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Ichthyotoxic principles against zebrafish embryos from the Indonesian marine sponge *Neopetrosia chaliniformis*

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ABSTRACT

The chemical ecology of *Neopetrosia chaliniformis* has never been studied before. A study on the extract of the Indonesian marine sponge *N. chaliniformis* for the ichthyotoxicity including its teratogenic effect against zebrafish (*Danio rerio*) embryos led to the identification of oxaquinolizidine alkaloids araguspongines C (**1**) and D (**2**). Their structure and conformation were determined on the basis of spectroscopic method and molecular modeling analysis. The ichthyotoxicity araguspongines fraction against zebrafish embryos showed LC_{50} 4.3 µg/ml (death, 48-hours post fertilization (hpf)) and 3.6 µg/ml (death, 72 hpf) as well as observation of its teratogenic effects including death, coagulation egg, tail, notochord, heart malformation, and yolk-sac edema at 24, 48, and 72 hpf. Because 76% cell death malformations in zebrafish embryos was observed, araguspongine rich fraction may serve as antimitotic-like drug in the treatment of cancer.

INTRODUCTION

Marine sponges of the genus *Neopetrosia* have been the subjects of numerous chemical investigations as they contain unique molecules with diverse biological activities (Skropeta and Wei, 2013; Blunt *et al*., 2013). In spite of the fact, its chemical ecology particularly ichthyotoxicity using zebrafish embryos (*Danio rerio*) has never been studied before. This aspect can trigger the discovery of bioactive molecules either as new molecules or possessing new activities. The zebrafish embryos (*D. rerio*) are powerful and versatile model for vertebrate development (Grunwald and Eisen, 2002), disease modeling (Lieschke and Currie, 2007), and drug discovery (Zon and Peterson, 2005) because of its morphological and physiological similarity to mammals including *Homo sapiens* (Aston *et al*., 2017), the existence of many genomic tools, and the ease with which large, phenotype-based screens can be performed (Zon and Peterson, 2005; Kari *et al*., 2007). More specifically, the teratogenic effect of zebrafish (*D. rerio*) embryos and mammalian are found to have significant correlation (Kari *et al.,* 2015) suggesting for an initial target identification of bioactive molecules. Zebrafish embryos toxicity including teratogenic effect have been used to screen a novel microtubule inhibitor from the synthetic library (Moon *et al*., 2002).

The metabolites isolated so far from *Neopetrosia* sponges can be grouped into three chemical classes: 1) alkaloids including tetrahydroisoquinolines (Oku *et al*., 2003), polycyclic amines (Liang *et al*., 2015; Li *et al*., 2011; Sorek *et al*., 2007), and pyridine

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nucleosides (Shubina *et al*., 2015), 2) tricyclic peptides (William *et al*., 2005), and 3) quinones including pentacyclic hydroquinones (Leone *et al*., 2008) and sesquiterpene benzoquinones (Winder *et al*., 2011). Their crude extracts and isolated compounds exhibited various bioactivities such as cytotoxic (Oku *et al*., 2003; Liang *et al*., 2015; Li *et al*., 2011; Sorek *et al*., 2007; Shubina *et al*., 2015; William *et al*., 2005; Winder *et al*., 2011), antibiotic (Leone *et al*., 2008), antiplasmodial (Wei *et al*., 2010), antibacterial (Liu *et al*., 2004), and antifungal activities (Qaralleh *et al*., 2010) to name a few. From the standpoint of chemistry, *Neopetrosia* metabolites e.g., polycyclic amines have challenged researchers to work because of difficulties in their purification, structure elucidation, and unstability of the molecules (Oku *et al*., 2003; Morinaka *et al*., 2011).

As the marine sponge *N. subtriangularis* was found to contain symbiotic microbes (Freeman *et al*., 2011), the symbionts could be the true producers of the bioactive molecules isolated from the host *Neopetrosia* sponges. In our continuing interests on bioactive molecules from marine organisms (Hanif *et al*., 2015), we examined the Indonesian marine sponge *N. chaliniformis* collected off Lampung, Indonesia. As its *n*-BuOH extract showed potent activity against zebrafish embryos (LC_{50} 61 µg/ml), the extract was separated to give an active fraction showing ichthyotoxicity against zebrafish embryos. Further chromatographic separation resulted in the isolation of a macrocyclic oxaquinolizidine alkaloid known as araguspongines C (**1**) and D (**2**) in which chemical and new biological result of active fraction are reported in this article.

