Investigation of Anti-dengue Virus (DeNV) Molecules from a Marine-Derived Bacterium

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Abstract. Dengue is a viral infection spread by mosquitos that occur in tropical and subtropical areas in the world. Presently, there is no directly acting drug against Dengue Virus (DeNV) for clinical use. Therefore, developing new medicines is required to control the severity of outbreaks. In this proceeding, several bioactive compounds **1**–**3** isolated from marine-derived bacterium will be reported. Compounds **1**–**3** were analyzed by several spectrophotometers and concluded as a cyclo(L-Leu-L-pro) (**1**), nocardamine (**2**) and cycloheximide (**3**). Compound **1** showed anti-DeNV activity with IC_{50} 0.38 mg/ml, CC_{50} 6.03 mg/ml, and SI 15.75. Compound **2** exhibited activity against DeNV with $IC_{50} > 10$ mg/ml, CC_{50} 7.96 mg/ml, and SI < 0.80. Compound **3** elicited an excellent activity with IC_{50} 0.02 mg/ml, CC_{50} 0.28 mg/ml, and SI 13.70.

Keywords: dengue · virus · bioactive compound · marine bacteria

1 Introduction

Dengue is one of the most serious arthropod-borne flavivirus infections in human beings named dengue virus (DeNV) and is transmitted by the *Aedes aegypti* mosquito [1]. There are four distinct stereotypes known as DeNV-1, 2, 3, and 4, and belonging to the genus *Flavivirus* in the Flaviviridae family [2]. The DeNV-2 has been known to be more lethal than other stereotypes. However, DeNV-1 or DeNV-3 resulting a more dangerous disease than infection by DeNV-2 or DeNV-4 [3]. Approximately 975 million of 2.5 billion people in tropical and sub-tropical countries, such as in Southeast Asia, the Pacific, and the Americas, are estimated at risk of infection with DeNV. The number of patients increases each year, reaching about 50 million dengue infections and about 500,000 individuals hospitalized with dengue hemorrhagic fever, mainly in Southeast Asia, the Pacific, and the Americas [4]. Globalization, urbanization, climate change, and jet travel are helping the virus move into more temperate zones. The Philippines alone records an average of 100,000 dengue virus infections every year resulting in hundreds of deaths. In 2010–2014, the Philippines Department of Health recorded 108,263 dengue

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cases nationwide and more than 300 deaths. A recent review in the Philippines showed that the incidence rate of dengue was highest among children of 5–14 years, with over 80% mortality occurring among those less than 20 years old [5]. While in Indonesia, in 2022, the Health Ministry stated that in the last two years during the pandemic, dengue fever cases had decreased. It might be caused by the discipline of the people in cleaning and healthy living behavior during the COVID-19 pandemic. There were 45,387 dengue cases were reported from 449 districts and cities spread across 34 provinces. The Indonesian government was also expecting that by 2030 Indonesia will be a zero-percent mortality rate. Therefore, research on anti-DeNV is emerging to be conducted to realize the target.

To date, there are many reports on anti-DeNV from plant-based natural medicinal products. World Health Organization (WHO) estimated that 80% of the world's population fulfills their healthcare needs from phytomedicinal sources [6]. Several traditional plants that have been used as traditional medicine systems to combat dengue diseases are *Carica papaya* and *Euphorbia hirta* [7]. Yet, the scientific reasons and the mechanism of action are lacking.

Presently, there is no directly acting drug against dengue for clinical use. Therefore, the development of new medicines is required to control the severity of outbreaks. Hence, drugs against DeNV are emerging to combat dengue diseases which is still a big problem, especially for tropical and subtropical countries. One of the potential sources of bioactive compounds which may have activity against DeNV is marine organisms and their symbionts, such as marine-derived bacteria [8].

