Studies on antibacterial and anti-HIV agents from Indonesian plants, marine sponges and fungi

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Abstract

An oxazinedione glycoside (**1**) was isolated from the methanol extract of *Spongia* sp. collected in Indonesia. Its chemical structure was elucidated by combination of extensive NMR spectroscopy, which suggested that the isolate may be new compound. To further elucidate the structure, enough amounts of the marine sponge are now collecting. In contrast, two new tetracyclic quassinoids, picrajavanicins A (**2**) and M (**3**) were isolated from the $CHCl_3$ soluble extract of *Picrasma javanica* bark collected in Myanmar. The structure of **2** and **3** were elucidated using spectroscopic techniques, including 1D and 2D NMR. The anti-HIV activity of the isolates were evaluated by inhibitory activity of Viral protein R (Vpr), which is an accessory gene of HIV-1 that plays important roles in viral pathogenesis, together with the antiprolifilative activity against a panel of five different human cancer cell lines. The $CHCl_3$-soluble extract of *P. javanica* and **2** and **3** showed mild anti-Vpr activity, in comparing with that of positive control, damnacanthal. In addition, **3** exhibited potent and selective antiproliferative activity against the human pancreatic cancer PANC-1 cell line.

1. Introduction

Nowadays, many bacteria infections have become impossible to treat due to antibiotic resistance. Despite the emergency and a lot of effort to find new antibiotics, only two new antibiotics have been approved since 2009. In addition, acquired immune deficiency syndrome (AIDS) also remains a serious health problem worldwide. Human immunodeficiency virus (HIV) is a retrovirus that causes AIDS. According to UNAIDS, globally, there were 34.0 million people living with HIV and 1.7 million people died of AIDS at the end of 2011.

Indonesia is a rich country in biodiversity due to the location in the tropical region. Abundant of medicinal plants are distributed in thousand islands of Indonesia. However, the most natural resources in Indonesia have remained to be investigated. Therefore, it is assumed that Indonesian natural resources such as medicinal plants, marine sponges, and fungi are good sources useful for discovering new drug lead candidates. In this research, we aimed to isolate the

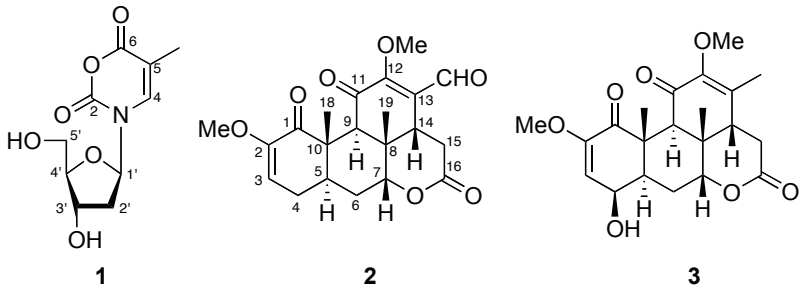

Fig. 1. Structures of oxazinedione derivative (**1**) and picrajavanicins A and M (**2** and **3**).

bioactive compounds from *Spondia* sp. collected in Indonesia. Simultaneously, we also performed the isolation of bioactive compounds from a fraction prepared from *Picrasma javanica* bark collected in Myanmar. The studies led to isolation of three compounds (**1-3**, Fig.1)

2. Materials and Methods

2.1 General experimental procedures

The ^{1}H, ^{13}C, and 2D NMR spectra were recorded using a JEOL JNM-LA400 spectrometer (Japan Spectroscopic Co. Ltd, Tokyo, Japan) with tetramethylsilane (Wako, Osaka, Japan) as an internal standard. Open column chromatography was performed with silica gel 60N, spherical, neutral, 40 − 50 μ m, (Kanto Chemical Co., Inc., Japan). Analytical TLC was carried out on precoated silica gel $60F_{254}$ (0.25 or 0.50 mm thickness, Merck). Analytical TLC was carried out on precoated silica gel $60F_{254}$ (0.25 or 0.50 mm thickness, Merck). The cell lines A549, HeLa, PSN-1, and MDA-MB-231 were available and maintained in our laboratory. Cell culture flasks and 96-well plates were from Corning (NY, USA).

2.2 Sponge material

The marine sponges were collected from the sea of Baranglompo in Indonesia and identified by Ms. Anna Manuputty in Research Center for Oceanography, LIPI (Indonesia Institute of Sciences) as *Spongia* sp. A voucher sample (SS-07-009) is preserved at the Bipharmaca Research Center of Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia.