MATERIAL AND METHODS

General

Optical rotation was obtained with a Jasco P-1010 digital polarimeter. NMR spectra were recorded on a Bruker Avance III 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts (δ) were referenced to TMS or $CDCl_3$ signals (^{1}H δ 7.26, ^{13}C δ 77.16). All chemical shifts (δ) are given in ppm and coupling constants (*J*) are in Hz. Multiplicities of ^{13}C NMR data were determined by DEPT experiments. ESIMS data were obtained on a Jeol JMS-T 100LP mass spectrometer. High performance liquid chromatography (HPLC) separations were carried out on a Hitachi L-6000 pump fitted with a Shodex RI-101 refractive index and a SPD-20A Shimadzu UV detectors. A column used for HPLC was Cosmosil $5C_8$-MS (4.6 × 250 mm), and analytical TLC was performed on Merck silica gel 60 F254 plates and visualized with Dragendorff reagent. All solvents used were reagent grade.

Animal material

The marine sponge was collected by hand using scuba off Lampung Bay, Indonesia in September 2014. The sponge was identified as *Neopetrosia chaliniformis* by one of us (NJdV). A voucher specimen has been deposited at the Department of Chemistry, Bogor Agricultural University, Indonesia (Code NH-G1-0001-14-a).

Extraction and isolation

The marine sponge specimen (wet, 700 g) stored in EtOH was extracted three times using MeOH (3 × 500 ml). The combined extracts were concentrated under reduced pressure, and the residue was partitioned using *n*-hexane and 90% aqueous MeOH. The latter layer was partitioned further between CH_2Cl_2 and 50% aqueous MeOH. Finally, the 50% aqueous MeOH layer was concentrated and added with H_2O. It was then extracted with *n*-BuOH. All layers (*n*-hexane, CH_2Cl_2, *n*-BuOH) were checked for their activity against zebrafish embryos, and we found the *n*-BuOH extract as the most toxic (LC_{50} 61 µg/ml). Purification of the extract (4.03 g) using silica gel 60 (0.040–0.063 mm) with gradient elution $CHCl_3$-MeOH gave six fractions **A**–**F**. The most ichthyotoxic fraction **F** (15.4 mg, LC_{50} 4.3 µg/ml) was further purified using HPLC C_8 with eluent H_2O:MeOH:NH_4OH (2:7:0.3) to give araguspongine C (**1**) (0.5 mg) and araguspongine D (**2**) (0.4 mg).

Zebrafish assay

Fertilized and normally developed embryos of the zebrafish *Danio rerio* were used for bioassay. The method used for zebrafish embryos assay was modified from Bai *et al*. (2016). For the first screening of the extracts, the zebrafish embryos were used at 72 hpf and exposed to the sample for 24 hours. Observation of the embryos was based on whether it is dead or live. To determine more precisely, the embryos (0 hpf) were used and the LC_{50} was determined at 48 hpf. Moreover, teratogenic effect was observed at 0, 24, 48, 72, and 96 hpf exposed with a series of sample concentrations at 0.5, 1, 2, 4, 6, and 8 µg/ml. The number of embryos used for the first screening was 10, while for the latter experiments was 20 for each well in 24 well microplates. Inverted microscope IX70 (Olympus) with objective power 1x–5x was used for observation of teratogenic effect. DMSO (1%) was used to improve the solubility of samples. Endpoints were assessed using binary responses (dead/live). Embryos and larvae were considered dead when coagulation had occurred and/or no heartbeat was detected. The LC_{50} was calculated by using statistical software SPSS IBM 23.0. The experiment was repeated for three times.

Conformational analysis

Conformational searches were performed with Spartan'14 (Wavefunction Inc.) using a commercially available PC. Energy-minimized molecule was obtained from calculation using MMFF with further optimization using semi-empirical method AM1. Initial conformers was set to 10,000, then the obtained conformer was optimized with AM1 semi-empirical method.

