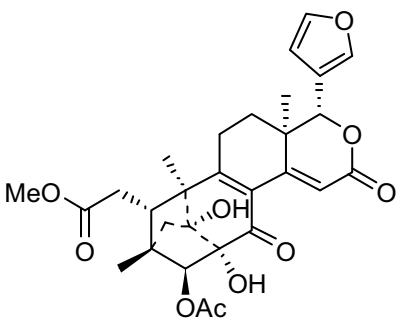
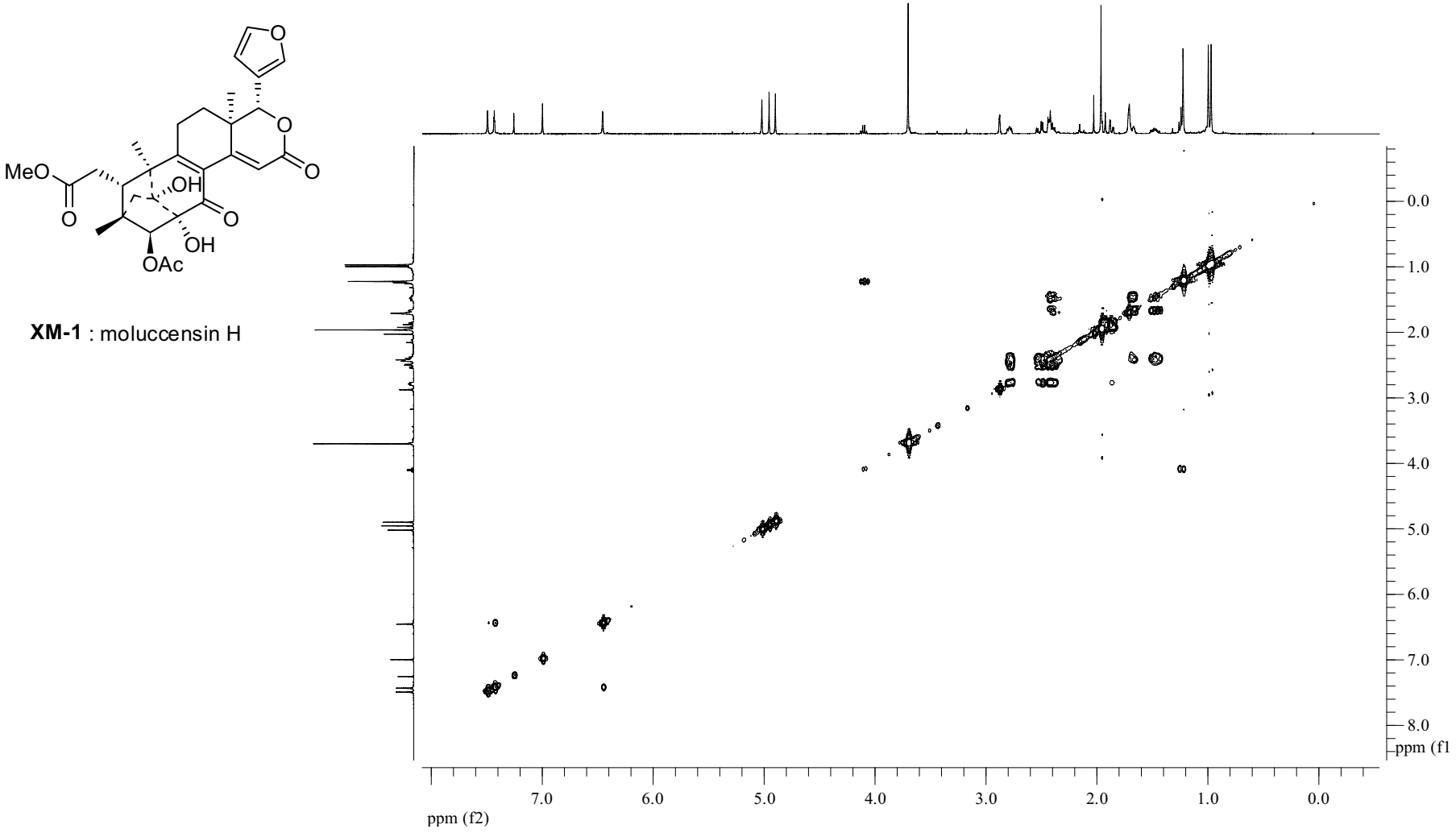


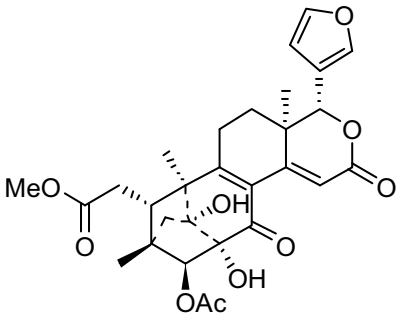
XM-1 : moluccensin H

Figure S42. ^{13}C NMR spectrum of **XM-1** in $CDCl_3$.



XM-1 : moluccensin H

Figure S43. COSY spectrum of **XM-1** in $CDCl_3$.



XM-1 : moluccensin H

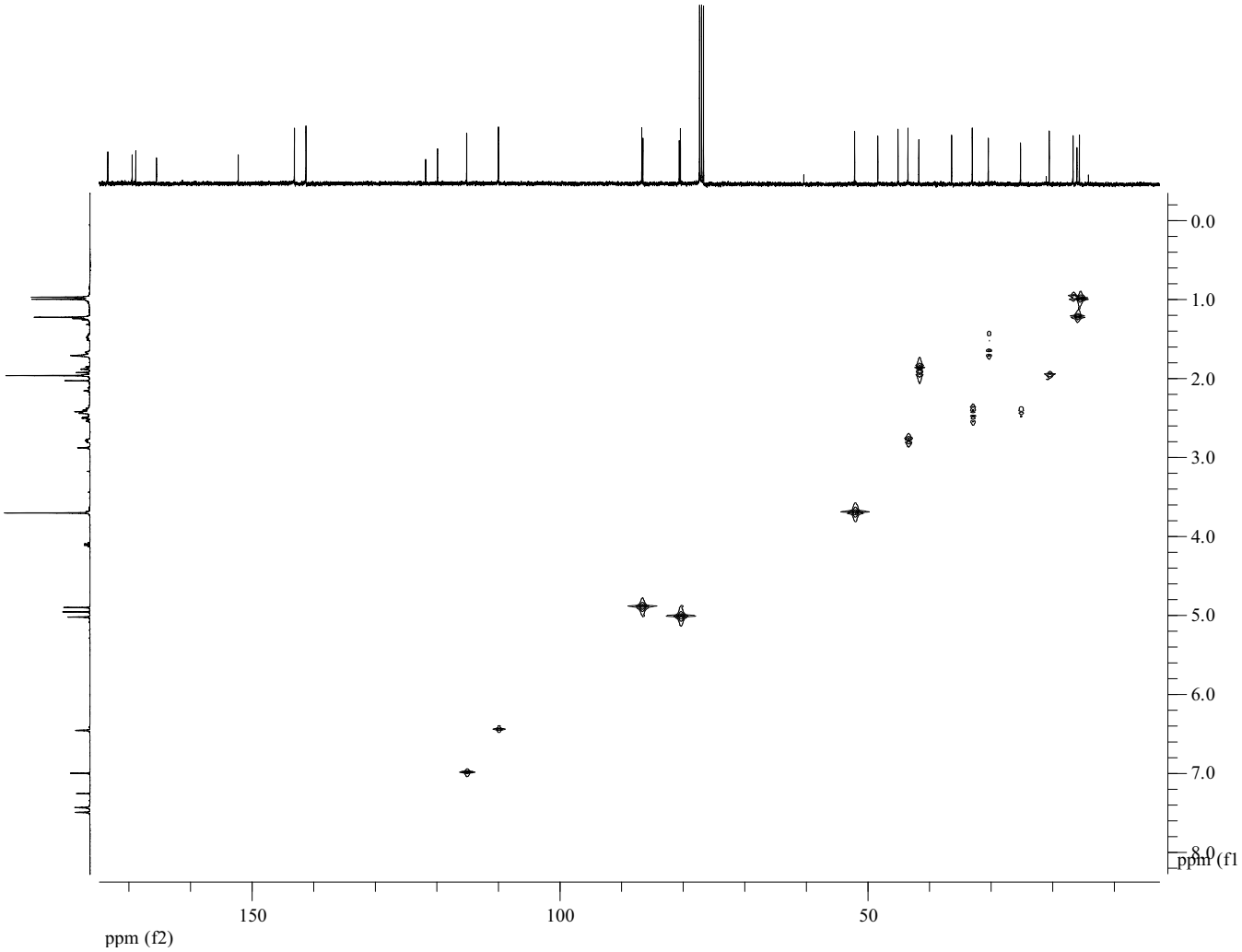
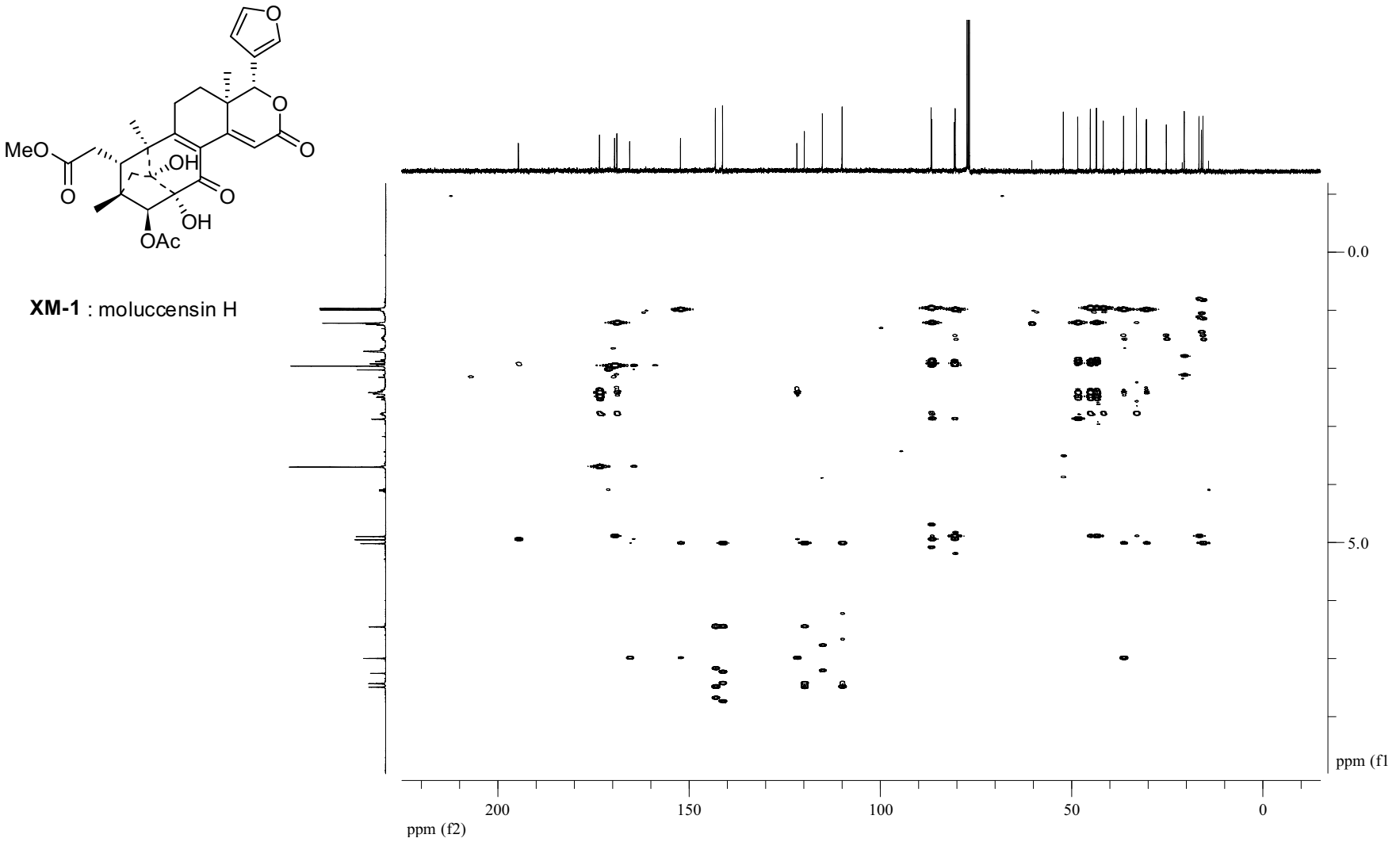
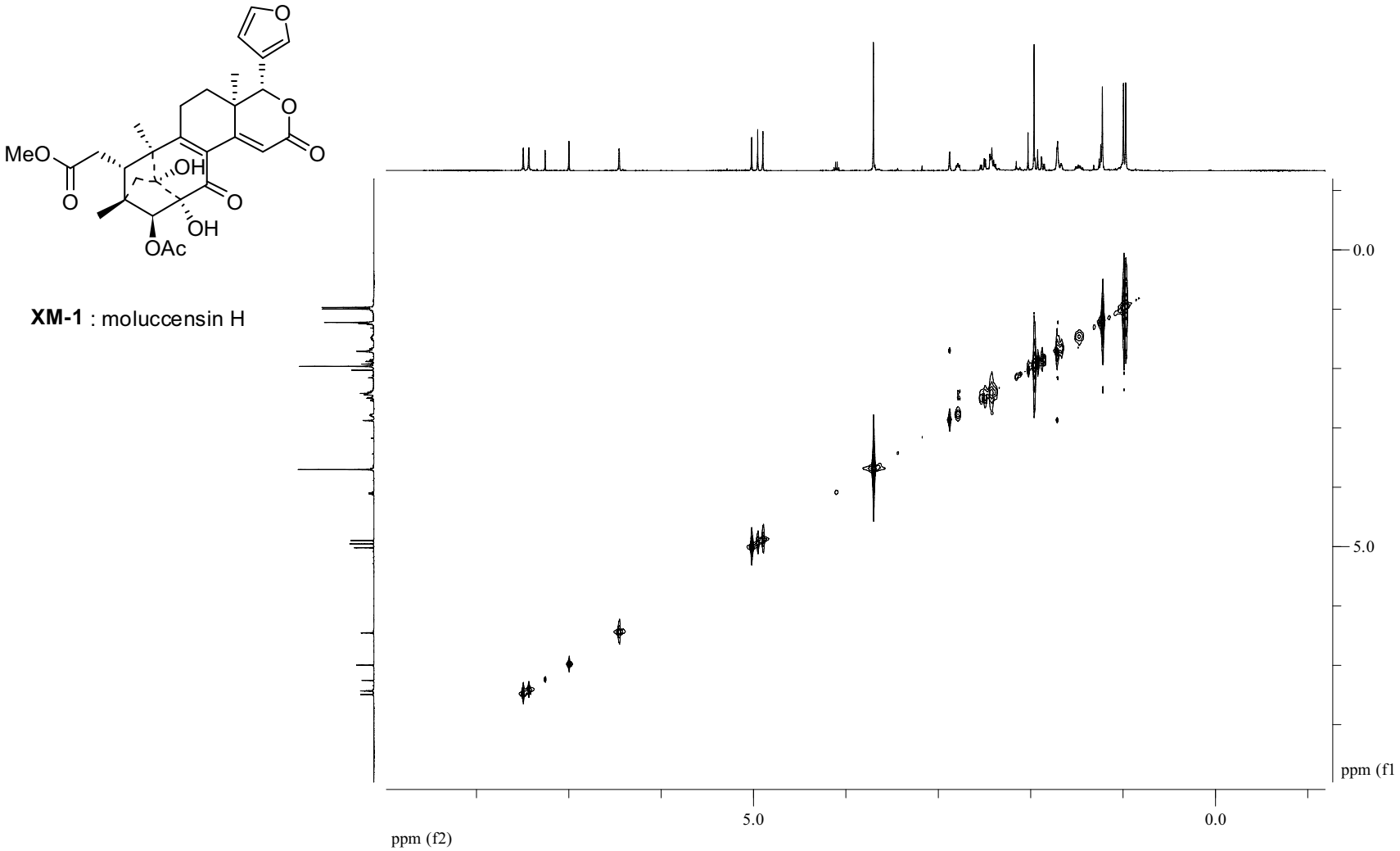


Figure S44. HSQC spectrum of **XM-1** in $CDCl_3$.



