Protoxylogranatin B, a Key Biosynthetic Intermediate from *Xylocarpus granatum*: Suggesting an *Oxidative Cleavage* Biogenetic Pathway to Limonoid

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Abstract: Protoxylogranatin B, a new protolimonoid identified from the seeds of a Chinese mangrove, *Xylocarpus granatum*, was proposed as the key biosynthetic intermediate. Its structure was established on the basis of spectroscopic techniques, including ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY spectra. The finding of this compound suggested an *oxidative cleavage* biogenetic pathway from protolimonoid to limonoid.

Keywords: Protoxylogranatin B, *Xylocarpus granatum*, limonoid, biosynthetic intermediate, biogenetic pathway, protolimonoid, gedunin.

1. INTRODUCTION

Limonoids, which have been found to date only in plants of the order Rutales, are triterpene derivatives from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton. They are classified by the type of four rings in the intact triterpene nucleus, usually oxidized and designated as A, B, C and D. Phragmalins, such as pseudrelones A_1-A_2 [1] isolated from *Pseudocedrela kotschyii* and khayanolides A-C [2] from *Khaya senegalensis*, have characteristic tricyclo [3.3.$1^{2,10}$.$1^{1,4}$] decane or tricyclo [4.2.$1^{10,30}$.$1^{1,4}$] decane ring systems.

The mangroves *Xylocarpus granatum* and *X. moluccensis* are distinguished for producing antifeedant limonoids, especially phragmalins and mexicanolides. Previous investigations on the seeds of the above two Meliaceae plants, afforded an obacunol, two phragmalins, three andirobins and fourteen mexicanolides, including xyloccensins A-K [3-7]. Recently, we have reported the isolation and identification of eight unique 8,9,30-phragmalin *ortho* esters and thirteen limonoids with a new carbon skeleton from the bark and seeds of a Chinese mangrove *X. granatum*, respectively [8-10]. To date, twenty-three phragmalins, including three 1,8,9-phragmalin *ortho* esters, eight 8,9,30-phragmalin *ortho* esters and twelve polyhydroxylated phragmalins, were isolated from the timber, seeds and fruits of *X. granatum* and *X. moluccensis*, together with forty-two mexicanolides [11]. In the current paper, we present the isolation and characterization of a new protolimonoid, protoxylogranatin B (**1**), from the seeds of a Chinese *Xylocarpus granatum*. The structure of protoxylogranatin B was established on the basis of spectroscopic techniques, including ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY spectra.

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2. RESULTS AND DISCUSSION

Protoxylogranatin B (**1**), a white amorphous powder, had the molecular formula $C_{32}H_{46}O_8$, as established by HR-TOFMS (*m/z* 581.3089, calcd for $[M + Na]^+$, 581.3085). Consequently, **1** had ten degrees of unsaturation. From the ^{1}H and ^{13}C NMR data (Table **1**) of **1**, it was clear that six of the ten elements of unsaturation came from two carbon-carbon double bonds and four carbonyls. Therefore, the molecule was tetracyclic. DEPT experiments revealed that **1** had eight methyls, six methylenes, eight methines (three olefinic) and ten quaternary carbons (including four carbonyls). Moreover, the ^{1}H and ^{13}C NMR data of **1** (Table **1**) showed the presence of an acetoxy (δ_H 1.95, s; δ_C 21.2 CH_3, 170.0 qC), a conjugated ketone (δ_C 204.5, s), a carboxyl carbon (δ_C 174.8 qC), an ester carbon (δ_C 177.2 qC) and three oxygenated carbons, being a methine (δ_H 5.23, br s; δ_C 74.6), a methylene [δ_H 4.43 (d, J = 8.5 Hz), 4.06 (dd, J = 10.8, 7.0 Hz); δ_C 66.9] and a quaternary carbon (δ_C 72.2).

The above NMR data coupled with seven methyls, four rings and thirty carbons in the nucleus of **1** suggested that it might be a protolimonoid. The ^{1}H and ^{13}C NMR data (Table **1**) of **1** were similar to those of protoxylogranatin A [12], isolated from the seeds of the same plant. Comparison of the above NMR data with those of protoxylogranatin A revealed that the four rings in the nucleus of **1** were the same as those in protoxylogranatin A. However, **1** had a linear side chain and an additional acetoxy group. The connection of the fragment 20-CH–22-CH_2–23-CH_2O- was confirmed by the homonuclear *proton-proton spin* system observed in the ^{1}H-^{1}H COSY spectrum of **1** (Fig. **1**). A terminal carboxyl group was linked to C-20 of the above fragment and this connection was corroborated by the HMBC correlation of H-20 to the carbon (δ_C 174.8) of this carboxyl group. The second fragment (from C-24 to Me-27) was characterized by HMBC studies. An oxygenated quaternary carbon (δ_C 72.2, C-25) bearing two symmetrical methyls (δ_H 1.44 s, Me-26 and Me-27) was linked with an ester carbonyl (δ_C 177.2, C-24). This connection was confirmed by HMBC interactions between H_3-26/C-25, H_3-27/C-25, H_3-26/C-24 and H_3-27/C-24.