2.3 Extraction and isolation of compounds from *Spongia* sp.

Marine *Spongia* sp. (100 g) were sliced and extracted with MeOH (200 mL) under sonication for 3 h. The extraction was repeated three times and then the combined extract was concentrated under reduced pressure and lyophilized to give a crude extract (3.2 g). The crude extract (3.2 g) was subjected to a silica gel open column chromatography eluted with *n*-hexane–EtOAc (80:20 to 0:100) followed by methanol elution to obtain 13 fractions. Fraction 13 (0.6 g) was chromatgraphed on a silica gel open column eluted with EtOAc–methanol (15:1 to 1:1) to give five further subfractions. The fraction 13-3 (7.5 mg) was furher separated by normal-phase preparative TLC with EtOAc–methanol–ammonia (15:1:0.1) to give compound **1** (0.6 mg).

2.4 Isolation of compounds from *P. javanica*

Fraction 7 (3.7 g), previously fractionated from the $CHCl_3$-soluble extract of the dried bark of *P. javanica*, was rechromatographed on a silica gel column chromatograph with *n*-hexane − $CHCl_3$ − EtOAc (1:1:1) to give four subfractions [7-1: 131 mg; 7-2: 534 mg; 7-3: 1.80 g; 7-4: 550 mg]. Purification of subfraction 7-2 using Cosmosil 75C18-OPN with MeCN − acetone − MeOH − H_2O (1:1:1:1) followed by a normal-phase preparative TLC with toluene − $CHCl_3$ − EtOAc (1:1:1) afforded picrajavanicin A (**2**, 18 mg).

Subfraction 7-4 (1.10 g), previously fractionated from the $CHCl_3$-soluble extract of the dried bark of *P. javanica*, was purified by chromatography on Sephadex LH20 with MeOH followed by normal-phase preparative TLC with $CHCl_3$–EtOA–propan-2-ol (2:2:0.5) (development four times) to afford picrajavanicin M (**3**, 15 mg).

2.5 Anti-Vpr assay

The established cell line, TREx-HeLa-Vpr (6,000 cells/well, 150 μL), was seeded in 48-well plates and incubated in α-minimal essential medium (α-MEM, Wako), supplemented with 10% fetal bovine serum (FBS, Nichirei Bioscience), 1% antibiotic antimycotic solution (Sigma-Aldrich), 5μg/mL of blasticidin (Invitrogen), and 50 μg/mL of zeocin, at 37°C under a 5% CO_2 and 95% air atmosphere, for 24 h. Since the expression of Vpr is regulated by tetracycline, the tetracycline-treated cells were designed to express Vpr by the addition of 50 μL of tetracycline (10 μg/mL). After 24 h incubation, 50 μL portions of various samples at different concentrations (1.25, 2.5, 5 μg/mL or μM) were added to the tetracycline-treated cells, and the wells without samples were used as controls. After 48 h incubation, 50 μL of 10% WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured, and the cell viability was calculated from the mean values of data from three wells, by the following equation.

$$(\%)\ \text{Cell viability} = 100 \times [\{Abs_{(\text{test samples})} - Abs_{(\text{blank})}\}/\{Abs_{(\text{control})} - Abs_{(\text{blank})}\}]$$

The inhibitory activity of the tested sample was obtained by comparing the number of viable cells treated with both tetracycline and sample to the number of viable cells treated with tetracycline without sample.

2.6 In vitro antiproliferative activity

The in vitro antiproliferative activity of **2** and **3** against the A549 (human lung cancer), HeLa (human cervix cancer), PANC-1 and PSN-1 (human pancreatic cancer), MDA-MB-231 (human breast cancer), and TIG-3 (normal human primary fibroblast) cell lines was evaluated by the procedure as described previously [1]. Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and cultured either in α-MEM or DMEM at 37°C under a 5% CO_2 and 95% air atmosphere for 24 h. After the cells were washed with PBS (Nissui Pharmaceuticals), serial dilutions of the samples to be tested were added. After 72 h incubation, the cells were washed with PBS, and 100 μL of α-MEM or DMEM containing 10% WST-8 cell counting kit solution

(Dojindo; Kumamoto, Japan) was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured. The different concentrations of the serial dilutions of the tested samples were 100 − 3.125 μg/mL for the crude extract, 100 − 3.125 μM for the isolated compounds, and 10 − 0.3125 μM for the positive control, respectively. Cell viability was calculated from the mean values of data from three wells by using the following equation, and the antiproliferative activity was expressed as the IC_{50} (50% inhibitory concentration) value.

