

Bioactive C₁₅ Acetogenins from the Red Alga *Laurencia obtusa*

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The petroleum ether extract of the red alga *Laurencia obtusa* afforded three new C₁₅ acetogenins (cyclic ether enyne): (12*Z*)-*cis*-maneone-D (1), (12*E*)-*cis*-maneone-E (2), and (12*Z*)-*trans*-maneone-C (3), along with one known *cis*-maneone-A (4). Blood neutrophils were prepared, cultured, and incubated for 24, 48, and 72 h in medium with and without isolated compounds. Blood neutrophils were prepared, cultured, and incubated for 24, 48 and 72 h in medium with and without the isolated compounds. Both morphology and DNA fragmentation methods assessed the percentage of neutrophils apoptosis in each culture. In the present study, several observations have been made concerning the apoptosis-inducing or inhibiting effect of 1 and 2. Both compounds had no inhibition of apoptosis but apoptosis was enhanced significantly by aging. However, 1 stimulated apoptosis of normal only at the initial 24 h. After that there was no significant difference in apoptosis with or without compound 1, while 2 stimulated apoptosis at all the times. The apoptosis induced by these two compounds was demonstrated by DNA fragmentation assay and microscopic observation. These observations suggest that compounds 1 and 2 may be involved in regulation of programmed death in the initiation and propagation of inflammatory responses.

Key words red algae; *Laurencia obtusa*; C₁₅ nonterpenoid halo ether; maneone; apoptosis

Species of the marine red algae *Laurencia*, are known to exhibit bromine and chlorine-containing C₁₅ nonterpenoid metabolites.¹⁾ Among these, are the unusual cyclic ethers, with a pentadec-3-en-1-yne carbon skeleton. The isolation of *trans* and *cis*-laurediols from *L. nipponica* indicated an evidence that, cyclic ether enynes were raised from linear C₁₅ acetylenic polyenes, through oxidation and cyclization processes.²⁾ Several maneones (halogenated cyclic ether enyne) were isolated from the genus *Laurencia*,^{3–9)} the fundamental differences between them is either geometry (through C₃–C₄ and/or C₁₂–C₁₃) and/or stereochemical configuration (C₅, C₆ and C₁₁).

Apoptosis is a programmed form of cell death by which unwanted cells are removed from the body without causing inflammation.¹⁰⁾ This contrasts with necrosis, which involves direct damage to cells and is associated with inflammation.¹¹⁾ Apoptosis may be an alternative and better method by which cells, such as neutrophils, are removed from an inflamed site.¹²⁾ An organism must also remove senescent, damaged, or abnormal cells that could interfere with organ function or develop into tumors.

In the present investigations, we describe the isolation and structural elucidation of three new (cyclic ether enynes) 1, 2 and 3 along with one known isomer 4^{5–7)} obtained from the petroleum ether extract of the red alga *L. obtusa*.

Results and Discussion

The petroleum ether extract of the air dried algal material was fractionated on aluminum oxide column using a stepped gradient of hexane and ether. The fractions were examined

by spot TLC chromatography and spray reagent methanol-sulfuric acid (50%) to give four compounds (1–4).

The spectral data of these four compounds (Table 1) showed their very close structural relationship and strongly suggested that they belong to the C₁₅ acetogenins. The structure of known (12*E*)-*cis*-maneone-A (4) was established by comparing its physical and spectral data with literature.^{5–7)}

Compound 1, was isolated as pale yellow oil. High resolution ionization-mass spectra (HR-EI-MS) established the molecular formula C₁₅H₁₆BrClO₂, implying 7 degrees of unsaturation. EI-MS exhibited a characteristic molecular-ion cluster at *m/z* 342/344/346 in a ratio 2:3:1, which clearly indicated the presence of one Br and one Cl atoms. The presence of acetylenic group, vinyl ether stretching, and two C–O functionalities were deduced from IR absorptions at ν_{\max} 3286, 1687, 1126, and 1190 cm⁻¹, respectively. Hence, both oxygen atoms could be involved in ether links. The ¹³C-NMR and distortionless enhancement by polarization transfer (DEPT) experiments allowed the determination of 1 methyl, 2 methylene, 9 methine and 3 quaternary carbon atoms. Moreover, ¹³C-NMR and heteronuclear single quantum coherence (HSQC) spectra displayed resonance for four carbons bearing oxygen, three of these were demonstrated by the signals at δ_C 78.2, 79.4, and 82.9; while the fourth is of vinyl ether carbon at δ_C 152.0.

