteraceae bacterium* Sp2.11strain was the same one that used in the previous study (Murniasih *et al*., 2013). This bacterium was isolated from *Aaptos* sp. collected from Barrang Lompo East Sulawesi Indonesia in June 2009. SYP medium that containing 5 g peptone and 1 g yeast extract per liter tropical seawater was used for culturing *Rhodobacteraceae bacterium*. After one-day cultivation, about 3 L of preculture solution of *Rhodobacteraceae bacterium* was inoculated into 27 L SYP medium. Incubation was done under aerobes condition at room temperature, pH 7.6. After 4 days cultivation, the cells were harvested and centrifuged at 6000 rpm for 15 min. Supernatant and pellet were separated and extracted using ethyl acetate and acetone.

Antimicrobial assay: Bacterial extract, results of open column fractions and purified compound were applied for antibacterial assay. Antimicrobial assay was done using pathogenic bacteria *Vibrio eltor*. Agar diffusion method was used for antibacterial assay (Bauer *et al*., 1966). Approximately 15 μL sample was dropped on antibiotic paper disc (6 mm) and dried the solvent. The pathogenic bacteria were inoculated in nutrient agar media than laid the paper disc on the agar media layer. Incubation was done at 30°C for over night. About 10 μg ampicillin was used as positive control. The clear zone around the paper disc indicate the bacterial growth inhibition.

Isolation of active compounds: Ethyl acetate extract of supernatant was evaporated and dried. The dried extract was applied in open silica gel column chromatography using the gradient system of n-hexane-dichloromethane-acetone. The potential antimicrobial fraction was continued for further separation using Perkin Elmer HPLC with UV detector. Sample was flashed into column ODS 10×250 mm ID with methanol. The purified active antimicrobial compound was characterized using LC-MS-MS (Liquid Chromatography-Mass Spectroscopy-Mass Spectroscopy), ^{1}H, ^{13}C and 2 D-NMR (Two dimentional Nuclear Magnetic Resonance) such as COSY (Correlation SpectroscopY), HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation).

RESULTS

Approximately 4.116 g supernatant extract was separated using silica open column into 11 fractions. Three fractions F2, F5 and F6 were active against *Vibrio eltor*. Further separation of F2 using HPLC resulted an active substance (F2.1). Figure 1 is the HPLC chromatogram of F2 fraction.

There are six peaks contained in fraction 2, peak 1, 2, 3 and 6 (F2.1, F2.2, F2.3 and F2.6) showed moderate active against *Vibrio eltor*. The diameter inhibition of compound F2.1 against *Vibrio eltor* was 8.2 mm, *Bacillus subtilis* was 7.5 mm and *Staphylococcus aureus* was 10.6 mm. Approximately 2.6 mg of fraction F2.1 was characterized for chemical structural analysis. Fraction F2.2 and F2.3 were continued for further purification.

NMR spectra data: Table 1 was the ^{1}H, ^{13}C, COSY, HMQC and HMBC data of isolated F2.1 compound. Considers to proton NMR data there are 16 proton environment. The ^{13}C data indicated that there were 23 carbons environment. COSY and HMBC correlation was discribed in Fig. 2.