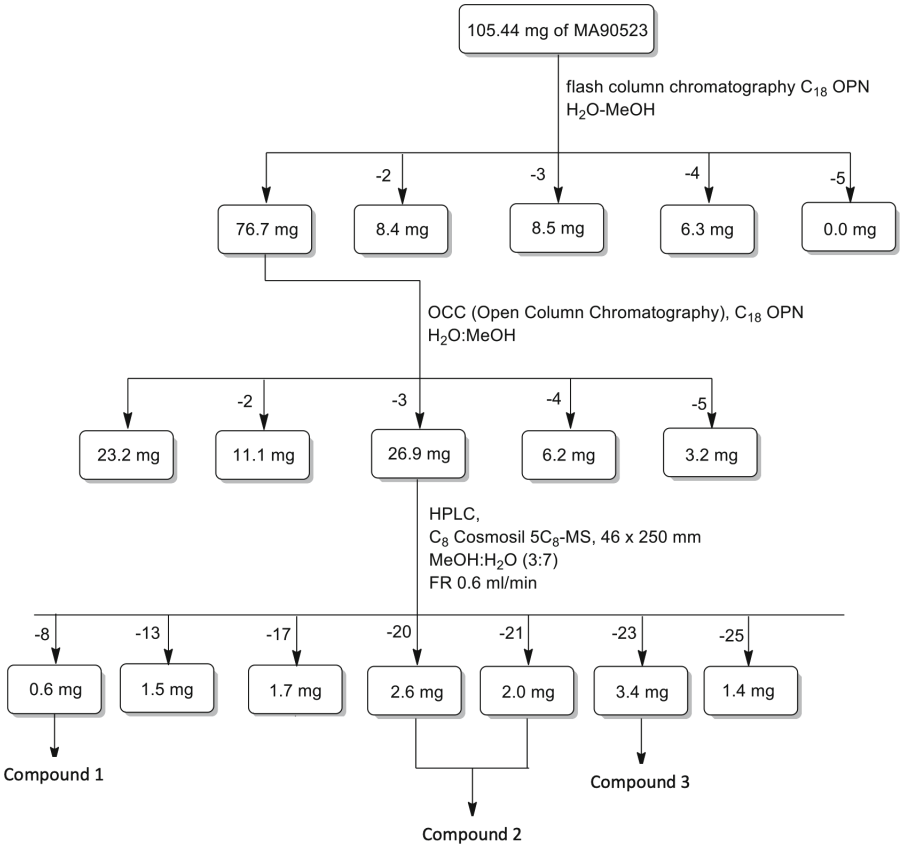
In this report, we examined a bacterial ferment to find new compounds possessing anti-DeNV properties. The bacterial extract (MA90523) was provided by OP BIO, Okinawa, Japan. After a second screening, some of them showed promising inhibition against the dengue virus. Herein, we will report bioactive compounds isolated from marine-derived bacteria having properties of anti-DeNV.

2 Materials and Methods

All commercially available reagents were used without further purification. HPLC purification was conducted on the Nacalai tesque reversed-phase column ($5C_8$-MS, 46 × 250 mm) (Kyoto, Japan) and two solvent systems, A: H_2O, and B: MeOH, were applied. The HPLC was equipped with a Shimadzu LC-10AD pump (Kyoto, Japan), a Shimadzu SPD-10A UV detector, a Shodex RI-101 refractive index detector (Tokyo, Japan). NMR spectra were recorded on a Bruker AVANCE III 500 Spectrometer (Billerica, MA, USA). ESI-mass spectra including high-resolution mass spectra (HRMS) were recorded on Jeol JMS-T 100LP mass spectrometer (Tokyo, Japan). Specific rotation was observed on a Jasco P-1010 polarimeter (Tokyo, Japan).

2.1 Isolation of Bioactive Compounds 1–3

The bioactive compounds were isolated based on bioassay-guided isolation (Scheme 1). A 105.44 mg of marine-derived bacteria extract was separated on a flash column chromatography C_{18} OPN and gradient eluted by MeOH:H_2O mixtures to give 5 subfractions.



Scheme 1. Flowchart purification of compounds **1**–**3**.

The first fraction showed strong activity against DeNV assay and was further purified on an open column chromatography C_{18} OPN and eluted with MeOH:H_2O mixtures to give 5 subfractions. The third fraction showed excellent activity against DeNV and purified by HPLC reversed-phased column by using C_8 Cosmosil 5C_8-MS, 46 × 250 mm, with solvent MeOH:H_2O (3:7) and flowrate 0.6 mL/min. The eighth, twenty-first, and twenty-third fractions were then identified as compound **1** (0.6 mg), compound **2** (4.6 mg), and compound **3** (3.4 mg).