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Table 1. ^{1}H (HSQC) and ^{13}C NMR Data for Compound 1 (500 and 125 MHz, $CDCl_3$)

No.	δ_H (*J* in Hz)	δ_C
1	7.15; d; 10.0	158.0 CH
2	5.87; d; 10.0	125.6 CH
3		204.5 qC
4		44.2 qC
5	2.20; m	46.2 CH_2
6α 6β	1.80; m 1.94; m	23.9 CH_2
7	5.23; brs	74.6 CH
8		42.8 qC
9	2.22; m	38.5 CH
10		39.9 qC
11α 11β	1.95; m 1.70; m	16.7 CH_2
12α 12β	1.90; m 1.56; m	34.3 CH_2
13		46.6 qC
14		159.1 qC
15	5.30; s	119.0 CH
16α 16β	2.05; m 2.24; m	34.7 CH_2
17	1.80; s	54.7 CH
18	1.09; s	19.8 CH_3
19	1.17; s	19.0 CH_3
20	2.47; m	35.8 CH
21		174.8 qC
22α 22β	2.38; m 2.52; m	35.0 CH_2
23	4.43; d; 8.5 4.06; dd; 10.8, 7.0	66.9 CH_2
24		177.2 qC
25		72.2 qC
26	1.44; s	27.3 CH_3
27	1.44; s	27.3 CH_3
28	1.07; s	27.4 CH_3
29	1.07; s	21.3 CH_3
30	1.18; s	27.4 CH_3
7-OAc	1.95; s	21.2 CH_3
		170.0 qC

Moreover, HMBC correlations of H_2-23 to C-24 linked the above two fragments *via* an ester. The presence of the strong HMBC correlation of H-7 (5.23, br s) to C-1′ (170.0 qC) of the acetoxy group suggested its location at C-7. Thus, the planar structure of **1** was characterized as shown in Chart **1**.

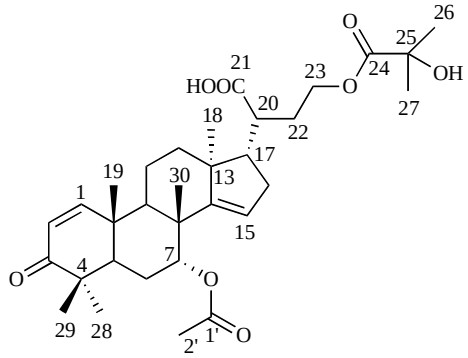


Chart 1. The structure of compound **1**.

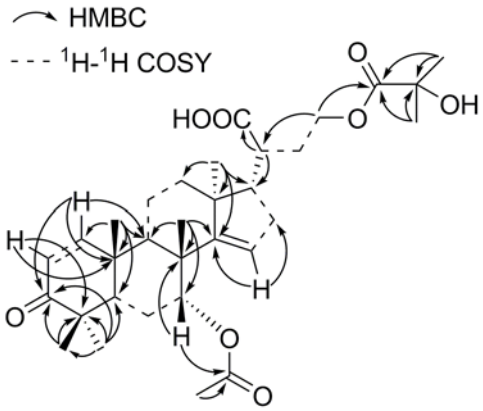


Fig. (1). Selected ^{1}H-^{1}H COSY and HMBC correlations for compound **1**.

The relative stereochemistry of **1** was established on the basis of NOE interactions as shown in Fig. (**2**). The significant NOE interactions between H-7/H_3-30, H-7/H-15, and H-7/H-6β, but not between H-7/H-5 and H-7/H_3-18, helped to establish this 7β-H and the corresponding 7α-acetoxy group. Similarly, NOE interactions between H-20/H_3-18 suggested the 20α-H. NOE interactions between H-17/H-15, H_3-18/H-9, H_3-19/H_3-30, H_3-28/H_3-19, H-5/H_3-29 and H-5/H-9 (Fig. **2**), indicated a *cis* orientation between these respective protons. Based on the above results, the relative stereochemistry of **1** was elucidated as shown in Fig. (**2**).