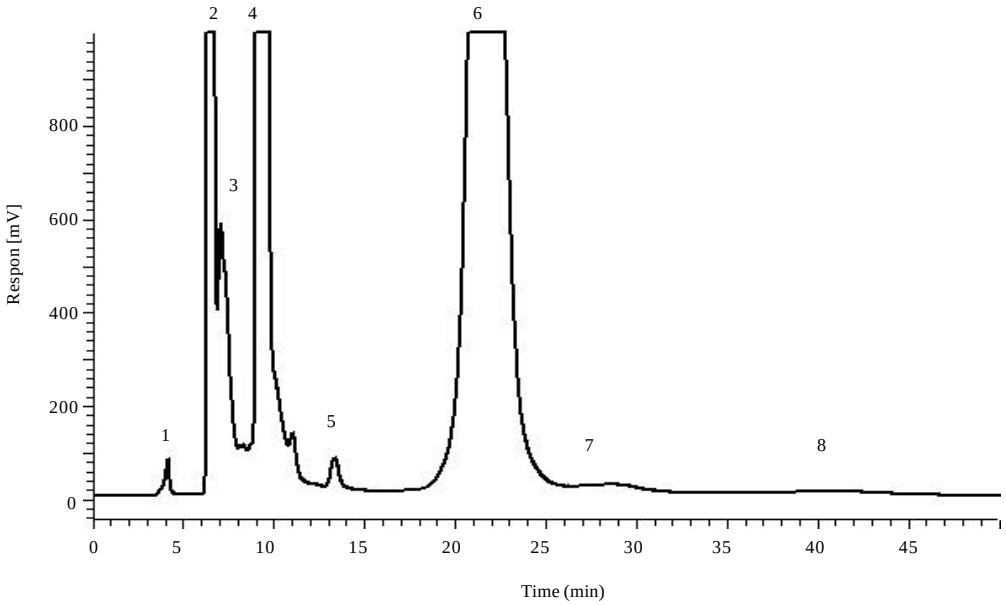

Fig. 1: HPLC chromatogram of fraction 2 in 100% methanol

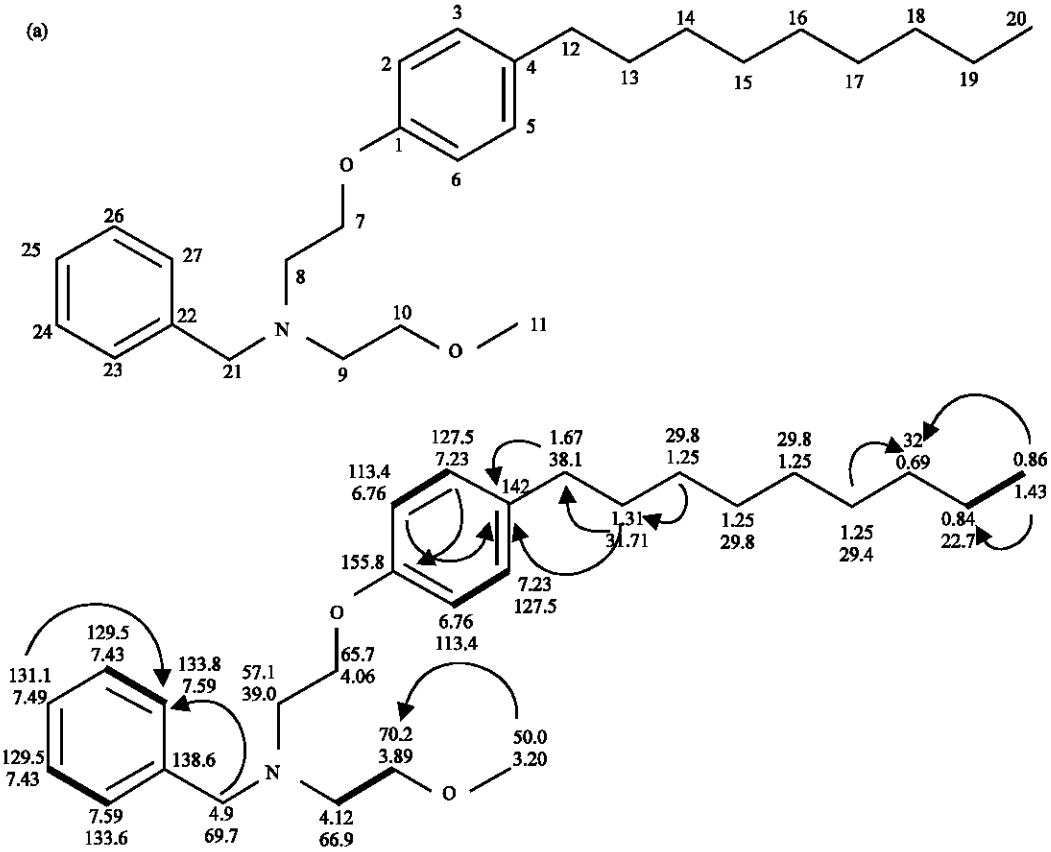

Fig. 2: Structure and its HMBC and COSYcorrelation of F2.1 compound

Table 1: NMR spectroscopy data (500 MHz, $CDCl_3$) of F 2.1 compound

Position	$\delta_{C,type}$	δ_H (J in Hz)	H-H COSY	HMBC H→C
1	155.8, C	-		
2 and 6	113.1,CH	6.76, d(8.4)	7.23	C4
3 and 5	127.4,CH	7.23*	6.74	C1
4	142, C	-		
7	65.7,CH_2	4.06		
8	57.1, CH_2	3.9, s		
9	66.9, CH_2	4.12, s	3.89	
10	70.2, CH_2	3.89		
11	50.9, CH_3	3.29		C10
12	38.1, CH2	1.67		C4
13	31.7, CH_2	1.3		C4, C12
14-16	29.8, CH_2	1.25		C18, C13
17	29.4, CH_2	1.25		
18	32, CH_2	0.69		
19	22.7, CH_2	0.84		
20	14.3, CH_3	0.86, t(6.49)	0.84	C19, C18
21	69.7, CH_2	4.9		C23
22	138.6, C			
23 and 27	133.8, CH	7.59, d(7.8)	7.43	C25
24 and 26	129.5, CH	7.43, t(7.1)		
25	131.1, CH	7.49, t(7.2)		C23