The presence of a terminal conjugated enyne moiety, which is frequently encountered in *Laurencia* C₁₅ acetogenins, was evident from the ¹H-NMR spectrum (Table 1) δ_H 2.63, 5.19 and 5.59. Interpretation of the ¹H–¹H correlation spectroscopy (COSY) spectrum confirmed the presence of halogenated petenyne group, in which, the olefinic (C-3) proton at δ_H 5.19 correlated to acetylenic (C-1) proton at δ_H 2.63 and to olefinic (C-4) proton at δ_H 5.59. H-4 is correlated

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Table 1. ^1H -NMR (600 MHz) and ^{13}C -NMR (125 MHz) in C_6D_6 Spectroscopic Data of Compounds 1—4

No. C	^{13}C	$^1\text{H}(\delta)$	Mult.	J (Hz)	^{13}C	$^1\text{H}(\delta)$	Mult.	J (Hz)	^{13}C	$^1\text{H}(\delta)$	Mult.	J (Hz)	^{13}C	$^1\text{H}(\delta)$	Mult.	J (Hz)
1	85.6	2.63	dd	2.4, 1.2	87.1	2.98	dd	2.4, 0.6	80.5	2.55	d	1.8	84.8	2.8	dd	2.4, 1.2
2	78.2	—	—	—	78.2	—	—	—	80.4	—	—	—	86.9	—	—	—
3	111.8	5.19	dd	10.8, 2.4	112.7	5.15	dd	10.8, 2.4	113.1	5.21	dd	15.6, 1.8	111.9	5.4	ddd	10.8, 2.4
4	141.5	5.59	ddd	10.8, 10.8, 1.2	141.7	5.35	ddd	10.8, 10.8, 0.6	142.1	5.79	dd	15.6, 9.6	142.0	6.1	ddd	10.8, 10.8, 1.2
5	58.4	4.92	dd	10.8, 10.8	56.9	4.89	dd	12.0, 10.8	63.6	3.70	dd	10.8, 9.6	58.3	5.1	dd	10.8, 10.8
6	58.3	1.72	dd	10.8, 1.8	52.8	2.25	dddd	12.0, 9.6, 4.2, 1.8	57.9	1.50	dd	10.8, 1.2	58.3	1.9	dd	10.8, 1.2
7	78.2	4.53	br d	5.4	79.4	4.51	dd	4.8, 4.2	77.8	3.66	dd	4.8, 1.2	78.3	4.6	d	5.4
8	38.6	1.34	ddd	13.2, 7.8, 5.4	35.5	1.37	dddd	14.4, 7.2, 4.8, 1.8	38.8	1.19	ddd	13.8, 7.8, 4.8	38.6	1.3	ddd	14, 7.8, 5.4
		1.08	d	13.2		1.75	d	14.4		0.93	d	13.8		1.0	d	14
9	79.4	4.08	dd	7.8, 4.8	80.0	4.13	dd	7.2, 4.8	78.1	4.07	dd	7.8, 4.8	80.4	4.1	dd	7.8, 4.8
10	82.9	4.41	dd	4.8, 4.8	85.1	4.42	dd	4.8, 4.8	82.4	4.32	dd	4.8, 4.8	82.2	4.4	dd	4.8, 4.8
11	44.1	2.58	br d	4.8	42.3	2.33	dd	9.6, 4.8	44.9	2.73	d	4.8	46.1	2.8	dd	5.0, 1.2
12	152.0	—	—	—	148.5	—	—	—	151.8	—	—	—	149.4	—	—	—
13	97.8	—	—	—	100.7	—	—	—	98.9	—	—	—	102.5	—	—	—
14	29.3	2.23	q	7.2	30.7	(14a)	dq	14.4, 7.2	29.2	2.57	q	7.2	26.7	(14a)	dq	15.0, 7.2
						1.92								2.60		
						(14b)	dq	14.4, 7.2						(14b)	dq	15.0, 7.2
						1.84								2.50		
15	13.3	1.03	t	7.2	14.0	1.02	t	7.2	13.9	1.19	t	7.2	13.0	1.0	t	7.2

