

CHAPTER II

EXPERIMENTAL

2.1 Plant Material

The Rhizomes of *Zingiber rubens* Roxb. were collected from Amphur Bankhai Rayong province during February 1995. The specimen was compared with The Bangkok Herbarium Number BK 59886 in the Botany-Weed Science Division Department of Agriculture Ministry of Agriculture and Co-operatives.

2.2 Instruments and Equipment

2.2.1 Rotary Evaporator

The Eyela rotary evaporator model N-1 was used for the rapid removal of large amounts of volatile solvents.

2.2.2 Fourier Transform - Infrared Spectrometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer model 1760 x Fourier Transform Infrared Spectrometer. Spectra of solid samples were recorded by incorporating the sample into a pellet of potassium bromide and spectra of liquid samples were recorded using a thin film.

2.2.3 ^1H and ^{13}C -Nuclear Magnetic Resonance Spectrometer

Routine ^1H and ^{13}C -NMR spectra were recorded on Bruker spectrometer model ACF 200 operated at 200.13 MHz for ^1H and 50.26 MHz for ^{13}C nuclei.

The chemical shifts in δ (ppm) were referenced to the signal from the residual proton in deuterated solvents. Assignments of ^{13}C spectra were assisted by Distortionless Enhancement by Polarization transfer (DEPT). Specialised NMR experiments (NOE, HMBC, HMQC, COSY, COLOC etc.) was performed on Jeol 500 MHz NMR model JNM-A500 by Dr. Sathorn Suwaiv.

2.2.4 Melting Point

The melting points were recorded on a Fisher-John melting point apparatus.

2.2.5 Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analyses were performed on a Fison Gas-Liquid Chromatograph model GC 8000 coupled with a Fison Mass Spectrometer model Trio 2000.

2.2.6 Gas Chromatography

The GC analyses were performed on a Hewlett Packard model 5890 series II gas chromatograph.

2.3 Solvents and Chromatographic Media

2.3.1 All solvents, except when they were reagent grade, were purified by distillation.

2.3.2 Merck's silica gel 60 Art. 7734.1000 (70-230 mesh ASTM) was used as an adsorbent for column chromatography.

2.3.3 Merck's silica gel Art.7729.1000 was used as adsorbent for Quick column chromatography.

2.3.4 Merck's TLC aluminium sheets, silica gel 60 F254 pre-coated 25 sheets, 20x20 cm², layer thickness 0.2 mm., were used for TLC analysis.

2.4 Physical Separation Techniques

2.4.1 Quick Column Chromatography

Quick Column Chromatography was performed as described in reference [18]

2.4.2 Column Chromatography (CC)

Column Chromatography was performed as described in reference [19]

2.4.3 Thin-Layer Chromatography (TLC)

Thin-Layer Chromatography was performed as described in reference [20,21]

2.5 Extraction

2.5.1 The dried and crushed Rhizomes of *Zingiber rubens* Roxb. (1.5 kg) was steam-distilled in the apparatus. Steam-distillation was carried out at atmospheric pressure. After 12 hours of distillation, the distillate was dried over anhydrous sodium sulfate and concentrated to give 7.88 g (0.53 % wt.by wt.) of the green-blue essential oil.