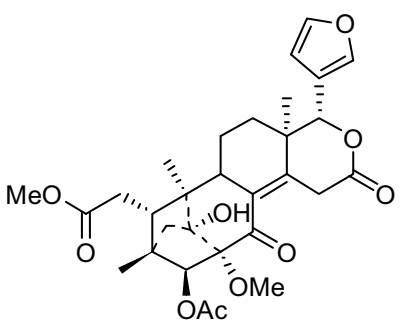
XM-1 : moluccensin H

Figure S45. HMBC spectrum of **XM-1** in $CDCl_3$.



XM-1 : moluccensin H

Figure S46. NOESY spectrum of **XM-1** in $CDCl_3$.



XM-2 : moluccensin I

Figure S47. ^{1}H NMR spectrum of **XM-2** in $CDCl_3$.

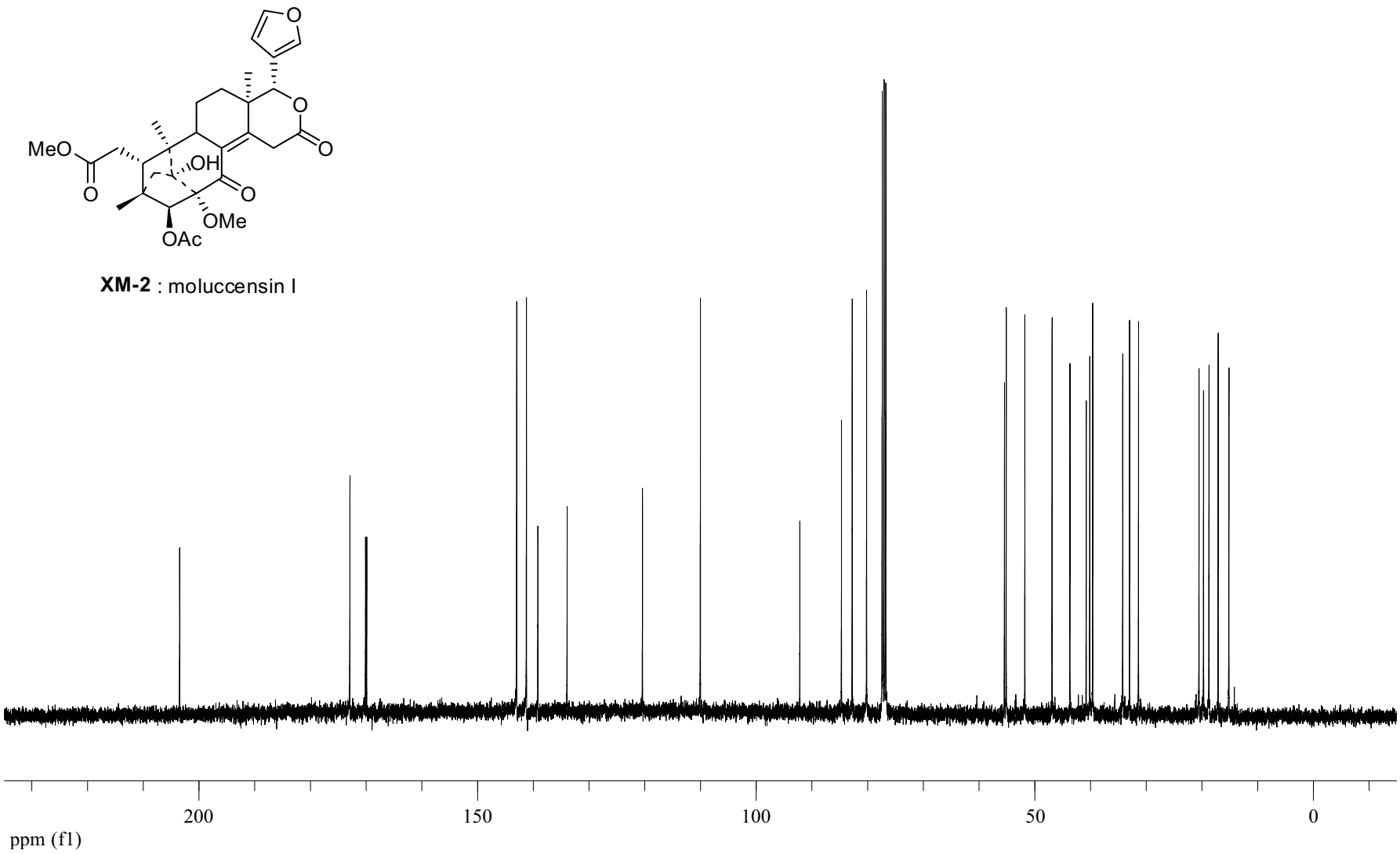
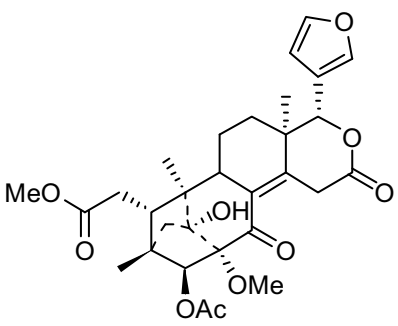


Figure S48. ^{13}C NMR spectrum of **XM-2** in $CDCl_3$.



XM-2 : moluccensin I

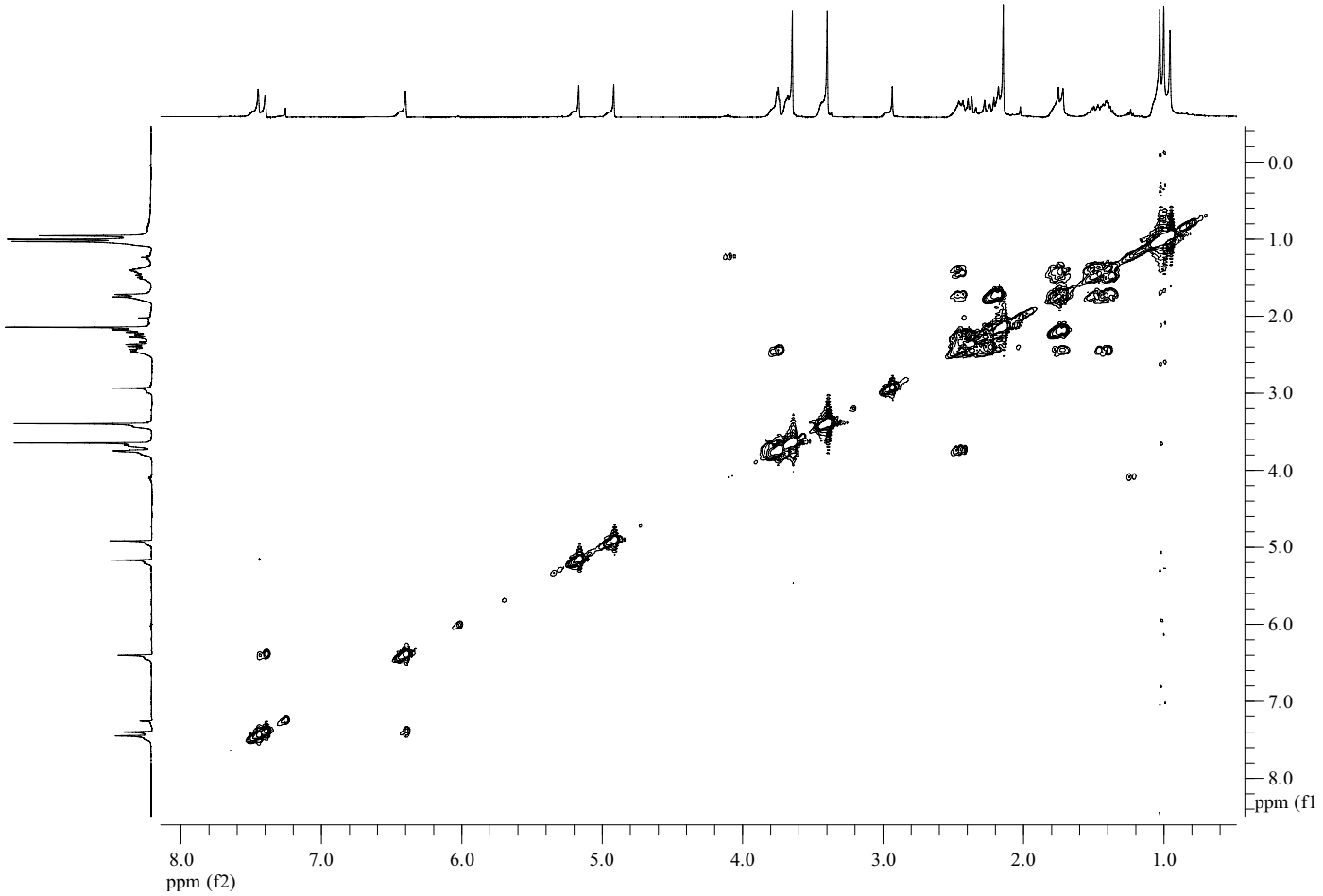


Figure S49. COSY spectrum of **XM-2** in $CDCl_3$.

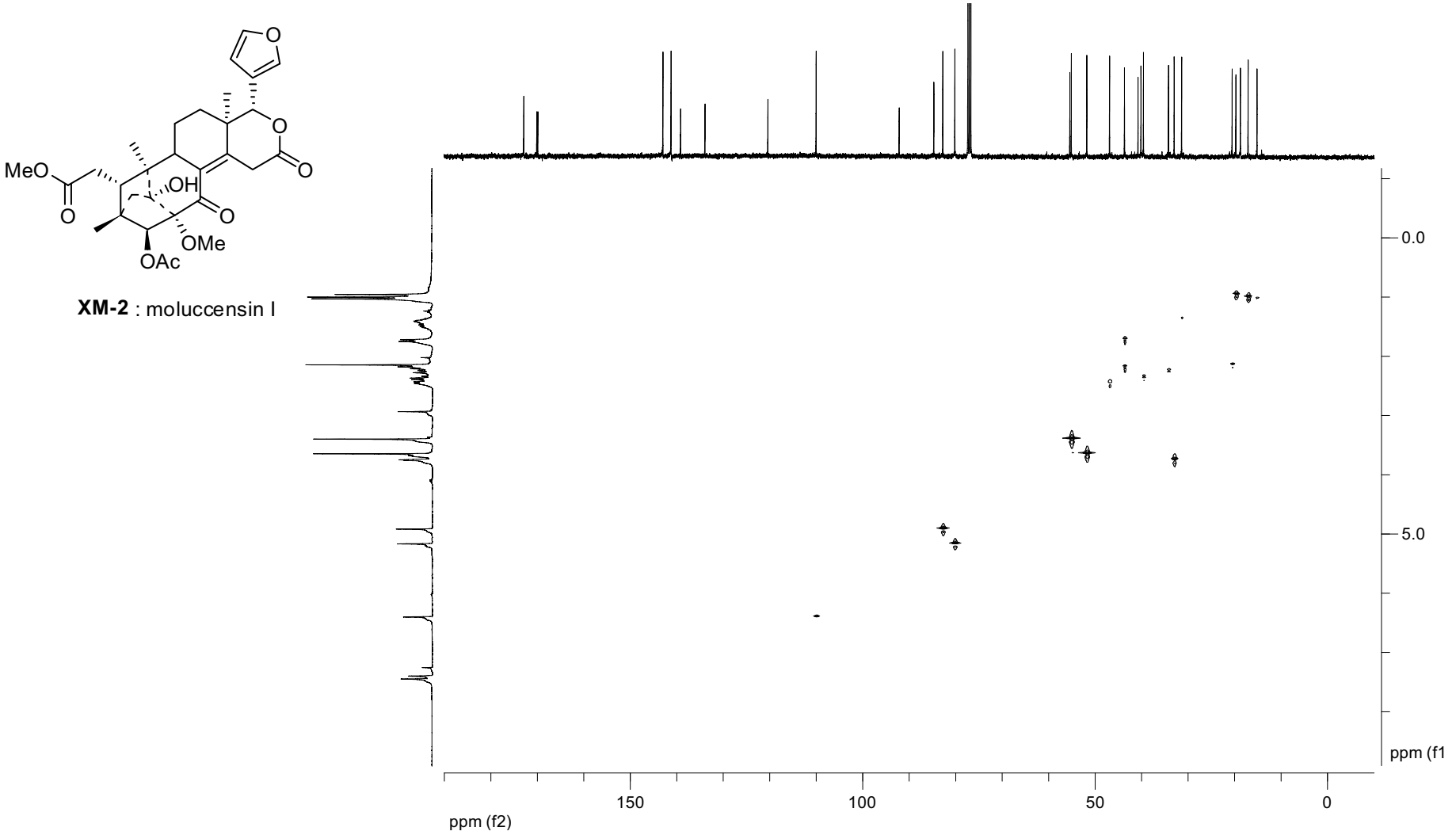
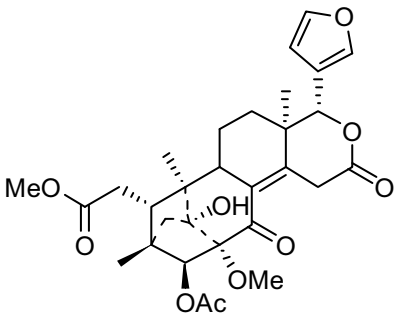


Figure S50. HSQC spectrum of **XM-2** in $CDCl_3$.