to a methine (C-5) proton at δ_{H} 4.92. The following observations can be concluded: The J -value (10.8 Hz) between H-3 and H-4 as well as the chemical shift value (δ_{H} 2.63) of the acetylenic proton indicated the geometry of the double bond at C-3 to be Z^{13} ; the low field chemical shift value of CH-5 (δ_{H} 4.92/ δ_{C} 58.4) implying that, such methine carbon should be attached to halogen atom; C-6 should be a part of cyclic system through its correlation with methine (C-7) proton resonated at δ_{H} 4.53, methine (C-11) proton resonated at δ_{H} 2.58 and C-5 proton; the isolated ethyl group, in which two methylene (C-14) protons appeared at δ_{H} 2.23 are correlated with methyl (C-15) protons appeared at δ_{H} 1.03 is allylic owing to the δ_{C} 29.3 (C-14); and the C-12/C-13 double bond is Z oriented owing to the value of chemical shift of allylic methylene $\delta_{\text{C}} < 30$ and the J values.¹⁴⁾

Furthermore, based on the chemical shift values of H-5 (δ_{H} 4.92/ δ_{C} 58.4), halogen atom was deduced to be attached to C-5 position. From heteronuclear multiple bond connectivity (HMBC) spectrum, structure connectivity's of compound **1** (Fig. 1) could be assigned as follow, cross peaks of C-7/H-10 and C-10/H-7 established the ether ring closure between C-7 and C-10. In addition, a cross peak between the terminal methyl protons and the vinyl carbon at δ_{C} 97.8 indicated that the ethyl group was attached to the tetra substituted double bond of C-12 and C-13 [$\text{C}=\text{C}$]. The chemical shift value (δ_{C} 97.8) of this vinyl carbon atom suggested that another substituent is a halogen atom.¹³⁾ Therefore, this double bond had to be inserted between C-9 and C-11 through