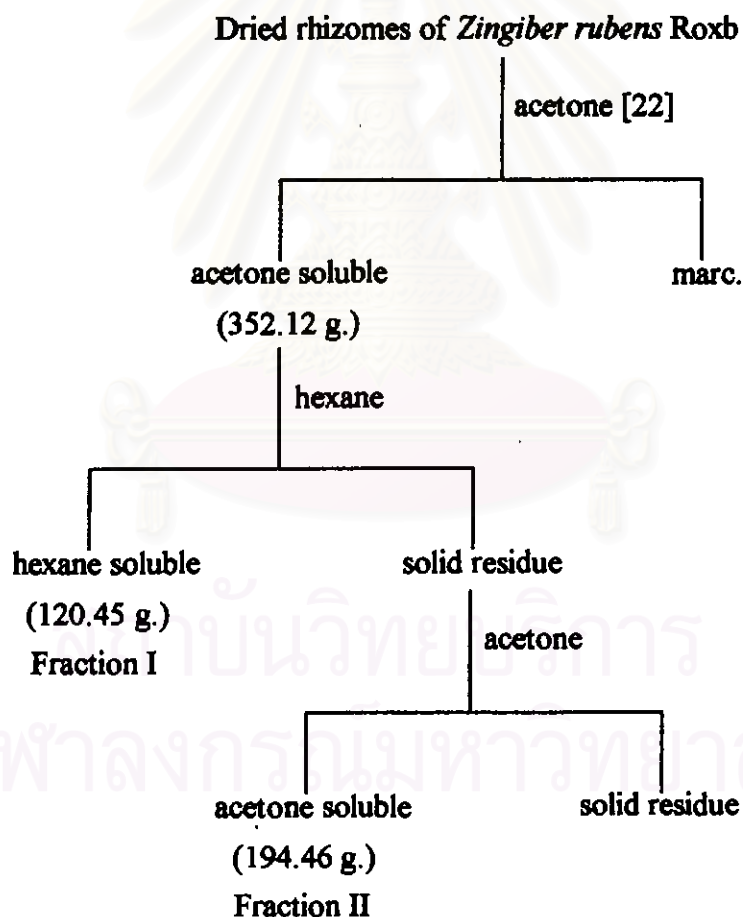
2.5.2 The dried Rhizomes of *Zingiber rubens* Roxb. (10 kg) were crushed and soaked in acetone for 4-5 days at room temperature for 3 times. The combined filtrate after evaporation of the solvent under reduced pressure gave an acetone extract of 352.12 g. (3.52 % wt. by wt.) as a dark-brown oil.

The acetone extract was re-extracted with hexane until the solution was colourless. The filtered hexane solution was evaporated to afford the hexane extract of 120.45 g (1.20 % wt. by wt.) as a dark-brown oil.

The hexane insoluble part was re-extracted with acetone repeatedly until the solution was clear. The combined acetone solution was concentrated on a rotary evaporator under reduced pressure to give the acetone extract of 196.46 g (1.96 % wt. by wt.) as a viscous dark-brown residue.

The procedure of the extractions is shown in Scheme 1.

Scheme 1 Extraction of the dried rhizomes of *Zingiber rubens* Roxb.



2.6 Isolation of the Chemical Constituents of the Rhizomes of *Zingiber rubens* Roxb.

2.6.1 Separation of hexane extract

The hexane extract (80 g) was chromatographed on silica gel Art 7734 using column chromatography. The column was eluted with hexane, hexane-chloroform, chloroform, chloroform-methanol and methanol respectively. Approximately 850 ml of eluant was collected for each fraction. It was concentrated on a rotatory evaporator to a volume of about 30 ml. Each fraction was monitored by TLC and the identical fractions were combined. The results of the separation of the hexane extract are presented in the Table 3.

Table 3 The results of the separation of hexane extract by column chromatography.

Eluent	Fraction No.	Remark	Weight (g)
5% CHCl ₃ -Hexane	1-2	yellow oil	1.43
	3-5	green-blue oil	0.87
10% CHCl ₃ -Hexane	6-14	yellow oil	2.52
20% CHCl ₃ -Hexane	15-18	yellow oil	0.92
	19-22	yellow oil (compound 3)	8.48
30% CHCl ₃ -Hexane	23-36	yellow oil	3.07
35% CHCl ₃ -Hexane	37-42	yellow oil	0.29
	43-49	semi solid in yellow oil	2.18

Table 3 (cont.)

Eluent	Fraction No.	Remark	Weight (g)
40% CHCl ₃ -Hexane	50-57	dark yellow oil	5.88
	58-65	dark yellow oil	3.46
50% CHCl ₃ -Hexane	66-69	yellow oil	0.79
	70-80	yellow oil	4.64
60% CHCl ₃ -Hexane	81-86	yellow oil	1.24
	87-95	yellow oil	2.58
70% CHCl ₃ -Hexane	96-103	white solid in yellow oil (mixture 1)	1.33
80% CHCl ₃ -Hexane	104-110	white solid in yellow oil	0.48
90% CHCl ₃ -Hexane	111-116	white solid in yellow oil	0.61
	117-122	white solid in yellow oil	0.54
100% CHCl ₃	123-132	brownish oil	4.37
2% MeOH-CHCl ₃	133-137	brown oil	0.54
5% MeOH-CHCl ₃	138-141	brown oil	1.07
10,20 % MeOH-CHCl ₃	142-146	brown oil	1.12
40,60 MeOH-CHCl ₃	147-150	dark-brown tar	2.01
100 % MeOH	151-153	dark brown tar	1.86

2.6.1.1 The separation of the eluted fraction No.19-22 (in Table 3)

Fraction No. 19-22 was obtained as a yellow oil 8.48 g (0.56 % wt. by wt.) eluted from the silica gel column by 20% CHCl₃-hexane, which was further purified by preparative GLC (SE 30 on chromosorb P). The analytical conditions were as follow: programmed temperature: 200 °C, 35 min then 200- 240 °C (20°C/min), TCD detector temperature 280 °C, injector temperature 280 °C, and flow rate N₂ 45 ml/min. The GLC chromatogram showed three main peaks in Fig.36 and Table 4 and each fraction was collected.

