# Chemical investigation on *Pseudoceratina purpurea* collected from Banyuwangi Indonesia

## Penelitian kandungan kimiawi *Pseudoceratina purpurea* yang dikoleksi dari Banyuwangi Indonesia

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### Abstrak

Telah dilakukan penelitian tentang komponen kimia spons laut *Pseudoceratina purpurea* yang dikoleksi dari Banyuwangi, Indonesia. Penelitian ini bertujuan untuk mengisolasi dan mengidentifikasi struktur metabolit sekunder spons tersebut dan menguji daya sitotoksisnya terhadap kultur sel limfoma tikus L5178Y.

Isolasi dilakukan dengan menggunakan beberapa teknik kromatografi yang berbeda. Metode spektroskopi resonansi magnetik inti serta spektrometri massa digunakan untuk mengidentifikasi struktur kimia isolat. Pengujian sitotoksisitas pada kultur sel limfoma tikus L5178Y dilakukan dengan menggunakan metode *microculture tetrazolium (MTT)*.

Penelitian ini mengungkap keberadaan lima alkaloid derivat tirosin, dua diantaranya yaitu aplysamine-2 (**1**) dan aeroplysinin-1 (**2**) menunjukkan aktivitas penghambatan pertumbuhan kultur sel limfoma tikus L5178Y dengan $IC_{50}$ berturut-turut sebesar 1.7 μg/mL dan 0.57 μg/mL.

**Kata kunci:** *Pseudoceratina purpurea*, spons, alkaloid.

### Abstract

Chemical investigation on marine sponge *Pseudoceratina purpurea* collected from Banyuwangi, Indonesia has been performed. This study was aimed to isolate and to identify structures of the sponge secondary metabolites as well as to test their cytotoxicity activity on mouse lymphoma cell line L5178Y.

Isolation procedure was performed by using different chromatography techniques. NMR spectroscopy and mass spectrometry methods were used to identify the compounds chemical structures. Cytotoxicity of the isolates was tested on mouse lymphoma cell line L5178Y by using the microculture tetrazolium (MTT) assay.

This study yielded five known tyrosine-derived alkaloids, two of which, aplysamine-2 (**1**) and aeroplysinin-1 (**2**) showed growth inhibition of mouse lymphoma cell line L5178Y with $IC_{50}$ value of 1.7 μg/mL and 0.57 μg/mL, respectively.

**Key words:** *Pseudoceratina purpurea*, sponge, alkaloids.

## Introduction

According to Van Soest (1989) Indonesian archipelago possesses almost 830 sponge fauna species (obvious synonym not counted) (Van Soest, 1989) exist in a high rate of dissimilarity among different area (Amir, 1992; Calcinai *et al*., 2005). Considering that sponges are predominant as the source of new marine natural products, studies on marine sponge-derived secondary metabolites collected from this area are highly required.

As part of our research on finding more biological active compounds from Indonesian marine sponges, investigation on the chemical profile of those sponges collected from different areas in Indonesia has been conducted. Combined approach of biological and chemical screening was applied as preliminary stage of this study. Sponge extracts from different location in Indonesia was screened for their chemical and biological profile. Several bioassay screening method were used i.e. protein kinase inhibitory, cytotoxicity, anti fungal and anti bacterial assays. In parallel to it, a chemical investigation was done by using DAD-HPLC and LC-MS equipments.

One of chemically interesting extract was sponge sample TRW14 collected from Banyuwangi on October 2003. Despite negative results showed by the bioassay screening test, this sponge extract was chosen to be investigated further as it exhibited an interesting chemical profile detected by the HPLC instruments.

## Methodology

### Sponge material

The sponge material was collected on October 2003 by SCUBA diving in Watudodol, Banyuwangi at a depth of 10–30 m and was given a code name of TRW14 (Fig.2). It was directly preserved in ethanol after harvesting. A voucher specimen is deposited in the Zoological Museum Amsterdam under reg. no. ZMAPOR17800.

Taxonomic identification on the TRW14 voucher specimen classify the sponge into the taxonomic class of Demospongiae; order Verongida; family Pseudoceratinidae; genus *Pseudoceratina* (Bergquist and Cook, 2002); species *Pseudoceratina purpurea* (Carter). It showed sticky surface, elastic and irregular in shape, a firm consistency, brown colour with dark blue and yellow spots.