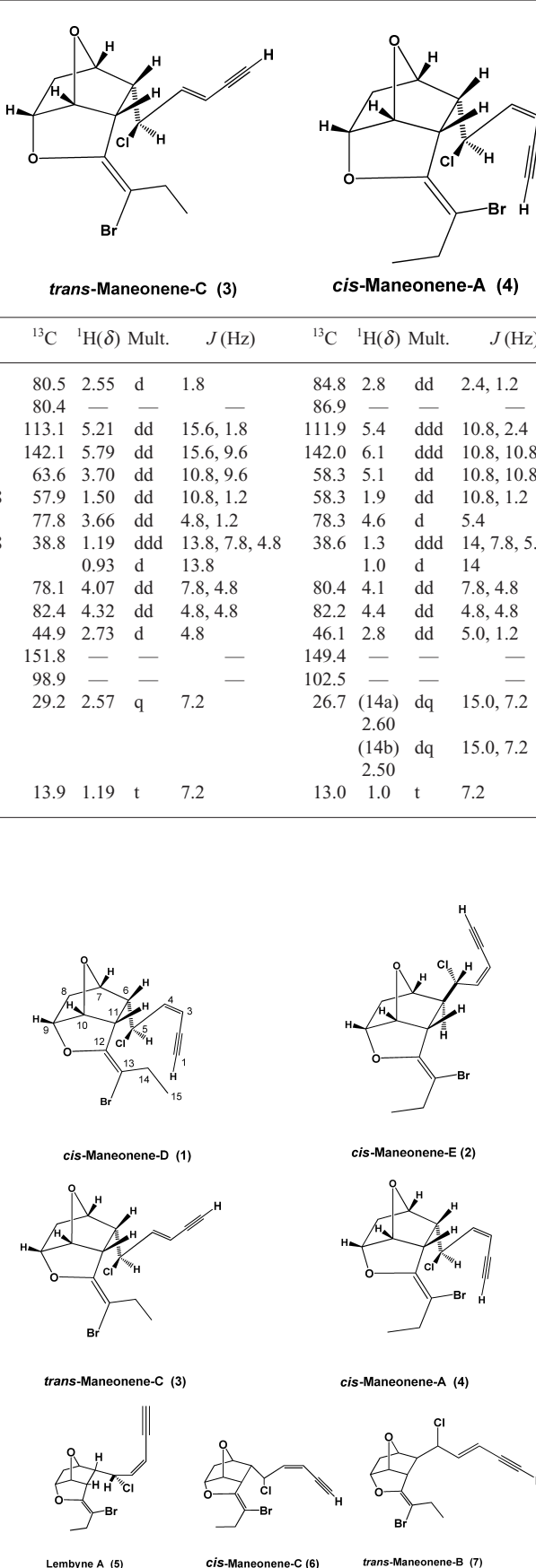


Fig. 1. Structure of Maneonenes 1—7

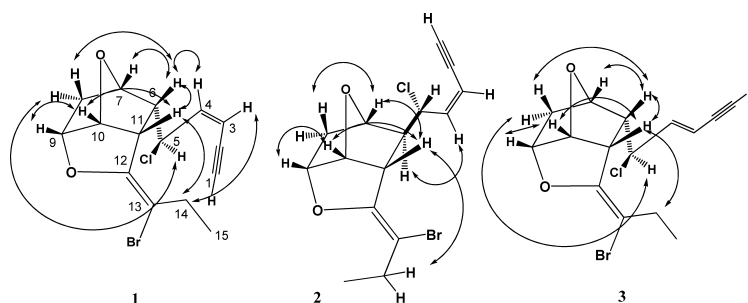


Fig. 2. Key NOESY of Compounds 1–3

ether linkage. A fragment ion of m/z 309, 307 $[M-Cl]^+$ in the EI-MS is an evident that Cl atom is attached to C-5, which strongly suggested the presence of a vinyl bromide moiety. 1H - and ^{13}C -NMR spectroscopic data of **1** (Table 1) are close to those of lembyne-A (**5**), which had been isolated from the Malaysian *Laurencia* species.⁸ The chemical shifts and coupling constants of the protons from C-1 to C-10 in the 1H -NMR spectrum of **1** are different from those of lembyne-A. However, distinct differences were observed in the chemical shift values of H-11 and H₂-14. Furthermore, the magnetic equivalence of the CH₂-14 in **1** is explained that, the ethyl group should be opposite to the oxygen atom of the vinyl ether moiety. Thus, the stereochemistry of the vinyl ether moiety in **1** is established as *Z*-configuration. In consequence, the compound **1** must be represented by formula **1** (Fig. 1). Again, both **1** and lembyne A have the same chemical structure but differ in chemical shift values, which is due to the difference in stereochemistry around C-5, C-6, and C-11. The relative stereochemistry was based on a nuclear Overhauser effect spectroscopy (NOESY) experiment (Fig. 2). The NOESY spectrum showed correlations between H-6/H-7, H-6/H-8 β and H-6/H-11. The H-11 correlates with H-10 and the H-9 correlates with H-10. From these correlations of all H-6, H-7, H-8 β , H-9, H-10 and H-11 are *cis* with each other indicating that this ring was cyclohexane boat form. Furthermore, the NOESY spectrum showed correlations between H-6 and H-4, and H-11 and 2H-14, and also H-3 correlates with H-14, indicating that the bromide atom should be *cis* with oxygen atom of furan ring. In view of the above mentioned data, compound **1** was named (12*Z*)-*cis*-maneonene-D.

Compound **2**, was isolated as colorless oil. The structure of compound **2** was shown to have *cis*-enyne function and formally a maneonene structure by the same approach of UV, IR, MS and NMR (*cf.* exp. and Table 1) data analysis of (12*Z*)-*cis*-maneonene-D (**1**). The main differences between compounds **1** and **2** were shown in 1H -NMR spectra of H-6 at δ 2.25 (dddd, $J=12.0, 9.6, 4.2, 1.8$ Hz, H-6) of compound **2** instead of δ 1.72 (dd, $J=10.8, 1.8$ Hz, H-6) of compound **1**. This difference allowed us to assign H-6 as *trans* geometry with both H-5, H-11 in compound **2**. Furthermore, the large magnetic nonequivalence of the CH₂-14 in **2** is attributed to the electronic influence of the oxygen atom that is *cis* to the ethyl group, which allowed us to establish the stereochemistry of the vinyl ether moiety in **2** as *E*-configuration. The deduced structure **2** is closely similar to that of **4** and *cis*-maneonene-C (**6**), which has been isolated from the red alga *Laurencia nidifica*,^{5,6} but distinct differences were observed