RESULT AND DISCUSSION

Two oxaquinolizidine alkaloids araguspongines C (**1**) and D (**2**) have been isolated from the active fraction **F** [LC_{50} 4.3 µg/ml (death, 48 hpf) and 3.6 µg/ml (death, 72 hpf)] showing positive results with Dragendorff reagent. Compound **1** was obtained as a glass with specific rotation $[\alpha]_D^{26}$ + 43º (0.042, MeOH). Its molecular formula was determined as $C_{28}H_{50}N_2O_4$ by observing its HRESIMS ion at *m/z* 479.38588 $[M+H]^+$ (calcd. for $C_{28}H_{51}N_2O_4$, Δ +1.0 mmu) indicating five degrees of unsaturation. Since the ^{13}C NMR spectrum showed only 14 signals, it was suggested that **1** is a symmetric molecule. The ^{1}H NMR ($CDCl_3$, 500 MHz) of **1** showed key signals at δ 3.56 (H2, t, *J* = 10.6); 1.76 (H_2-3a, m);

1.07 (H_2-3b, m); 3.11 (H4-a, brt, J = 12.4); 2.97 (H4-b, dd, J =13.6, 4.1); 2.35 (H6-a, dd, J = 8.5, 2.2); 3.04 (H6-b, td, J = 11.8, 2.6); 1.80 (H_2-7a, m); 1.52 (H_2-7b, m); 1.40 (H_2-8a, m); 1.30 (H_2-8b, m); 4.07 (H-10, s); 1.62 (H_2-11a, m); 1.30 (H_2-11b, m); 1.31 (H_2-15a, m); 1.55 (H_2-15b, m); 1.32 (H_2-16a, m); 1.55 (H_2-16b, m), while its ^{13}C NMR ($CDCl_3$, 125 MHz) exhibited major signals at δ 76.7 (CH, C2), 26.2 (CH_2, C3), 52.6 (CH_2, C4), 44.2 (CH_2, C6), 21.0 (CH_2, C7), 22.7 (CH_2, C8), 70.9 (C, C9), 90.5 (CH, C10), 38.7 (CH_2, C11), 29.8 (CH_2, C12), 32.4 (CH_2, C13), 31.6 (CH_2, C14), 25.1 (CH_2, C15), 36.5 (CH_2, C16). All the above data are in agreement with those reported for araguspongine C (Kobayashi *et al*., 1989; Orabi *et al*., 2002). The relative stereochemistry of **1** was confirmed by coupling constants, NOEs, and conformational analyses using Spartan '14 as in Figure 1 which was 2*R**, 9*R**, 10*S**, 2'*R**, 9'*R**, 10'*S** (Figure 2).

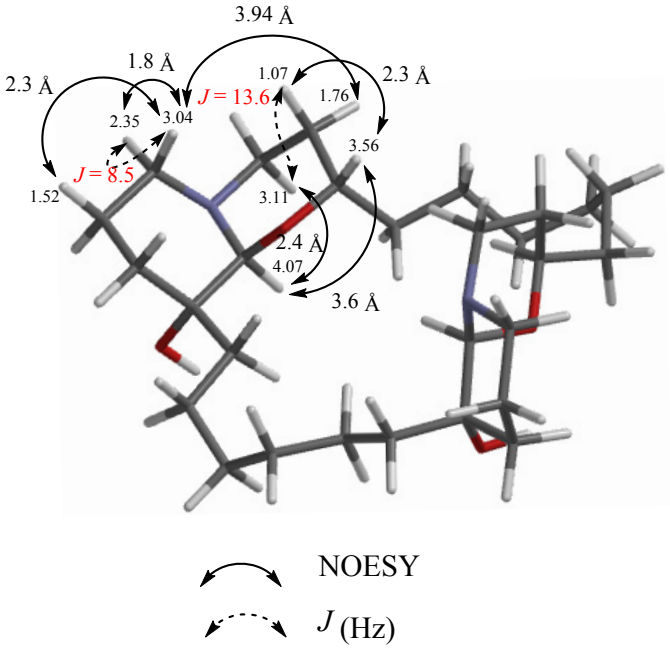

Fig. 1: Key NOESY correlations, analysis of coupling constants, and calculated distance (Å) of **1** between two selected hydrogens for energy-minimized molecule obtained from calculated MMFF and AMI semi-empirical methods.

