Bioactive C\textsubscript{15} Acetogenins from the Red Alga *Laurencia obtusa*

Seif-Eldin Nasr AYYAD,\textsuperscript{a,∗} Khalid Omar AL-FOOTY,\textsuperscript{a} Walid Mohamed ALARIF,\textsuperscript{b} Tariq Rashad SOBAHI,\textsuperscript{a} Salim Ahmed BASSAIF,\textsuperscript{a} Mohamed Saleh MAKKI,\textsuperscript{a} Abdullah Mohamed ASIRI,\textsuperscript{a} Amani Yahya AL HALAWANI,\textsuperscript{a} Adel Farid BADRIA,\textsuperscript{c} and Farid Abd Al-raheem BADRIA\textsuperscript{d}

\textsuperscript{a}Department of Chemistry, Faculty of Science, King Abdulaziz University; P.O. Box 80203, Jeddah 21589, KSA, Saudi Arabia; \textsuperscript{b}Marine Chemistry Department, Faculty of Marine Sciences, King Abdulaziz University; P.O. Box 80207, Jeddah 21589, KSA, Saudi Arabia; \textsuperscript{c}Tissue Engineering Laboratory, Alexandria University; Alexandria 21311, Egypt; and \textsuperscript{d}Pharmacognosy Department, Faculty of Pharmacy, Mansoura University; Mansoura 33516, Egypt.

Received May 11, 2011; accepted July 21, 2011; published online July 25, 2011

The petroleum ether extract of the red alga *Laurencia obtusa* afforded three new C\textsubscript{15} acetogenins (cyclic ether enyne); (12Z)-\textit{cis}-maneonene-D (1), (12E)-\textit{cis}-maneonene-E (2), and (12Z)-\textit{trans}-maneonene-C (3), along with one known \textit{cis}-maneonene-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*; C\textsubscript{15} nonterpenoid halo ether; maneonene; apoptosis

Species of the marine red algae *Laurencia*, are known to exhibit bromine and chlorine-containing C\textsubscript{15} nonterpenoid metabolites.\textsuperscript{1} Among these, are the unusual cyclic ethers, with a pentadec-3-en-1-yl carbon skeleton. The isolation of \textit{trans} and \textit{cis}-laurediols from *L. nipponica* indicated an evidence that, cyclic ether enynes were raised from linear C\textsubscript{15} acetylenic polyenes, through oxidation and cyclization processes.\textsuperscript{2} Several maneonenes (halogenated cyclic ether enyne) were isolated from the genus *Laurencia*;\textsuperscript{3–9} the fundamental differences between them is either geometry (through C\textsubscript{12}–C\textsubscript{13} and/or C\textsubscript{12}–C\textsubscript{13}) and/or stereochemical configuration (C\textsubscript{15}, C\textsubscript{11} and C\textsubscript{7}).

Apoptosis is a programmed form of cell death by which unwanted cells are removed from the body without causing inflammation.\textsuperscript{10} This contrasts with necrosis, which involves direct damage to cells and is associated with inflammation.\textsuperscript{11} Apoptosis may be an alternative and better method by which unscheduled cells, such as neutrophils, are removed from an inflamed site.\textsuperscript{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\textsuperscript{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 C\textsubscript{15} acetogenins. The structure of known (12E)-\textit{cis}-maneonene-A (4) was established by comparing its physical and spectral data with literature.\textsuperscript{5–7}

Compound 1, was isolated as pale yellow oil. High resolution-electron ionization-mass spectra (HR-El-MS) established the molecular formula C\textsubscript{15}H\textsubscript{26}BrClO\textsubscript{2}, implying 7 degrees of unsaturation. El-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\textsubscript{max} 3286, 1687, 1126, and 1190 cm\textsuperscript{−1}, respectively. Hence, both oxygen atoms could be involved in ether link. The \textsuperscript{13}C-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, \textsuperscript{13}C-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\textsubscript{c} 78.2, 79.4, and 82.9; while the fourth is of vinyl ether carbon at δ\textsubscript{c} 152.0.

