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 (I) 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 LC50 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, aragusponge 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 Curie, 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
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; Sorok 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 (LC50 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 araguspongine 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 1H and 125 MHz for 13C). Chemical shifts (δ) were referenced to TMS or CDCl3 signals (1H δ 7.26, 13C δ 77.16). All chemical shifts (δ) are given in ppm and coupling constants (J) are in Hz. Multiplicities of 13C NMR data were determined by DEPT experiments. ESIMS data were obtained on a Jeol JMS-T100LP mass spectrometer. High performance liquid chromatography (HPLC) separations were carried out on a Hitachi L-6000 pump fitted with a Shimadzu UV detectors. A column used for HPLC was Cosmosil 5C18 (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 (NIdV). 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 CH2Cl2 and 50% aqueous MeOH. Finally, the 50% aqueous MeOH layer was concentrated and added with H2O. It was then extracted with n-BuOH. All layers (n-hexane, CH2Cl2, n-BuOH) were checked for their activity against zebrafish embryos, and we found the n-BuOH extract as the most toxic (LC50 61 μg/ml). Purification of the extract (4.03 g) using silica gel gel 60 (0.040–0.063 mm) with gradient elution CHCl3-MeOH gave six fractions A–F. The most ichthyotoxic fraction F (15.4 mg, LC50 4.3 μg/ml) was further purified using HPLC C18 with eluent H2O:MeOH:NH4OH (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 LC50 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 LC50 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 [LC50 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 [α]26 + 43° (0.042, MeOH). Its molecular formula was determined as C17H16NO4 by observing its HRESIMS ion at m/z 479.38588 [M+H]+ (calcd. for C17H15NO4, Δ +1.0 mmu) indicating five degrees of unsaturation. Since the 13C NMR spectrum showed only 14 signals, it was suggested that 1 is a symmetric molecule. The 1H NMR (CDCl3, 500 MHz) of 1 showed key signals at δ 3.56 (H2, t, J = 10.6); 1.76 (H-3a, m);
Compound 2 isolated after 1 was eluted and analyzed for C_{25}H_{39}NO_{5} 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 1H NMR (CDCl₃, 500 MHz) of 2 showed key signals at δ 3.35 (H2, t, J = 10.6); 1.68 (H3-3a, m); 1.47 (H3-3b, 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 (H7-a, m); 1.58 (H7-b, m); 1.68 (H8-a, m); 1.25 (H8-b, m); 1.61 (H-9, m); 3.06 (H-10, d, J = 8.4); 1.59 (H-11a, m); 1.34 (H-11b, m); 1.40 (H-12a, m); 1.18 (H-12b, m); 1.30 (H-13a, m); 1.18 (H-13b, m); 1.26 (H-14a, m); 1.16 (H-14b, m); 1.58 (H-15a, m); 1.35 (H-15b, m); 1.60 (H-16a, m); 1.40 (H-16b, m), while its 13C NMR exhibited primary signals at δ 75.3 (CH, C2), 32.3 (CH, C3), 54.3 (CH, C4), 54.1 (CH, C6), 24.9 (CH, C7), 29.0 (CH, C8), 40.6 (CH, C9), 95.9 (CH, C10), 36.2 (CH, C11), 28.8 (CH, C12), 31.6 (CH, C13), 25.2 (CH, C14), 25.3 (CH, C15), 35.4 (CH, C16). The observed specific optical rotation of 2 was [α]_{D}^{20} + 12.1º (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.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>2 (%)</th>
<th>[%] (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body axis</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Brain</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Caudal fin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Circulation</td>
<td>3</td>
<td>3.2</td>
</tr>
<tr>
<td>Eye</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heart</td>
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<td>15</td>
</tr>
<tr>
<td>Jaw</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Notochord</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Otic vesicle</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Somite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trunk</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yolk-sac</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Embryo with cell death</td>
<td>72</td>
<td>76</td>
</tr>
</tbody>
</table>

*The number of embryos affected teratogenic effect with all of concentration for 96 hpf.

*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 parameters 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 cell 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 chemoeological effects of marine chemical defensive substances.

CONCLUSION

Araguspongines 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 chemoeological agents. This is the first report that araguspongines C (1) and D (2) were isolated from *N. chaliniformis* and have a new biological activity of active fraction against zebrafish (*Danio rerio*) embryos.

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