2.2 Cell-Based Assay for Evaluation of Anti-dengue Virus Activity

Baby hamster kidney fibroblast (BHK-21) cells were seeded in a 96-well plate at 3.0×10^4 cells/well, and cultured at 37°C in a 5% CO_2 incubator for 24 h. The medium of the plate in which the cells were seeded was removed with an aspirator, and the diluted drug was added to the corresponding well at a concentration of 100 μL/well from the lower concentration. MEM medium was added to the wells of uninfected cells at 100 μL/well, and the dengue virus (serotype 2) solution was added to the wells of infected cells at

100 μL/well (1,000 TCID50/ml). The plate was placed in a 5% CO_2 incubator at 37°C and cultured for 72 h. The medium was removed with an aspirator, 200 μL/well of 70% ethanol was added, and the mixture was left at room temperature for 5 min to fix the cells. After fixation, ethanol was removed by decantation and the remaining viable cells were stained with crystal violet. After staining, the staining solution was removed by decantation, and the plate was washed with tap water. After drying at room temperature, the absorbance at a wavelength of 560 nm was measured with a microplate reader. IC_{50} (50% virus inhibitory concentration) and CC_{50} (50% cell toxicity concentration) were calculated by GraphPad Prism (Prism v. 5.01, GraphPad Software).

3 Results

3.1 Isolation and Elucidation Structure

The extract of the fermented cultured bacteria was purified on the basis of bioassay-guided isolation with consecutive reversed-phase column chromatography, C_{18}-OPN and C_8 to give three bioactive compounds **1** (0.6 mg), **2** (4.6 mg), and **3** (3.4 mg). The isolated bioactive compounds (**1**–**3**) were then determined their structures based on spectroscopy analysis and 1D-2D NMR analysis.

Compound **1** (Fig. 1) was isolated as a white powder (0.6 mg). The ^1H (Fig. 2) and ^{13}C (Fig. 3) data were shown in Table 1. The ^1H NMR spectrum of compound **1** showed a characteristic signal at δ 5.65 (brs) indicating an amide which was supported by the signal at δ_C 170.0 (s). Two *N*-methine protons were observed at δ 4.01 (dd, $J = 3.6$, 9.6 Hz, H-3) and 4.10 (dd, $J = 8.2$, 8.2 Hz, H-6). Signals at δ_H 0.95 (d, $J = 6.6$ Hz) and 1.00 (d, $J = 6.6$ Hz) were identified as methyls. In addition, DEPT data showed signals at δ 23.3, 28.2, 38.6, and 59.0 as methylenes. From this observed data, compound **1** was elucidated as a diketopiperazine. After database search and reference, the absolute configuration of **1** was determined by comparison of its specific rotation value ($[\alpha]_D$ -22 (c 0.04, MeOH)) with reported cyclo(L-leu-L-pro) ($[\alpha]_D$ -139.4 (c 0.16, EtOH)) and cyclo(D-leu-D-pro) ($[\alpha]_D$ + 128 (c 0.11, EtOH) [9–11]. Although the values were not exactly the same, compound **1** showed the same sign as cyclo(L-Leu-L-Pro) [10]. Therefore, the absolute configuration of **1** was elucidated as depicted.

Compound **2** was isolated as a white powder. The ESI-MS data of **2** showed a protonated ion at *m/z* 601.35663 $[M + H]^+$ (Δ − 0.80 ppm) indicating the chemical formula $C_{27}H_{49}N_6O_9$ with seven degrees of unsaturation.

The NMR spectra (Figs. 5 and 6) of compound **2** showed simple and characteristic signals of two amide carbonyls at δ 172.0, 172.5, an *N*-hydroxyl signal at δ_H 9.60 (brs),

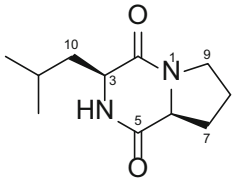


Fig. 1. The chemical structure of compound **1**.

