New Cytotoxic Pregnane-type Steroid from the Stem Bark of *Aglaia elliptica* (Meliaceae)

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**Abstract:** A new pregnane-type steroid, 2α-hydroxy-3α-methoxy-5α-pregnane (1), together with three known dammarane-type triterpenoid, 3β-acetyl-20S,24S-epoxy-25-hydroxydammarane (2), 20S,24S-epoxy-3α,25-dihydroxydammarane (3), and eichlerianic acid (4) have been isolated from the stem bark of *Aglaia elliptica*. The structures were determined by spectroscopic methods including the 2D-NMR techniques. Compound 1-4 showed moderate cytotoxic activity against P-388 murine leukemia cells.

**Keywords:** Pregnane-type steroid; *Aglaia elliptica*; cytotoxic activity; Meliaceae.

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1. Introduction

*Aglaia* is the largest genus belong to Meliaceae family contain about 150 species, and more than 65 species of them were grown in Indonesia [1,2]. Recently, *Aglaia* genus used traditionally for treatment some disease. In Thailand, *A. odorata* used for the treatment of traumatic injury, bruises, febrifuge, heart disease and toxin by causing vomiting [3] and the bark of *A. eximia* used by Indonesian people for treating coughs, skin, reducing fever, and contused wound [4]. This genus distributed in Indo-Malaysian region, especially in tropical and subtropical forest [1]. Previous phytochemical investigation in this genus reported contain interesting secondary metabolites with biological activity, including antifungal and antitumor sesquiterpenoid [5,6], cytotoxic and anti-inflammatory diterpenoid [7], cytotoxic and anti-retroviral triterpenoid [3,8-11], cytotoxic steroid [4], cytotoxic alkaloid [12,13], anti-inflammatory and cytotoxic rocaglamide [14-16]. During the course of our continuing search for anticancer compounds from *Aglaia* plant, the methanolic extract...
from the stem bark of *Aglaia elliptica* showed moderate cytotoxic activity against P-388 murine leukemia cells. *A. elliptica* is a rain forest plant, mainly distributed in some part in Kalimantan island in central part of Indonesia [17]. This plant has been used as traditional medicine for healing of tumor, skin and contused wound diseases [18]. Previous phytochemical study from this plant reported the presence of diamide and cycloartane-type triterpenoid from leaves [19] and cytotoxic 1H-cyclopenta[b]benzofuran from fruits [18]. Herein, we describe the isolation and structure determination of the new pregnane-type steroid, 2α-hydroxy-3α-methoxy-5α-pregnane (1) together with the cytotoxic activity of compounds 1–4 against P-388 murine leukemia cells.

2. Materials and Methods

2.1. General

Optical rotation was recorded on a Perkin-Elmer 341 (Waltham, MA, USA). Melting points were measured on IA9000 electrothermal melting point (Bibby Scientific Limited, Staffordshire, UK). The IR spectra were recorded on a 1760X Perkin-Elmer FT-IR using KBr as pellet (Waltham, MA, USA). Mass spectra were obtained with a HR-MS XEVO™ Water Qtof mass spectrometer (Waters, MA, USA). 1H- and 13C-NMR spectra were measured with a JEOL ECZ-600 and JEOL JNM A-500 spectrometer (Tokyo, Japan) using TMS as an internal standard. Column chromatography was conducted on silica gel 60 (Kanto Chemical Co., Inc., Japan) and Sephadex LH-20 (Sigma-Aldrich). TLC plates with silica gel GF254 (Merck, 0.25 mm) and compound detection by spraying with 10% H2SO4 in ethanol, continued by heating.

2.2. Plant Material

The stem bark of *A. elliptica* were obtained in Bogor Botanical Garden, West Java, Indonesia in June 2015. The plant was identified and classified by the staff of Herbarium Bogoriense, Indonesia and a voucher specimen (No. Bo-1294562) was deposited at the herbarium.