XM-2 : moluccensin I

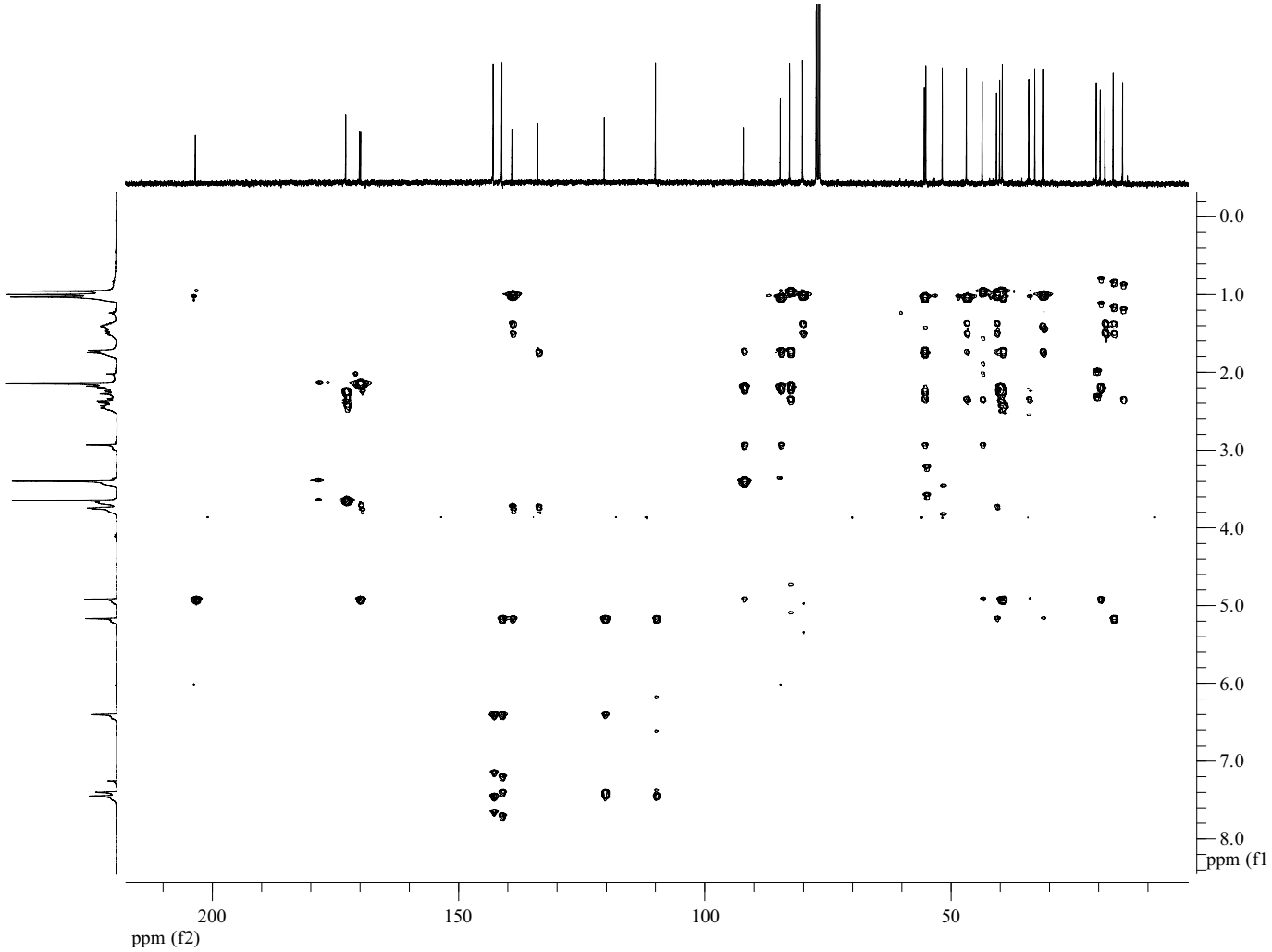
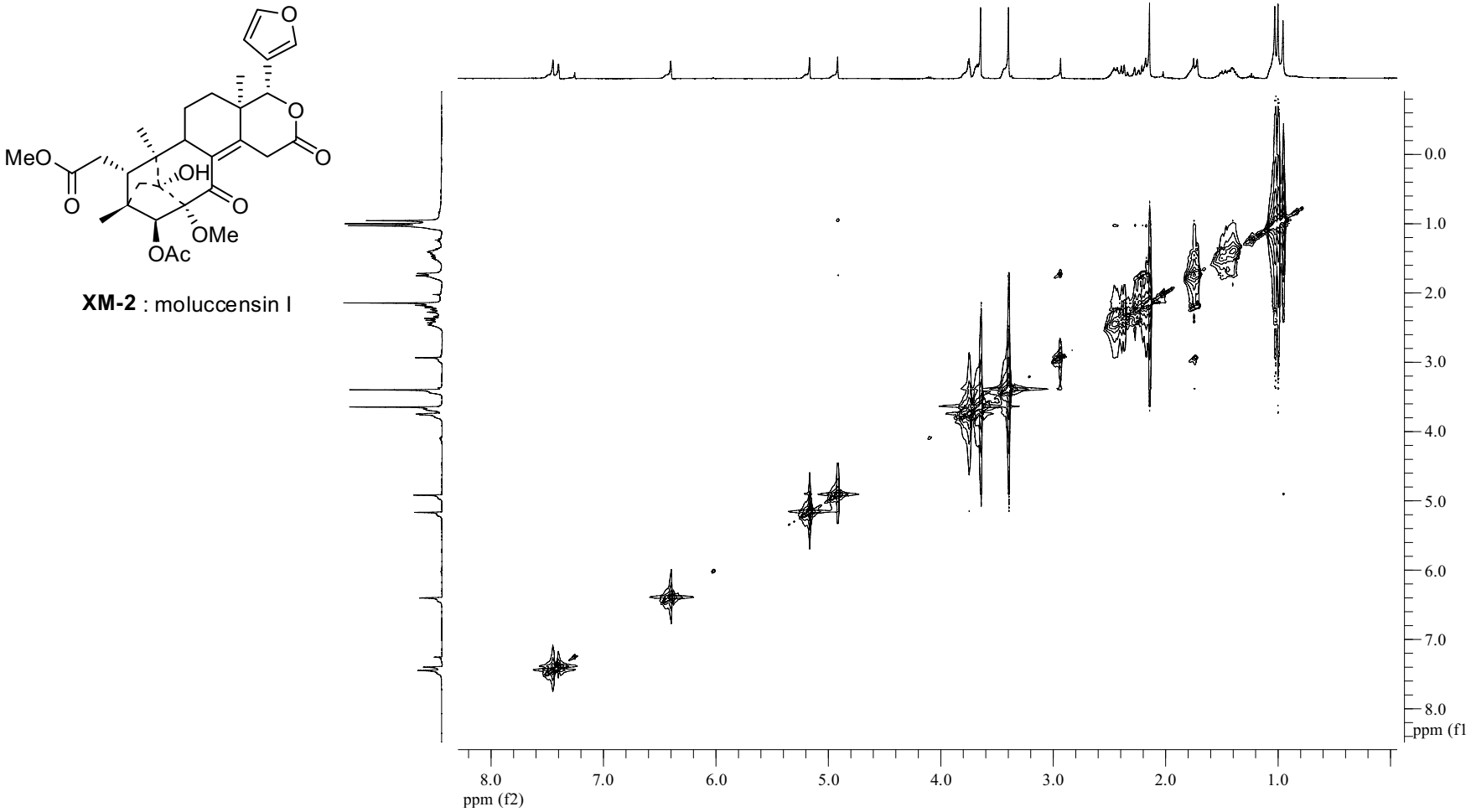
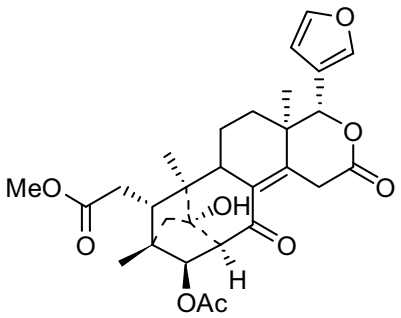


Figure S51. HMBC spectrum of **XM-2** in $CDCl_3$.



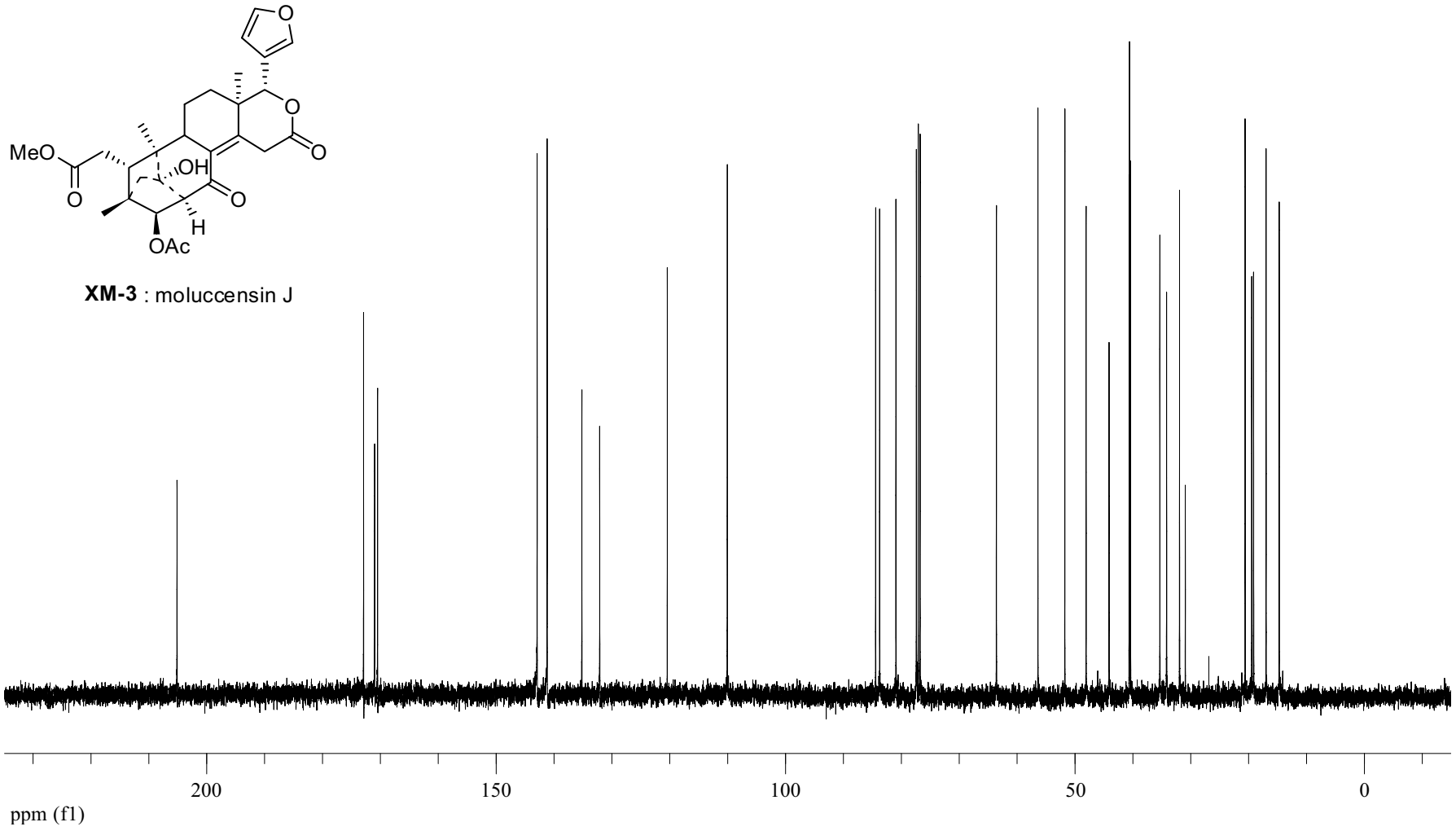
XM-2 : moluccensin I

Figure S52. NOESY spectrum of **XM-2** in $CDCl_3$.



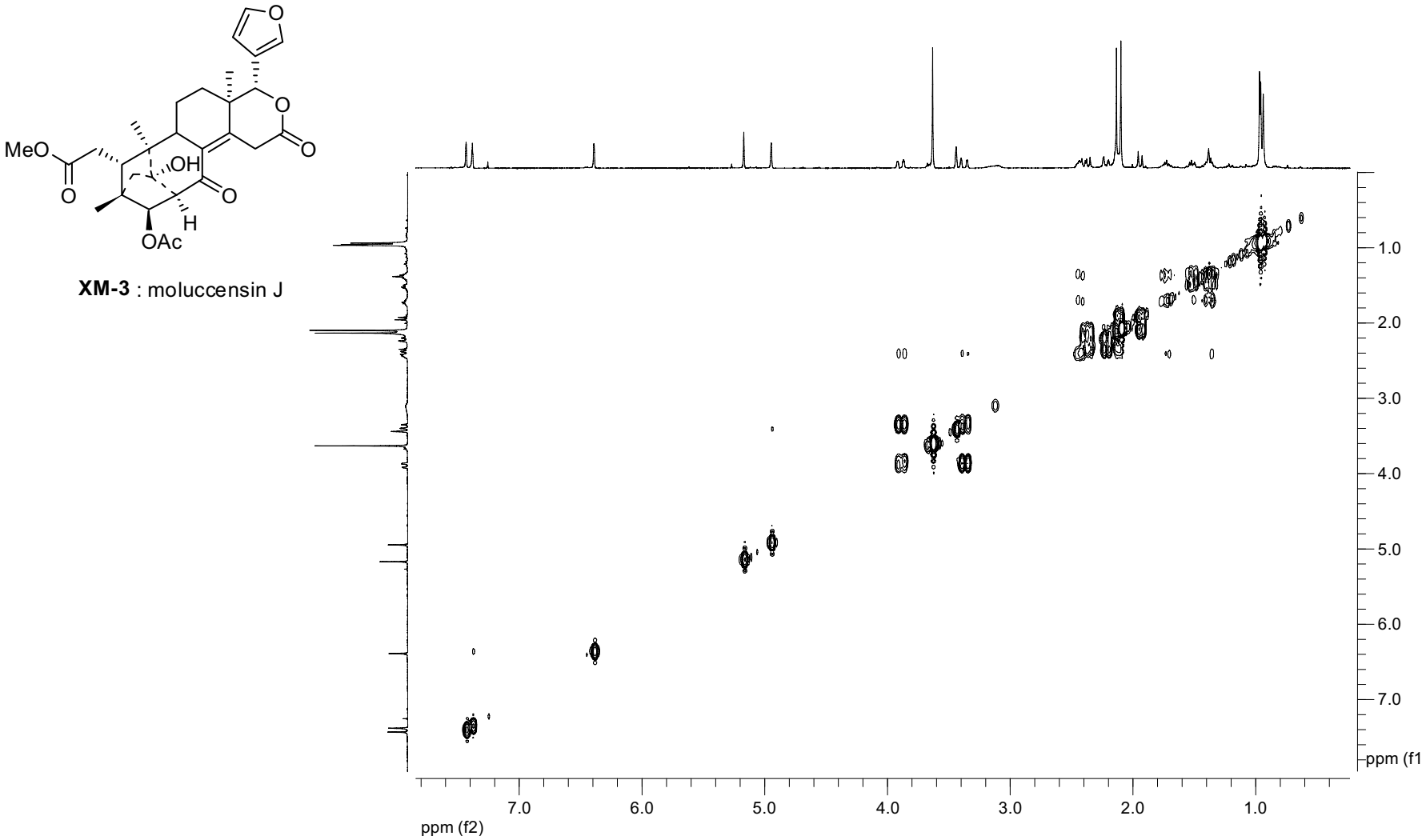
XM-3 : moluccensin J

Figure S53. ^{1}H NMR spectrum of **XM-3** in $CDCl_3$.



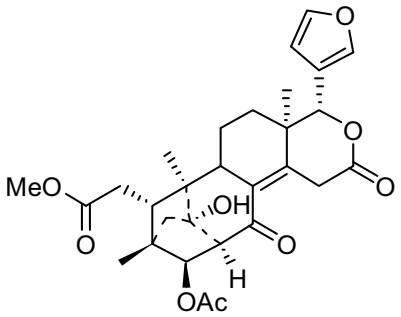
XM-3 : moluccensin J

Figure S54. ^{13}C NMR spectrum of **XM-3** in $CDCl_3$.



XM-3 : moluccensin J

Figure S55. COSY spectrum of **XM-3** in $CDCl_3$.