Protoxylogranatin B (**1**) was proposed as a key biosynthetic intermediate and the finding of this compound suggested an *oxidative cleavage* biogenetic pathway from protolimonoid to gedunin, a well-known limonoid obtained from plants of *Xylocarpus* genus. A probable biogenetic pathway was depicted in Scheme **1**. The biogenetic origin of **1** might be spitacin [13], a protolimonoid obtained from the seeds of the same plant [14]. Dehydration of 23- and 24- two hydroxyls in spitacin could form a 23, 24-oxirane ring, thus generating the intermediate **a**, whose further oxidation could afford the intermediate **b**. The cleavage of the ester bond of **b** and then atomic rearrangement would produce the key intermediate, protoxylogranatin B (**1**). Moreover, the loss of a 2-hydroxy-2-methyl propanoyl group in protoxylogranatin B and cyclic ester formation could generate the intermediate **d**, whose atomic rearrangement, dehydroxylation and hydrogenation would lead to the formation of a 17-β-furyl ring E, thus generating the intermediate **g**, being a typical limonoid. Final two oxidative steps of the ring D in **g** could form a δ-lactone and a 14,15-oxirane ring, resulting in the target limonoid, gedunin.

To our knowledge, this is the first report of a protolimonoid possessing a 2-hydroxy-2-methyl propanoyloxy group as the terminal four carbons of C24 to C27 in its side chain. This study also demonstrated that *X. granatum* is a new source for the production of novel protolimonoids.

3. EXPERIMENTAL SECTION

3.1. General Experimental Procedures

Optical rotations were recorded on a POLAPTRONIC HNQW5 automatic high-resolution polarimeter (Schmidt & Haensch Co. Ltd.). UV spectra were obtained on a Beckman DU-640 UV spectrophotometer and matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) spectra were measured on a Bruker APEX II spectrometer in positive ion mode. NMR spectra were recorded in $CDCl_3$ a Bruker AV-500 spectrometer (500

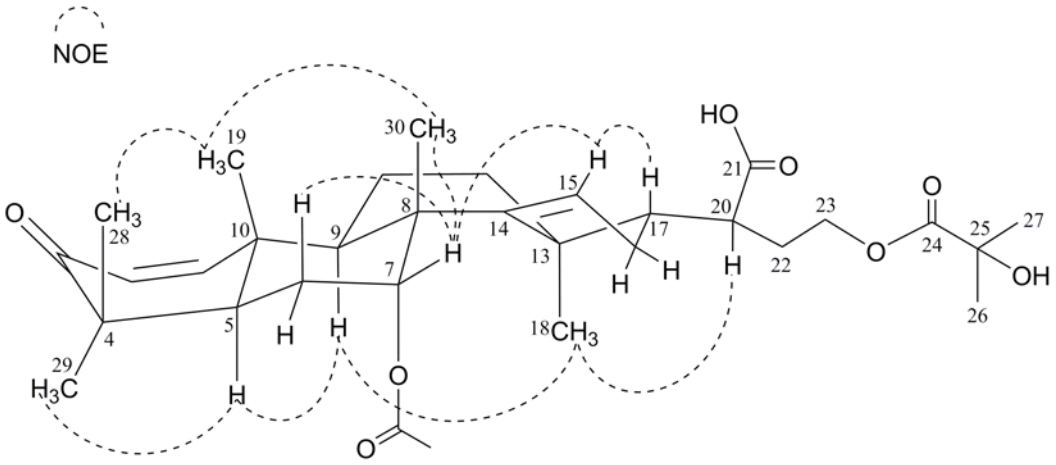
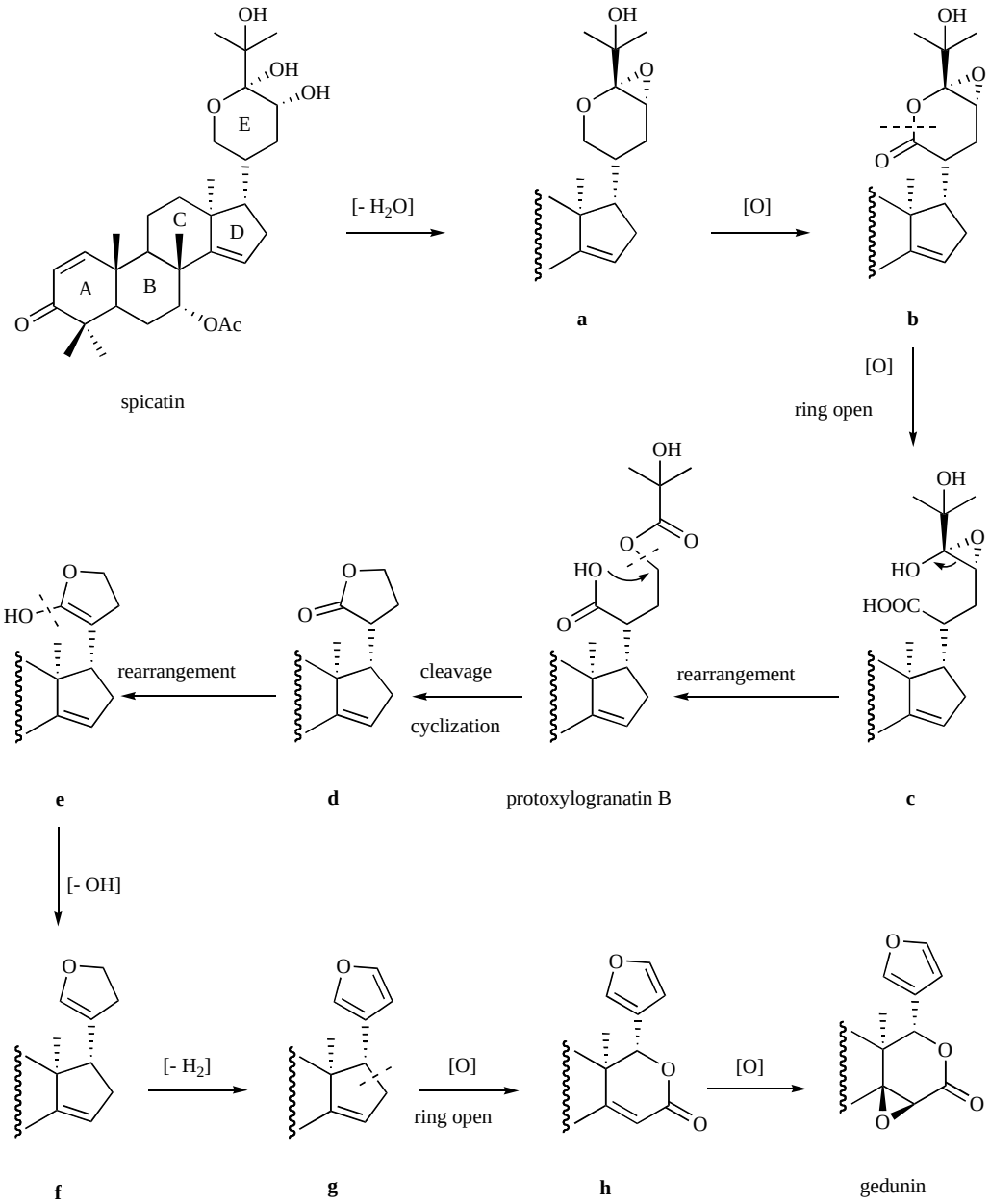