in the chemical shift values of H-11 and H-14 (up-field shift in **2**), that was attributed to the shielding effect being caused by the bromine atom at C-13 that is close to the H-11, and also the small difference in chemical shifts of protons from C-4 to C-10 in the 1H -NMR spectrum indicated that conformational structure of **2** (Fig. 1) is different from that of *cis*-maneonene-C (**6**). Both **2** and **6** have the same chemical structure but differ in chemical shift which is due to the difference in stereochemistry around C-5, C-6, and C-13. The relative stereochemistry was based on a NOESY experiment (Fig. 2). The NOESY spectrum showed correlations between H-7/H-8 β , H-11/H-10 and H-9/H-10. From these correlations of all H-7, H-8 β , H-9, H-10 and H-11 are *cis* with each other and *trans* with H-6 indicating that this ring is cyclohexane boat form. The NOESY spectrum showed correlations between H-6/H-4, and H-11/only one proton of CH₂-14 which resonated at δ 1.84 (1H, dq, $J=14.4, 7.2$ Hz, H_b-14), indicating that the bromide atom should be *trans* with oxygen atom of furan ring and the double bond established as *E*-configuration. In view of the above mentioned data, compound **2** was named (12*E*)-*cis*-maneonene-E.

Compound **3**, was isolated as colorless oil. Its structure was established by a comparison of its spectral data with that of **1** (*cf.* exp. and Table 1), the only difference is of the double bond at C-3 with the $J=15.6$ Hz, which led us to assign compound **3** as *trans* geometry instead of $J=10.8$ Hz of *cis*-maneonene D (**1**). In addition both (12*Z*)-*trans*-maneonene-C (**3**) and *trans*-maneonene-B (**7**) have the same chemical structure but different in the chemical shift average δ (0.3 to 0.1) that is due to the difference in the structure confirmation around C-5 and C-6. Both **3** and **7** have the same chemical structure (Fig. 1), but differ in chemical shift which is due to the difference in stereochemistry around C-5 and C-6. The relative stereochemistry was based on a NOESY experiment (Fig. 2). The NOESY spectrum showed correlations between H-6/H-7, H-6/H-8 β and H-6/H-11. The H-11 correlated with H-10 and the H-9 correlated with H-10. From these correlations all of H-6, H-7, H-8 β , H-9, H-10 and H-11 are *cis* with each other indicating that this ring has cyclohexane boat form. The NOESY spectrum showed correlations between H-11/H-14 and also H-5 correlates with H-7. Furthermore the magnetic equivalence of the CH₂-14 in **3** is attributed to the ethyl group should be opposite to the oxygen atom. Thus the stereochemistry of the vinyl ether moiety in **3** is established as *Z*-configuration. Consequently, the compound **3** must be represented as (12*Z*)-*trans*-maneonene-C (**3**).

Pharmacology In the present study, several observations have been made concerning the apoptosis-inducing or in-

hibiting effect of (12*Z*)-*cis*-maneone-D (**1**) and (12*E*)-*cis*-maneone-E. Degradation of DNA into a specific fragmentation pattern is a characteristic feature of apoptosis. In contrast to the random fragmentation with necrosis, apoptosis-associated DNA fragmentation is characterized by cleavage of the DNA at regular intervals, visualized on agarose gel electrophoresis as a DNA ladder consisting of multimers of approximately 200 base pairs. Freshly isolated neutrophils were incubated with **1** and **2** in comparison. **1** stimulates apoptosis of normal cells only at the initial 24 h and after that there is no significant difference in apoptosis with or without treatment with **2** stimulated apoptosis all time. The IC₅₀ of the tested compounds after 24 h of incubation were 23 μM for **1**, and 15 μM for **2** versus dexamethasone 0.9 μM. In view of the above data, it is suggested that compounds **1** and **2** may be involved in regulation of programmed cell death in the initiation and propagation of inflammatory responses.

Experimental

General Chromatographic Material: Aluminum oxide for column chromatography, neutral type 507 C, 100–125 mesh was used; for thin layer chromatography silica gel GF 254 was used; and for PTL aluminum sheets 20×20. Optical rotations were measured on ATAGO POLAX-L 2 polarimeter. Electron impact mass spectra were determined at 70 eV on a Kratos EIMS-25 instrument. Nuclear Magnetic Resonance spectra were recorded for 1D and 2D on Bruker 600 MHz ¹H-NMR and 150 MHz ¹³C-NMR, DEPT spectrometer. Chemical shifts are given δ (ppm) relative to TMS as internal standard (S.S.P.).