XM-3 : moluccensin J

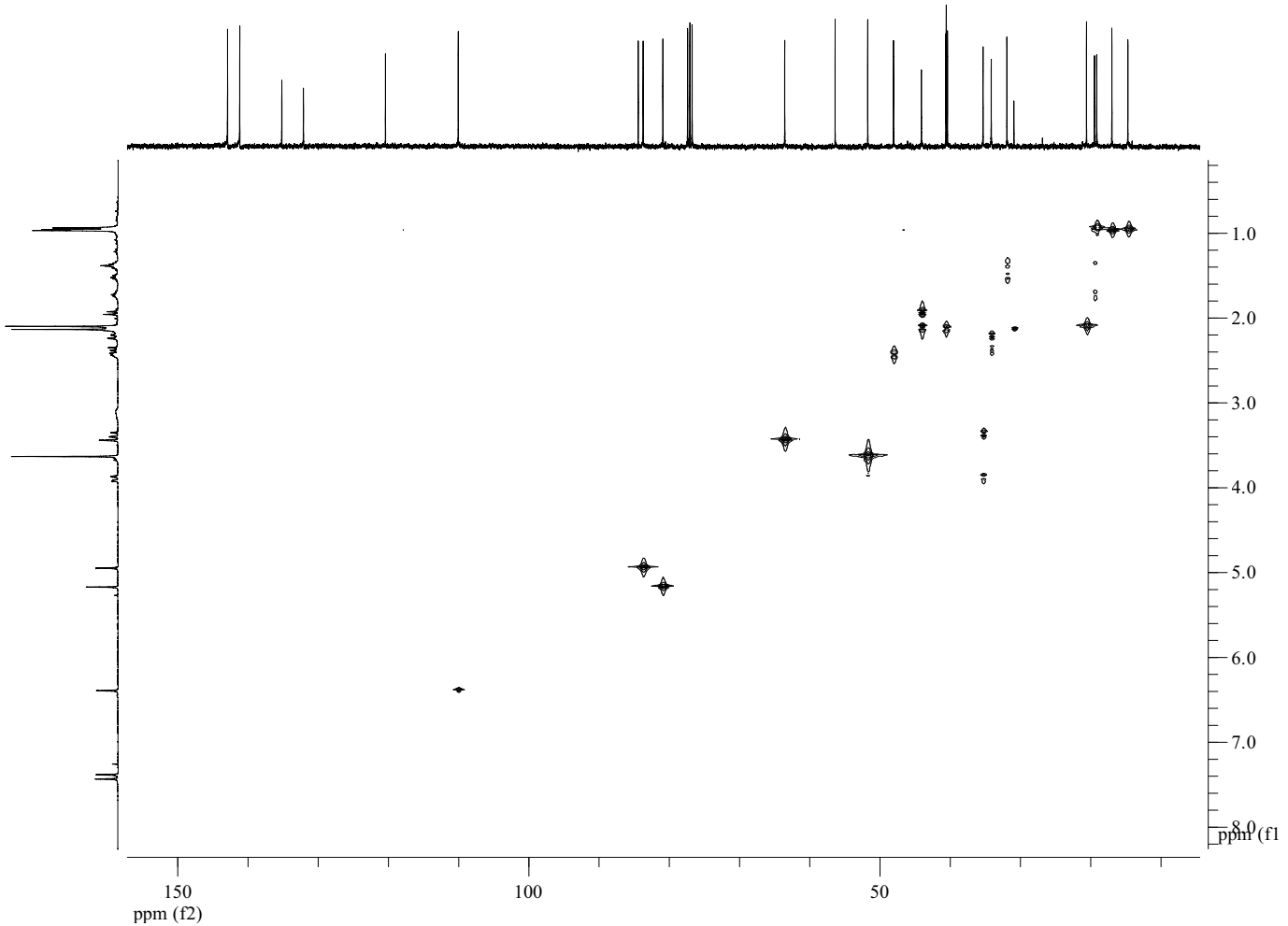
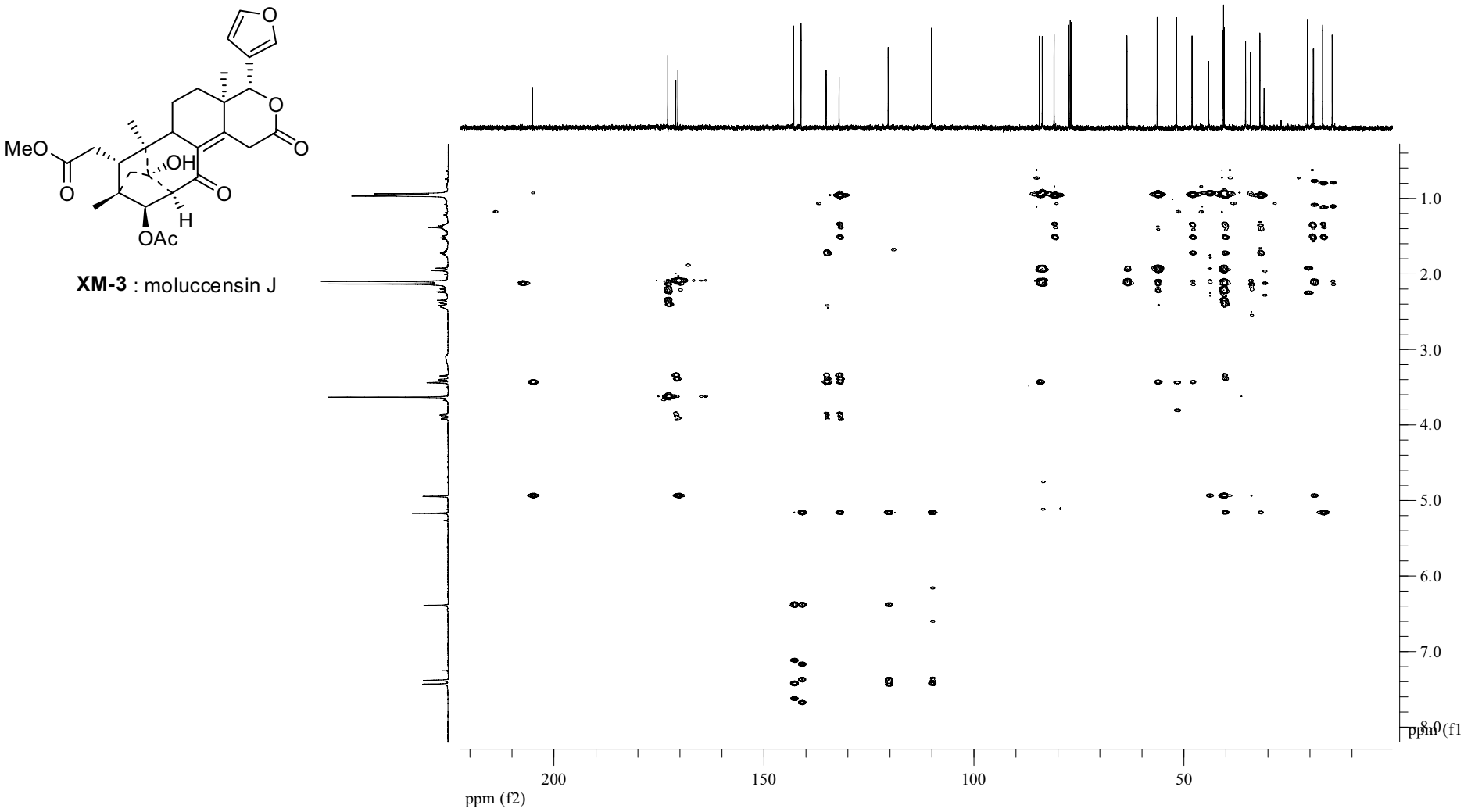


Figure S56. HSQC spectrum of **XM-3** in $CDCl_3$.



XM-3 : moluccensin J

Figure S57. HMBC spectrum of **XM-3** in $CDCl_3$.

ภาคผนวก

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Moluccensins H-J, 30-Ketophragmalin Limonoids from *Xylocarpus moluccensis*

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Three new phragmalin limonoids, moluccensins H-J (**1**-**3**), were isolated from the seed kernels of the cedar mangrove, *Xylocarpus moluccensis* (Lamk.) Roem. Their structures were established by extensive analysis of spectroscopic data. A plausible biosynthetic pathway for their formation is also proposed. All three compounds revealed no cytotoxic activity against all five human tumor cell lines tested in tissue culture, while compound **2** displayed a weak antibacterial activity against *Staphylococcus hominis* and *Enterococcus faecalis*.

Limonoids are a class of tetranorterpenoids, are moderately polar, insoluble in water and hexane but soluble in hydrocarbons, alcohols and ketones. Their occurrence in the plant kingdom are most abundant in the Sapindales order and especially the mahogony (Meliaceae) and citrus (Rutaceae) families. Limonoid research from the Meliaceae family (meliacins) is of growing interest due to a range of biological activities, such as insect antifeedants and growth regulators, antibacterial, antifungal, antimalarial, anticancer and antiviral activities on humans. [1-3] In addition, this variety of chemically unique limonoids, including the phragmalins, the ring B,D-seco limonoids with characteristic tricyclo[3.3.$1^{2,10}$.$1^{1,4}$]decane or tricyclo[4.2.$1^{10,30}$.$1^{1,4}$]decane ring systems, that characterizes the structural diversity and potential biological significance of limonoids, [4-6] is greater in the Meliaceae than in the Rutaceae.[7,8] Indeed, several phragmalin limonoids with novel skeletons have been isolated from *X. granatum* in recent years.[9-12]

Limonoid derivatives have been found in all *Xylocarpus* plants studied, but their distribution and content may vary both between different plant species, and between parts or geocultivars of the same species. This, combined with their wide ranging structural diversity and potential biological significance across this plant family, prompted us to investigate another plant in this genus, the cedar mangrove or puzzlenut, *Xylocarpus moluccensis* (Lamk.) Roem. *X. moluccensis* seeds have been used as a cure for elephantitis, to cure scabies and kudis and is reported to have antifilarial activity[13-15] and to affect the central nervous system,[16] whilst the bark has been used for abdominal problems such as dysentery and diarrhea and has antibiotic activity against diverse Gram-negative and Gram-positive bacteria.[17] It has been found to produce a number of limonoids[18-20] including, recently, the isolation from the seeds of a further seven novel phragmalins from the moluccensis group with a conjugated C-30 carbonyl group.[21]

In our investigation on the chemical constituents of *X. moluccensis*, three new 30-ketophragmalin limonoids, moluccensins H-J (**1**-**3**), were isolated from the kernel seeds of this plant, collected from the Samutsongkram Province, Thailand. These new compounds differ from all other reported limonoids and possess an unprecedented structure that features a characteristic α,β-unsaturated ketone moiety at C-30. Herein, we report the isolation and structural elucidation of these three new compounds (**1**-**3**) and outline a plausible biosynthetic pathway for their formation.

Results and Discussion

Moluccensin H (**1**) was isolated as a colorless gum, and its molecular formula was established as $C_{29}H_{32}O_{10}$ by HRESIMS (*m/z* 563.1888 $[M+Na]^+$, calcd. 563.1893), which indicated 14 degrees of unsaturation. The IR absorptions at 3452, 1737 and 1682 cm^{-1} implied the existence of hydroxyl and ester groups. The ^1H NMR spectrum (Table 1) displayed resonances of a β-substituted furanyl ring (δ_H 7.49, 7.44 and 6.45), an olefinic proton (δ_H 7.00), three tertiary methyl (δ_H 1.22, 0.99 and 0.97), a methoxyl (δ_H 3.70) and an acetoxyl (δ_H 1.96) groups. In the ^{13}C NMR spectrum, 29 nonequivalent carbon resonances were observed, including four carbonyl group (δ_C 194.6, 173.4, 169.5 and 165.5), eight olefinic carbons (δ_C 168.9, 152.3, 143.1, 141.3, 121.8, 119.9, 115.2 and 110.0), and five methyl carbons (δ_C 52.2, 20.6, 16.7, 16.1 and 15.7). The remaining carbons were assigned to four methylenes, three methines, and five quaternary carbons, based on the results of HSQC based analysis. These NMR data indicated that eight of the 14 units of unsaturation come from four carbon-carbon double bonds and four carbonyls. Therefore, the remaining six degrees required **1** to be a hexacyclic core. The data from decouplings and the subsequent 2D NMR studies (HMBC and HSQC) suggested that **1** was a phragmalin limonoid. Two protons at δ_H 1.94 and 1.87 correlating in the HSQC spectrum to a methylene signal at δ_C 41.7 were indicative of the H-29 protons of the characteristic 4,29,1-ring bridge of phragmalin limonoids.[8-11] This was confirmed by the HMBC correlations (Figure 1) observed from the H-29 protons to the tertiary carbon at δ_C 43.5 (C-5) and to the quaternary carbons at δ_C 86.6 (C-1), 45.1 (C-4) and 48.4 (C-10). The HMBC correlations between C-7 (δ_C 173.4) and H_2-6 (δ_H 2.52 and 2.40), and the methoxyl protons at δ_H 3.70 also confirmed the typical C-6-C-7 appendage of phragmalins.[22-25] A singlet proton at δ_H 5.02 was assignable to H-17 by correlations with the furanyl carbon at δ_C 119.9 (C-20) along with C-18 methyl carbon at δ_C 15.7. The vinylic proton at δ_H 7.00 assigned to H-15 exhibited significant HMBC correlation to both bridgehead carbons, C-13 (δ_C 36.4) and C-14 (δ_C 152.3), and the lactone carbonyl carbon at δ_C 165.5. Further, this δ-lactone was conjugated to the $\Delta^{8,9}$ double bond to form a conjugated diene lactone system, which was confirmed by the HMBC correlation of H-15/C-8 and Me-19/C-9. The $\Delta^{8,9}$ double bond was also conjugated to the C-30 ketone carbonyl carbon, responsible for the high-field signal at δ_C 194.6. This ketone was

assigned for C-30 due to the HMBC cross-peak from the D_2O exchangeable proton at δ_H 4.95 which correlated to C-2 at δ_C 80.6. The analyses above, and other 1D and 2D NMR information, led us to suggest that the gross structure of **1** depicted in Figure 1, which possess an unprecedented structure with a characteristic diene lactone-conjugated ketone moiety at C-30.

The relative stereochemistry of **1** was elucidated by NOESY experiment (Figure 1). Limonoids are stereochemically homogenous compounds since they have a prototypical structure that either contains or is derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton.[26] The H-17 configuration had been found to be exclusively β in all known phragmalins.[9-12] Consistent with this in that for compound **1**, the cross-peaks in the NOESY spectrum from H-17 and H-5 to H-12β, and H-5 to Me-28 indicated a β-orientation of these protons. NOESY correlations of Me-18 with H-12α, 1-OH with Me-19, and H-3 with H-29 and 2-OH all suggested that Me-18, Me-19, H-3, H_2-29, 1-OH, and 2-OH were in the α-orientation. Thus, the relative stereochemistry of **1** is demonstrated to be as depicted.

Moluccensin I (**2**) was isolated as a light yellow gum with the molecular formula $C_{30}H_{36}O_{10}$ as determined by HRESIMS ion at *m/z* 579.2201 $[M+Na]^+$ (calcd. 579.2206). From the molecular formula, 13 unsaturation units in compound **2** were calculated. The ^1H and ^{13}C NMR as well as the 2D NMR spectra suggested that **2** is also a 30-ketophragmalin limonoid with the same basic skeleton as that of **1**. The obvious difference was the absence of the olefinic proton at C-15 in **1** and the presence of only one double bond between C-8 and C-14, confirmed by the HMBC correlations of Me-18/C-14, H_2-15/C-8 and H_2-11/C-8. Without the long conjugative effect seen in **1**, the ketone carbonyl at C-30 was significantly shifted downfield to a δ_C of 203.5. Furthermore, a detailed analysis of NMR data revealed the presence of the methoxyl group (δ_H 3.40 and δ_C 55.1) at C-2 in place of the hydroxyl group of **1**. The similar NOESY correlations between **2** (Figure 2) and **1** (Figure 1) were indicative of the same stereochemistry of the core skeleton of **2** compared to **1**. The key NOE cross-peak for 2 between Me-19 and H-9 confirmed the α-orientation of H-9.

Moluccensin J (**3**) was isolated as a light yellow gum with an evaluated molecular formula of $C_{29}H_{34}O_9$ by HRESIMS ion at *m/z* 549.2100 $[M+Na]^+$ (calcd. 549.2101). The ^1H and ^{13}C NMR data of **3** were virtually identical to those of **2**. The

absence of the methoxyl signal, along with resonance indicating an additional methine at δ_H 3.44 coupled to the carbon resonance at δ_C 63.6 in the HSQC spectrum suggested that the methoxyl group had been replaced by the methine group at C-2. This was confirmed by the ^{1}H-^{1}H COSY cross-peak of H-2/H-3 and the HMBC correlations from H-2 at δ_H 3.44 to C-30 at δ_C 205.1 and C-1 at δ_C 84.4. The relative configuration was determined the same as **2** by the NOESY spectrum.