Table 4 The composition of the fraction No.19-22 as analyzed by preparative GLC

Peak Number	Remark	Retention time (min)
fraction I	colorless oil (compound 3A)	18.5
fraction II	green-blue oil	27.5
fraction III	green-blue oil	41.7

Each fraction was further analyzed by GC/MS. A capillary column DB wax was used. The analytical conditions were as follows: programmed temperature 60°C, 5 min then 60-200°C (4°C/min), injector temperature 220 °C, ion source temperature 200 °C, ionization voltage 70 ev. The results are shown in Fig.37 and Table 5.

Table 5 GC-MS analysis of each fraction from the fraction No.19-22

Sample number	Remark	R _t (min)	EI-MS pattern
*Fraction No. 19-22	yellow oil	32.33	204,189,121,107,93,81,67,59
		34.54	222,204,189,161,147,133,105, 91,81,67,59
		35.85	204,189,161,149,109,93
		36.07	204,164,149,109,95,81,59
		36.68	202,162,159,147,119,105,91
Fraction I	colorless oil	18.60	204,189,161,147,121,107,93
		20.01	204,189,161,121,107,93,91,67
Fraction II	green-blue oil	20.04	204,189,161,133,119,105,93
		21.16	204,189,161,147,133,119,105, 91,81,79
		22.47	204,189,175,161,147,121,107, 93,79,67
		22.60	204,189,175,161,147,133,107, 93,81,79
		24.17	204,189,161,133,122,107,91

* Fraction No. 19-22 was used for preparative GLC

2.6.2 Separation of acetone extract

Quick column chromatography was used for separating the acetone extract into various fractions. Silica gel Art.7729 as adsorbent was packed to the height of about 6.0 cm. Crude acetone extract 60 g was applied on the top of the column and was eluted with hexane-chloroform, chloroform, chloroform-methanol and methanol respectively.

After the eluate was collected to approximately 300 ml. for each fraction, it was concentrated on a rotatory evaporator to a volume of about 30 ml. Each fraction was monitored by TLC and fractions containing similar components were combined.

The results of the separation of the acetone extract are presented in Table 6

Table 6 The results of the separation of acetone extract by Quick column chromatography

Eluent	Fraction No.	Remark	weight (g)
30% CHCl ₃ -Hexane	1-4	brownish oil	0.30
	5-16	brownish oil	1.51
	17-41	brownish oil	3.47
40% CHCl ₃ -Hexane	42-50	brownish oil	0.63
	51-59	yellow solid in brown oil	1.37
	60-73	yellow solid in brown oil	2.40
50% CHCl ₃ -Hexane	74-85	yellow solid in brown oil	1.95
60% CHCl ₃ -Hexane	86-99	yellow solid in brown oil	2.56

Table 6 (cont.)

Eluent	Fraction No.	Remark	Weight (g)
80% CHCl ₃ -Hexane	100-107	yellow solid in brown oil (compound 4)	0.84
	108-125	yellow solid in brown oil (compound 4)	2.85
	126-155	yellow solid in brown oil (compound 4)	3.46
90% CHCl ₃ -Hexane	156-173	brown oil	3.63
100% CHCl ₃	174-193	brown oil	2.18
	194-210	solid in brown oil	2.72
2% MeOH-CHCl ₃	211-222	solid in brown oil	2.24
2%, 5% MeOH-CHCl ₃	223-237	solid in brown oil	2.51
5% MeOH-CHCl ₃	238-242	brown oil	0.68
10% MeOH-CHCl ₃	243-252	brown oil	1.23
20% MeOH-CHCl ₃	253-259	brown oil	0.74
40%,60% MeOH-CHCl ₃	260-267	dark brown tar	1.63
100% MeOH	268-270	dark brown tar	0.95

2.6.2.1 The separation of the eluted fraction No. 100-155 (in Table 6)

TLC indicated that fraction No. 100-107, 108-125 and 126-155 should be combined. The combined fractions No.100-155 appeared as a yellow solid in brown oil weighed 10.40 g and was chromatographed on silica gel. The column was eluted with hexane-chloroform, chloroform, chloroform-methanol and methanol respectively. Approximately 200 ml of each fraction was collected and concentrated by rotary evaporator to a volume of about 25 ml. Each fraction was checked by TLC and identical fractions were combined. The results of the separation of fractions No. 100-155 are shown in Table 7.