Extraction and Isolation The red alga *Laurencia obtusa* was collected in June 2009, off the Saudi Arabia Red Sea Coast at Jeddah. Voucher sample (JAD 03060) was deposited at the Marine Chemistry Department, King Abdulaziz University, Jeddah, Saudi Arabia. It was washed with water and dried in the shade at room temperature. The dried material (200 g) was extracted with petroleum ether at room temperature. The extract was partitioned between ether and water. The crude organic residue (7 g) was subjected to column chromatography of aluminum oxide as adsorbent material, using the petroleum ether–ether mixture. The course of fractionation was followed by thin-layer chromatographic studies. Further separation of the various constituents of each fraction was performed by preparative TLC using aluminum oxide chromatoplates.

(12*Z*)-*cis*-Maneone D (**1**): The fraction B, *R*_f=0.68 (30 mg) was purified by preparative TLC using the solvent system *n*-hexane–ether (9 : 1). The band with *R*_f=0.43 (brown color with sulfuric acid–methanol) was taken to give pale yellow color oil (8 mg, 0.004%), [α]_D²⁰ – 10 (*c*=0.05, CHCl₃). IR ν_{max} (film) cm⁻¹: 3286, 2969, 1687, 1456, 1221, 1137, 1190, 1026, 983, 899, 800, 776. HR-EI-MS: *m/z* 342.0016 (Calcd 342.0022) C₁₅H₁₆BrClO₂; EI-MS (70 eV) *m/z* (rel. int.): 342, 344, 346 (2 : 3 : 1) [M]⁺ (C₁₅H₁₆BrClO₂), 309, 307 (66 : 64), [M–Cl]⁺, 265, 263 (8 : 22), [M–Br]⁺, 228 (50), [M–Br+Cl]⁺ 199 (72), 184 (90), 141 (56), 129 (94), 115 (76), 108 (70), 91 (72), 79 (64), 77 (100), 69 (92), 65 (60). ¹H- and ¹³C-NMR (C₆D₆) (Table 1).

(12*E*)-*cis*-Maneone E (**2**): The fraction C, *R*_f=0.36 (50 mg) was purified by preparative PTL using the solvent system *n*-hexane–ether (8.5 : 1.5). The band with *R*_f=0.32 (blue color with sulfuric acid–methanol) was taken to give colorless oil (8 mg, 0.01%), [α]_D²⁰ – 13.6 (*c*=0.05, CHCl₃). IR ν_{max} (film) cm⁻¹: 3286, 2970, 2929, 2100, 1734, 1687, 1456, 1438, 1344, 1266, 1267, 1204, 1182, 1128, 1117, 1086, 1044, 1027, 990, 974, 959, 916, 889, 829, 816, 797, 776, 724, 655. H-REI-MS: *m/z* 342.0012 (Calcd 342.0022) C₁₅H₁₆BrClO₂; EI-MS (70 eV) *m/z* (rel. int.): 346, 344, 342 (8 : 12 : 4) [M]⁺ (C₁₅H₁₆BrClO₂), 309, 307 (80 : 82) [M–Cl]⁺, 263 (62), 227 (26) 199 (50), 159 (60), 141 (54), 128 (58), 115 (70), 108 (56), 91 (70), 77 (100), 69 (66), 65 (52). ¹H- and ¹³C-NMR (C₆D₆) (Table 1).

(12*Z*)-*trans*-Maneone C (**3**): The fraction D, *R*_f=0.29 (30 mg) was purified by preparative TLC using the solvent system *n*-hexane–ether (85 : 15). The band with *R*_f=0.30 (violet color with sulfuric acid–methanol) was taken to give colorless oil (10 mg, 0.005%), [α]_D²⁰ – 20 (*c*=0.90, CHCl₃). IR ν_{max} (film) cm⁻¹: 3280, 2972, 2280, 1686, 1618, 1453, 1330, 1220, 1183, 1161, 1124, 1044, 1028, 991, 959, 889, 812, 797, 774. HR-EI-MS: *m/z* 342.0024 (Calcd 342.0022) C₁₅H₁₆BrClO₂; EI-MS (70 eV) *m/z* (rel. int.): 346, 344, 342 (35 : 50 : 15) [M]⁺ (C₁₅H₁₆BrClO₂), 309, 307 (20 : 20) [M–Cl]⁺, 281, 263 (24 : 20) [M–Br]⁺, 228 (35), 207 (52), 199 (50), 184 (50), 141 (55), 129