(%) Cell viability = 100 × [{$Abs_{(test\ samples)}$ − $Abs_{(blank)}$}/{$Abs_{(control)}$ − $Abs_{(blank)}$}]

Results

The oxazinedione glycoside (**1**) was isolated as a white powder. Its molecular formula was determined to be $C_{10}H_{13}NO_6$ by HRFABMS, in conjunction with an NMR analysis data. The ^{1}H NMR spectrum (Table 1) showed signals of an olefinic proton [δ_H 7.80, d (J = 1.2, 1.2 Hz, H-4)], an anomeric proton [δ_H 6.26, t (J = 6.58 Hz, H-1′)], two oxygenated methine protons [δ_H 4.38, m (H-3′), 3.88, dd (J = 6.7, 3.2 Hz, H-4′)], an oxygenated methylene protons with geminal coupling as a methine proton [δ_H 3.78, 3.71, both dd (J = 12.0, 3.2 Hz, H_2-5′)], a methylene proton [δ_H 2.22, m (H_2-2′)], and a tertiary methyl proton [δ_H 1.86, d (J = 1.2 Hz, H_3-7)]. The ^{13}C NMR (Table 1) and HMQC spectra showed 10 carbon signals, including two carbonyl carbons (δ_C 166.6, 152.5), an oleofinic carbon (δ_C 138.3), a quaternary oleofinic carbon (δ_C 111.6), two oxygenated methine carbons (δ_C 88.9, 72.4), an anomeric carbon (δ_C 86.4), an oxigenated methylene carbon (δ_C 63.0), a methylene carbone (δ_C 41.3), and a methyl carbon (δ_C 12.6).

The ^{1}H − ^{1}H COSY and HMBC spectra (Fig. 2) revealed the connectivities between H-1′ (δ_H 6.26, t), H-3′ (δ_H 4.38, m), H-4′ (3.88, dd), H_2-5′ (δ_H 3.78, 3.71, both dd), and H_2-2′ (δ_H 2.22, m) and C-4′ (δ_C 88.9), C-1′ (δ_C 86.4), C-3′ (δ_C 72.4), C-5′ (δ_C 63.0), and C-2′ (δ_C 41.3), suggesting the presence of a pentose moiety. This also confirmed that the pentose moiety is C-2′ deoxy type. However, the HMBC correlations from H-4 (δ_H 7.80, d) to C-6 (δ_C 166.6), C-2 (δ_C 152.5), and C-5 (δ_C

Table 1. ^{1}H NMR Spectroscopic Data (600 MHz, $CDCl_3$) of oxazinedione glycoside (1) (δ in ppm and J Values in (Hz) in Parentheses)

	1	
position	δ_H	δ_C
2		152.5
4	7.80, d (1.2)	138.3
	2.31, m	
5		111.6
6		166.6
7	1.86, d (1.2)	12.6
1'	6.26, t (6.58)	86.4
2'	2.22, m	41.3
3'	4.38, m	72.4
4'	3.88, dd (6.7, 3.2)	88.9
5'	3.78, dd (12.0, 3.2)	63.0
	3.71, dd (12.0, 3.2)	

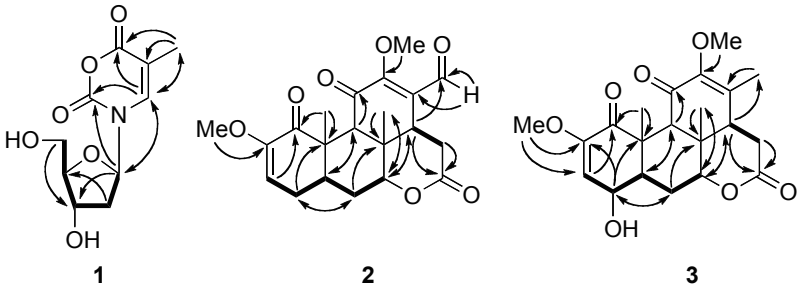

Fig. 2. COSY (bold lines) and key HMBC ($^{1}H \rightarrow ^{13}C$) (arrows) correlations in compounds **1**–**3**.

116.6) and from H_3-7 (δ_H 1.86, d) to C-4 (δ_C 138.3), C-5, and C-6 indicated the presence of a heterocycle oxazinedione moiety as the partial structure of **1**. The HMBC spectrum also displayed correlations from H-1′ on the pentose moiety to C-2 and C-4 on the oxazinedione moiety. These results suggest that the pentose moiety directly linked to nitrogen atom at 3 positon of the oxazinedione moiety, as well as the confirmation of the strcture of aglycone part of **1** to be 5-methyl-2,3-dihydro-6*H*-1,3-oxazine-2,6-dione.