MHz: Mega hertz, $CDCl_3$: Deutero chloroform, •: Overlapped with H_2O

LC-MS-MS data: High Resolution Mass Spectrum data showed that compound F2.1 has molecular ion $(M+H)^+$ = 412.3231 or this compound has molecular weight 411.3231 g moL^{-1}. Corresponding molecular formula was $C_{27}H_{41}NO_2$. LC-MS-MS fragmentations showed m/z: 412.3 (16.25%), 381.2 (1.25%), 320.2 (100%), 300.2 (7.5%), 233.2 (3.75%), 208.1 (10%), 177.1 (1%), 149.1 (3.50%), 135.1 (7.5%), 118.1 (13.75%). Fragmentation pattern was proposed in Fig. 3. Three patterns of molecular fragmentation were proposed in this data.

DISCUSSION

Proton NMR data consisted two methyls group, one was attached to aliphatic chain at δ 0.86 ppm (triplet, J = 6.49 Hz) another one as methoxy group (-OCH_3) at δ 3.29 ppm (singlet). Proton methylenes (-CH_2) devided into 2 groups, one was aliphatic chain at δ 0.84, 0.69, 1.25, 1.3 and 1.6 ppm. Second one was methylenes that attached to nitrogen amine (N-) and oxigen as ether (-OR) at δ 3.90; 3.89; 4.03; 4.11 and 4.91 ppm. Proton aromatic shown at δ 6.74 ppm (doublet, J = 8, 4 Hz); 7.43 ppm (triplet, J = 7, 1 Hz), 7.49 ppm (triplet, J = 7, 2 Hz) and 7.59 ppm (doublet, J =7.8 Hz). HMQC experiment data was shown in Table 1.

^{13}C. NMR: Approximately 7 aliphatic carbons were at δ: 14.3; 22.9; 29.4; 29.8; 31.7; 32 and 38.1 ppm. Chemical shift at δ: 50.9; 57.1; 65.57; 66.8; 69.7 and 70.2 ppm) were carbon that attached to more electronegative atoms, such as oxygen and nitrogen. Aromatic carbon were identified at δ 113.4; 127.4; 129.5; 131.11; 133.8; 138.6; 142 and 155.8 ppm.

DEPT: (Distortion Enhancement by Polarization Transfer) data showed that C alkanes number 14-19 were CH_2 and C number 20 (δ 14,3 ppm) was CH_3 or CH. The DEPT signal for carbon that attached with C-OR or C-NR2 showed that most of them were methylenes, except C number 11 (δ 50.9 ppm) that has methyl or methine group. DEPT signals for aromatic carbon group indicated that all of them were CH signals.

COSY (correlation spectroscopy): From H-H COSY experiment data was shown in Fig 2. In this data, there is H-H aromatic coupling between proton number 2 or 6 at (δ 6.74 ppm) with proton number 3 or 5 at (δ 7.23 ppm). Another COSY correlation in aromatic chain was between proton number 23 or 27 (δ 7.59 ppm) with proton number 24 or 26 at (δ 7.43 ppm). Correlation H-H COSYin alkanes was occurred between proton number 19 at (δ 0.86 ppm) with proton number 20 at (δ 0.84 ppm) and proton number 9 at (δ 4.11 ppm) with H number 10 at (δ 3.89 ppm). H-C HMBC correlation was shown at Fig. 2.

Mass spectroscopy analysis (Fig. 3) showed that there are 3 pathways of fragmentation: First pathway is parent peak m/z 412.3 lost of $^{\cdot}OCH_2$ and led to m/z 381.2. Second pathway is parent peak that lost a benzyl ($PhCH_2$) group and led to base peak m/z: 320.3 continued to m/z 233.2 (3.75%); 177.1 (1%); 149.1 (3.50%) and 135.1%. The third is parent peak lost an acyclic carbon chain $CH_3(CH_2)6CH_2$- become the molecule ion with m/z : 208.1 (10%), continued to 118.1 (13.75%).

Compilation of NMR and LC-MS-MS data could be predicted that the isolated compound: F2.1 was N-benzyl-2-methoxy-N-(2-(4-nonylphenoxy) ethanamine. This new compound was 92% similar with Ph-CH_2-N((CH_3)2-CH_2-CH_2-O-Ph-(CH_2)11-Me (Buchi *et al.*, 1951). The stucture of antibiotic metabolite in host organisms (*Aaptos* sp.) compared to this structure didn't showed a similarity correlation structure. Similar study of Sponge-associated α Proteobacteria reported that cis-vaccenic acid from *Rhodopseudomonas capsulata* (Hirotani *et al.*, 1991; Chandrasekaran and Ashok Kumar, 2011) and topodithietic acid (TDA) acid from *Pseudovibrio* sp., D323 (Penesyan *et al.*, 2011) were inhibitting phatogenic bacterial growth.