The presence of a terminal conjugated enyne moiety, which is frequently encountered in *Laurencia* C\textsubscript{15} acetogenins, was evident from the \textsuperscript{1}H-NMR spectrum (Table 1) δ\textsubscript{H} 2.63, 5.19 and 5.59. Interpretation of the \textsuperscript{1}H–\textsuperscript{1}H correlation spectroscopy (COSY) spectrum confirmed the presence of halogenated petenyne group, in which, the olefinic (C-3) proton at δ\textsubscript{H} 5.19 correlated to acetylenic (C-1) proton at δ\textsubscript{H} 2.63 and to olefinic (C-4) proton at δ\textsubscript{H} 5.59. H-4 is correlated with δ\textsubscript{H} 2.63 and with δ\textsubscript{H} 5.59.
to a methine (C-5) proton at $\delta_H$ 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 ($\delta_H$ 2.63) of the acetylenic proton indicated the geometry of the double bond at C-3 to be Z; the low field chemical shift value of CH-5 ($\delta_H$ 4.92/$\delta_C$ 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 $\delta_H$ 4.53, methine (C-11) proton resonated at $\delta_H$ 2.58 and C-5 proton; the isolated ethyl group, in which two methylene (C-14) protons appeared at $\delta_H$ 2.23 are correlated with methyl (C-15) protons appeared at $\delta_H$ 1.03 is allylic owing to the $\delta_C$ 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 $\delta_C$<30 and the $J$ values.14)

Furthermore, based on the chemical shift values of H-5 ($\delta_H$ 4.92/$\delta_C$ 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 $\delta_C$ 97.8 indicated that the ethyl group was attached to the tetra substituted double bond of C-12 and C-13 [C=C=]. The chemical shift value ($\delta_C$ 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

Table 1. $^1$H-NMR (600 MHz) and $^{13}$C-NMR (125 MHz) in C$_6$D$_6$ Spectroscopic Data of Compounds 1—4

<table>
<thead>
<tr>
<th>No.</th>
<th>$^1$C ($\delta$)</th>
<th>$^1$H ($\delta$)</th>
<th>$^1$H ($\delta$)</th>
<th>$^1$C ($\delta$)</th>
<th>$^1$H ($\delta$)</th>
<th>$^1$H ($\delta$)</th>
<th>$^1$C ($\delta$)</th>
<th>$^1$H ($\delta$)</th>
<th>$^1$H ($\delta$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85.6 2.63 dd 2.4, 1.2</td>
<td>87.1 2.98 dd 2.4, 0.6</td>
<td>80.5 2.55 d 1.8</td>
<td>84.8 2.8 dd 2.4, 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>78.2 — — — —</td>
<td>78.2 — — — —</td>
<td>80.4 — — — —</td>
<td>86.9 — — — —</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>111.8 5.19 dd 10.8, 2.4</td>
<td>112.7 5.15 dd 10.8, 2.4</td>
<td>113.1 5.21 dd 10.8, 2.4</td>
<td>111.9 5.4 dd 10.8, 2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>141.5 5.59 ddd 10.8, 10.8, 1.2</td>
<td>141.7 5.35 ddd 10.8, 10.8, 0.6</td>
<td>142.1 5.79 dd 10.8, 9.6</td>
<td>142.0 6.1 dd 10.8, 10.8, 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>58.4 4.92 dd 10.8, 10.8</td>
<td>56.9 4.89 dd 12.0, 10.8</td>
<td>63.6 3.70 dd 10.8, 9.6</td>
<td>58.0 5.1 dd 10.8, 10.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>58.3 1.72 dd 10.8, 1.8</td>
<td>52.8 2.25 ddd 12.0, 9.6, 4.2, 1.8</td>
<td>57.9 1.50 dd 10.8, 1.2</td>
<td>58.3 1.9 dd 10.8, 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>78.2 4.53 brd 5.4</td>
<td>79.4 4.51 dd 4.8, 4.2</td>
<td>77.8 3.66 dd 4.8, 1.2</td>
<td>78.3 4.6 d 5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>38.6 1.34 ddd 13.2, 7.8, 5.4</td>
<td>35.5 1.37 ddd 14.4, 7.2, 4.8, 1.8</td>
<td>38.8 1.19 ddd 13.8, 7.8, 4.8</td>
<td>38.6 1.3 dd 14.7, 7.8, 5.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>79.4 4.08 dd 7.8, 4.8</td>
<td>80.0 4.13 dd 7.2, 4.8</td>
<td>78.1 4.07 dd 7.8, 4.8</td>
<td>80.4 4.1 dd 7.8, 4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>82.9 4.41 dd 4.8, 4.8</td>
<td>85.1 4.42 dd 4.8, 4.8</td>
<td>82.4 4.32 dd 4.8, 4.8</td>
<td>82.4 4.4 dd 4.8, 4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>44.1 2.58 brd 4.8</td>
<td>42.3 2.33 dd 9.6, 4.8</td>
<td>44.9 2.73 dd 4.8</td>
<td>46.1 2.8 dd 5.0, 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>152.0 — — — —</td>
<td>148.5 — — — —</td>
<td>151.8 — — — —</td>
<td>149.4 — — — —</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>97.8 — — — —</td>
<td>100.7 — — — —</td>
<td>98.9 — — — —</td>
<td>102.5 — — — —</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>29.3 2.23 q 7.2</td>
<td>30.7 (14a) dq 14.4, 7.2</td>
<td>29.2 2.57 q 7.2</td>
<td>26.7 (14a) dq 15.0, 7.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>1.92</td>
<td>1.92</td>
<td>1.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>(14b) dq 14.4, 7.2</td>
<td>1.84</td>
<td>(14b) dq 15.0, 7.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>13.3 1.03 t 7.2</td>
<td>14.0 1.02 t 7.2</td>
<td>13.9 1.19 t 7.2</td>
<td>13.0 1.0 t 7.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Structure of Maneonenes I—7
ethyl group, which allowed us to establish the stereochemistry of the vinyl ether moiety. The magnetic equivalence of the CH$_2$-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, 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, 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 date, compound 1 was named (12Z)-cis-maneonene-D.