### Isolation procedure

Solvents used for separation techniques were acetone; acetonitrile; dichloromethane; ethyl acetate; *n*-hexane; methanol. These solvents were purchased from the Institute of Chemistry HHU Düsseldorf. They were distilled before using. Others solvent used were *n*-butanol and acetone (Fluka, Seelze, Germany), ethanol (Merck, Darmstadt, Germany).

Column chromatography was performed on silica gel (0.040-0.063 mm; Merck, Darmstadt, Germany) or on Sephadex LH20. For HPLC analysis, samples were injected into an HPLC system equipped with a photodiode array detector (Dionex, München, Germany). Routine detection was at λ 235, 254, and 340 nm. The separation column (125 x 4 mm i.d.) was prefilled with Eurospher 100-C18, 5µm (Knauer, Berlin, Germany). Separation was achieved by applying a linear gradient from 90% $H_2O$ (pH 2.0) to 100% MeOH over 40 min. TLC analysis was carried out using aluminium sheet precoated silica gel 60 F254 or on glass precoated RP-18 F254 plates (Merck, Darmstadt, Germany). Chemicals used in the detection and isolation methods were anisaldehyde (4-methoxybenzaldehyde), hydrochloric acid and concentrated sulphuric acid (all provided by Merck, Darmstadt, Germany); ammonium hydroxide, glacial acetic acid, (Fluka, Seelze, Germany).

Sponge tissue was separated from the supernatant (ethanol) and dried at room temperature. Dried sponge tissue was ground and extracted exhaustively with methanol. After removing the solvent under reduced pressure, the resulting methanol extract was combined with the ethanol extract to obtain a total weight of 26 g. This total crude extract was partitioned between *n*-hexane and 90% MeOH-water to obtain the hexane fraction (516 mg). The residue was partitioned further between ethyl acetate and $H_2O$ to obtain the ethyl acetate fraction (2.6 g), and finally the water phase was partitioned against *n*-BuOH to yield the butanol fraction (1.2 g).

The ethyl acetate fraction was subjected to Sephadex LH20 column with MeOH as eluent, to yield **aplysamine-2** (**1**, 11 mg) and other 9 fractions. Fraction 3 was chromatographed over a Sephadex LH20 column (MeOH) to obtain **aeroplysinin-1** (**2,** 79 mg). Fraction 4 was subjected to a Sephadex LH20 column (MeOH) to obtain **5-[3,5-dibromo-4-[(2-oxo-5-oxazolidinyl)methoxy]phenyl]-2-oxazolidinone** (**3**, 26.0 mg).

The butanol fraction was chromatographed over a Sephadex LH20 column by using MeOH as eluent resulted to 28 fractions. Fraction 4 was then purified by using semi-preparative HPLC RP18 in gradient composition of methanol and water to



Figure 1. Isolated compounds.

obtain **dienone dimethoxyketal** (**4**, 70 mg) and **dienone methoxy-ethoxy ketal** (**5**, 2 mg). The HPLC solvent system program was run as follows, 0 – 5': 10% MeOH; 5' – 20': 10% to 100% MeOH; 20' – 23': 100% MeOH; 23' – 25': 10% MeOH.

### Structure Identification

$^{1}$H NMR and $^{1}$H - $^{1}$H COSY spectra were measured on a Bruker Unity 500 MHz spectrometer using deuterated methanol or DMSO-$d_6$ as solvents. ESI mass spectra were obtained on a Thermo Finnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. Optical rotation was determined on a Perkin-Elmer 241 MC polarimeter by measuring the angle of rotation at a wavelength of 589 nm of a Na/Hg vapour lamp. Samples were measured in a 0.5 mL cuvette with 0.1 dm length and unless stated different was run at room temperature (25° C). Solvent used in this method were spectroscopy grade of methanol (Sigma-Aldrich, Steinheim, Germany).

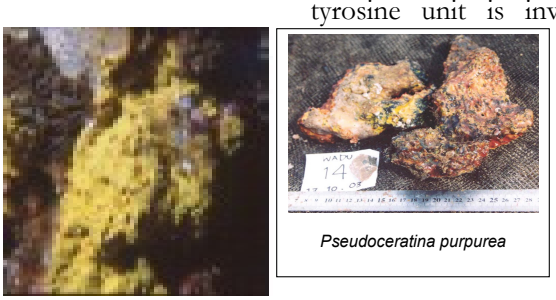


Figure 2. Sponge picture under water (left) and directly after harvesting (right) (Pictures were taken by Juswono).

### Cytotoxicity assay

Cytotoxicity assay against L5178Y mouse lymphoma cells was determined by using microculture tetrazolium (MTT) assay (Carmichael *et al*., 1987).