2.3. Extraction and Isolation

The crushed and dried powder of stem bark of *A. elliptica* (2.3 kg) was macerated with methanol (12 L x 5) at room temperature. After removing the solvent in vacuum condition, the dark brown concentrated of MeOH extract (321.5 g) was obtained. This extract was first dissolved in H2O and partitioned with n-hexane, EtOAc, and n-butanol, respectively. The n-hexane fraction (22.6 g) was fractionated by vacuum liquid chromatography on silica gel using a gradient combination of n-hexane and EtOAc (10:0, 9.5:0.5, 9:1, 8.5:1.5,...,0:10). Based on TLC analyses, similar fractions were combined to yield five main fractions (A-E). Fraction C (5 g) was separated on a chromatography column of silica gel, eluted with a combination of gradient of n-hexane–EtOAc (10:0–1:1), to give five subfractions (C1–C5). Subfraction C2 was separated on a chromatography column of silica gel, eluted with n-hexane–EtOAc (10:0–5:5), to give three subfractions (C2A-C2C). Subfraction C2B was separated on a preparative TLC, eluted with combination of n-hexane–EtOAc (8:5:1.5), to give 2 (10.3 mg). Subfraction C5 was separated on a chromatography column of silica gel, eluted with n-hexane–EtOAc (10:0–7:3), to give three subfractions (C5A-C5C). Subfraction C5B was recrystallized in MeOH, to give 3 (19.5 mg). The EtOAc soluble fraction (20 g) was fractionated by column chromatography on silica gel using a gradient combination of n-hexane and EtOAc to give fractions F–J. Fraction F (627 mg) was separated on a chromatography column of silica gel, eluted with CHCl3–EtOAc (10:0-7:3), to give four subfractions (F1–F4). Subfraction F4 was chromatographed on sephadex LH-20, eluted with CHCl3–MeOH (7:3), to give 4 (119.2 mg). Fraction H (1.76 g) was chromatographed on a column of silica gel, eluted with CHCl3–MeOH (10:0-7:3), to give seven subfractions (H1–H7). Subfraction H2 was separated on a preparative TLC, eluted with CHCl3–EtOAc (5:5), to give 1 (5 mg).
2.3.1. 2α-hydroxy-3α-methoxy-5α-pregnane (I)

Colorless oil; [α]°D +17.1° (c, 0.3, CHCl₃); IR (KBr) νmax 3403, 2980, 1067, 1073 cm⁻¹. ¹H NMR (CDCl₃, 600 MHz), see Table 1; ¹³C NMR (CDCl₃, 150 MHz), see Table 1; HR-ESI-TOFMS (negative ion mode) m/z 333.2425 [M-H]⁻, (calcd. for C₂₂H₃₈O₂, m/z 334.2422).

2.4. Determination of Cytotoxic Activities

The P388 murine leukemia cells were seeded into 96-well plates at an initial cell density of approximately 3 x 10⁴ cells cm⁻². After 24 h incubation for cell attachment and growth, variation concentration of samples were added. The compounds in DMSO solvent added at the required concentration. Subsequent six desirable concentrations were prepared using PBS (phosphoric buffer solution, pH = 7.30 - 7.65), the negative control is only DMSO. After a 48 h incubation period, the assay was terminated by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and the incubation was continued for another 4 h, in which the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added and another 24 h incubation was conducted using a micro plate reader at 550 nm, optical density was read, the plotted graph of percentage live cells compared to control (%), receiving only PBS and DMSO, versus the tested concentration of compounds will give the IC₅₀ values in µg/mL. The IC₅₀ value is the concentration required for 50% growth inhibition. Each experiment was run in triplicate and averaged.