Table 2. Effect of the Isolated Compounds on Apoptosis of Peripheral Blood Neutrophils

Compound	% Apoptotic neutrophils (mean±S.D.)			
	0 h	24 h	48 h	72 h
(Control)	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01
1	0.58±0.59	26.35±4.25	30.92±5.22	37.41±4.88
2	0.64±0.05	20.67±2.20	40.07±1.11	57.27±2.30

(80), 115 (78), 108 (65), 91 (78), 79 (65), 77 (100), 69 (100). ¹H- and ¹³C-NMR (C₆D₆) (Table 1).

(12*E*)-*cis*-Maneone A (**4**): The fraction A, *R*_f=0.76 (10 mg) was purified by preparative TLC using the solvent system *n*-hexane–ether (9 : 1), the band with *R*_f=0.18 (gray color with sulfuric acid–methanol) was taken to give pale yellow color oil (2 mg, 0.001%), [α]_D²⁰ +38 (*c*=0.95, CHCl₃). IR ν_{max} (film) cm⁻¹: 3260, 3010, 2900, 1670, 1453, 1162, 1125, 812. HR-EI-MS: *m/z* 342.0008 (Calcd 342.0022) C₁₅H₁₆BrClO₂; EI-MS (70 eV) *m/z* (rel. int.): 346, 344, 342 (3 : 10 : 6), [M]⁺ (C₁₅H₁₆BrClO₂), 310, 309, 307 (10 : 45 : 43), [M–Cl]⁺, 265, 263, 249, 235, 228 (10 : 22 : 10 : 10 : 30), [M–Cl+Br]⁺, 207 (45), 199 (50), 184 (65), 129 (75), 77 (100), 69 (94), 65 (60). ¹H- and ¹³C-NMR (C₆D₆) (Table 1).

Biological Evaluation of the Isolated Compounds¹⁵ All compounds were dissolved in dimethyl sulfoxide (DMSO) at concentration less than 0.01% v/v.

Preparation of Blood Neutrophils: Neutrophils (>98% pure on May-Giemsa stain) were isolated from peripheral blood of normal healthy volunteer donors by a combination of dextran sedimentation and centrifugation through discontinuous plasma percoll gradients as described by Haslett *et al.*¹⁶

Culture of Neutrophils: Neutrophils were resuspended in an appropriate volume of RPMI 1640 medium with 10% autologous PRPDS and 100 μl of penicillin and streptomycin and divided into five equal volumes each put in culture tube. Cells were incubated (at 37 °C in a 5% carbon dioxide) as follows: (1) Only cells, (2) cells+DMSO at 0.01%v/v, (3) cells+each compound in DMSO at dose of 50 mM/ml culture. The age of neutrophils in culture was calculated at the start of culture (zero time or base line), 24, 48, and 72 h.

Assessment of Cell Viability: At time 0 and then at subsequent times, cells were removed from culture and counted on a haemocytometer. Cell viability was determined by trypan blue dye exclusion test; one volume of trypan blue (0.4% GIBCO) was added to 5 volumes of cells at room temperature for 5 min. The IC₅₀ of isolated compounds were determined in comparison to dexamethasone.

Measurements of Apoptosis: The neutrophils apoptosis in each culture was assessed:

(i) **Morphological Assessment of Apoptosis:** At time 0 and at subsequent times, cells were removed from each culture, fixed in methanol, harvested on slides and slides were stained with May Grunwald Giemsa and examined by oil immersion light microscope. For assessment of the percentage of cells showing morphology of apoptosis 500 cells /slide were examined for each case at different times (0, 24, 48, 72 h) in presence or absence of the drugs used. Neutrophils were considered apoptotic if they exhibited the highly characteristic morphological features of chromatin aggregation, nuclear pyknosis and cytoplasmic vacuolation. The apoptotic neutrophils percentage at different times was calculated for normal cells in presence or after addition of isolated compounds and the results were then compared statistically using *F* test and student *t*-test. One drop from cell suspension was added to one drop of AO solution (10 μg/ml in PBS), mixed gently on a slide, and immediately examined with an Olympus HB-2 microscope with fluorescence attachment. Green fluorescence was detected between 500 and 525 nm. Cells exhibiting bright green fluorescent condensed nuclei (intact or fragmented) were interpreted as apoptotic cells and expressed as a percentage of the total cell number viable cells, were interpreted as cells which exhibited a green, diffusely stained intact nucleus.

