SUPPLEMENTARY MATERIAL

Synthesis and biological activity of new derivatives with the preserved carane system

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Abstract

Terpenoid derivatives, which contain a preserved carane system in their structure, exhibit a broad spectrum of biological activities. Among them, we can distinguish insecticides, structures with pharmacological application etc. In the presented paper, the substrate - (–)-*cis*-caran-*trans*-4-ol was transformed using the reactions of typical organic synthesis to obtain novel derivatives. Most importantly, bromolactone ((–)-(1*R*,4*R*,6*S*)-2'-(bromomethyl)-4,7,7-trimethylspiro[bicyclo[4.1.0]heptan-3,3'-furan]-5'(4'H)-one) with the preserved carane system was synthesized. This bromolactone was tested for antifeedant activity against storage pest insects. In addition, its moderate antibacterial activity was observed against the *Bacillus subtilis* strain (with Minimal Inhibitory Concentration of 200 µg/mL).

Keywords: (+)-carene, bromolactone, antifeedant activity, antibacterial activity

Experimental section

Feeding deterrent activity. To determine the feeding deterrent activity of the examined compounds, the previously described standard method of choice and no-choice tests were used (Dancewicz et al. 2016). Oat flakes purchased from Melvit S. A. (Warsaw, Poland) were used as the test food. For the feeding assays, acetone solutions of the test compounds at a concentration of 10 mg/mL were prepared. 1 mL of solution or acetone alone as a control was applied to 1 g of flakes using a micropipette. After evaporation of the solvent (30 min of air-drying), the flakes were weighed and placed into Petri dishes (15 cm in diameter) with ten approximately 25-30-day-old larvae or ten unsexed 7-10-day-old adults. In choice tests (insects could choose either control or treated food), control and treated flakes in Petri dishes were separated by a thin glass capillary. In the no-choice test, insects were exposed to only one type of food-treated or control. Four replicates for each type of test and each compound were performed for each insect life stage. Petri dishes were maintained in a rearing chamber at 29 ± 1ºC in the dark for 3 days. After this period, the remaining uneaten oat flakes were re-weighed, and the average weight of the food eaten was calculated. This experimental design was the basis for calculating the deterrence coefficients.

To evaluate the deterrence potential of bromolactone against insects, the peach potato aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) was used. *M. persicae* is extremely polyphagous, highly effective in transmitting plant viruses, and resistant to several classes of insecticides (Szczepanik et al. 2008). Aphids (maintained as a multiclonal colony) and plants (Chinese cabbage *Brassica pekinensis*) were reared in laboratory at 20°C, 65% relative humidity., and 16:8 (Light/Dark) photoperiod. One- to seven-day-old apterous females of *M. persicae* and 3-week-old plants with 4-5 fully developed leaves were used for the experiments. All experiments were performed under the same conditions of temperature, relative humidity, and photoperiod. The bioassays began at 10-11 a.m. The procedures have been described in detail by Grudniewska et al. (2013). Briefly, this bioassay enables the study of aphid host preferences under semi-natural conditions, where aphids are given free choice between control and treated leaves. The **5** compounds were applied to one leaf of a plant by immersing it in 0.1% ethanolic solution of a given compound for 30 s. Control leaves of a similar size were immersed in 70% ethanol, which was used as a solvent for the 5 compounds studied. Treated and control leaves were allowed to dry for 1 h before the start of the experiment to permit the evaporation of the solvent. Treated and control leaves were placed in a Petri dish and allowed to dry for 1 h to permit the evaporation of the solvent. Next, aphids

were placed in the dish along the line that divided the area into two halves such that the aphids could choose between treated (on one half of a Petri dish) and control leaves (on the other half of the dish). Aphids that settled, i.e., they did not move, and the position of their antennae indicated feeding on each leaf, were quantified at 1 h, 2 h, and 24 h intervals after access to the leaf (8 replicates, 20 viviparous apterous females/replicate). Aphids that were moving or had moved out of any of the leaves were not quantified.

The relative deterrence coefficient R was calculated using the following formula:

$$R = \frac{(C-E)}{(C+E)} x100$$

where C and E are the weights of the control and treated foods consumed by the insects in the choice test, respectively. The absolute deterrence coefficient A was calculated using the same formula, but C and E were obtained from the no-choice test. The total coefficient of deterrence (T =A+R), which ranged from -200 to 200, served as the index activity. Compounds with T-values ranging from 151 to 200 are very good deterrents, those with values 101-150 are good deterrents, and those with values of 51-100 are only average antifeedants. T-values lower than 50 indicated weak deterrent activity. Negative T-values indicate attractant properties of the compound. Student's t-test (Microsoft Office 2010, Excel) was used to compare the values of deterrence coefficients.

Antibacterial activity. All bacterial strains (*Escherichia coli* PCM 2057, *Bacillus subtilis* PCM 2027 and *Staphylococcus aureus* PCM 2054) were obtained from the Polish Academy of Sciences.

Screening for antibacterial activity was performed on 96-well microplates in Mueller-Hinton Broth. Microplates were inoculated with bacterial suspensions adjusted to $5 \cdot 10^5$ CFU/mL. Stock solutions of the examined compounds were prepared in ethanol and added into the bacterial suspension at a final concentration of 200 µg/mL. Cultures were incubated overnight at 37°C with gentle shaking. Optical density was measured at 650 nm with a Tecan Sunrise microplate reader coupled with Magellan software.

Final antibacterial characteristics were obtained using the microdilution method and reported as Minimal Inhibitory Concentration (MIC) values. The applied methodology was based on CLSI Standard (2012). Compounds within the range of 25-300 µg/mL were tested. Ethanol was incorporated as a solvent and added to the untreated control.

General. Macherey-Nagel, ALUGRAM SIL G / UV254 tiles were used for rapid analysis of thin-layer chromatography (TLC).

To clean the compounds, a CombiFlash Rf + Lumen with a RediSept 12 g column of Silica Flash column or a 50 centimetre classical chromatography column of Macherey-Nagel with a pore size of 0.04-0.063 mm was used. Measurements using gas chromatography were performed on an Agilent 7890A GC. Mass spectrometry was performed on the WATERS GCT Premier system, which consisted of a high-resolution mass spectrometer with a flight time (TOF) spectrometer. Infrared spectrum was performed on a Bruker VERTEX 70V equipped with ATR Platinium, and the results were analysed using Bruker OPUS. The optical rotation was measured with a polAAr 31 polarimetre. The measurements were made in a methanol solution at 24°C and at a wavelength of 589 nm, and the cuvette length was 100 mm.

NMR analysis was performed on a Bruker Avance DRX 600 nuclear magnetic resonance spectrometer. MestReNova version 6.0.2 was used to describe the NMR spectra obtained.

The starting compound - (–)-cis-caran-trans-4-ol (**1**) - was obtained before in the research group of prof. Lochyński. In brief, it was synthesized directly from (+)-3-caren in a two-step reaction of hydroboration and oxidation with Brown-Garg reagent (Brown and Zweifel 1964), that resulted in the formation of a crystalline alcohol.