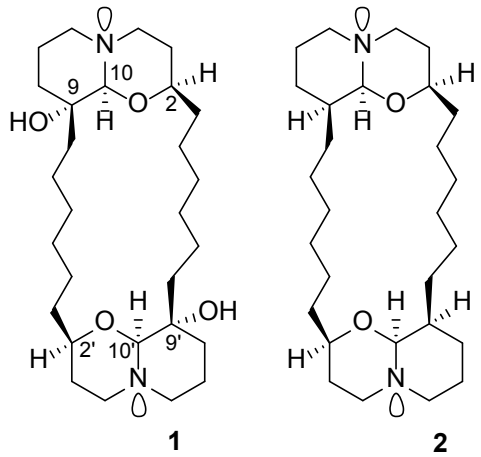

Fig. 2: Structure of **1** and **2** with its relative stereochemistry.

Compound **2** isolated after **1** was eluted and analyzed for $C_{28}H_{50}N_2O_2$ by HRESIMS indicating it to be a bis-1-oxaquinolizidine alkaloid with the absence of two hydroxy groups at C9 and C9' (δC 40.56). The ^{1}H NMR ($CDCl_3$, 500 MHz) of **2** showed key signals at δ 3.35 (H2, t, J = 10.6); 1.68 (H_2-3a, m); 1.47 (H_2-3b, m); 2.18 (H4-a, ddd, J = 3.3, 11.9, 11,9); 2.94 (H4-b, dd, J =11.5, 1.5); 1.99 (H6-a, ddd, J = 11.7, 11.7, 3.0); 2.76 (H6-b, d, J = 11.5); 1.65 (H_2-7a, m); 1.58 (H_2-7b, m); 1.68 (H_2-8a, m); 1.25 (H_2-8b, m); 1.61 (H-9, m); 3.06 (H-10, d, J = 8.4); 1.59 (H_2-11a, m); 1.34 (H_2-11b, m); 1.40 (H_2-12a, m); 1.18 (H_2-12b, m); 1.30 (H_2-13a, m); 1.18 (H_2-13b, m); 1.26 (H_2-14a, m); 1.16 (H_2-14b, m); 1.58 (H_2-15a, m); 1.35 (H_2-15b, m); 1.60 (H_2-16a, m); 1.40 (H_2-16b, m), while its ^{13}C NMR exhibited primary signals at δ 75.3 (CH, C2), 32.3 (CH_2, C3), 54.3 (CH_2, C4), 54.1 (CH_2, C6), 24.9 (CH_2, C7), 29.0 (CH_2, C8), 40.6 (CH, C9), 95.9 (CH, C10), 36.2 (CH_2, C11), 28.8 (CH_2, C12), 31.6 (CH_2, C13), 25.2 (CH_2, C14), 25.3 (CH_2, C15), 35.4 (CH_2, C16). The observed specific optical rotation of **2** was $[\alpha]_D^{26.7}$ + 12.1° (0.033, MeOH). The relative configuration of **2** has been analyzed using NOE and coupling constants (Kobayashi *et al*., 1998) to conclude the structure as in **2** (Figure 1).

Table 1: Teratogenic effect of **F** against zebrafish embryos.

Abnormality	Σ[a]	[%][b]
Body axis	18	19
Brain	0	0
Caudal fin	0	0
Circulation	3	3.2
Eye	0	0
Heart	14	15
Jaw	0	0
Notochord	12	13
Otic vesicle	0	0
Pigmentation	0	0
Somite	0	0
Trunk	0	0
Yolk-sac	16	17
Embryo with cell death	**72**	**76**

[a] The number of embryos affected teratogenic effect with all of concentration for 96 hpf.
[b] Percentage malformation obtained from total embryos affected teratogenic effect with all of concentration for 96 hpf.

Due to the small amount of **1** and **2**, we did not determine each compound for teratogenic activity against zebrafish, but the activity of the fraction **F** containing **1** and **2** was evaluated against zebrafish embryos from 0 to 96 hpf. The fraction **F** exhibited ichthyotoxicity included teratogenic effect such as cells death, coagulation of eggs, tail, notochord, and heart malformation as well as yolk-sac edema (Figure 3, Table 1). One major malformation was concluded as zebrafish embryos with cell death (76%) (Table 1). Those paramaters were in agreement with the endpoint used for assessing the embryo toxicity (Bai *et al*., 2016). At 24 hpf, normal embryos began with cells death at 2 µg/ml and had an increasing number of cells death (initial coagulation) at 4 µg/ml (48 hpf). Body axis malformation was further observed at