A plausible biosynthetic pathway for the formation of compounds **1**-**3** is proposed as shown in Scheme 1. Their biosynthetic origin should be the 1,8,9-phragmalin ortho esters (**4**). De-*ortho*-acetylation of **4** would produce a key intermediate, polyhydroxy compound **5**, named tabulalin, which has also been found in *Chukrasia tabularis*, another plant in the Meliaceae family.[27] Tabulalin could be involved in the oxidation of the hydroxyl group at C-30 and dehydroxylation to generate compound **1**. Further reduction of a double bond of **1** followed by 1,3-hydride shift might lead to another intermediate (**6**), which, after methylation and demethoxylation, would give **2** and **3**, respectively.

Compounds **1**-**3** were tested for their cytotoxic effects against five human tumor cell lines (BT474, CHAGO, Hep-G2, KATO-3 and SW-620) as well as for their antibacterial properties toward *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus epidermidis, Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus valgaris*, and *Salmonella typhimurium*. All compounds were inactive against all five tested cell lines (IC_{50} > 10 μg/mL), while only compound **2** displayed a weak antibacterial activity against only *S. hominis* and *E. faecalis* with MIC values of 256 μg/mL.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at wavelength 589 nm, and UV data were recorded on a Shimadzu UV-160 spectrophotometer. IR spectra were recorded on a Bruker vector22 Fourier transform infrared spectrophotometer. HRESIMS spectra were obtained using a Bruker micrOTOF mass spectrometer. The NMR spectra were recorded on a Varian YH400 spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS (trimethylsilane) as the internal standard.

Plant Material. Fruits of *X. moluccensis* were collected from Samutsongkram Province, Thailand in May 2009. A voucher specimen (BKF 159046) of *X. moluccensis* has been deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Extraction and Isolation. The kernel seeds (1.50 kg) of *X. moluccensis* were extracted with MeOH (2 L x 2, each for two days) at room temperature. After removing the solvent in vacuo, the combined MeOH crude extract was suspended in H_2O (250 mL), then partitioned with EtOAc (500 mL x 3) to obtain the EtOAc crude extract (56.8 g). This crude extract was chromatographed on a silica gel column eluted with a gradient of acetone-hexane (from 1:0 to 0:1) to yield seven fractions (I-VII). Fraction IV (10.2 g) was subjected to silica gel column chromatography and eluted with a gradient system of acetone-benzene to give 15 subfractions (IV.1-IV.15). Subfraction IV.8 (2.1 g) was further subjected to column chromatography over silica gel using a mixture of acetone-hexane (1:2) to afford nine fractions, IV.8.1-IV.8.9. Fraction IV.8.5 was further chromatographed on a silica gel column, eluted with acetone-hexane (1:2) to yield **1** (60.8 mg), while fraction IV.8.9 was purified by column chromatography with MeOH-CH_2Cl_2 (3:97) to give **2** (128.3 mg) and **3** (156.0 mg), respectively.

Moloccensin H (1): colorless gum; $[\alpha]^{20}_D$ +140 (*c* 0.10, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 265 (4.16) nm; IR (KBr) ν_{max} 3452, 2957, 1737, 1682, 1636, 1376, 1227, 1029 and 875 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 563.1888 $[M+Na]^+$ (calcd. $C_{29}H_{32}O_{10}Na$, 563.1893).

Moloccensin I (2): light yellow gum; $[\alpha]^{20}_D$ -12 (*c* 0.10, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 215 (4.12) nm; IR (KBr) ν_{max} 3458, 2944, 1743, 1504, 1460, 1373, 1228, 1163, 1111, 1024, 913, 875 and 731 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 579.2201 $[M+Na]^+$ (calcd. $C_{29}H_{32}O_{10}Na$, 579.2206).

Moloccensin J (3): light yellow gum; $[\alpha]^{20}_D$ -4 (*c* 0.10, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 290 (3.13) nm; IR (KBr) ν_{max} 3452, 2944, 1741, 1505, 1461, 1437, 1372, 1236, 1164, 1025, 912, 875 and 731 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 549.2100 $[M+Na]^+$ (calcd. $C_{29}H_{34}O_9Na$, 549.2101).

Cytotoxicity Bioassays.[28,29] All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000 cells per well with compounds added from DMSO-diluted stock.

After three days in culture, attached cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide). The absorbency at 540 nm was measured using a microplate reader after solubilizing the bound dye. The mean IC_{50} is the concentration of agent that inhibits cell growth by 50% under the experimental conditions and is the average from at least six independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: human breast ductol carcinoma ATCC No. HTB 20 (BT474), undifferentiated lung carcinoma (CHAGO), liver hepatoblastoma (Hep-G2), gastric carcinoma ATCC No. HTB 103 (KATO-3), and colon adeno carcinoma ATCC No. CCL 227 (SW-620). All cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University and were cultured in RPMI-1640 supplemented with 25 mM HEPES, 0.25% (w/v) sodium bicarbonate, 5% (v/v) fetal bovine serum, and 100 μg/mL kanamycin.

Antibacterial Assays. A broth microdilution method was used to determine the MIC according to the NCCLS protocol.[30] Reference strains were five Gram positive bacteria: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Staphylococcus hominis* ATCC 27844, *Staphylococcus epidermidis* ATCC 12228 and *Enterococcus faecalis* ATCC 29212; and four Gram negative bacteria: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Proteus valgaris* ATCC 13315 and *Salmonella typhimurium* ATCC 13311. All tests were performed in Mueller Hinton Broth. Serial doubling dilutions of the compound, prepared in a 96-well microtiter plate, ranged from 0.5 to 256 μg/mL. The final concentration of each strain was adjusted to $5x10^5$ CFU/mL. The MIC was defined as the lowest concentration of the compound at which the microorganism does not demonstrate visible growth as determined by turbidity.

Acknowledgement. This work was supported by the Thailand Research Fund (MRG4980028). Thanks are due to Miss Supichar Chokpaiboon and Miss Poonlap Pompeng from Program in Biotechnology, Faculty of Science, Chulalongkorn University, for cytotoxic and antibacterial assays.

Supporting Information Available: IR, HRESIMS, and ^1H and ^{13}C NMR spectra of compounds **1-3** are available free of charge via the internet at http://pubs.acs.org.

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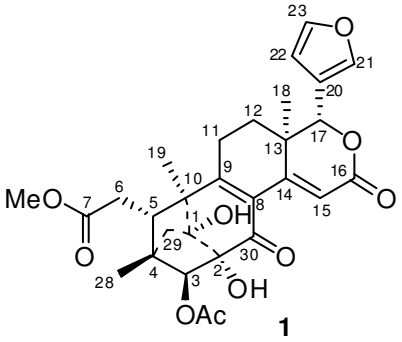
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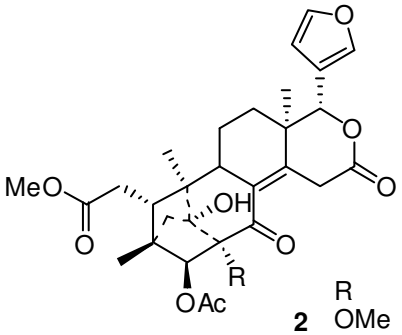
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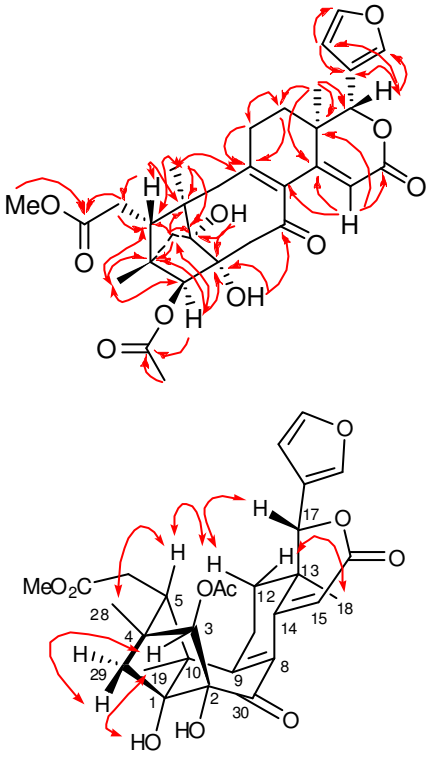


Figure 1. Key HMBC and NOESY correlation of **1**.

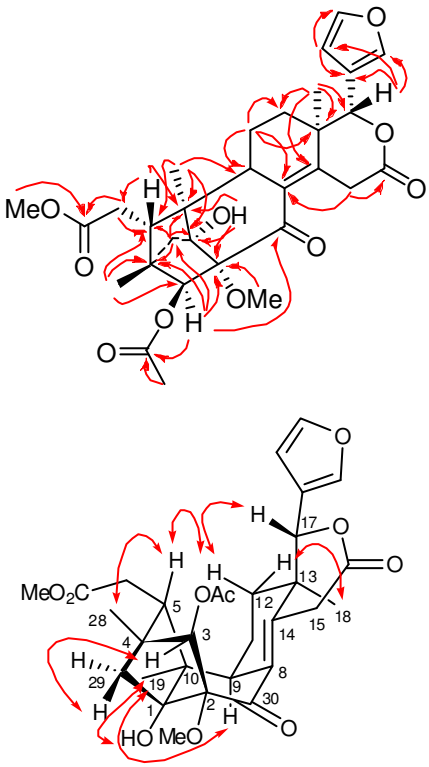
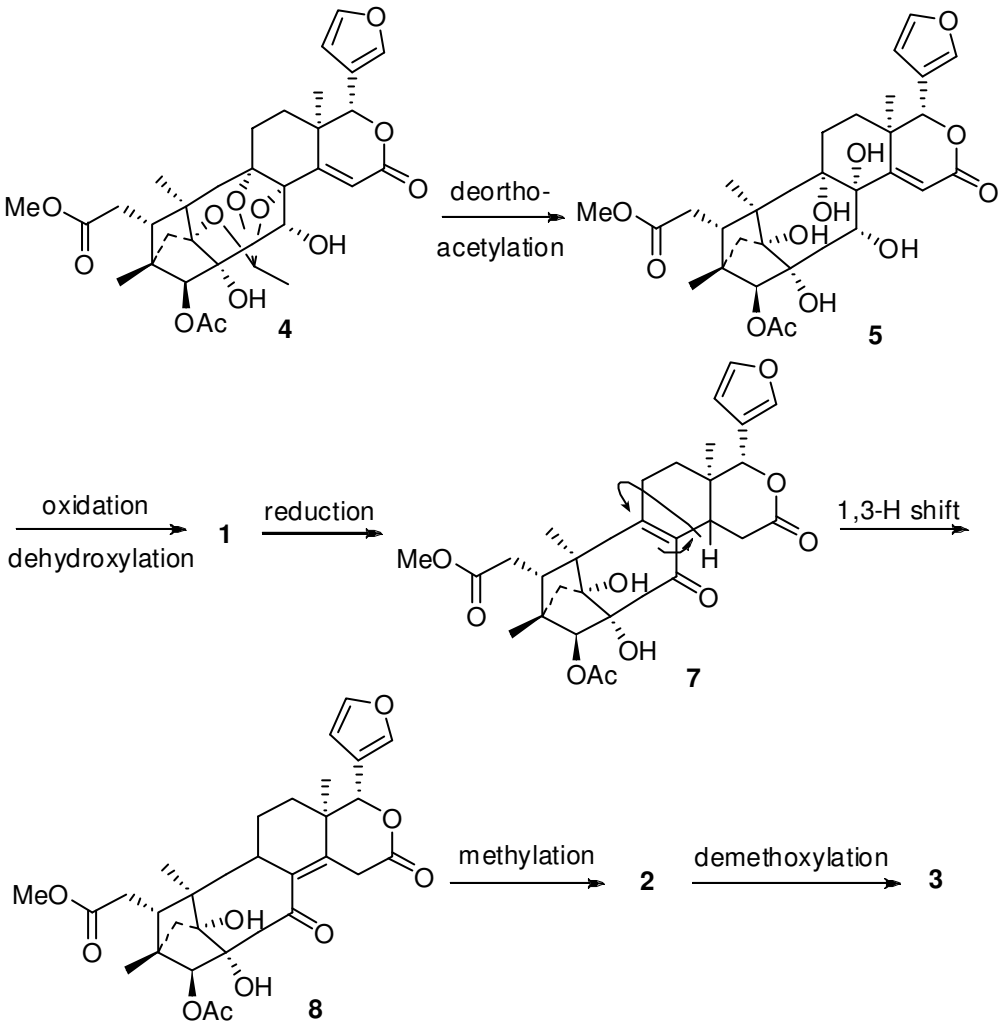


Figure 1. Key HMBC and NOESY correlation of **2**.



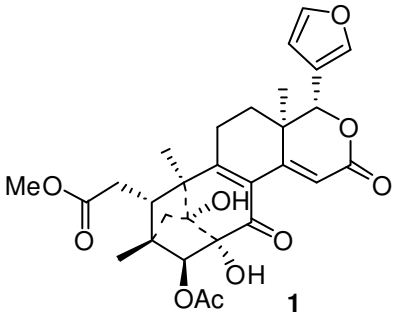
Scheme 1. Plausible biosynthetic origin of **1**-**3**.