Compound 2, was isolated as colorless oil. The structure of compound 2 was shown to have cis-enzyme function and formally a maneonene structure by the same approach of UV, IR, MS and NMR (cf. exp. and Table 1) data analysis of (12Z)-cis-maneonene-D (1). The main differences between compounds 1 and 2 were shown in $^1$H-NMR spectra of H-6 at $\delta$ 2.25 (dd, $J$=12.0, 9.6, 4.2, 1.8 Hz, H-6) of compound 2 instead of $\delta$ 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 CH$_2$-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,\(3,6)\) 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 $^1$H-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, H-11/H-10 and H-9/H-10. From these correlations of all H-7, H-8, H-9, H-10 and H-11 are cis with each other and trans with H-6 indicating that this ring is cyclo-hexane boat form. The NOESY spectrum showed correlations between H-6/H-4, and H-11/only one proton of CH$_2$-14 which resonated at $\delta$ 1.84 (1H, dq, $J$=14.4, 7.2 Hz, H$_4$-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 (12E)-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 (12Z)-trans-maneonene-C (3) and trans-maneonene-B (7) have the same chemical structure but different in the chemical shift average $\delta$ (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, 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, 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 CH$_2$-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 (12Z)-trans-maneonene-C (3).

**Pharmacology** In the present study, several observations have been made concerning the apoptosis-inducing or in-
hibiting effect of (12Z)-cis-maneone D (1) and (12E)-cis-
maneone-E. Degradation of DNA into a specific fragmen-
tation 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 IC\textsubscript{50} of the tested compounds after 24 h of incubation were 23 \textmu M for 1, and 15 \textmu M for 2 versus demethanethoxane 0.9 \textmu M. 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 chromato-
graphy, neutral type 507 C, 100—125 mesh was used; for thin layer chromosome silica gel GF 254 was used; and for PTLC aluminum sheets 20x20. Optical rotations were measured on ATAGO POLAX-L 2 polarime-
ter. 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,

**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 Ab-
dulaziz University, Jeddah, Saudi Arabia. It was washed with water and dried in the shade at room temperature. The dried material (200 g) was ex-
tracted with petroleum ether at room temperature. The extract was parti-
tioned between ether and water. The crude organic residue (7 g) was sub-
dicted 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.

(12Z)-cis-Maneone D (1): The fraction B, R\textsubscript{f}=0.68 (30 mg) was puri-
ified by preparative TCL using the solvent system n-hexane–ether (9:1). The band with R\textsubscript{f}=0.32 (blue color with sulfuric acid–methanol) was taken to give pale yellow color oil (8 mg, 0.004%), [\textalpha]\textsubscript{D}\textsubscript{20}+38 (c=0.95, CHCl\textsubscript{3}), IR \nu\textsubscript{max} (film) cm\textsuperscript{-1}: 3286, 2969, 1687, 1456, 1211, 1137, 1190, 1026, 983, 899, 800, 776. HR-ESI-MS: m/z 342.0016 (Calcd 342.0022) C\textsubscript{15}H\textsubscript{16}BrClO\textsubscript{2}; EI-MS (70 eV) m/z (rel. int.): 346, 344, 342 (3:10:6), [M\textsuperscript{+}]\textsuperscript{+} (C\textsubscript{15}H\textsubscript{16}BrClO\textsubscript{2}), 310, 309, 307 (10:45:43), [M–Cl]\textsuperscript{+}, 265, 263, 249, 235, 228 (10:22:10:30), [M–Cl+Br]\textsuperscript{+}, 207 (45), 199 (50), 184 (65), 129 (75), 77 (100), 69 (94), 65 (100). 1H- and 13C-NMR (CD\textsubscript{3}OD) (Table 1).