Fig. (2). Diagnostic NOE correlations for compound **1**.



Scheme 1. Proposed biogenetic pathway from spicatin to gedunin *via* protoxylogranatin B.

MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane as the internal standard. Preparative HPLC was carried out on ODS columns (250 × 10 mm i.d. and 250 × 4.6 mm i.d.,YMC) with a Waters 2998 photodiode array detector. For CC, silica gel (200-300 mesh) (Qingdao Mar. Chem. Ind. Co. Ltd.) and RP C_{18} gel (Cosmosil C18-PREP 140 μm, Nacalai Tesque, Kyoto, Japan) were used.

3.2. Plant Material

The seeds of the mangrove *Xylocarpus granatum* were collected in May 2006 from Hainan Island, southern China. The identification of the plant was performed by Prof. Jun Wu, Key Laboratory of Marine Bio-resources Sustainable Utilization, South China Sea Institute of Oceanology, Chinese Academy of Sciences. A voucher sample (NO. GKLMMM-002-3) is maintained in the Herbarium of the South China Sea Institute of Oceanology.

3.3. Extraction and Isolation

The dried seeds (5 kg) of *X. granatum* were extracted with hot 95% ethanol three times. The extract was concentrated under reduced pressure, followed by suspension in water. After defatting with *n*-hexane, the aqueous layer was further extracted with ethyl acetate. The ethyl acetate extract (150 g) was chromatographed by CC on Si gel and eluted using a chloroform-methanol system (100:0~2:1) to yield 100 fractions. Fractions 39 to 45 (2.2 g) were combined and further purified with preparative HPLC (YMC-Pack ODS-5-A, 250 × 20 mm i.d., acetonitrile–water 45:55 to 50: 50) to afford protoxylogranatin B (**1**, 3 mg).

Protoxylogranatin B (1)

White, amorphous powder; $[\alpha]_D^{25}$ − 2.5 (*c* 0.04, acetone); UV (MeCN) λ_{max} 214, 230 nm; For ^{1}H and ^{13}C NMR data

(see Table **1**); HR-TOFMS *m/z* 559.3249 [calcd for $C_{32}H_{47}O_8$ $[M + H]^+$, 559.3265], *m/z* 581.3089 [calcd for $C_{32}H_{46}O_8Na$ $[M + Na]^+$, 581.3085], *m/z* 597.2877 [calcd for $C_{32}H_{46}O_8K$ $[M + K]^+$, 597.2824].

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