(–)-(1*R*,4*R*,6*S*)-4,7,7-Trimethylbicyclo[4.1.0]heptan-3-one (2)

cis-Caran-*trans*-4-ol **1** (14.5 g, 0.094 mol) was added to 200 mL of diethyl ether and gentle drop, while stirring. Next, 174.5 mL of BG reagent was added at 4-10°C. The reaction was controlled using TLC and run until the product appeared (24 h). After completion, 190 mL of distilled water was added to the reaction mixture, and the mixture was extracted three times with 250 mL portions of diethyl ether. The organic phase was washed with $NaHCO_3$ and NaCl and then dried with anhydrous $MgSO_4$. The crude product was purified using flash chromatography (silica gel, hexane-ethyl acetate 5:3) to obtain pure compounds **2**: $\mathbf{[\alpha]_D^{24}}$ = -134.9^0 [c = 1.0 MeOH] **IR** (ATR, cm^{-1}): 2929(m), 1711(vs.), 1456(m)

^{1}H NMR: (600 MHz, $CDCl_3$, δ, ppm): 0.63 (t, *J* = 4.0 Hz, 2H, at C-1 and C-6), 1.08 (d, *J* = 6.6 Hz, 3H, at C-10), 1.16 (s, 6H, at C-8 and C-9), 1.39-1.46 (m, 1H, at C-5), 2.02 (dd, *J* = 4.4, 2.8 Hz, 1H, at C-5), 2.04 (dd, *J* = 7.7, 4.1 Hz, 1H, at C-2), 2.45 (d, *J* = 2.5 Hz, 1H, at C-

2), 2.46–2.48 (m, 1H, at C-3) **^{13}C NMR: (150 MHz, $CDCl_3$, δ, ppm):** 17.63 (C-10), 20.06-20.47 (C-1 and C-6), 22.63 (C-8), 22.96 (C-9), 24.33 (C-7), 27.55 (C-5), 37.25 (C-2), 39.56 (C-3), 208.43 (C-4) **HRMS:** (TOFMS ES+) Anal. Calcd for [$C_{10}H_{16}O$] 153.1279, Found: 153.1281

(–)-Ethyl [(1*R*,4*R*,6*S*)-4,7,7-trimethylbicyclo[4.1.0]hept-3-ylidene]acetate (3)

NaH 0.45 g (0.019 mol) was added to 10 mL of tetrahydrofuran. Next, 8.0 g (0.036 mol) of triethyl phosphonoacetate was mixed with 10 mL of tetrahydrofuran, and the mixture was added dropwise to the flask. After 15 minutes, a solution of 1.9 g (0.012 mol) of ketone **2** in 20 mL of tetrahydrofuran was added dropwise, while stirring. The mixture was diluted with water (45 mL), and the product was extracted three times with hexane (70 mL). The compound was dried with anhydrous $MgSO_4$. The crude product was purified by flash column chromatography (silica gel, hexane - ethyl acetate 5: 3) to obtain pure compound **3**:

$[\alpha]_D^{24}$ = -34.6^0 [c = 1.0 MeOH] **IR** (ATR, cm^{-1}): 2925(m), 1713(vs.), 1457(m), 1143(m)

^{1}H NMR: (600 MHz, $CDCl_3$, δ, ppm): 0.82 (m, 2H, at C-1 and C-6), 1.02 (dd, *J* =11.4, 4.4 Hz, 1H, at C-5), 1.13 (d, *J* = 5.6, 3H, at C-10), 1.18 (s, 6H, at C-8 and C-9), 1.53 (t, *J* = 10.1 Hz, 3H, at C-14), 1.62–1.70 (m, 1H, at C-5), 1.81–1.86 (m, 2H, at C-2), 2.26–2.28 (m, 1H, at C-4), 4.27 (q, *J* = 6.2 Hz, 2H, at C-13), 5.47 (s, 1H, at C-11) **^{13}C NMR: (150 MHz, $CDCl_3$, δ, ppm):** 15.20 (C-14), 18.26 (C-7), 20.11 (C-1), 21.03 (C-10), 23.05 (C-8 i C-9), 24.48 (C-6), 27.45 (C-2), 30.85 (C-5), 36.07 (C-4), 61.39 (C13), 115.91 (C-11), 164.56 (C-3), 169.73 (C-12) **HRMS:** (TOFMS ES+) Anal. Calcd for [$C_{14}H_{22}O_2$] 223.1698, Found: 223.1703

(–)-2-[(1*R*,4*R*,6*S*)-4,7,7-Trimethylbicyclo[4.1.0]hept-3-ylidene]ethanol (4)

In a round bottom flask, 0.2 g (0.9 mmol) of ester **3**, (0.1 g, 2.6 mmol) of $LiAlH_4$ and 4.0 mL of pure tetrahydrofuran were added. Upon completion of the reaction, 1:1 distilled water was added to the reaction mixture, which was extracted three times with diethyl ether (10 mL). The product was dried with anhydrous $MgSO_4$. The crude product was purified by column chromatography (silica gel, hexane - ethyl acetate 5:3) to obtain 2-[(1*R*,4*R*,6*S*)-4,7,7-trimethylbicyclo[4.1.0]hept-3-ylidene]ethanol **4**:

$[\alpha]_D^{24}$ = -4.7^0 [c = 1.0 MeOH] **IR** (ATR, cm^{-1}): 3440(w), 2925(vs.), 1455(m), 1068(s)

^{1}H NMR: (600 MHz, $CDCl_3$, δ, ppm): 0.85 (s, 2H, at C-6 and C-1), 0.99-1.04 (m, 1H, at C-5), 1.06 (d, *J* = 8.1 Hz, 3H, at C-10), 1.14 (s, 6H, at C-8 and C-9), 1.60 (t, *J* = 8.4 Hz, 1H, at

C-5), 1.83 (dd, *J*=9.5, 5.9 Hz, 1H, at C-2), 2.04 (s, 1H, at OH), 2.11 (dd, *J* = 8.2, 4.1 Hz, 1H, at C-2), 2.22 – 2.30 (m, 1H, at C-4), 4.36 (d, *J* = 7.6 Hz, 2H, at C-12), 5.47 (t, *J* =9.2 Hz, 1H, at C-11)**^{13}C NMR: (150 MHz, $CDCl_3$, δ, ppm):** 18.56 (C-10), 20.05 (C-1), 20.74 (C-7), 23.69 (C-8 i C-9), 24.58 (C-6), 27.83 (C-2), 30.87 (C-5), 36.16 (C-4), 59.94 (C-12), 123.23 (C-11), 147.21 (C-3) **HRMS:** (TOFMS ES+) Anal. Calcd for [$C_{14}H_{20}O$] 181.1592, Found: 181.1252

(–)-Ethyl [(1*R*,4*R*,6*S*)-3-ethenyl-4,7,7-trimethylbicyclo[4.1.0]hept-3-yl]acetate (5)