Table 1. ^{1}H and ^{13}C data of reference and compound **13** ($CDCl_3$, 500 MHz)

No.	Reference [9]			**13**	
	δ_C		δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)
1	N				
2	166.1	s		nd	
3	53.4	d	4.00 (dd, 9.5, 3.9)	53.4	4.01 (dd, 9.6, 3.6)
4	NH		5.67 (s)		5.65 (s)
5	170.1	s		170.0	
6	59.0	d	4.10 (dd, 8.1, 8.1)	59.0	4.10 (dd, 8.2, 8.2)
7	28.1	t	2.2–1.8 (m)	28.1	2.38–1.87 (m)
8	23.3	t		23.3	
9	45.5	t	3.55 (m)	45.5	3.56 (m)
10	38.6	t	1.70 (m)	38.7	1.71 (m)
			1.50 (m)		1.52 (m)
11	24.7	d	2.34 (m)	24.8	2.34 (m)
12	22.7	q	0.99 (d, 6.7)	22.7	1.00 (d, 6.6)
13	21.2	q	0.94 (d, 6.5)	21.2	0.95 (d, 6.6)

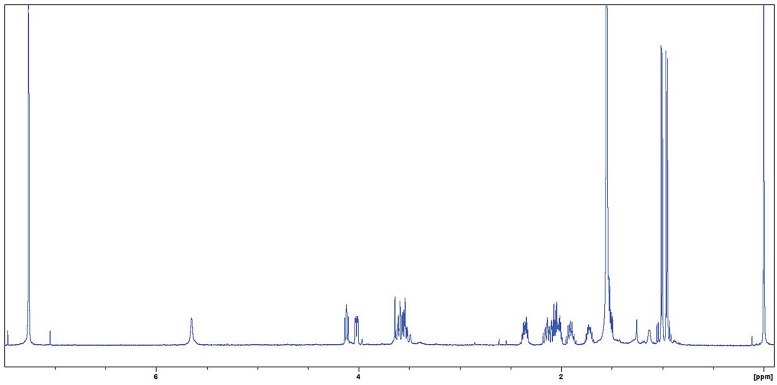


Fig. 2. ^{1}H NMR spectrum of compound **13** ($CDCl_3$, 500 MHz).

an amide proton at δ 7.73, and seven methylenes at δ_H 3.47, δ_C 47.4; δ_H 3.01, δ_C38.8; δ_H 2.60, δ_C 28.0; δ_H 2.29, δ_C 30.4; δ_H 1.50, δ_C 26.4; δ_H 1.38, δ_C 29.0; and δ_H 1.21, δ_C 23.6. The COSY data showed correlations for H-3/H-4, H-7/H-6,8 and HMBC data showed correlations for H-3/C-1,4,5; H-4/C-3, H-5/C-4,7; H-6/C-4,7; H-7/C-4,5,6,9; H-10/C-9,11; H-11/C-1,10 indicating that five methylenes were adjacent each other, while two others were separated by amide carbonyls and expected to be a cyclic molecule.

By comparing the molecular formula with the number of NMR signals (Table 2), it was suggested that the compound is a trimer. After a database search, compound **2** was

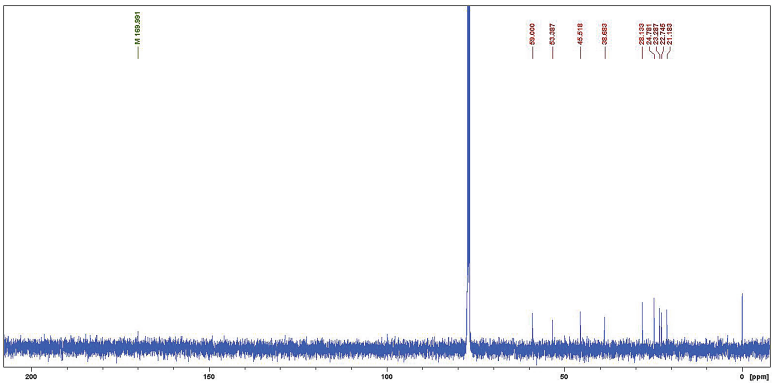


Fig. 3. ^{13}C NMR spectrum of compound **13** ($CDCl_3$, 125 MHz).

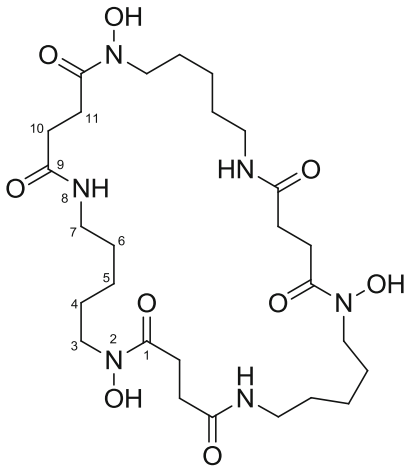


Fig. 4. Nocardamine (**2**)

identified as a nocardamine [12–14]. The planar structure of compound **2** was depicted as shown (Fig. 4).