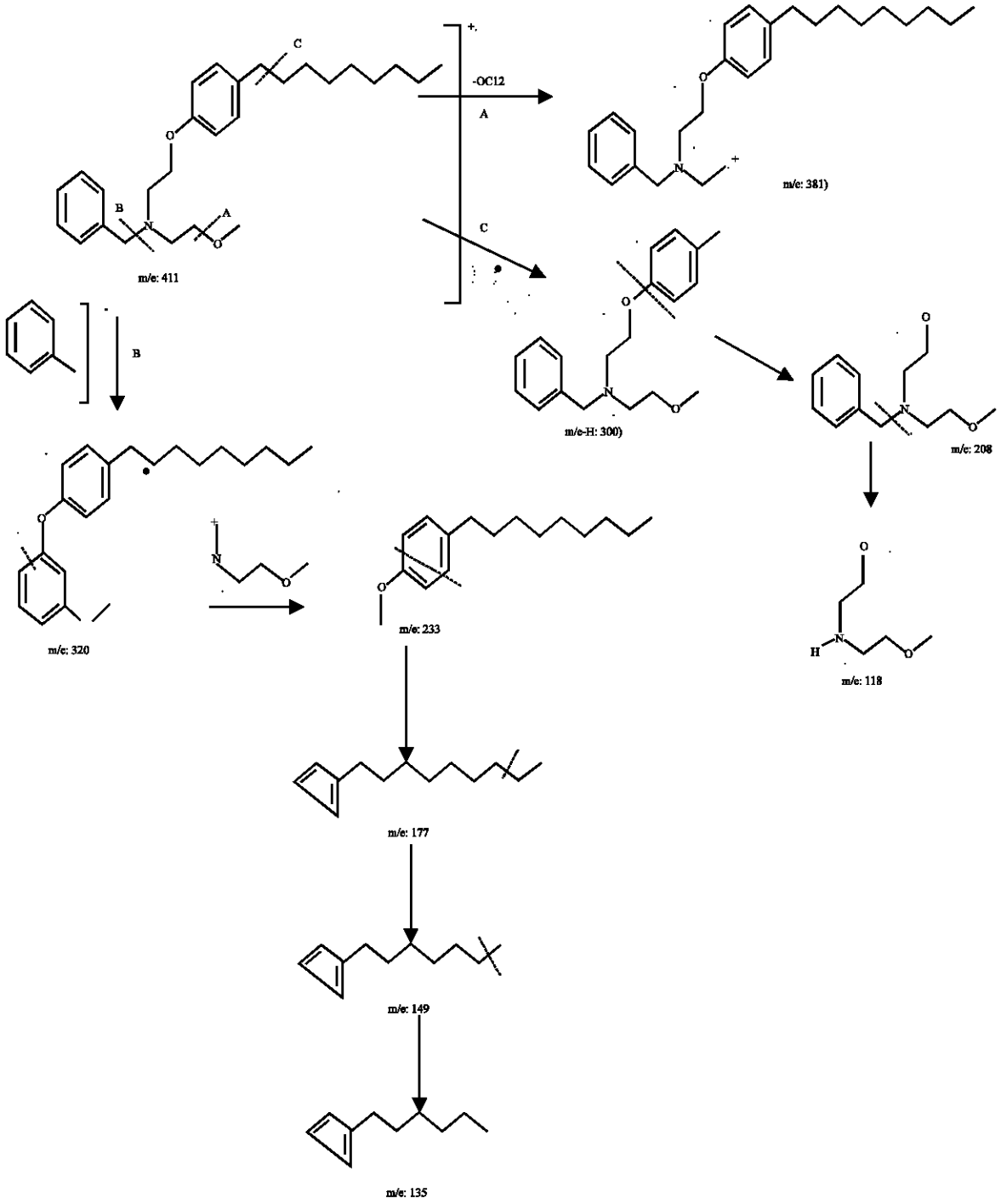

Fig. 3: Suggestion of fragmentation pattern of isolated compound F2.1

CONCLUSION

In this study reported that *Rhodobacteraceae bacterium* that was isolated from sponge *Aaptos* sp., contained antibacterial metabolite. The chemical structural analysis of isolated compound indicated a new amina compound N-benzyl-2-methoxy-N-(2-(4- nonylphenoxy) ethanamine. This compound was moderately inhibit against phatogenic *Vibrio eltor*, *Bacillus subtilis* and *Staphylococcus aureus*.

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