In a two-necked round bottom flask, 0.24 g (1.3 mmol) of 2-[(1*R*,4*R*,6*S*)-trimethylbicyclo[4.1.0]hept-3-ylidene]ethanol **4**, (0.05 g, 0.67 mmol) propionic acid and 4 mL triethyl orthoacetate were added. The reaction was performed for approximately 5 hours at 137°C until the reaction was complete, and monitored by thin layer chromatography (TLC). After completion of the reaction, excess triethyl orthoacetate was distilled. The crude product was purified by column chromatography (silica gel, hexane - ethyl acetate 5: 3) to obtain ethyl-[(1*R*,4*R*,6*S*)-3-ethenyl-4,7,7-trimethylbicyclo[4.1.0]hept-3-yl]acetate **5**:

$[\alpha]_D^{24}$ = -5.74^0 [c = 1.0 MeOH] **IR** (ATR, cm^{-1}): 2931(m), 1736(vs.), 1446(m), 1179(m)

^{1}H NMR: (600 MHz, $CDCl_3$, δ, ppm): 0.76 (dt, *J* = 8.7, 4.4 Hz, 2H, at C-1 i C-6), 0.84 (d, *J* = 4.1 Hz, 3H, at C-10), 1.08 (s, 7H, at C-5, C-8 and C-9), 1.22 (ddd, *J* = 11.4, 6.1, 2.3 Hz, 1H, at C-2 and C-5), 1.38 (t, *J* = 8.4 Hz, 3H, at C-14), 1.45-1.51 (m, 1H, at C-4), 1.64 (dt, *J* = 11.2, 8.1 Hz, 1H, at C-5), 1.85 (d, *J* = 5.0 Hz, 1H, at C-2), 2.04 (d, *J* = 7.9 Hz, 1H, at C-11), 2.30 (d, *J* = 6.7 Hz, 1H, at C-11), 4.16 (q, *J* = 8.3 Hz, 2H, at C-13), 4.96 (dd, *J* = 7.3, 2.1 Hz, 1H, at C-15), 5.03 (dd, *J* = 12.3, 2.1 Hz, 1H, at C-15), 5.81 (dd, *J* = 14.9, 8.9Hz, 2H, at C-16). **^{13}C NMR: (150 MHz, $CDCl_3$, δ, ppm):** 15.22 (C-14), 16.39 (C-7), 17.47 (C-10), 21.15 (C-6), 23.03(C-8 i C-9), 23.28 (C-1), 28.25 (C-5), 34.07 (C-2), 37.73 (C-3), 38.07 (C4), 39.09 (C-11), 61.69 (C-13), 116.47 (C-15), 144.28 (C-16), 173.62 (C-12) **HRMS:** (TOFMS ES+) Anal. Calcd for [$C_{16}H_{26}O_2$] 251.1698, Found: 251.1704

(–)-(1*R*,4*R*,6*S*)-2'-(Bromomethyl)-4,7,7-trimethylspiro[bicyclo[4.1.0]heptan-3,3'-furan]-5'(4'*H*)-one (6)

A mixture of 10 mL THF, 1 mL distilled water and 5.0 mg (0.028 mmol) of *N*-bromosuccinimide was added to ethyl [(1*R*,4*R*,6*S*)-3-ethenyl-4,7,7-trimethylbicyclo[4.1.0]hept-3-yl]acetate **5 (**0.15 g, 0.5 mmol). The mixture was stirred for 24 hours using a magnetic stirrer at room temperature, and the reaction was controlled using thin

layer chromatography (TLC). The combined extracts were washed twice with saturated $NaHCO_3$ and distilled water and dried with anhydrous $MgSO_4$. The crude product was purified by column chromatography (silica gel, hexane - ethyl acetate 5: 3) to give –)-(1*R*,4*R*,6*S*)-2'-(bromomethyl)-4,7,7-trimethylspiro[bicyclo[4.1.0]heptan-3,3'-furan]-5'(4'*H*)-one **6**.

$[\alpha]_D^{24}$ = -25.67^0 [c = 1.0 MeOH] **IR** (ATR, cm^{-1}): 2823 (s), 1778 (m), 1482 (m), 1189 (s), 668 (m)

^{1}H NMR: (600 MHz,$CDCl_3$, δ, ppm) 0.75-0.8 (m, 2H, at C-1 and C-6), 0.85 (d, *J* = 6.6 Hz, 3H, at C-10), 1.10 (s, 6H, at C-8 and C-9), 1.13-1.19 (m, 1H, at C-2), 1.33 (dd, *J* = 8.8, 4.0 Hz, 1H, at C-5), 1.35-1.38 (m, 1H, at C-2), 1.39-1.43 (m, 1H, at C-4), 1.57-1.63 (m, 1H, at C-5), 2.07 (d, *J* = 8.8 Hz, 1H, at C-4'), 2.33 (d, *J* = 7.9 Hz, 1H, at C-4'), 3.42 (dd, *J* = 19.3, 8.2 Hz, 1H, at C-6'), 3.62 (dd, *J* = 19.4, 8.8 Hz, 1H, at C-6'), 4.54 (t, *J* = 6.0 Hz, 1H, at C-2').**^{13}C NMR: (150 MHz, $CDCl_3$, δ, ppm)** 16.44 (C-7), 17.18 (C-10), 20.98 (C-1), 21.07 (C-6), 23.08(C-8 and C-9), 29.12 (C-5), 31.49 (C-2), 32.05 (C-6'), 39.01 (C-4'), 39.19 (C4), 40.84 (C-6), 80.47 (C-2'), 178.45 (C-5') **HRMS:** (TOFMS ES+) Anal. Calcd for [$C_{14}H_{21}BrO_2Na$] 323.0310, Found: 323.0303

Table S1. Feeding deterrence activity of lactone tested against glisten, *Alphitobius diaperinus* Panzer.

Compound	Deterrence factors Larvae			Imago		
	A	R	*T*	A	R	*T*
6	51.64±17.8	53.02±6,96	104.66±21.56	-12.59±9.38	66.58±9.25	53.99±10.16
Student Test	P=0.05	P=0.00088	P=0.0012	P=0.202	P=0.019	P=0.3069

A - absolute deterrent activity coefficients (non-selective test)

R - relative deterrent activity coefficients (selection test)

T = A + R total deterrent activity factor

Table S2. Feeding deterrent activity of the tested lactone in relation to the peach potato aphid *Myzus persicae* (Sulzer).