(12E)-cis-Maneone E (2): The fraction C, R\textsubscript{f}=0.36 (50 mg) was puri-
ified by preparative TCL using the solvent system n-hexane–ether (8.5:1.5). The band with R\textsubscript{f}=0.32 (blue color with sulfuric acid–methanol) was taken to give colorless oil (8 mg, 0.001%), [\textalpha]\textsubscript{D}\textsubscript{20}–13.6 (c=0.05, CHCl\textsubscript{3}), IR \nu\textsubscript{max} (film) cm\textsuperscript{-1}: 3286, 2970, 2929, 2100, 1734, 1687, 1456, 1348, 1344, 1326, 1267, 1204, 1182, 1128, 1117, 1064, 1044, 1027, 990, 959, 916, 889, 829, 816, 797, 776, 724, 655. H-REI-MS: m/z 342.0012 (Calcd 342.0012) C\textsubscript{15}H\textsubscript{16}BrClO\textsubscript{2}; EI-MS (70 eV) m/z (rel. int.): 346, 344, 342 (2:3:1) [M\textsuperscript{+}]\textsuperscript{+} (C\textsubscript{15}H\textsubscript{16}BrClO\textsubscript{2}), 309, 307 (66:64), [M–Br]\textsuperscript{+}, 265, 263 (8:22), [M–Br+Cl]\textsuperscript{+}, 228 (50), [M–Br+2Cl]\textsuperscript{+}, 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 (CD\textsubscript{3}OD) (Table 1).

**Biological Evaluation of the Isolated Compounds**  All compounds were dissolved in dimethyl sulfoxide (DMSO) at concentration less than 0.1% v/v.

Preparation of Blood Neutrophils: Neutrophils (>98% pure on May-
Gierna stain) were isolated from peripheral blood of normal healthy volun-
tee donors by a combination of dextran sedimentation and centrifugation through discontinuous plasma percoll gradients as described by Haslett et al.\textsuperscript{(5)}

Culture of Neutrophils: Neutrophils were resuspended in an appropriate volume of RPMI 1640 medium with 10% autologous PRPDS and 100 \mu l of penicillin and streptomycin and divided into five equal volumes each put in culture tube. Cells were incubated at 37 \degree 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 mmol/l culture. The age of neutrophils in cul-
ture 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 temper-

ture for 5 min. The IC\textsubscript{50} of isolated compounds were determined in comparison to dexamethasone.

Measurement 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, washed 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 py-
knosis and cytoplasmatic vaculation. 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 \mu g/ml in PBS), mixed gently on a slide, and imme-
diately examined with an Olympus HB-2 microscope with fluorescence at-
tachment. Green fluorescence was detected between 500 and 525 nm. Cells displaying 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 thy-
ocytes. Cells (2.5x10\textsuperscript{7}) were washed three times and resuspended in a 0.15 mol/l NaCl solution. The cells were chilled at 4 \degree 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 \texttimes g at 4 \degree C

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Apoptotic neutrophils (mean±S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h</td>
</tr>
<tr>
<td></td>
<td>24h</td>
</tr>
<tr>
<td></td>
<td>48h</td>
</tr>
<tr>
<td></td>
<td>72h</td>
</tr>
<tr>
<td>(Control)</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>1</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>2</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.39±0.01</td>
</tr>
</tbody>
</table>
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 mg/ml, and the sample was incubated for a further 24 h at 37 °C. The DNA was isolated as described in the text, electrophoresed on a 1% agarose gel, and stained with 0.5 mg/ml ethidium bromide and were visualized under UV light.

6. cis-Maneonene-C (5): 1H-NMR (C6D6, 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, Hαa-8), 1.90 (1H, d, J=14 Hz, Hαa-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, Hb-12), 2.61 (1H, m, J=7 Hz, Hb-14), 1.35 (3H, t, J=7 Hz, H-15).
7. trans-Maneonene-B (6): 1H-NMR (C6D6, 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).