Compound **3** (Fig. 7) was isolated as white amorphous substance. The ESI-MS data of compound **3** showed a sodiated ion at *m/z* 304.15128 (Δ + 3.90 ppm) indicating the molecule formula $C_{15}H_{23}NO_4$ with five degrees of unsaturation.

Table 2. ^{1}H and ^{13}C NMR data of compound **2** in DMSO-d_6.

No.	δ_C		δ_H (m, J in Hz)
1	172.0	s	
2	N-OH		9.60 (brs)
3	47.4	t	3.47 (t, 6.7)
4	26.4	t	1.50 (quintet, 6.7)
5	23.6	t	1.21 (m)
6	29.0	t	1.38 (p, 6.3)
7	38.8	t	3.0 (dt, 6.3)
8	NH		7.73 (brs)
9	172.5	s	
10	30.4	t	2.29 (dd, 6.8, 6.8)
11	28.0	t	2.60 (dd, 6.3, 6.8)

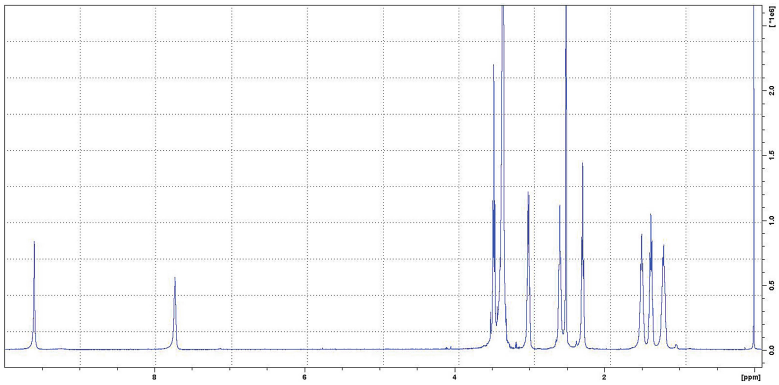


Fig. 5. ^{1}H NMR spectrum of compound **15** (DMSO-d_6, 500 MHz).

The ^{13}C NMR spectrum (Fig. 8) showed three carbonyls at δ 214.9, 174.3, and 174.1, an oxymethine at δ 66.2, five methylene carbons, four methines, and two methyls. The ^{1}H NMR (Fig. 9) showed an oxymethine proton at δ 4.02 (dt, $J = 5.8$, 7.2 Hz), methyl doublets at δ 1.26 and 0.94, and a deshielded methine at δ 2.56 (dt, $J = 5.8$, 13.0 Hz). After a database search, compound **3** was identified as cycloheximide (Table 3) [15].

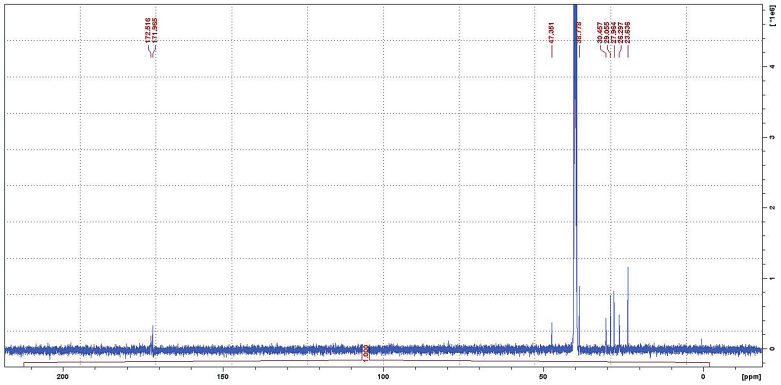


Fig. 6. ^{13}C NMR spectrum of compound **15** (DMSO-d_6, 125 MHz).

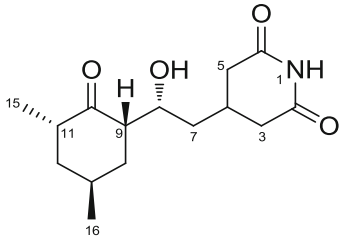


Fig. 7. Cycloheximide (**3**).