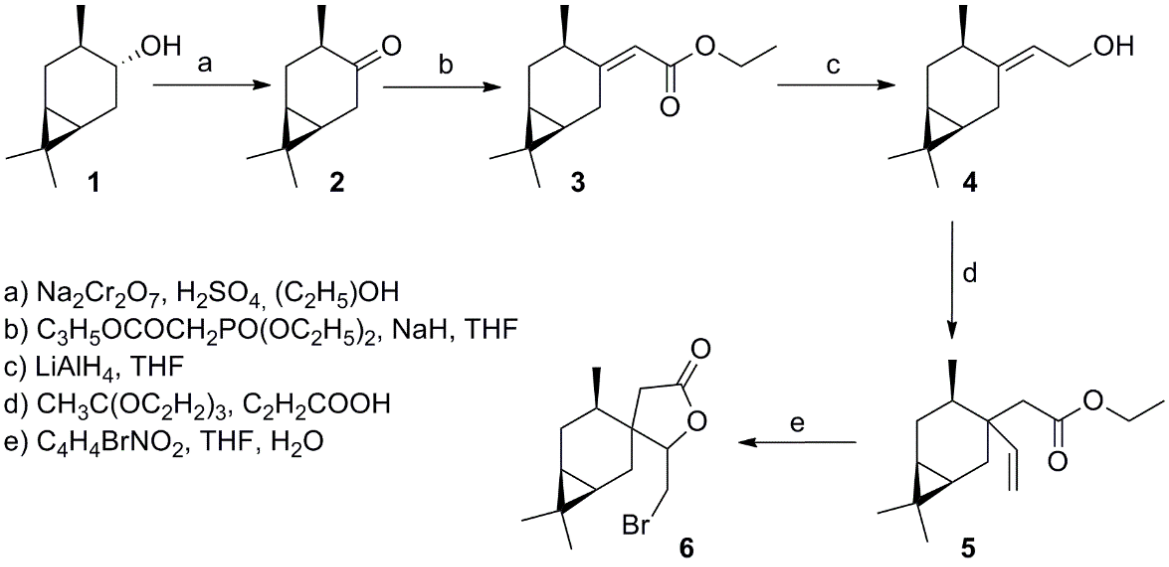
Time	number of aphids on control leaves	number of aphids on treated leaf	p	ID
1 h	8.6 (±1.7)	2.3 (±0.3)	0.0028	0.59
2 h	6.2 (±1.7)	1.0 (±0.4)	0.0106	0.72
24 h	8.3 (±1.9)	3.3 (±1.0)	0.0326	0.43

p – Student's test results (statistically significant biological activity: $p<0.05$)

ID – Index of Deterrence (ID < 0, attractant; ID > 0, deterrent)

Table S3. Antibacterial activity of compounds **2**, **3** and **6** presented as Minimal Inhibitory Concentration [µg/mL]. The MIC was defined as the lowest concentration of tested compound that restricted the growth of bacteria by 50% or 90%.

Compound	*Escherichia coli* PCM 2057		*Bacillus subtilis* PCM 2027		*Staphylococcus aureus* PCM 2054	
	MIC_{50} [µg/mL]	MIC_{90} [µg/mL]	MIC_{50} [µg/mL]	MIC_{90} [µg/mL]	MIC_{50} [µg/mL]	MIC_{90} [µg/mL]
2	250	>300	>300	>300	100	175
3	>300	>300	>300	>300	200	>300
6	>300	>300	100	200	200	>300



Scheme S1. Synthesis of bromolactone **6**.

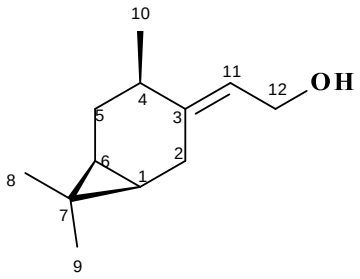


Figure S1. Compound **4**.

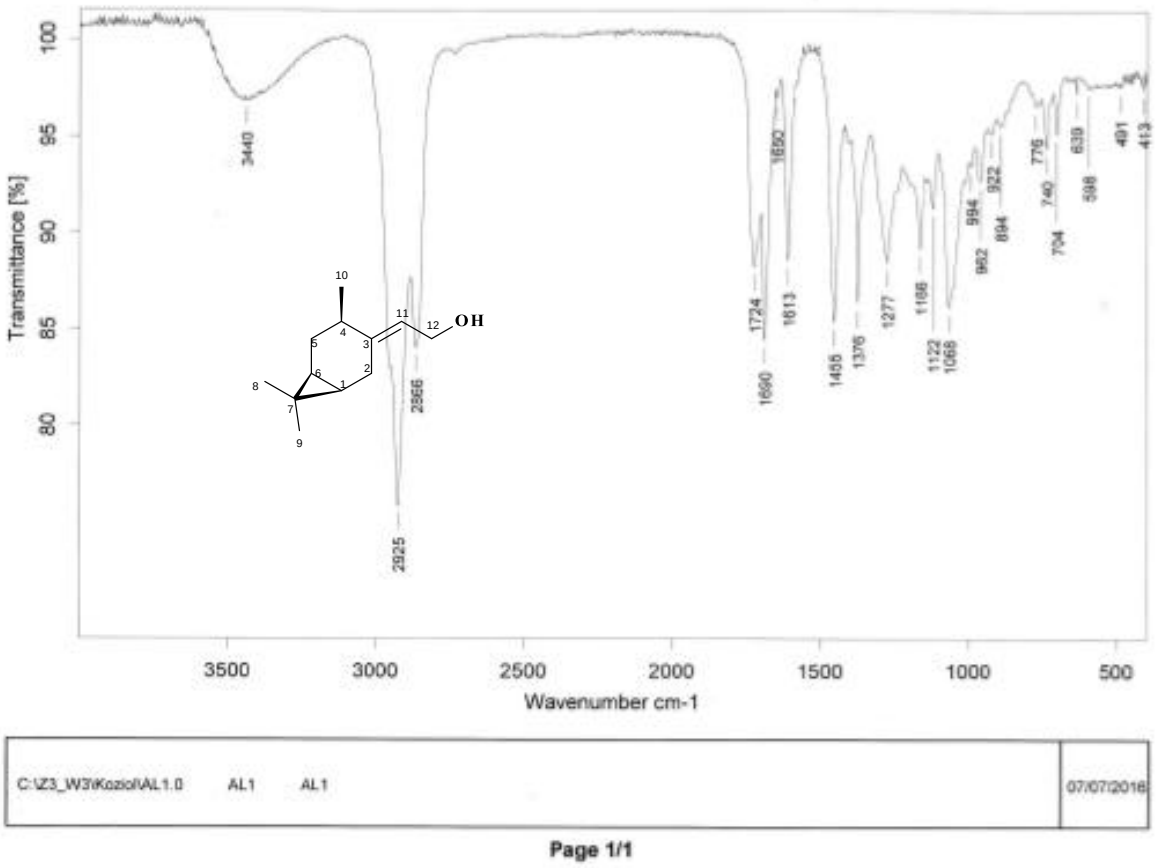


Figure S2. IR spectrum of the compound **4**.