## Result and Discussion

Sponge sample in this research was later identified as *Pseudoceratina purpurea*, a sponge from order Verongida. Undamaged sponge exhibited yellow colour underwater which turned slowly to dark colour when exposed to the air (Fig. 2). This might be due to a possession of an unstable pigment as was shown by another Verongid sponge, *Verongia aerophoba* Schmidt (Cimino *et al.,* (1984). By transferring the *V. aerophoba* to the water surface, its yellow pigment, uranidine (3,5,8-trihydroxy-4-quinolon) will be slowly oxidized to an unstable blue quinone. Furthermore, this blue substance will be quickly polymerized to an insoluble black material (Cimino *et al*., 1984).

Order Verongida is well-known due to their tyrosine biogenetically related compounds with interesting biological activities. It was reported by Simmon and collaborators (2005) that these constituents may be produced by associated bacteria. The unusually large number of biosynthetically related compounds has been linked to the potentially large number of chemical variations that are possible within the aromatic ring and/or side chains of the tyrosine moiety (Tabudravu and Jaspars, 2002). The aromatic ring can be either maintained, reduced, or oxidized (Capon and MacLeod, 1987) or mono- or dibrominated (Minale *et al*., 1976; Yagi *et al*., 1993). In many cases, a single tyrosine unit is involved, as with dienone (dibromoverongiaquinol) (D'Ambrosio, *et al*., 1982 and 1984), and aeroplysinin-1 (Fattorusso *et al*., 1972).

Chemical profile of the ethyl acetate fraction by DAD HPLC in comparison to the internal spectra library revealed a presence of several tyrosine derived compounds. ESIMS data extracted from LC-MS spectra of compounds **1** - **5** informed that those compounds are brominated as the molecular ions of bromine-bearing compounds will show multiple peaks due to the fact that the halogen exists in nature as two stable isotopes, $^{79}$Br and $^{81}$Br in relatively equal abundance (Smith, 2005).

ESIMS data of **1** revealed pseudo molecular ion cluster of 647/649/651/

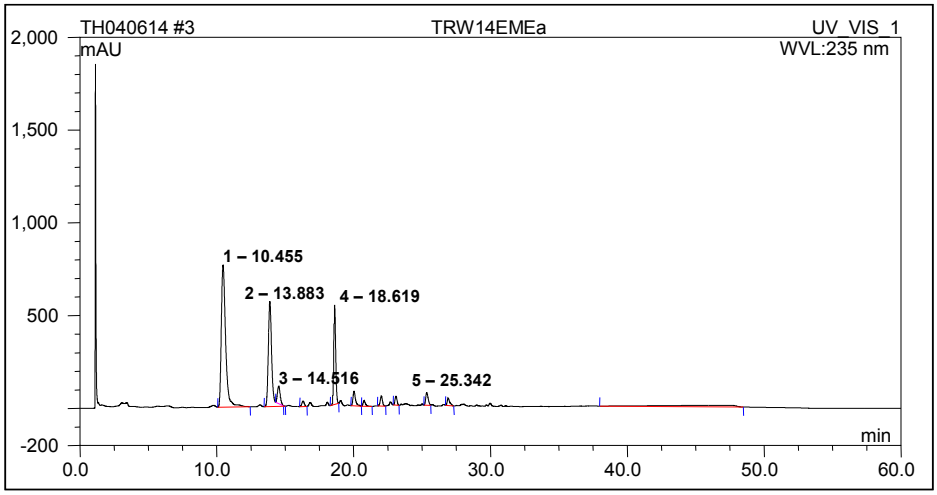


Figure 4. Chemical profile of *Pseudoceratina purpurea* ethyl acetate fraction in DAD HPLC at 235 nm.
Peak 1: dienone dimethoxy ketal (**4**); peak 2: aeroplysinin-1(**2**); peak 3: dienone methoxy- ethoxy ketal (**5**); peak 4: bisoxazolidinone derivative (**3**); peak 5: aplysamine-2 (**1**)

653 g/mol in an intensity ratio of 1:2:2:1, which indicated a possession of three bromines. It corresponds to the molecular formula $C_{23}H_{28}Br_3N_3O_4$ of aplysamine-2. On the other hand, ESIMS isotopic ion cluster of a dibrominated compound at *m/z* 338/340/342 $[M+H]^+$ having an intensity ratio of 1:2:1 corresponds to the molecular formula $C_9H_9Br_2NO_3$ of aeroplysinin-1 (**2**). Compound **3** showed similar UV absorption pattern to **1** suggesting a presence of a similar chromophore. It showed ESIMS isotopic pseudo molecular ion peaks at *m/z* 435/437/439 $[M+H]^+$ with an intensity ratio of 1:2:1. These finding was compatible with molecular formula $C_{13}H_{12}Br_2N_2O_5$ of a 5-(3,5-dibromo-4-((2-oxooxazolidin-5yl)methoxy)phenyl) oxazolidin-2-one.