Figure 1. Structures of compounds 1-4.
3. Results and Discussion

3.1. Structure Elucidation

Compound 1 was a colorless oil form, with $[\alpha]_D^{20} + 17.1^\circ$ ($c$ 0.3, CHCl$_3$). The molecular composition is determined as C$_{22}$H$_{38}$O$_3$, from the HR-TOF-MS with molecular ion peak found $m/z$ 333.2425 [M-H], (calcd. for C$_{22}$H$_{39}$O$_3, m/z$ 334.2422) and together with NMR data (Table 1). The IR spectrum showed the functional group of a hydroxyl (3403 cm$^{-1}$) and ether groups (1067 and 1073 cm$^{-1}$). In $^{13}$C NMR spectrum, its resonated of 22 carbons, which were classified as 4 methyl (one methoxy), 9 methylenes, 7 methines (two oxygenated), and 2 quarternary carbons. This molecule have four degrees of unsaturation, which consistent with tetracyclic pregnane-type steroid skeleton. The presence of singlets of two tertiary methyls ($\delta_{HI}$ 0.67 and 0.86, each 3H), one primary methyl ($\delta_{HI}$ 1.00, 3H), one methoxy ($\delta_{HI}$ 3.95, 3H), and two oxymethine protons ($\delta_{HI}$ 3.60 and 3.40, each 1H) were observed in $^1$H NMR spectrum and supported the presence of pregnane-type steroid skeleton of compound 1. The spectral data of 1 was similar to aglatomin A, isolated from A. tomentosa [20], except the absence of carbonyl ketone and different position of hydroxy and methoxy groups in 1. The confirmed structure of 1 was obtained from the HMBC and $^1$H-$^1$H COSY experiments (Figure 2). The skeleton of pregnane-type steroid was determined by HMBC correlation of tertiary methyls. The correlation of CH$_3$-19 ($\delta_{HI}$ 0.86) to C-1 ($\delta_C$ 44.9), C-5 ($\delta_C$ 54.2), C-10 ($\delta_C$ 37.7) and C-9 ($\delta_C$ 38.6) and correlation of CH$_3$-18 ($\delta_{HI}$ 0.67) to C-12 ($\delta_C$ 38.2), C-13 ($\delta_C$ 42.2), C-14 ($\delta_C$ 50.5) and C-17 ($\delta_C$ 65.4), showed the characteristic of tetracyclic core of pregnane-type steroid [21]. The presence of ethyl group in side chain of 1, was proved by typical of $^1$H NMR of primary methyl CH$_2$-21 ($\delta_{HI}$ 1.00, t, $J = 6.6$ Hz) and HMBC correlation between CH$_3$-21 ($\delta_{HI}$ 1.00) to C-20 ($\delta_C$ 17.7) and C-17 ($\delta_C$ 65.4), this correlation also indicated that the ethyl group was linked at C-17. The absence of ketone group at C-16 in 1, instead the presence of typical methylene group at $\delta_{HI}$ 1.25 ($\delta_C$ 44.8). The position of hydroxy and methoxy groups were proved by HMBC and $^1$H-$^1$H COSY correlations. HMBC cross peak which observed between H-1 ($\delta_{HI}$ 2.00) to oxymethine at C-2 ($\delta_C$ 73.0) and C-3 ($\delta_C$ 76.4), and COSY correlation between H-1/H-2/H-3/H-4 suggested that the oxymethins were located at position C-2 and C-3, respectively. Position of methoxy group at C-3 established from correlation at methoxyl signal at $\delta_{HI}$ 3.95 to C-3 ($\delta_C$ 76.4), consequently, the hydroxy group was attached at C-2. Configuration of 5a was proved by the upfield shift of CH$_3$-19 [22] and the NOESY correlation H-5a/H-4a. The NOESY experiment confirmed the configuration of H-14a and H-17a, which was the usual configurations from pregnane derivatives. Finally the H-2b and H-3b configurations were deduced from the cross peaks of NOESY H-2/H-19 and H-2/H-3 (Figure 2). Therefore, the structure of new pregnane-type steroid, 2α-hydroxy-3α-methoxy-5α-pregnane was thus elucidated to be 1.

![Figure 2](image-url)

**Figure 2.** Selected HMBC, $^1$H-$^1$H COSY, and $^1$H-$^1$H NOESY correlations for 1.

The known compounds $3\beta$-acetyl-20$\delta,24\delta$-epoxy-25-hydroxydammarane (2) [23], 20$\delta,24\delta$-epoxy-3α,25-dihydroxydammarane (3) [23], and eichlerianic acid (4) [23], were confirmed by comparison and biogenetic analysis of these compounds with reported values.
3.2 Cytotoxic Activity

The cytotoxicity values of the four isolated compounds 1-4 against the P388 murine leukemia cells were conducted based on experiment in previous paper [8,24,25] and artonin E (IC_{50} 0.75 µg/mL) used as a positive control [26].

Compounds 1-4 were evaluated for their cytotoxicity against the P-388 murine leukemia cells and showed IC_{50} values of 6.07 ± 0.02, 4.09 ± 0.09, 11.03 ± 0.10, and 5.59 ± 0.10 µg/mL, respectively. Among those isolated compound, 3β-acetyl-20S,24S-epoxy-25-hydroxydammarane (2) showed strongest activity whereas eichlerianic acid (4) having open chain A-ring showed stronger activity than 20S,24S-epoxy-3α,25-dihydroxydammarane (3) suggested that acetyl group and open chan A-ring in dammarane-type triterpenoids may be play an important role for cytotoxic activity.

Table 1. NMR Data (600 MHz for ^1H and 150 MHz for ^13C, in CDCl3) for 1.

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<th>Position</th>
<th>^1C NMR δc (mult.)</th>
<th>^1H NMR δH (Integral, mult., J=Hz)</th>
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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/RNP
Pregnane-type steroid from *Aglaia elliptica*

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References