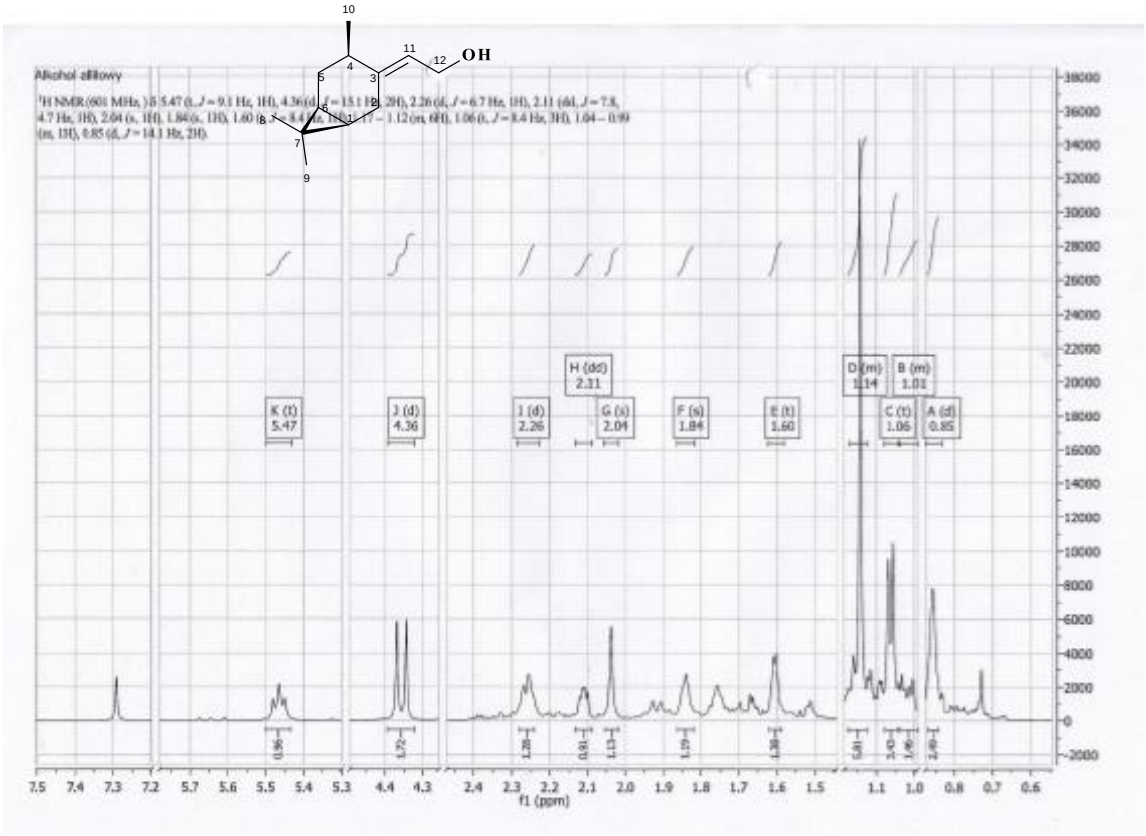


Figure S3. ^{1}H NMR spectrum of the compound **4**.

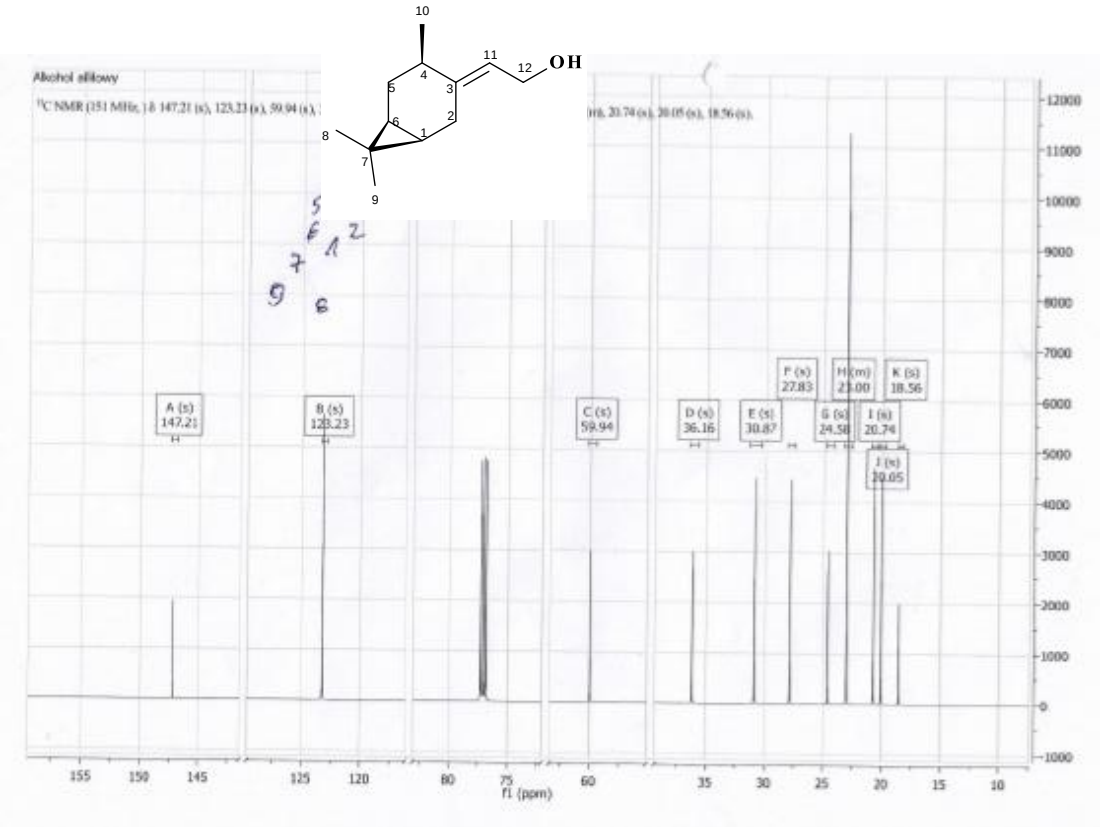


Figure S4. ^{13}C HNMR spectrum of the compound **4**.

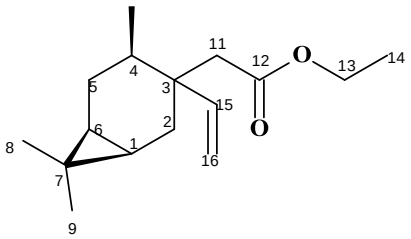


Figure S5. Compound **5**.