A positive ESIMS spectrum of **4** exhibited pseudo molecular ion peaks at *m/z* 338/340/342 $[M+H-MeOH]^+$ having an intensity ratio of 1:2:1 and suggesting the presence of two bromines in the molecule. The molecular weight was in agreement with the molecular formula $C_{10}H_{13}Br_2NO_4$ of 2-(3.5-dibromo-1-hydroxy-4,4-dimethoxycyclohexa-2,5-dienyl)acetamide or dienone dimethoxy-ketal. On the other hand, ESIMS pseudo molecular ion peaks at *m/z* 429/431/433 $[M-H+HCOOH]^-$ having an intensity ratio of 1:2:1 was exhibited by compound **5** and compatible with the molecular formula $C_{11}H_{15}Br_2NO_4$ of 2-(3,5-dibromo-4-ethoxy-1-hydroxy-4-methoxy-cyclohexa-2,5-dienyl)acetamide or dienone methoxy-ethoxyketal).

Careful interpretation of the compounds **1-5** spectral data in comparison to the literatures confirmed the proposed structures. The NMR data of those compounds could be seen in table I – III. Correlations found in HMBC and COSY spectra of **1** and **3** were as described in Fig.5.

Considering their chemical structures, compounds **1** – **4** are considered to be optically active as could be seen by the results of their optical rotation measurements. Aeroplysinin-1 is optically active due to a chiral center on C-1. It showed a $[\alpha]_D^{20}$ value of +110.91° ± 0.46° (*c* 0.5, $CH_3OH$). The absolute configuration of the dextrorotatory enantiomer as shown in Fig.1 was provided by the determination of the CD curve by Fulmor and collaborators (1970). On the other hand, compounds **4** and **5** exhibited $[\alpha]_D^{20}$ value of -4.0° ± 0.6° (*c* 0.65, $CH_3OH$) and +1.2° ± 0.4° (*c* 0.39, $CH_3OH$), respectively.

Concerning the presence of two chiral carbon atoms in compound **3** (C-3 and C-11), four stereoisomers are possible, i.e, (*R*, *R*), (*R*, *S*), (*S*, *R*), (*S*, *S*). Analysis of coupling constants

of **3** in DMSO-$d_6$ could not clearly differentiate its possible relative configuration, since *J* values for hydrogen couplings in both carbamoyl side chains were ca. 8 Hz. However, a tentative assignment of configuration could be made by comparison of specific rotations. The absolute configuration of the levorotatory (*R*,*R*) isomer $[\alpha]_D$ -33°, *c* 1.1, MeOH was established unambiguously by single-crystal X-ray diffraction by Norte and collaborators (1988). Another levorotatory enantiomer showing a $[\alpha]_D$ value of -9.2° (*c* 0.35, MeOH) was determined to have a relative configuration (*R**,*S**) (Kossuga *et al*., 2004). Rogers *et al.* (2005) reported later its absolute configuration as 11*R*, 6*S* (note different numbering scheme) by using microscale hydrolysis followed by derivatization with Marfey's reagent. Since compound **3** exhibited magnitude of rotation value of $[\alpha]_D^{20}$ -6.6° ± 0.6° (*c* 0.28, $CH_3OH$), its relative configuration was suggested to be (*R**,*S**) as shown in Figure 1.

The presence of aeroplysinin-1 (**2**) along with a bisoxazolidinone derivative (**3**) and dienone ketals (**4** and **5**) in *P. purpurea* sponge might be in relevance to the previously reported chemical defense mechanism for Verongid sponges e.g., *Aplysina aerophoba*. This sponge, as well as other Verongid sponge produces isoxazoline alkaloids such as isofistularin-3 and aerophobin, a group of compounds possessing antifeeding activity. Bioconversion of these alkaloids into smaller products such as aeroplysinin-1 is paralleled to an increase of their antibiotic and cytotoxic activity in *Aplysina* sponges (Teeyapant *et al*., 1993; Weiss *et al*., 1996) but in contrast, feeding deterrent activity of the bioconversion products against the Mediterranean fish *Blennius sphinx* was significantly weaker (Thoms *et al*., 2004).