(ii) **DNA Fragmentation Assay:** Assessment of chromatin fragmentation in neutrophils was done by modification of methods previously used for thymocytes. Cells (2.5×10.7) were washed three times and resuspended in a 0.15 mol/l NaCl solution. The cells were chilled at 4 °C and lysed by adding 4.5 ml of 10 mmol/l of Tris/HCl buffer, pH 8.0, containing 100 mmol/l of ethylenediaminetetraacetic acid (EDTA) and 0.2% volume/volume Triton X-100 (Lysis buffer). After 4 h, the lysate was centrifuged at 35000 *g* at 4 °C

for 20 min. The supernatants were collected into tubes and precipitated with 0.1 volume of 5 mol/l of NaCl and 2 volumes of absolute ethanol. The DNA was precipitated for 24 h at 4 °C. The precipitate was centrifuged at 12500 *g* at 6 °C for 15 min. The pellet was resuspended in 1 ml of 10 mmol/l of Tris-HCl buffer pH 8 containing 100 mmol/l EDTA and 0.1 mmol/l of sodium dodecyl sulfate. Proteinase K was added to a final concentration of 20 mg/ml, and the sample was incubated for a further 24 h at 37 °C. The DNA was extracted with phenol and chloroform and re-precipitated with absolute ethanol. The pellet was re-dissolved in 20 ml of lysis buffer and 10 μ l of RNase. Compounds **1** and **2** were treated with neutrophils at 10, 20, or 40 mg/ml for 24 h. DNA was isolated as described in the text, electrophoresed on a 1% agarose gel, and stained with 0.5 mg/ml ethidium bromide, control (0.3% DMSO), 40 mg/ml of **1**, 20 mg/ml of **2**. Each sample of the purified DNA (20 μ l) was subjected to electrophoresis in 1% agarose gel containing 200 ng/ml ethidium bromide and were visualized under UV light. The sizes of the fragments were confirmed by reference to a 1-kb DNA ladder (Gibco/BRL).

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- cis*-Maneone-C (**5**): $^1\text{H-NMR}$ (C_6D_6 , 270 MHz) δ : 2.83 (1H, d, $J=2.3$ Hz, H-1), 5.15 (1H, dd, $J=10.5$, 2.3 Hz, H-3), 5.56 (1H, t, $J=10.5$ Hz, H-4), 5.01 (1H, t, $J=10.5$ Hz, H-5), 2.61 (1H, m, $J=10.5$, 9.5, 4.5 Hz, H-6), 3.98 (1H, t, $J=4.5$, 4.5 Hz, H-7), 1.42 (1H, td, $J=14$, 7, 4.5 Hz, H_{exo} -8), 1.90 (1H, d, $J=14$ Hz, H_{endo} -1), 4.31 (1H, dd, $J=7$, 5 Hz, H-9), 4.65 (1H, t, $J=5$, 5 Hz, H-10), 3.52 (1H, dd, $J=9.5$, 5 Hz, H-11), 2.97 (1H, sextet, $J=7$, 7 Hz, H_a -14), 2.61 (1H, m, $J=7$ Hz, H_b -14), 1.35 (3H, t, $J=7$ Hz, H-15).
- trans*-Maneone-B (**6**): $^1\text{H-NMR}$ (C_6D_6 , 270 MHz) δ : 2.76 (1H, d, $J=2$ Hz, H-1), 5.43 (1H, dd, $J=15.5$, 2 Hz, H-3), 6.05 (1H, dd, $J=15.5$, 9 Hz, H-4), 3.90 (1H, dd, $J=11$, 9 Hz, H-5), 1.62 (1H, dd, $J=11$, 2 Hz, H-6), 3.87 (1H, d, $J=5$ Hz, H-7), 1.40 (2H, m, H-8), 4.27 (1H, dd, $J=6$, 5 Hz, H-9), 4.57 (1H, t, $J=5$, 5 Hz, H-10), 2.87 (1H, dd, $J=5$, 2 Hz, H-11), 2.70 (2H, m, H-14), 1.23 (3H, t, $J=7$ Hz, H-15).
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