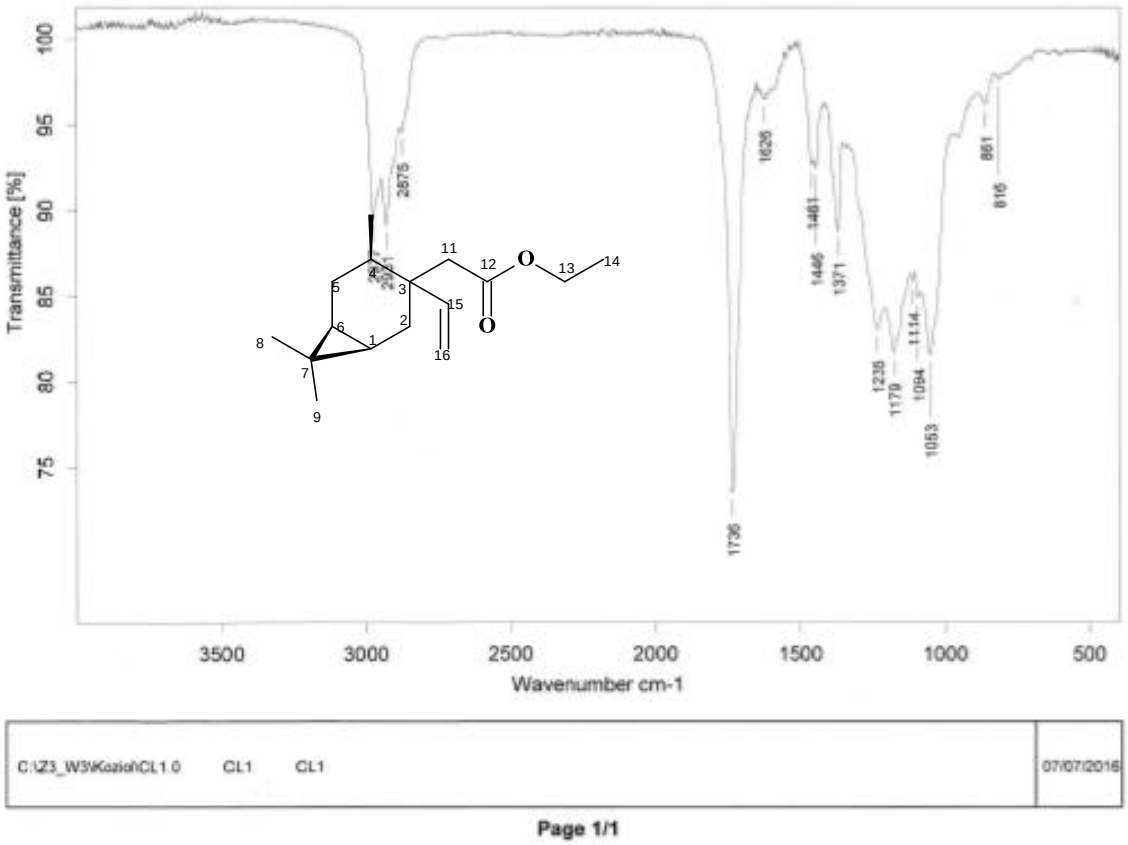


Figure S6. IR spectrum of the compound **5**.

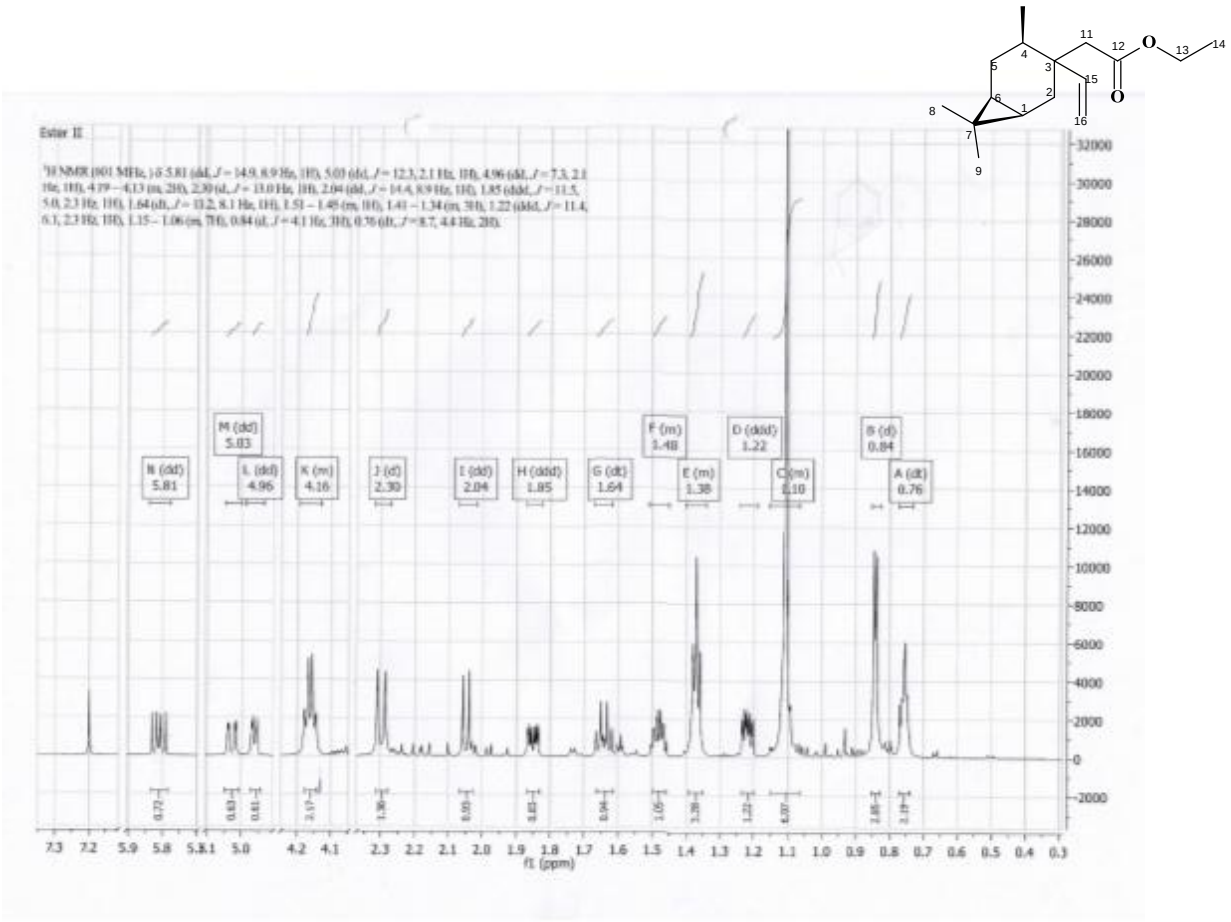


Figure S7. ^{1}H NMR spectrum of the compound **5**.