The above bioconversion seems to be specific for Verongid sponges (Thoms *et al*., 2006). It is typically catalyzed by enzymes that are usually separated from their substrates (precursors) by compartmentalization. Upon tissue damage, these compartments are disrupted, allowing contact between substrates and enzymes, which in turn induces the bioconversion. In many cases, the compounds resulted from such reactions arise from cleavage of the precursors (Thoms *et al*., 2006). This kind of compartmentalization was observed in the mesohyl tissue of *Aplysina* sponges, where the brominated isoxazoline alkaloids are mainly stored in spherulous cells (Thompson *et al*., 1983; Turon *et al*., 2000; Thoms *et al*., 2006).

Absence of the isoxazolines and on the contrary the presence of aeroplysinin-1, bioxazolidinone derivative and the ketals in *P. purpurea* studied here may be due to the isolation procedure performed on this sponge. Regarding the biotransformation of the isoxazolines happens so fast (45 second to 1 minute) (Ebel *et al*., 1997; Thoms *et al*., 2006), the compounds could no longer be detected in the ethanol extract. But the presence of isofistularin-3 in the fresh sponge can be traced from its bioconversion products, the aeroplysinin-1 and the bisoxazolidinone derivative (Thoms *et al*., 2006). Meanwhile, a dienone which is the conversion product of aeroplysinin-1 in the presence of methanol and water during the isolation procedure will form the ketals (Andersen and Faulkner, 1973). Therefore, dienone dimethoxy ketal and dienone ethoxy methoxy ketal in this sponge extract were suspected to be artefacts formed during the extraction and or purification. On the other hand, aplysamine-2 is not an isoxazoline derivative. Therefore it experienced neither this biotransformation nor the degradation by solvent and therefore could be isolated from the methanol extract.

Aplysamine-2 was reported for the first time by Xynas and Capon (1989) from the Australian sponge *Aplysina* sp. In 2005, Kijjoa and collaborators reported its presence in *Pseudoceratina purpurea* collected in the Gulf of Thailand as well as its moderate dose dependent growth inhibitory effects against human tumour cell lines, MCF-7 (breast); NCI-H460 (lung) and SF-268 (CNS) (Kijjoa *et al*., 2005). In this study, pronounced cytotoxicity activity against mouse lymphoma cell (L5178Y) was observed with $IC_{50}$ 1.7 μg/mL. Interestingly, antifouling assay on aplysamine-2 towards *Balanus improvisus* Darwin resulted in inhibition activity without cytotoxic effect encountered at 10 μM (Ortlepp, 2007) which makes this compound a promising candidate to be further developed not only as cytotoxic but also as an antifouling agent.

Besides aplysamine-2, in this study aeroplysinin-1 also showed pronounced cytotoxic activity against mouse lymphoma cell line (L5178Y) with $IC_{50}$ 0.57 μg/mL. In fact this compound has already known to be a potential cytotoxic agent against several human cell lines (Koulman *et al*., 1996; Kreuter *et al*., 1989, 1990).

Table IV. List of cytotoxicity assay results

| Name of compounds or extracts (10μg/mL) | Growth of L5178Y in % [a)] | $IC_{50}$ μg/mL |
|---|---|---|
| *Pseudoceratina purpurea* crude extract | 97.2 | n.t |
| Aplysamine-2 | 1.0 | 1.7 |
| Aeroplysinin-1 | -0.1 | 0.57 |
| Bisoxazolidinone derivative | 89.6 | n.t |
| Dienone dimethoxyketal | 96.5 | n.t. |
| Dienone methoxyethoxyketal | 87.4 | n.t. |

[a)] 10 μg/mL sample concentration; negative control = 100%; n.t.: not tested

## Conclusion

Extract of the sponge *Pseudoceratina purpurea* collected in Watudodol, Banyuwangi, yielded five brominated tyrosine derivatives, aplysamine-2 (**1**) accompanied with isofistularin-3-bioconversion products, (+)-aeroplysinin-1 (**2**), bisoxazolidinone derivatives (**3**), and possibly artefacts **4** and **5**. Compounds **1** – **3** play an important role in the sponge chemical defense mechanism. Compounds **1** and **2** showed growth inhibition of the mouse lymphoma cell line L5178Y with $IC_{50}$ value of 1.7 μg/mL and 0.57 μg/mL, respectively.

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