Table 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for compounds **1**-**3**

	1		**2**		**3**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		86.6		84.7		84.4
2		80.6		92.2	3.44 (s)	63.6
3	4.90 (s)	87.6	4.92 (s)	82.8	4.95 (s)	83.7
4		45.1		40.1		40.6
5	2.79 (m)	43.5	2.37 (t, 10.0)	39.6	2.13 (m)	40.5
6	2.40 (m)	33.1	2.25 (d, 12.0)	34.2	2.22 (dd, 2.8, 15.6)	34.2
	2.52 (dd, 5.6, 17.2)		2.43 (m)		2.38 (dd, 4.4, 15.6)	
7		173.4		172.9		172.9
8		121.8		133.9		135.2
9		168.9	2.46 (m)	46.9	2.43 (br m)	48.1
10		48.4		55.4		56.4
11	2.43 (m)	25.2	1.43 (m)	18.7	1.40 (m)	19.5
			1.74 (m)		1.73 (m)	
12	1.49 (m)	30.5	1.41 (m)	31.4	1.38 (m)	31.9
	1.67 (br m)		1.49 (m)		1.52 (m)	
13		36.4		40.8		40.4
14		152.3		139.2		132.1
15	7.00 (s)	115.2	3.75 (m)	33.0	3.37 (d, 19.2)	35.3
					3.89 (d, 19.2)	
16		165.5		169.9		171.0
17	5.02 (s)	80.4	5.17 (s)	80.2	5.17 (s)	80.9
18	0.99 (s, 3H)	15.7	1.00 (s, 3H)	17.1	0.97 (s, 3H)	17.0
19	1.22 (s, 3H)	16.1	1.03 (s, 3H)	15.1	0.96 (s, 3H)	14.7
20		119.9		120.4		120.4
21	7.49 (s)	141.3	7.45 (s)	141.2	7.43 (s)	141.2
22	6.45 (s)	110.0	6.40 (s)	110.0	6.39 (s)	110.1
23	7.44 (s)	143.1	7.40 (s)	143.0	7.38 (s)	142.9
28	0.97 (s, 3H)	16.7	0.96 (s, 3H)	19.7	0.94 (s, 3H)	19.1
29	1.87 (dd, 2.0, 11.2)	41.7	1.72 (m)	43.7	1.94 (d,	44.1
	1.94 (d, 11.2)		2.20 (d, 13.2)		2.12 (br s)	
30		194.6		203.5		205.1
7-OCH_3	3.70 (s, 3H)	52.2	3.65 (s, 3H)	51.8	3.63 (s, 3H)	51.7
3-OAc		169.5		170.1		170.5
	1.96 (s, 3H)	20.6	2.15 (s, 3H)	20.5	2.10 (s, 3H)	20.6
2-OCH_3			3.40 (s)	55.1		
2-OH	4.95 (s)					
1-OH	2.88 (br s)		2.93 (br s)			

Moluccensins A-C, 30-Ketophragmalin Limonoids from *Xylocarpus moluccensis*

Khanitha Pudhom,* Damrong Sommit, Paulwatt Nuclear, Nattaya Ngamrojanavanich, and Amorn Petsom



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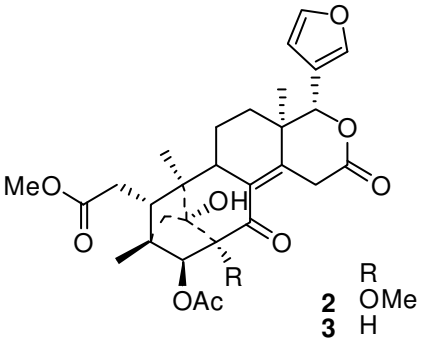

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3 R = H

Protoxylocarpins F−H, Protolimonoids from Seed Kernels of *Xylocarpus granatum*

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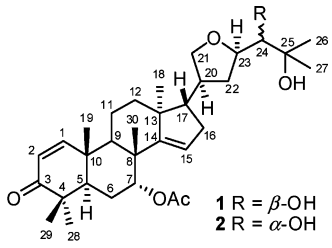
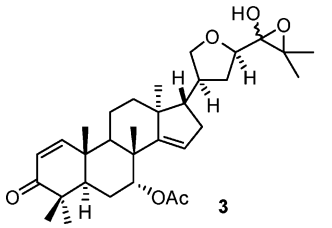
Three new protolimonoids, protoxylocarpins F−H (**1**−**3**), along with 11 known limonoids, were isolated from seed kernels of *Xylocarpus granatum*. Their structures were elucidated on the basis of extensive spectroscopic data analyses. All compounds isolated were evaluated for cytotoxic activity against five human tumor cell lines.

Research on limonoids from the Meliaceae family is of interest due to their range of biological activities, such as insect antifeedant and growth regulator, antibacterial, antifungal, antimalarial, anticancer, and antiviral activities.[1−3] Members of the genus *Xylocarpus*, in particular the cannonball mangrove *Xylocarpus granatum* Koenig. *X. granatum* (Meliaceae) are used as folk medicines in Southeast Asia for the treatment of diarrhea, cholera, viral diseases such as influenza, and malaria. They are also used as insect antifeedants or insecticides.[4] To date, more than 40 limonoid derivatives have been isolated from *X. granatum*, and these are classified as phragmalin, mexicanolide, and andirobin types.[5] In our search for structurally and biologically interesting metabolites from plant resources, we describe herein the isolation and structural elucidation of three new protolimonoids (**1**−**3**) along with 11 known limonoids from the kernel seeds of *X. granatum* collected from Samutsongkram Province, Thailand. The compounds were all evaluated for cytotoxicity against five human tumor cell lines.





The MeOH extract of *X. granatum* seed kernels was partitioned between EtOAc and H_2O to afford an EtOAc extract, which was subjected to silica gel column chromatography. Further separations by repeated normal column chromatography (CC) and preparative thin-layer chromatography (PTLC) gave three new protolimonoids, protoxylocarpins F−H (**1**−**3**), and 11 known limonoids, xyloccensins K (**4**),[5k] O (**5**), and P (**6**),[5i] xylogranatin C (**7**),[5d] mexicanolide (**8**),[6] methyl angolensate (**9**),[7] proceranolide (**10**),[8] 7-oxo-7-deacetoxygenudin (**11**),[9] 7-deacetylgenudin (**12**),[10] chisocheton F (**13**),[11] and 21-acetyloxy-21,23:24,25-diepoxy-7-hydroxy-4,4,8-trimethylcholest-14-en-3-one (**14**).[12] The structures of the known compounds were determined by comparison of their NMR spectroscopic data with those in the literature.

Protoxylocarpin F (**1**) was isolated as a colorless gum, and its molecular formula was assigned as $C_{32}H_{48}O_6$ on the basis of the $[M + Na]^+$ peak at *m/z* 551.3348 (calcd 551.3349) in the HRESIMS, requiring nine degrees of unsaturation. IR absorptions implied the presence of α,β-unsaturated ketone (1733 cm^{-1}) and OH (3543 cm^{-1}) groups. The ^1H NMR spectrum (Table 1) showed signals of seven tertiary methyls [δ 1.24, 1.12, 1.10 (Me × 2), 1.00 (Me × 2), and 0.91], an olefinic proton (δ 5.23), an acetyl methyl (δ 1.88), and an α,β-unsaturated ketone moiety indicated by a pair of doublets at δ 7.10 and 5.97. A combined analysis of ^{13}C NMR (Table 1) and HSQC spectra revealed 32 nonequivalent carbon resonances due to one carbonyl (δ 204.8), four olefinic carbons (δ 159.2, 158.5, 125.4, and 119.0), seven methyl carbons (δ 27.4, 27.0, 26.3, 22.4, 21.3, 20.0, and 19.0), and the acetyl carbons (δ 170.2 and 21.2) together with six methylenes and seven methines, accounting for four double-bond equivalents. The remaining five degrees required **1** to be pentacyclic. These data suggested that the structure of **1** possessed a protolimonoid skeleton. The NMR data (Table 1) of **1** were similar to those of hilstinone C,[13b] a protolimonoid isolated from *Turraea holstii* (Meliaceae), except for the presence of additional methylene [δ_H 3.44 (dd, J = 2.8, 13.6 Hz), 3.54 (d, J = 12.4 Hz); δ_C 64.2] and acetyl groups [δ_H 1.88 (s); δ_C 21.2 and 170.2] and the absence of the OCH_3 and C-21 hemiacetal methine resonances present in hilstinone. The HMBC cross-peak from H-7 (δ_H 5.16, s) to the acetyl carbon (δ_C 170.2) (Figure 1) indicated that the above acetoxyl group was attached to C-7 and suggested that **1** was a 7-acetyl-21-demethoxy analogue of hilstinone C. The full assignments (Table 1) were determined by COSY and HMBC correlations (Figure 1).

The relative configuration of **1** was assigned on the basis of NOESY data as depicted in Figure 2. In the tetracyclic nucleus, the NOESY correlations of Me-29/Me-19, Me-19/Me-30, and Me-30/H-7 indicated their β-orientations, while those of H-5/H-9 and H-9/Me-18 confirmed that H-5, H-9, and Me-18 were α-oriented. Since H-17 was assigned to the β-form, as observed in all protolimonoids,[5a,13] the NOE correlations of H-20/Me-18, H-20/H-23, and H-23/H-24 combined with the noninteraction of H-17/H-20 allowed assignment of the α-orientation to Me-18, H-20, H-23, and H-24. The absolute configuration at C-24 of **1** was assigned using the modified Mosher method.[15] The difference in

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1**−**3** in $CDCl_3$

	1		**2**		**3**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	7.10 d (10.0)	158.5	7.14 d (10.4)	158.5	7.14 d (10.0)	158.3
2	5.97 d (10.0)	125.4	5.80 d (10.4)	125.5	5.83 d (10.0)	125.4
3		204.8		204.8		204.8
4		44.1		44.2		44.1
5	2.10 m	46.2	2.16 m	46.4	2.14 m	46.1
6	1.64 m; 1.70 m	23.8	1.80 m; 1.90 m	24.0	1.76 m	23.8
7	5.16 br s	74.7	5.20 br s	74.8	5.19 br s	74.6
8		42.7		42.8		42.6
9	2.13 m	38.5	2.20 m	38.7	2.16 m	38.4
10		39.8		39.7		39.8
11	1.58 m; 1.83 m	16.7	1.55 m; 2.00 m	16.8	1.49 m; 1.73 m	16.7
12	1.60 m; 1.83 m	34.0	2.26 m	34.9	1.92 m; 2.16 m	33.9
13		46.3		46.2		46.5
14		159.2		159.0		158.7
15	5.23 br d (2.4)	119.0	5.30 br d (2.4)	119.6	5.25 br s	119.2
16	1.93 m; 2.21 m	35.0	2.26 m	35.0	2.14 m	29.8
17	1.84 m	54.2	2.00 m	52.3	1.40 m	57.1
18	0.91 s	20.0	0.90 s	20.4	1.04 s	20.4
19	1.09 s	19.0	1.14 s	19.1	1.13 s	19.0
20	1.84 m	36.3	1.88 m	35.8	1.76 m	34.5
21	3.44 dd (2.8, 13.6)	64.2	3.42 dd (2.0, 12.0)	70.0	3.60 m; 3.79 m	65.3
	3.54 br d (12.4)		3.98 br d (11.6)			
22	1.55 m; 1.91 m	37.9	1.52 m; 2.04 m	36.2	1.69 m	32.8
23	3.74 ddd (2.4, 8.8, 10.4)	68.0	3.86 ddd (2.8, 8.8, 13.2)	64.4	3.85 m	67.5
24	3.36 d (8.8)	80.7	2.98 d (8.8)	86.5		95.5
25		76.2		74.3		76.3
26	1.24 s	26.3	1.30 s	28.6	1.39 s	24.3
27	1.10 s	22.4	1.25 s	23.8	1.25 s	23.1
28	1.00 s	21.3	1.05 s	21.3	1.04 s	21.2
29	1.00 s	27.0	1.05 s	27.0	1.04 s	27.0
30	1.12 s	27.4	1.18 s	27.3	1.13 s	27.3
7-OCO*CH*$_3$	1.88 s	21.2	1.92 s	21.2	1.92 s	21.1
7-O*CO*CH$_3$		170.2		170.2		170.2

chemical shift values ($\Delta\delta$) for its diastereomeric esters, (*R*)-MTPA (**1a**) and (*S*)-MTPA (**1b**), indicated the *S*-configuration at C-24, as shown in Figure 3.

Protoxylocarpin G (**2**) had the molecular formula $C_{32}H_{48}O_6$, the same as that of **1**. The ^{1}H and ^{13}C NMR spectra of **2** were nearly identical to those of **1**. The obvious difference was observed in the carbon resonance of C-24 (δ_C 86.5 for **2** and 80.7 for **1**). The relative configuration of **2** was determined by NOESY correlations (Figure 2) and indicated that all of the chiral carbons had configurations the same as those of **1**, except for that of C-24. Due to the lack of an NOE cross-peak between H-23 and H-24, compound **2** was assigned as the C-24 epimer of **1**. This assignment was supported by the opposite signs of their specific rotations ($[\alpha]_D$ −57.0 for **1** vs +38.0 for **2**).

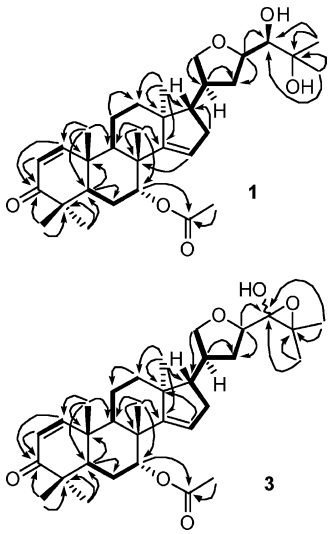

Figure 1. COSY (bold lines) and selected HMBC (arrows) correlations for **1** and **3**.

Protoxylocarpin H (**3**) was isolated as a colorless gum and had the molecular formula $C_{32}H_{46}O_6$ as determined by HRESIMS at *m*/*z* 526.3297 (calcd 526.3294), which indicated an additional double-bond equivalent relative to **1** and **2**. Similar to compounds **1** and **2**, the ^{1}H and ^{13}C NMR data of **3** were characteristic of protolimonoids, except for the presence of the hemiacetal quaternary carbon (δ 95.5) instead of the oxygenated methine carbon in **1** (δ 80.7) and **2** (δ 86.5) in the aliphatic part. An epoxide ring was assigned between C-24 (δ_C 95.5) and C-25 (δ_C 76.3) due to the additional degree of unsaturation and the HMBC correlations of H-23/C-24, Me-26/C-24, and Me-27/C-24 (Figure 1). The relative configuration of **3** was determined to be the same as **1** by NOESY data.

All compounds isolated (**1**−**14**) were tested for cytotoxicity toward CHAGO (lung carcinoma), SW-620 (gastric carcinoma), KATO-3 (colon carcinoma), BT-474 (breast carcinoma), and Hep-G2 (hepatocarcinoma). Compound **12** showed cytotoxic activity against CHAGO and Hep-G2 cells with IC_{50} values of 16.00 and 10.26 μM, respectively. Compound **7** was active against CHAGO cells with an IC_{50} value of 9.16 μM, and compound **11** was cytotoxic toward Hep-G2 cells with an IC_{50} value of 16.17 μM, whereas compounds **1**−**6**, **8**−**10**, and **13**−**14** were not cytotoxic to any of the cell lines tested.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at a wavelength 589 nm, and UV data were recorded on a Shimadzu UV-160 spectrophotometer. Melting points were measured using a Fisher-Johns melting point apparatus. IR spectra were recorded on a Perkin-Elmer model 1760X Fourier transform infrared spectrophotometer. HRESIMS

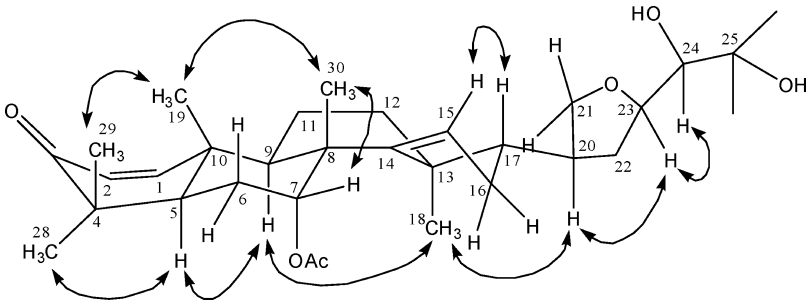

Figure 2. Selected NOESY correlations for **1**.

spectra were obtained using a Bruker micrOTOF mass spectrometer. The NMR spectra were recorded on a Varian YH400 spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS (trimethylsilane) as an internal standard.