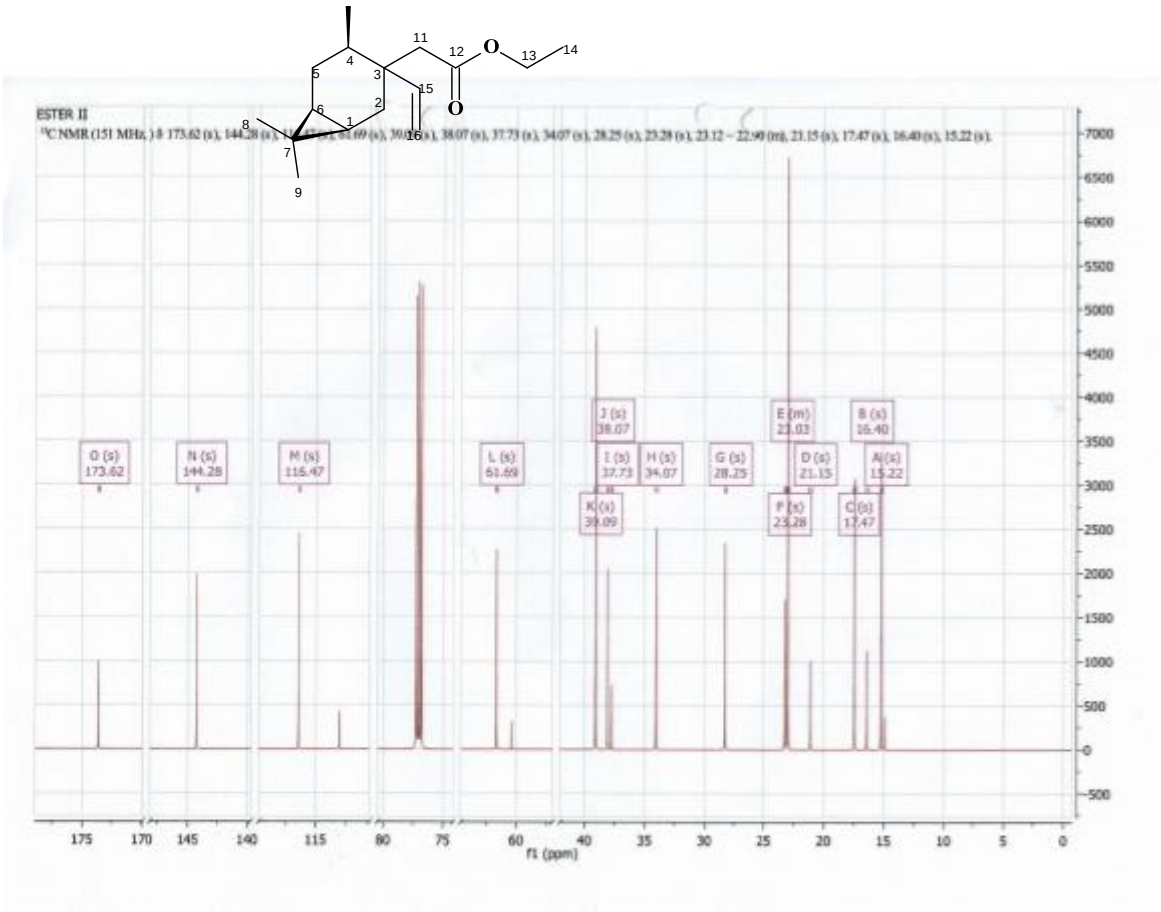


Figure S8. ^{13}C HNMR spectrum of the compound **5**

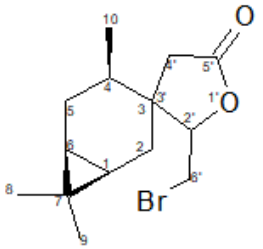


Figure S9. Compound **6**.

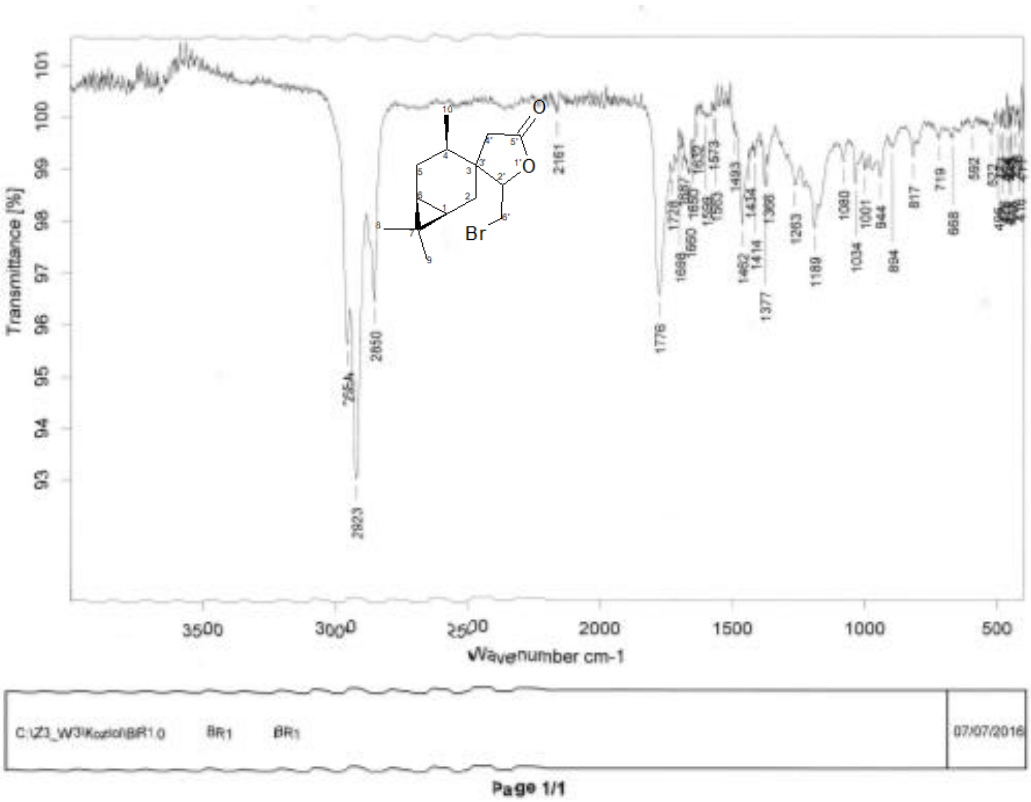


Figure S10. IR spectrum of the compound **6**.