Remaining relative configuration of sugar moiety of **1** assigned on the basis of 2D NOESY experiment, which showed the NOESY correlations between H-1′ and H-4′ and between H-3′ and H_2-5′, whereas not represented any correlations between H-1′, H-4, and H-5. These data suggested that the configuration of the pentose moiety is β at the anomeric proton (Fig.1). Further acid hydrolysis of **1** is now under way.

Picrajavanicin A (**2**) was obtained as a pale yellow amorphous solid, and its molecular formula was determined as $C_{21}H_{24}O_7$ from the HREIMS and ^{13}C NMR data. Spectroscopic analysis revealed UV absorption bands at 264 nm due to a conjugated enone system. The IR spectrum displayed absorption bands at 1730, 1702, and 1666 cm^{-1}, indicative of the presence of δ-lactone and α, β -unsaturated carbonyl functionalities. The ^{1}H NMR data (Table 2) displayed signals corresponding to an olefinic proton [δ_H 5.58, dd (J = 5.7, 2.3 Hz, H-3)], an oxymethine [δ_H 4.33, t (J = 3.0 Hz, H-7 β)], three methines [δ_H 2.30, m (H-5α), 2.97, dd (J = 11.1, 7.3 Hz, H-14 β), 3.06, s (H-9α)], three methylenes, two methoxy groups [δ_H 3.61, s (MeO-2), 4.00, s (MeO-12)], two tertiary methyls [δ_H 1.16, s (H_3-19), 1.56, s (H_3-18)], and an aldehyde group [δ_H 10.27, s (CHO-13)]. The ^{13}C NMR data (Table 2) included 21 signals for four olefinic carbons [δ_C 110.4 (C-3), 129.8 (C-13), 148.9 (C-2), 158.5 (C-12)], an oxymethine [δ_C 81.7 (C-7)], three methines [δ_C 35.8 (C-5), 37.9 (C-14), 48.0 (C-9)], three methylene carbons [δ_C 26.8 (C-4), 29.0 (C-6), 32.1 (C-15)], two methoxy groups [δ_C 55.0 (MeO-2), 60.8 (MeO-12)], two tertiary methyl groups [δ_C 11.4 (C-18), 22.1 (C-19)], two quaternary carbons [δ_C 37.3 (C-8), 45.8 (C-10)], an aldehyde carbon [δ_C 191.7 (CHO-13)], two carbonyls [δ_C 194.5 (C-11), 196.9 (C-1)], and one lactone carbonyl carbon [δ_C168.5 (C-16)]. These data suggested that **1** is a tetracyclic quassinoid similar to javanicin F [2], which was reported by Koike et al. However, there were significant differences found, including the absence of a tertiary methyl group and the presence of an aldehyde group in **1**. The $^{1}H - ^{13}C$ HMBC correlations (Fig. 2) of an aldehyde proton (δ_H 10.27, s) to C-13 (δ_C 129.8) and of H-14 [δ_H 2.97, dd (J = 11.1, 7.3 Hz)] to

Table 2. ^{1}**H NMR Spectroscopic Data (600 MHz, $CDCl_3$) of Picrajavanicins A and M (2, 3) (δ in ppm and *J* Values in (Hz) in Parentheses)**

	2		**3**	
position	δ_H	δ_C	δ_H	δ_C
1		196.9		196.8
2		148.9		149.1
3/3α	5.58, dd (5.7, 2.3)	110.4	5.56, d (2.5)	112.8
4/4α	2.21, m	26.8		68.2
4β	2.31, m		4.35, m	
5α	2.30, m	35.8	1.99, m	46.0[a]
6/6α	1.80, dt (14.6, 3.0)	29.0	2.42, dd (14.9, 3.7)	24.6
6β	2.02, td (14.6, 2.3)		1.97, dd (14.9, 1.9)	
7/7β	4.33, t (3.0)	81.7	4.33, t (1.9)	81.6
8		37.3		37.1
9/9α	3.06, s	48.0	3.01, s	46.0[a]
10		45.8		47.0
11		194.5		190.4
12		158.5		148.2
13		129.8		137.9
14/14β	2.97, dd (11.1, 7.3)	37.9	2.43, dd (11.6, 7.2)	46.5
15/15α	2.46, dd (18.7, 11.1)	32.1	2.63, dd (18.8, 11.6)	31.5
15β	3.05, dd (18.7, 7.3)		3.00, dd (18.8, 7.2)	
16		168.5		169.0
17		11.4		12.8
18	1.56, s	22.1	1.55, s	22.2
19	1.16, s	55.0	1.22, s	55.2
MeO-2	3.61, s		3.63, s	
MeO-12	4.00, s	60.8	3.67, s	59.3
Me-13			1.89, s	15.4
CHO-13	10.27, s	191.7		