Plant Material. The fruits of *X. granatum* were collected from Samutsongkram Province, Thailand, in December 2008. A voucher specimen (BKF 159047) has been deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Extraction and Isolation. Air-dried and powdered seed kernels of *X. granatum* (1.5 kg) were extracted with MeOH (5 L × 3, each 2 days) at room temperature. After removing the solvent in vacuo, the combined MeOH crude extract was suspended in H_2O (250 mL), then partitioned with EtOAc (500 mL × 3) to afford the crude EtOAc extract (52.6 g). The EtOAc extract was chromatographed on a silica gel column eluted with a gradient of hexane−acetone (from 1:0 to 0:1) to yield nine fractions (I−IX). Fraction III was subjected to CC over silica gel eluting with MeOH−CH_2Cl_2 (1:19) to give 10 subfractions (IIIa−IIIj). Subfraction IIIc was subsequently separated on a silica gel column (EtOAc−hexane, 1:3 to 1:1) followed by PTLC (EtOAc−benzene, 1:8) to afford **12** (12.5 mg) and **13** (6.2 mg), while subfraction IIId was chromatographed on a silica gel column (acetone−hexane, 1:3) to yield **8** (322.6 mg). Subfraction IIIe was separated on a reversed-phase silica gel (C_{18}) column using a mixture of MeOH−H_2O (8:2) to afford **2** (86.4 mg). Fraction IV was subjected to silica gel CC (EtOAc−hexane, 1:2 to 1:1) to give 15 subfractions. Subfraction IVd was separated on a silica gel column (EtOAc−benzene, 1:6) to yield **11** (20.0 mg) and **7** (19.1 mg); subfractions IVe and IVj were separated in the same manner eluted with MeOH−CH_2Cl_2 (2:98) and acetone−benzene (1:5) to afford **9** (25.1 mg) and **1** (30.8 mg), respectively. Fraction V was chromatographed on a silica gel column using MeOH−CH_2Cl_2 (2:98 and 3:97) to furnish 13 subfractions (Va−Vl). Subfraction Vb was rechromatographed on a silica gel column (EtOAc−hexane, 1:2) to give **8** (6.7 mg). Subfraction Vc was subjected to silica gel CC (MeOH−$CHCl_3$, 1:99); then fraction Vc.2 was separated in the same manner, eluted with EtOAc−hexane (1:1), to yield **5** (3.2 mg) and **6** (14.7 mg). Subfraction Vh was separated into six fractions by CC over silica gel (acetone−hexane, 1:2), and fraction Vh.5 was further purified by reversed-phase (C_{18}) silica gel CC using a mixture of MeCN−H_2O (1:1) to afford **3** (62.8 mg). Fractions VI and VII were combined and then recrystallized from EtOAc to obtain **4** (12.6 g).

Protoxylocarpin F (1): colorless gum; $[\alpha]^{25}_D$ −57.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 235 (4.10); IR (KBr) ν_{max} 3453, 2937, 1733, 1668, 1458, 1381, 1250, 1058, and 732 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 531.3348 (calcd for $C_{32}H_{48}O_6$ + Na, 531.3349).

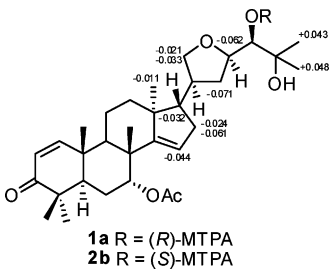

Figure 3. $\Delta\delta$ values (in ppm) = $\delta_S - \delta_R$ obtained for (*S*)- and (*R*)-MTPA esters **1b** and **1a**.

Protoxylocarpin G (2): colorless gum; $[\alpha]^{25}_D$ +38.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (4.08); IR (KBr) ν_{max} 3460, 2950, 1728, 1460, 1437, 1381, 1255, 1170, 1026, and 732 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 531.3345 (calcd for $C_{32}H_{48}O_6$ + Na, 531.3349).

Protoxylocarpin H (3): colorless gum; $[\alpha]^{25}_D$ +13.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (4.09); IR (KBr) ν_{max} 3454, 2941, 1736, 1667, 1461, 1381, 1250, 1028, and 826 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 549.3190 (calcd for $C_{32}H_{46}O_6$ + Na, 549.3192).

Preparation of (*R*)-MTPA Ester (1a) and (*S*)-MTPA Ester (1b). A reaction mixture of **1** (5 mg), (*S*)-MTPA Cl (10 μL), and DMAP (catalytic amount) in pyridine (0.5 mL) was stirred at room temperature overnight. After removing the solvent under reduced pressure, the (*S*)-MTPA ester (**1a**) was purified by mini-column chromatography on silica gel with EtOAc−hexane (1:8): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.12 (1H, d, J = 10.2 Hz, H-1), 5 0.98 (1H, d, J = 10.2 Hz, H-2), 5.26 (1H, br s, H-15), 5.18 (1H, m, H-7), 3.77 (1H, ddd, J = 2.7, 8.6, 10.0 Hz, H-23), 3.55 (1H, br d, J = 12.0 Hz, H-21a), 3.47 (1H, dd, J = 2.8, 12.9 Hz, H-21b), 3.40 (1H, d, J = 8.8 Hz, H-24), 2.14 (1H, m, H-9), 2.22 (1H, m, H-16a), 2.15 (1H, m, H-5), 1.99 (1H, m, H-16b), 1.97 (1H, m, H-22a), 1.87 (3H, s, 7-OCO*CH$_3$*), 1.88 (2H, m, H-17 and H-20), 1.84 (2H, m, H-11a and H-12a), 1.72 (1H, m, H-6a), 1.69 (1H, m, H-6b), 1.61 (2H, m, H-11b and H-12b), 1.54 (1H, m, H-22b), 1.22 (3H, s, Me-26), 1.18 (3H, s, Me-30), 1.11 (3H, s, Me-19), 1.10 (3H, s, Me-27), 1.05 (6H, s, Me-28 and Me-29), 0.94 (3H, s, Me-18).

Similarly, the reaction mixture of **1** (5 mg), (*R*)-MTPA Cl (10 μL), and pyridine (0.5 mL) was processed as described above for **1a** to afford **1b**; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.12 (1H, d, J = 10.2 Hz, H-1), 5.97 (1H, d, J = 10.2 Hz, H-2), 5.22 (1H, br s, H-15), 5.14 (1H, m, H-7), 3.71 (1H, ddd, J = 2.6, 8.9, 10.2 Hz, H-23), 3.53 (1H, br d, J = 12.4 Hz, H-21a), 3.44 (1H, dd, J = 2.6, 13.2 Hz, H-21b), 3.35 (1H, d, J = 8.8 Hz, H-24), 2.12 (1H, m, H-9), 2.20 (1H, m, H-16a), 2.09 (1H, m, H-5), 1.93 (1H, m, H-16b), 1.90 (1H, m, H-22a), 1.87 (3H, s, 7-OCO*CH$_3$*), 1.85 (2H, m, H-17 and H-20), 1.83 (2H, m, H-11a and H-12a), 1.70 (1H, m, H-6a), 1.66 (1H, m, H-6b), 1.59 (2H, m, H-11b and H-12b), 1.56 (1H, m, H-22b), 1.28 (3H, s, Me-26), 1.16 (3H, s, Me-30), 1.15 (3H, s, Me-27), 1.14 (3H, s, Me-19), 1.05 (6H, s, Me-28 and Me-29), 0.93 (3H, s, Me-18).

In Vitro Cytotoxicity Assays.[15,16] All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000 cells per well with compounds added from DMSO-diluted stock. To minimize potential solvent effects on cell growth, the final concentrations of DMSO in all culture wells were less than 0.05%. After three days in culture, attached cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide). The absorbency at 540 nm was measured using a microplate reader after solubilizing the bound dye. The mean IC_{50} is the concentration of agent that inhibited cell growth by 50% under the experimental conditions and is the average from at least six independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: human breast ductol carcinoma ATCC No. HTB 20 (BT474), undifferentiated lung carcinoma (CHAGO), liver hepatoblastoma (Hep-G2), gastric carcinoma ATCC No. HTB 103 (KATO-3), and colon adenocarcinoma ATCC No. CCL 227 (SW-620). All cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, and cultured in RPMI-1640 supplemented with 25 mM HEPES, 0.25%

(w/v) sodium bicarbonate, 5% (v/v) fetal bovine serum, and 100 μg/mL kanamycin.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1**−**3** are available free of charge via the Internet at http://pubs.acs.org.

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Protoxylocarpins F-H, Protolimonoids from the Kernel Seeds of *Xylocarpus granatum*

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Three new protolimonoids, protoxylocarpins F-H (**1**-**3**), along with 11 known limonoids, were isolated from the kernel seeds of *Xylocarpus granatum*. Their structures were elucidated on the basis of extensive spectroscopic data analysis. All compounds isolated were evaluated for cytotoxic activity against five human tumor cell lines.

Limonoid research from the Meliaceae family is of growing interest due to a range of biological activities, such as insect antifeedants and growth regulators, antibacterial, antifungal, antimalarial, anticancer and antiviral activities on humans.[1-3] Such a focused interest upon limonoids from the family Meliaceae has already resulted in the discovery of several limonoids with novel skeletons, mostly, but not exclusively, from within the genus Xylocarpus, and, in particular, the cannonball mangrove, *Xylocarpus granatum* Koenig. *X. granatum* is used as a folk medicine in Southeast Asia for the treatment of diarrhea, cholera, viruses, such as influenza, and fevers, such as malaria, as well as an antifeedant based insecticide.[4] To date, more than 40 liminoid derivatives have been isolated from *X. granatum*, and are classified as phragmalin, mexicanolide and andirobin types.[5] In continuing search for structurally and biologically interesting metabolites from plant resources, we described herein the isolation and structural elucidation of three new protolimonoids (**1**-**3**) along with 11 known limonoids from the kernel seeds of *X. granatum* collected from the Samutsongkram Province, Thailand, and the evaluation for their cytotoxicity on five human tumor cell lines.

Results and Discussion

The MeOH extract of the kernel seeds of *X. granatum* was partitioned between EtOAc and H_2O to afford an EtOAc extract, which was subjected to silica gel column chromatography using acetone-hexane mixtures of increasing polarity as eluent. Further purification by repeated normal column chromatography and preparative thin-layer chromatography gave three new protolimonoids, protoxylocarpins F-H (**1**-**3**), and 11 known limonoids, xyloccensins K (**4**),[5k] O (**5**) and P (**6**),[5i] xylogranatin C (**7**),[5d] mexicanolide (**8**),[6] methyl angolensate (**9**),[7] proceranolide (**10**),[8] 7-oxo-7-deacetoxygenudin (**11**),[9] 7-deacetylgenudin (**12**),[10] chisocheton F (**13**)[11] and 21-acetyloxy-21,23:24,25-diepoxy-7-hydroxy-4,4,8-trimethylcholest-14-en-3-one (**14**).[12] The structures of the known compounds were determined by the comparison of their NMR spectroscopic data with those in the literature.

Protoxylocarpin F (**1**) was isolated as a colorless gum and its molecular formula was assigned as $C_{32}H_{48}O_6$ on the basis of the $[M+Na]^+$ peak at *m/z* 551.3348 (calcd. 551.3349) in the HRESIMS, requiring nine degrees of unsaturation. The IR absorptions implied the presence of an α,β-unsaturated ketone (1733 cm^{-1}) and

hydroxyl (3543 cm^{-1}) groups. The ^1H NMR spectrum (Table 1) showed signals of seven tertiary methyls [δ_H 1.24, 1.12, 1.10 (Me x 2), 1.00 (Me x 2) and 0.91], an olefinic proton (δ_H 5.23), an acetyl methyl (δ_H 1.88), and an α,β-unsaturated ketone moiety indicated by a pair of doublets at δ_H 7.10 and 5.97. A combined analysis of ^{13}C NMR (Table 1) and HSQC spectra revealed 32 nonequivalent carbon resonances due to one carbonyl (δ_C 204.8), four olefinic carbons (δ_C 159.2, 158.5, 125.4 and 119.0), seven methyl carbons (δ_C 27.4, 27.0, 26.3, 22.4, 21.3, 20.0 and 19.0), the acetyl carbons (δ_C 170.2 and 21.2) together with six methylenes, and seven methines, accounting for four double-bond equivalents. The remaining five degrees required **1** to be a pentacyclic at the central core. These data suggested that the structure of **1** possessed a protolimonoid skeleton. The NMR data (Table 1) of **1** were similar to those of hilstinone C,[13b] a protolimonoid isolated from *Turraea holstii*, another plant in the Meliaceae family, except for the presence of an additional metylene [δ_H 3.44 (dd, J = 2.8, 13.6 Hz), 3.54 (d, J = 12.4 Hz); δ_C 64.2] and acetyl group [δ_H 1.88 (s); δ_C 21.2 and 170.2] as well as the absence of the methoxyl group and C-21 hemiacetal methine resonance in hilstinone. The HMBC cross-peak from H-7 (δ_H 5.16, s) to the acetyl carbon (δ_C 170.2) (Figure 1) indicated that the above acetoxyl group was attached to C-7. These data suggested that **1** was a 7-acetyl-21-demethoxy analogue of hilstinone C. The full assignments and connectivity were determined by COSY and HMBC correlations shown in Figure 1.

The relative stereochemistry of **1** was assigned on the basis of NOESY data as depicted in Figure 2. In tetracyclic nucleus, the NOESY correlations of Me-29/Me-19, Me-19/Me-30 and Me-30/H-7 indicated their β-orientations, while those of H-5/H-9 and H-9/Me-18 confirmed that H-5, H-9 and Me-18 were α-oriented. Since H-17 was exclusively assigned to the β-form as observed in all protolimonoids,[5a,13] the NOE correlations of H-20/Me-18, H-20/H-23 and H-23/H-24 combined with the non-interaction of H-17/H-20 allowed the assignment of α-orientation for Me-18, H-20, H-23 and H-24. The absolute configuration at C-24 of **1** was assigned by the modified Mosher method.[15] The difference in chemical shift values ($\Delta\delta$) for its diastereomeric esters, (*R*)-MTPA (**1a**) and (*S*)-MTPA (**1b**), suggested the *S*-configuration at C-24 as shown in Figure 3.

Protoxylocarpin G (**2**) had the molecular formula $C_{32}H_{48}O_6$, the same as that of **1**. The ^1H and ^{13}C NMR spectra of **2** were nearly identical to those of **1**. The

obvious difference was observed in the carbon resonance of C-24 (δ_C 86.5 for **2** and 80.7 for **1**). The relative configuration of **2** was determined by NOESY correlations (Figure 2) and indicated all of the chiral carbons had configurations the same as those of **1**, except for that of C-24. Due to the lack of the NOE cross-peak between H-23 and H-24, compound **2** was assigned to be the C-24 epimer of **1**. This postulation was supported by the opposite signs of their specific rotations ($[\alpha]_D$ -57.0 for **1** vs +38.0 for **2**).

Protoxylocarpin H (**3**) was isolated as a colorless gum and had the molecular formula $C_{32}H_{46}O_6$ as determined by HRESIMS at *m/z* 526.3297 (calcd. 526.3294), which indicated an additional double-bond equivalent relative to **1** and **2**. Similar to compounds **1** and **2**, the ^1H and ^{13}C NMR data of **3** were characteristic of protolimonoid skeleton, except for the presence of the hemiacetal quaternary carbon (δ_C 95.5) instead of the oxygenated methine carbon in **1** (δ_C 80.7) and **2** (δ_C 86.5) in the linear aliphatic part. The epoxide ring was assigned in the linear aliphatic system between C-24 (δ_C 95.5) and C-25 (δ_C 76.3) due to the existence of the above additional degree of unsaturation and the HMBC correlations of H-23/C-24, Me-26/C-24 and Me-27/C-24 (Figure 1). The relative stereochemistry of **3** was determined the same as **1** by NOESY data.

All compounds isolated (**1**-**14**) were tested for their cytotoxicity toward CHAGO (lung carcinoma), SW-620 (gastric carcinoma), KATO-3 (colon carcinoma), BT-474 (breast carcinoma) and Hep-G2 (hepatocarcinoma). Compound **12** showed moderate cytotoxic activity against CHAGO and Hep-G2 cells with IC_{50} values of 7.05 and 4.52 μg/mL, respectively. Compound **7** was active on CHAGO cells with IC_{50} values of 4.97 μg/mL, while compound **11** displayed the activity toward Hep-G2 with IC_{50} values of 7.09 μg/mL.

Experimental Section

General Experiment Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at wavelength 589 nm, and UV data were recorded on a Shimadzu UV-160 spectrophotometer. Melting points were measured using a Fisher-Johns melting point apparatus. IR spectra were recorded on a Perkin-Elmer model 1760X Fourier transform infrared spectrophotometer. HRESIMS spectra were obtained using a Bruker micrOTOF mass spectrometer. The NMR

spectra were recorded on a Varian YH400 spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS (trimethylsilane) as internal standard.