2 µg/ml (48 hpf) and tail malformation was seen at 4 µg/ml (72 hpf). Notochord malformation was monitored at 1 µg/ml (96 hpf). The remaining observation for 96 hpf was heart malformation and yolk-sac edema at 2 µg/ml. As the fraction **F** showed cells death at the embryo development (2 µg/ml, 24 hpf) and led coagulation of eggs, the araguspongine-rich fraction **F** could be used as an antimitotic agent (van Vuuren *et al*., 2015). Furthermore, the ichthyotoxicity fraction of the sponge *N. chaliniformis* is also benefit for the understanding of the chemoecological effects of marine chemical defensive substances.

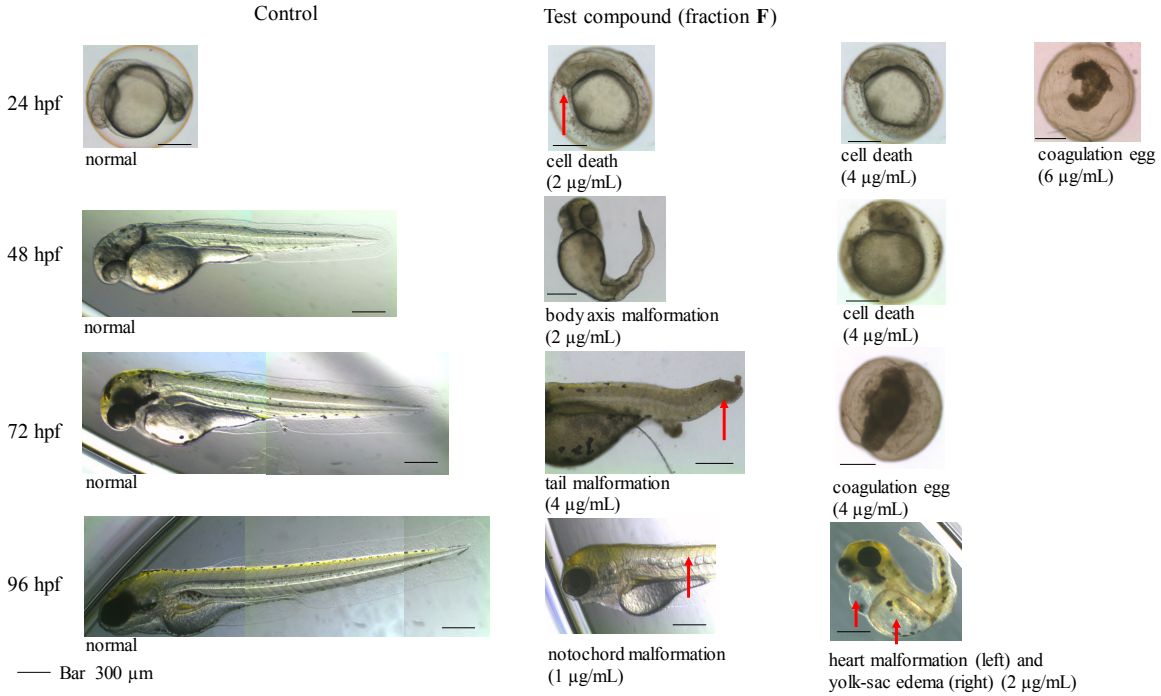

Fig. 3: Zebrafish embryos morphology at 24, 48, 72, 96 hpf with the corresponding concentration at 1, 2, 4, and 6 µg/ml.

CONCLUSION

Aragusponginеs C (**1**) and D (**2**) have been isolated from the Indonesian marine sponge *Neopetrosia chaliniformis*. Compounds **1** and **2** were characterized by NMR, HRMS and their optical rotation data as well as comparison with literature values. Furthermore, compound **1** was analyzed its stereochemistry using NMR and molecular modeling. The active fraction **F** containing araguspongine molecules was shown to have ichthyotoxicity included teratogenic effect against zebrafish embryos, which could be used as antimitotic and chemoecological agents. This is the first report that aragusponginеs C (**1**) and D (**2**) were isolated from *N. chaliformis* and have a new biological activitity of active fraction against zebrafish (*Danio rerio*) embryos.

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