The absolute configuration of compound **3** was determined by comparing the specific rotation to those of the commercially available cycloheximide and reference [15]. The ^{1}H NMR spectrum of **3** exactly matched with reference cycloheximide. The specific rotation of compound **3** was $[\alpha]_D^{25}$ -14 (*c* 0.1, MeOH), while cycloheximide was reported as $[\alpha]_D^{25}$ -2.8 (*c* 9.6, MeOH)). Even though they were not exactly the same value, they have the same negative sign. Therefore, the absolute configuration of compound **3** was that of (-) cycloheximide.

3.2 Anti-deNV Activity

The isolated compounds (**1**–**3**) were evaluated against DeNV (Table 4). Compound **3** showed the highest inhibition among the isolated compounds with IC_{50} 0.02 μg/mL and SI 13.70 followed by a diketopiperazine **1**, with IC_{50} 0.38 μg/mL and SI 15.75. While nocardamine (**2**) showed weak activity with IC_{50} value $>$ 10.00 μg/mL and SI $<$ 0.80.

Table 3. 1D and 2D data of compound **3** (MeOH-d_4, 500 MHz).

No.	δ_C		δ_H (m, J in Hz)	HMBC	COSY	NOE
1		NH				
2	174.1	s				
3	38.0	t	2.63 (m)	1, 3, 4	3, 4a, 4b, 15	
4	27.6	d	2.37 (m)	1, 5		H-7
5	36.5	t	2.34 (m)	2, 3, 5, 6	2, 3	
			2.75 (m)			
6	174.3	s				
7	39.7	t	1.45 (m)	1, 4, 5, 7		
8	66.2	d	4.02 (dt, 5.8, 7.2)	3, 4, 8, 9, 12	6, 8	H-8
9	50.8	d	2.56 (brdt, 5.8, 13.0)	6, 7, 9, 10, 13		
10	214.9	s				
11	40.3	d	2.67 (m)	9, 11, 14	11, 14	
12	42.7	t	1.59 (dt, 4.8, 13.0)	6, 9, 13, 14, 15	10, 12	
			1.91 (m)	13		
13	27.0	d	2.17 (m)			
14	35.0	t	1.75 (dt, 4.8, 13.0)	7, 8, 9, 11, 12, 15	8, 12	
			2.07 (m)			
15	17.2	q	0.94 (d, 6.4)	9, 10, 11		H-8
16	13.4	q	1.26 (d, 7.2)	11, 12, 13		

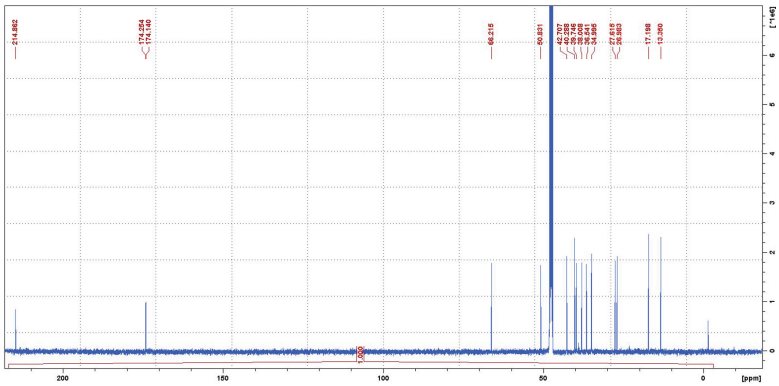


Fig. 8. ^{13}C NMR spectrum of compound **3** (DMSO-d_6, 125 MHz).

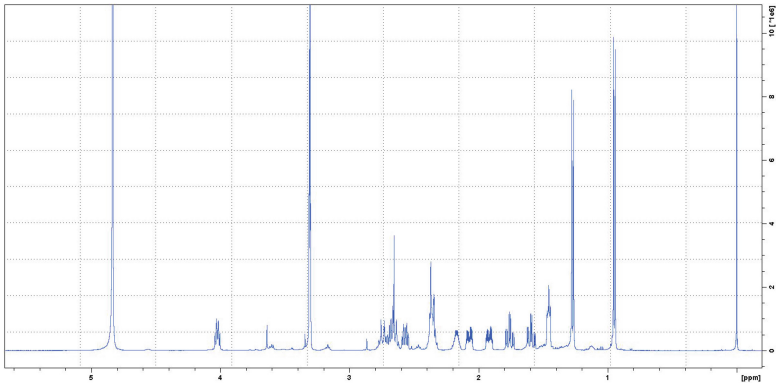


Fig. 9. ^{1}H NMR spectrum of compound **3** (DMSO-d_6, 500 MHz).