Plant Material. The fruits of *X. granatum* were collected from the Samutsongkram Province, Thailand in December 2008. A voucher specimen (BKF 159047) has been deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Extraction and Isolation. The air-dried powder of the kernel seeds of *X. granatum* (1.5 kg) was extracted with MeOH (5 L x 3, each 2 days) at room temperature. After removing the solvent in vacuo, the combined MeOH crude extract was suspended in H_2O (250 mL), then partitioned with EtOAc (500 mL x 3) to afford EtOAc crude extract (52.6 g). This crude extract was chromatographed on silica gel column eluted with a gradient of hexane-acetone (from 1:0 to 0:1) to yield nine fractions (I-IX). Fraction III was subjected to column chromatography over silica gel eluting with MeOH-CH_2Cl_2 (1:19) to give 10 subfractions (IIIa-IIIj). Subfractions IIIc were subsequently separated on silica gel column (EtOAc-hexane, 1:3 to 1:1) and preparative TLC (EtOAc-benzene, 1:8) to afford **12** (12.5 mg) and **13** (6.2 mg), while subfraction IIId was chromatographed on silica gel column (acetone-hexane, 1:3) to yield **8** (322.6 mg). Subfraction IIIe was applied on a reversed-phase silica gel column chromatography (C-18) using a mixture of MeOH-H_2O (8:2) to afford **2** (86.4 mg). Fraction IV was subjected to silica gel column chromatography (EtOAc-hexane, 1:2 to 1:1) to give 15 subfractions. Then, subfractions IVd was separated on a silica gel column (EtOAc-benzene, 1:6) to yield **11** (20.0 mg) and **7** (19.1 mg), subfractions IVe and IVj were separeated by the same manner eluted with MeOH-CH_2Cl_2 (2:98) and acetone-benzene (1:5) to afford **9** (25.1 mg) and **1** (30.8 mg), respectively. Fraction V was chromatographed on a silica gel column using MeOH-CH_2Cl_2 (2:98 and 3:97) to furnish 13 subfractions (Va-Vl). Subfraction Vb was rechromatographed on a silica gel column (EtOAc-hexane, 1:2) to give **8** (6.7 mg). Subfraction Vc was subjected to silica gel column chromatography (MeOH: $CHCl_3$, 1:99), then fraction Vc.2 was purified by the same manner eluted with EtOAc-hexane (1:1) to yield **5** (3.2 mg) and **6** (14.7 mg). Subfraction Vh separated into six fractions by column chromatography over silica gel (acetone-hexane, 1:2) and fraction Vh.5 was further purified by reversed-phase silica gel column chromatography (C-18) using a mixture of MeCN-H_2O (1:1) to afford **3** (62.8 mg). Fractions VI and VII was combined and then recrystallized from EtOAc to obtain **4** (12.6 g).

Protoxylocarpin F (1): colorless gum; $[\alpha]^{25}_{D}$ -57.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 235 (4.10); IR (KBr) ν_{max} 3453, 2937, 1733, 1668, 1458, 1381, 1250, 1058, and 732 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 531.3348 (calcd. for $C_{32}H_{48}O_6$ + Na, 531.3349).

Protoxylocarpin G (2): colorless gum; $[\alpha]^{25}_{D}$ +38.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 215 (4.08); IR (KBr) ν_{max} 3460, 2950, 1728, 1460, 1437, 1381, 1255, 1170, 1026 and 732 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 531.3345 (calcd. for $C_{32}H_{48}O_6$ + Na, 531.3349).

Protoxylocarpin H (3): colorless gum; $[\alpha]^{25}_{D}$ +13.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 230 (4.09); IR (KBr) ν_{max} 3454, 2941, 1736, 1667, 1461, 1381, 1250, 1028 and 826 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 549.3190 (calcd. for $C_{32}H_{46}O_6$ + Na, 549.3192).

Preparation of (*R*)-MTPA ester (1a) and (*S*)-MTPA ester (1b). A reaction mixture of **1** (5 mg), (*S*)-MTPA Cl (10 μL) and DMAP (catalytic amount) in pyridine (0.5 mL) was stirred at room temperature overnight. After removing the solvent under reduced pressure; the (*R*)-MTPA esters (**1a**) was purified by mini-column chromatography on silica gel with EtOAc-hexane (1:8); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.12 (1H, d, *J* = 10.2 Hz, H-1), 5 .98 (1H, d, *J* = 10.2 Hz, H-2), 5.26 (1H, br s, H-15), 5.18 (1H, m, H-7), 3.77 (1H, ddd, *J* = 2.7, 8.6, 10.0 Hz, H-23), 3.55 (1H, br d, *J* = 12.0 Hz, H-21a), 3.47 (1H, dd, *J* = 2.8, 12.9 Hz, H-21b), 3.40 (1H, d, *J* = 8.8 Hz, H-24), 2.14 (1H, m, H-9), 2.22 (1H, m, H-16a), 2.15 (1H, m, H-5), 1.99 (1H, m, H-16b), 1.97 (1H, m, H-22a), 1.87 (3H, s, 7-OCO*CH*$_3$), 1.88 (2H, m, H-17 and H-20), 1.84 (2H, m, H-11a and H-12a), 1.72 (1H, m, H-6a), 1.69 (1H, m, H-6b), 1.61 (2H, m, H-11b and H-12b), 1.54 (1H, m, H-22b), 1.22 (3H, s, Me-26), 1.18 (3H, s, Me-30), 1.11 (3H, s, Me-19), 1.10 (3H, s, Me-27), 1.05 (6H, s, Me-28 and Me-29), 0.94 (3H, s, Me-18).

Similarly, the reaction mixture of **1** (5 mg), (*R*)-MTPA Cl (10 μL) and pyridine (0.5 mL) was processed as described above for **1a** to afford **1b**; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.12 (1H, d, *J* = 10.2 Hz, H-1), 5.97 (1H, d, *J* = 10.2 Hz, H-2), 5.22 (1H, br s, H-15), 5.14 (1H, m, H-7), 3.71 (1H, ddd, *J* = 2.6, 8.9, 10.2 Hz, H-23), 3.53 (1H, br d, *J* = 12.4 Hz, H-21a), 3.44 (1H, dd, *J* = 2.6, 13.2 Hz, H-21b), 3.35 (1H, d, *J* = 8.8 Hz, H-24), 2.12 (1H, m, H-9), 2.20 (1H, m, H-16a), 2.09 (1H, m, H-5), 1.93 (1H, m, H-16b), 1.90 (1H, m, H-22a), 1.87 (3H, s, 7-OCO*CH*$_3$), 1.85 (2H, m, H-17

and H-20), 1.83 (2H, m, H-11a and H-12a), 1.70 (1H, m, H-6a), 1.66 (1H, m, H-6b), 1.59 (2H, m, H-11b and H-12b), 1.56 (1H, m, H-22b), 1.28 (3H, s, Me-26), 1.16 (3H, s, Me-30), 1.15 (3H, s, Me-27), 1.14 (3H, s, Me-19), 1.05 (6H, s, Me-28 and Me-29), 0.93 (3H, s, Me-18).

In Vitro Cytotoxicity Bioassays.[15,16] All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000 cells per well with compounds added from DMSO-diluted stock. After three days in culture, attached cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide). The absorbency at 540 nm was measured using a microplate reader after solubilizing the bound dye. The mean IC_{50} is the concentration of agent that inhibits cell growth by 50% under the experimental conditions and is the average from at least six independent determinations that were reproducible and statistically significant. The following human tumor cell lines were used in the assay: human breast ductol carcinoma ATCC No. HTB 20 (BT474), undifferentiated lung carcinoma (CHAGO), liver hepatoblastoma (Hep-G2), gastric carcinoma ATCC No. HTB 103 (KATO-3), and colon adenocarcinoma ATCC No. CCL 227 (SW-620). All cell lines were obtained from Institute of Biotechnology and Genetic Engineering, Chulalongkorn University and cultured in RPMI-1640 supplemented with 25 mM HEPES, 0.25% (w/v) sodium bicarbonate, 5% (v/v) fetal bovine serum, and 100 μg/mL kanamycin.

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Supporting Information Available: The ^{1}H and ^{13}C NMR spectra of compounds **1-3** are available free of charge via the internet at http://pubs.acs.org.

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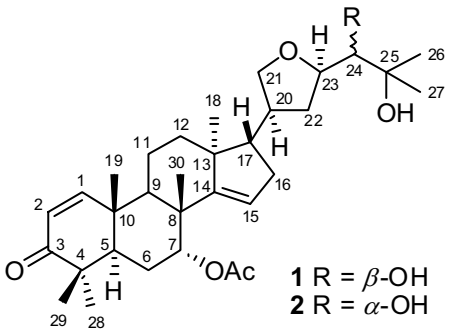

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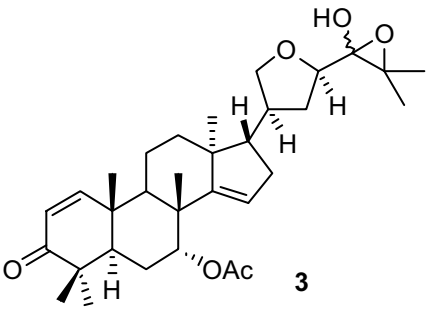

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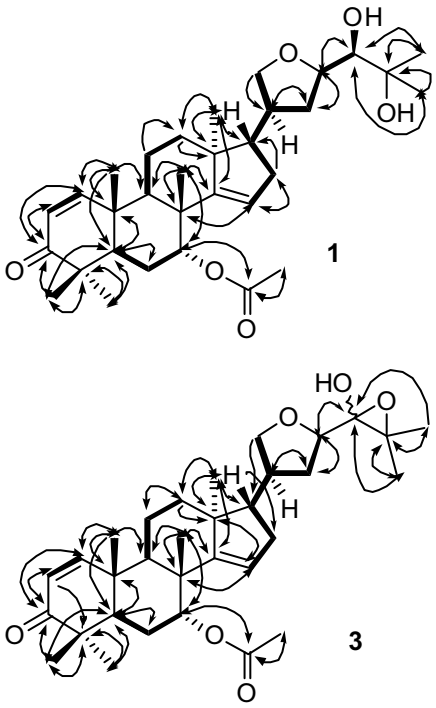

Figure 1. COSY (bold lines) and selected HMBC (arrows) correlations for **1** and **3**.

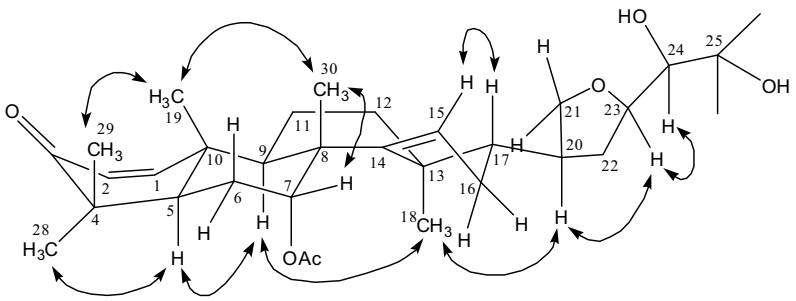

Figure 2. Selected NOESY correlations for **1**.

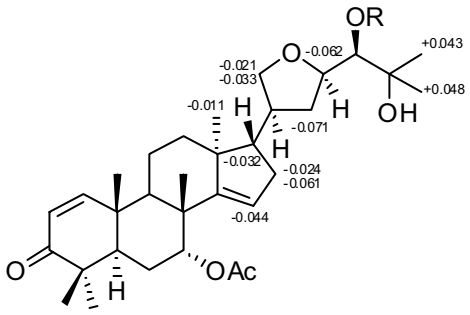

Figure 3. $\Delta\delta$ values (in ppm) = $\delta_S - \delta_R$ obtained for (*S*)- and (*R*)-MTPA esters **1b** and **1a**.

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1**-**3** in $CDCl_3$.

	1		**2**		**3**	
position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	7.10 d (10.0)	158.5	7.14 d (10.4)	158.5	7.14 d (10.0)	158.3
2	5.97 d (10.0)	125.4	5.80 d (10.4)	125.5	5.83 d (10.0)	125.4
3	-	204.8	-	204.8	-	204.8
4	-	44.1	-	44.2	-	44.1
5	2.10 m	46.2	2.16 m	46.4	2.14 m	46.1
6	1.64 m; 1.70 m	23.8	1.80 m; 1.90 m	24.0	1.76 m	23.8
7	5.16 br s	74.7	5.20 br s	74.8	5.19 br s	74.6
8	-	42.7	-	42.8	-	42.6
9	2.13 m	38.5	2.20 m	38.7	2.16 m	38.4
10	-	39.8	-	39.7	-	39.8
11	1.58 m; 1.83 m	16.7	1.55 m; 2.00 m	16.8	1.49 m, 1.73 m	16.7
12	1.60 m; 1.83 m	34.0	2.26 m	34.9	1.92 m, 2.16 m	33.9
13	-	46.3	-	46.2	-	46.5
14	-	159.2	-	159.0	-	158.7
15	5.23 br d (2.4)	119.0	5.30 br d (2.4)	119.6	5.25 br s	119.2
16	1.93 m; 2.21 m	35.0	2.26 m	35.0	2.14 m	29.8
17	1.84 m	54.2	2.00 m	52.3	1.40 m	57.1
18	0.91 s	20.0	0.90 s	20.4	1.04 s	20.4
19	1.09 s	19.0	1.14 s	19.1	1.13 s	19.0
20	1.84 m	36.3	1.88 m	35.8	1.76 m	34.5
21	3.44 dd (2.8, 13.6); 3.54 br d (12.4)	64.2	3.42 dd (2.0, 12.0) 3.98 br d (11.6)	70.0	3.60 m, 3.79 m	65.3
22	1.55 m; 1.91 m	37.9	1.52 m; 2.04 m	36.2	1.69 m	32.8
23	3.74 ddd (2.4, 8.8, 10.4)	68.0	3.86 ddd (2.8, 8.8, 13.2)	64.4	3.85 m	67.5
24	3.36 d (8.8)	80.7	2.98 d (8.8)	86.5	-	95.5
25	-	76.2	-	74.3	-	76.3
26	1.24 s	26.3	1.30 s	28.6	1.39 s	24.3
27	1.10 s	22.4	1.25 s	23.8	1.25 s	23.1
28	1.00 s	21.3	1.05 s	21.3	1.04 s	21.2
29	1.00 s	27.0	1.05 s	27.0	1.04 s	27.0
30	1.12 s	27.4	1.18 s	27.3	1.13 s	27.3
7-OCO*CH*$_3$	1.88 s	21.2	1.92 s	21.2	1.92 s	21.1
7-O*CO*CH$_3$	-	170.2	-	170.2	-	170.2

Protoxylocarpins F-H, Protolimonoids from the Kernel Seeds of *Xylocarpus granatum*

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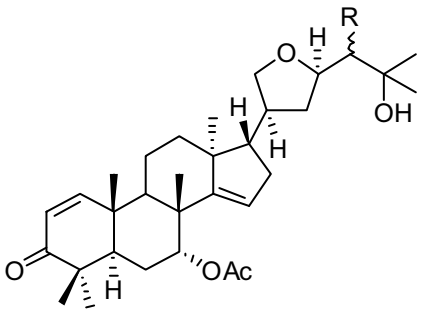

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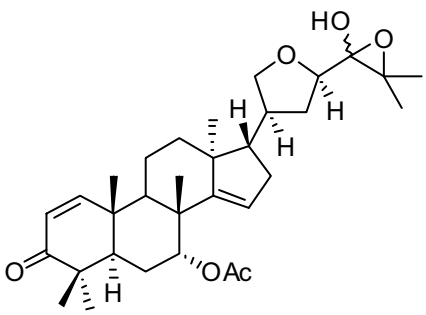

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