[a] Overlapping resonances within the same column.

an aldehyde carbonyl carbon (δ_C 191.7) suggested that an aldehyde group is located at C-13. The relative configuration of **2** was assigned on the basis of a 2D NOESY NMR experiment. The NOESY correlations between H-5 and H-9 suggested α orientations of these protons, whereas those between H-7 and H-14/H_3-19 and between H_3-18 and H_3-19 suggested that they are β-oriented. Hence, the structure of **2** was assigned as shown, and was assigned the trivial name as picrajavanicin A (Fig. 1) [3]. To the best of our knowledge, this is the first report of the substitution by an aldehyde group at C-13 in a compound bearing the des-4-methylated picrasane skeleton.

Compound **3** was obtained as amorphous solids. The molecular formula, $C_{21}H_{26}O_7$, and the ^{1}H and ^{13}C NMR spectroscopic data (Tables 2) of **3** were similar to those of picrajavanicin L. The COSY and HMBC correlations (Fig. 2) confirmed that **3** is a C-4 isomer of picrajavanicin L. The significant difference was the α orientation of the C-4 hydroxy group, which was established from the NOESY correlations between H-4 (δ_H 4.35, m) and H_3-18 (δ_H 1.55, s). With respect to the absolute structure of picrajavanicin L, the absolute configuration at C-4 in **3** was deduced to be S. Accordingly, the structure of **3** was established, and it was named picrajavanicin M (Fig. 1) [4].

Since the $CHCl_3$-soluble extract of the *P. javanica* bark exhibited anti-Vpr activity at 5 μg/mL concentration, the anti-Vpr activity of **2** and **3** was evaluated against TREx-HeLa-Vpr cells. Damnacanthal [5], a natural product that is a potent inhibitor of Vpr-induced cell death, was used as a positive control in the present study. **2** and **3** showed the mild anti-Vpr activity (i.e. no. of cell viability treated with 1.25-5 μM of **2** and **3** is lesser than that of treated with damnacanthal). However, **2** exhibited no inhibitory activity against any of the cancer cells used (IC_{50} values > 10 μM) [3]. In contrast, **3** exhibited potent and selective antiproliferative activity against the human pancreatic cancer PANC-1 cell line with an IC_{50} value of 7.37 μM [4].

Discussion

The isolation of the secondary metabolites from a methanolic extract of *Spongia* sp. gave the oxazinedione glycoside (**1**). Although further careful strcutre elucidaton of **1** is requried due to less amount of this compound, **1** was likely to be the new compound, 3-(2-deoxy- β -D-erythro-pentofuranosyl)-5-methyl-2,3-dihydro-6H-1,3-oxazine-2,6-dione. Further structure elucidation and bioactvity of **1** would provide new insight into the marine natural products.

In contrast, further separation of the fraction previosuly prepared from the $CHCl_3$-soluble extract of the dried bark of *P. javanica* led to the isolation of picrajavanicins A (**2**) and M (**3**). **3** had potent and selective antiproliferative activity against the human pancreatic cancer PANC-1 cell line. Interestingly, most of previously isolated picrajavanicins A–L [3, 4] and quassinoids such as javanicins B [6], F [2], and I [7], picrasin A [8], and 2′-isopicrasin A [8] exhibited potent and selective activity against PANC-1 cells. Structure comparison of **2** and **3** with the other qussinoids indicated that the substitution at C-4 by carbonyl, hydroxy or methyl group was important for the antiproliferative activity. The presence of one of these functional groups at C-4 resulted in the increased potency [picrajavanicin K > 2′-isopicrasin A > picrasin A > **2** > picrajavanicin L > javanicin F (IC_{50} > 100 μM)]. Furthermore, the presence of $-CH_2OH$ at C-13 has been shown to increase the activity [picrajavanicin H > javanicin F (IC_{50} > 100 μM); **2** > picrajavanicin B (IC_{50} > 100 μM)]. These structure–activity relationships (SAR) suggested that the substituents at C-4 and C-13 are the critical factors for antiprolieferative activities of this class of quassinoids. However, **2** and **3** exhibited mild anti-Vpr activity in comparing with that of positive control, damnacanthal. To further clarify SAR of qussinoids regarding anti-Vpr activity, further isolations of qussinoids having anti-Vpr activity investigation will be necessary.

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