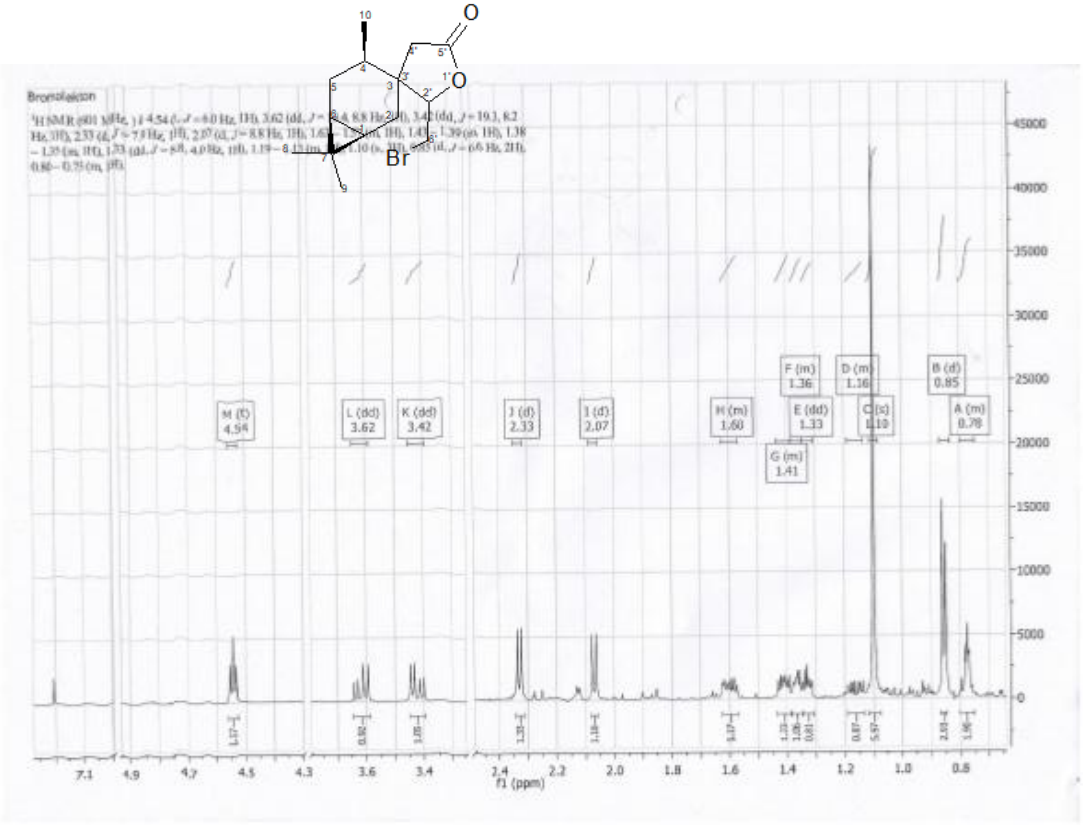


Figure S11. [1]H NMR spectrum of the compound **6**.

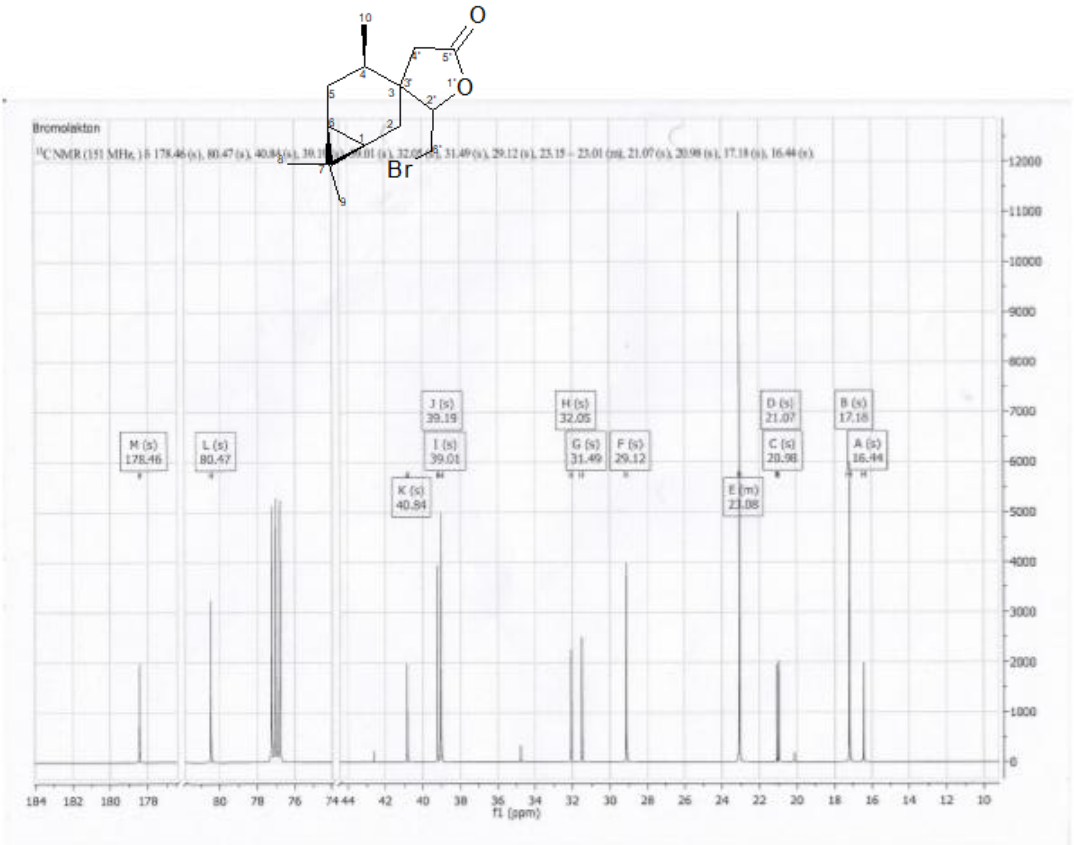


Figure S12. ^{13}C HNMR spectrum of the **6**.

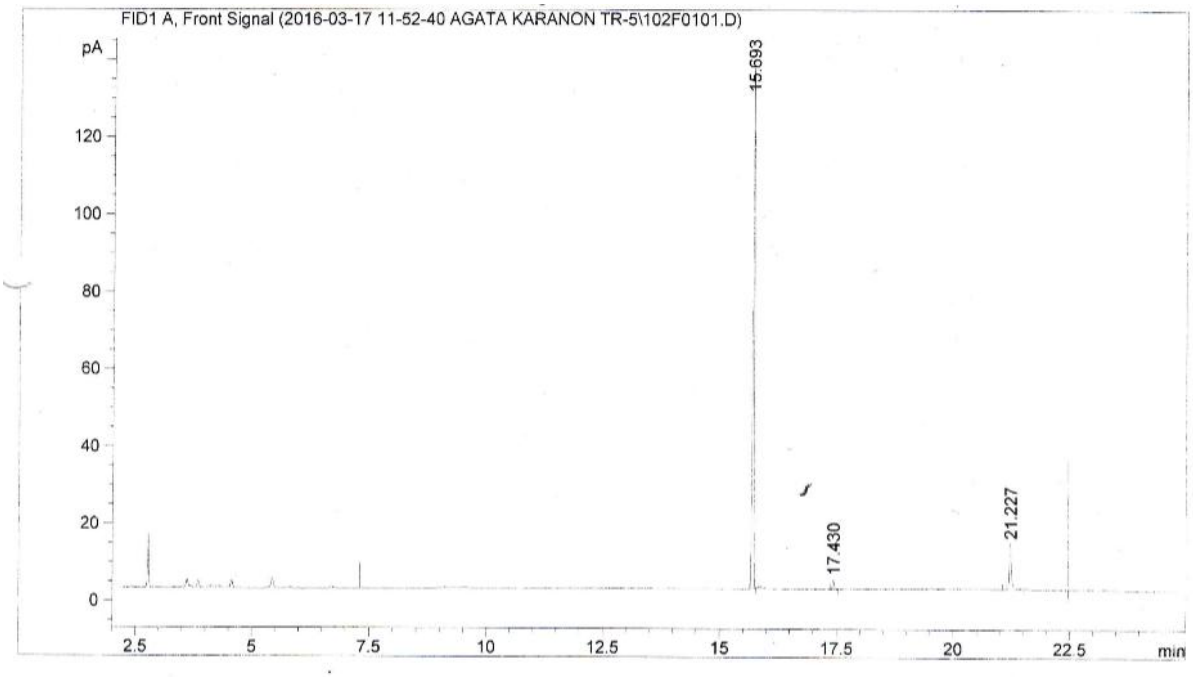


Figure S13. Exemplary GC spectrum obtained for compound **3**.

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