Table 4. The anti DeNV activity of the isolated compounds (**1**–**3**).

No.	Compound	IC_{50} (μg/mL)	CC_{50} (μg/mL)	SI
1	Cyclo(L-Leu-L-Pro) (**1**)	0.38	6.03	15.75
3	Nocardamine (**2**)	>10.00	7.96	<0.80
4	Cycloheximide (CHX, **3**)	0.02	0.28	13.70

4 Discussion

In this project, we examined the possibility of marine-derived bacteria as a source of bioactive compounds against DeNV. From a fermented bacteria extract provided by OP Bio, Okinawa, Japan, we successfully isolated and elucidated the 3 structures of bioactive compounds which were responsible for the activity against DeNV. The three isolated bioactive compounds were then identified as cyclo(L-leu-L-pro) (**1**), nocardamine (**2**), and cycloheximide (**3**).

Compound **1** was isolated as slightly more polar compared to **2** and **3**, this was seen during the purification of compound **1** was eluted in reversed-phase HPLC faster than compounds **2** and **3**. The moieties profiling compound **1** were amino acids consisting of L-Leu and L-Pro. Although the configurations of **1** were determined without Marfey's method, the specific rotation of the isolated compound was a match to the reported one, cyclo(L-Leu-L-Pro) [9], with specific rotation value $[a]_D$ -22 (c 0.04, MeOH) and supported by their identical ^{1}H and ^{13}C NMR data (Table 1).

The fraction of compound **2** was collected after compound **1** in reversed-phased HPLC indicating that compound **2** is less polar than compound **1**. In ^{1}H and ^{13}C NMR data of compound **2** showed that the number of protons and carbon that consisted in compound 2 were only 16 and 9, respectively. However, in the ESI-MS data exhibited *m/z* 601.35663 $[M + H]^+$ indicating chemical formula $C_{27}H_{49}N_6O_9$ with seven degrees

of unsaturation. From these data, we concluded that compound **2** is a trimer and later was identified as nocardamine (**2**) [12–14].

Compound **3** was collected after fractions of compounds **1** and **2** in reversed-phased HPLC indicating that compound **3** was less polar than **1** and **2**. Compound **3** was identified as a small molecule by showing *m/z* 304.15128 $[M + Na]^+$. The NMR data spectra and specific rotation of compound **3** were compared to the reported results in an identical sign. Therefore compound **3** and its absolute configurations were determined as (-) cycloheximide [15].

The structure characteristics of compound **1** were one secondary amide and one tertiary amide moiety. Compound **2** consisted of three tertiary amides, three secondary amides, and three *N*-alcohol moieties. While characteristic of compound **3** was one carbon ketone, two amides sharing the same N-H (isolated), and one oxymethine. From these specific moieties contained in each isolated compound and their correlation with their activities against DeNV, where the activity of **3** was higher than that of **1** and **2**, respectively, we could expect that number of moieties profiling in the structure of bioactive compounds affected their activities. In this case, the carbonyl, isolated N-H, and oxymethine moieties in cycloheximide were presumed to be responsible as active sites against DeNV. Compounds **1**–**3** were previously reported as an antifungal agent [16], antimalarial [17], and antibiotic, cell migration inhibition activity [18, 19], respectively.

In conclusion, we isolated three bioactive compounds (**1**–**3**) from a fermented bacteria extract and evaluated their activity against DeNV. The results were surprisingly excellent as we got cycloheximide (**3**) elicited strong inhibition against DeNV. Cycloheximide (**3**), which has long been known as an antibiotic, showed excellent inhibition with an IC_{50} 0.02 mg/mL. From this work, we presume there are many possibilities that remain to explore marine organisms and their symbionts as a source of bioactive compounds for biological active targets for anti-infectious diseases, such as anti-DeNV. Therefore, our work of direction still has a long way to go and remains a promising area to work to discover unsolved problems in the scientific world, especially the health aspect and infectious diseases.

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