Table 7 The results of the separation of the eluted fractions No. 100-155 by silica gel

Eluent	Fraction No.	Remark	weight (g)
40% CHCl ₃ -Hexane	1-4	yellow oil	0.09
	5-9	trace solid in yellow oil	0.85
50% CHCl ₃ -Hexane	10-12	solid in yellow oil (compound 5)	2.03
	13-19	trace solid in yellow oil	0.86
	20-27	trace solid in yellow oil	1.10
	28-43	brownish oil	0.99
70% CHCl ₃ -Hexane	44-62	brown oil	1.03
80% CHCl ₃ -Hexane	63-90	brown oil	1.20
100% CHCl ₃	91-107	brown oil	1.46

Table 7 (cont.)

Eluent	Fraction No.	Remark	Weight (g)
5% MeOH-CHCl ₃	108-113	brown oil	0.53
10% MeOH-CHCl ₃	114-118	brown tar	0.09
40% MeOH-CHCl ₃	119-122	dark brown tar	0.06
100% MeOH	123-125	dark brown tar	0.02

2.6.2.2 The separation of the eluted fraction No. 194-237 (inTable 6)

TLC indicated that fraction No.194-121, 211-222 and 223-237 should be combined. The combined fraction No.194-237 appeared as a solid in brown oil 19.58 g and was chromatographed on silica gel.

The column was eluted with hexane-ethyl acetate, ethyl acetate and methanol-ethyl acetate respectively. Approximately 25 ml of each fraction was collected and was concentrated by rotatory evaporator to a volume of about 25 ml. Each fraction was checked by TLC and identical fractions were combined. The results of the separation of fraction No. 194-237 are shown in Table 8.

Table 8 The results of the separation of the eluted fractions No. 194-237 by silica gel

Eluent	Fraction No.	Remark	Weight (g)
20% EtOAc-Hexane	1-7	brownish oil	0.71
30% EtOAc-Hexane	8-27	brown oil	1.34
40% EtOAc-Hexane	28-66	brown oil	1.73
50% EtOAc-Hexane	67-111	brown oil	2.57
60% EtOAc-Hexane	112-145	brown oil	1.92
70% EtOAc-Hexane	146-158	white solid in brown oil (mixture 2)	1.84
	159-180	white solid in brown oil	1.21
80% EtOAc-Hexane	181-214	brown oil	2.68
100% EtOAc	215-243	brown oil	1.44
1% MeOH-EtOAc	244-260	dark brown oil	1.06
2% MeOH-EtOAc	261-265	dark brown oil	0.05
5% MeOH-EtOAc	266-270	dark brown tar	0.38
10% MeOH-EtOAc	271-275	dark brown tar	0.31
20% MeOH-EtOAc	276-280	dark brown tar	0.14
50% MeOH-EtOAc	281-285	dark brown tar	0.41

2.7 Purification and properties of the essential oil by steam-distillation and elution of compounds by column chromatography

2.7.1 Purification and properties of essential oil

The essential oil was obtained by steam-distillation as a green-blue oil 7.88 g. The essential oil is soluble in hexane, ether, chloroform, and methanol.

ν_{\max} (liquid film, cm^{-1}) 3400-3360(m), 3080(w), 2929,2867(s), 1712(w), 1642, 1450,1377(m), 913,888(m). GC-MS analyzed a capillary column DBwax Temperature programs 50-200 °C, 3°C/min, 200-240 °C, 10°C/min, and held to 240 °C,15 min, injector temperature 220 °C, ion source temperature 220 °C, ionization voltage 70 eV.

The GC-MS spectrum in retention time of each component: 14.00 (5.08 %), 16.64 (3.45 %), 25.70 (1.88 %), 26.64 (1.17 %), 30.87 (2.44 %), 31.61(11.59 %), 35.49 (2.22 %), 35.68 (1.42 %), 38.02 (1.22 %), 38.24 (2.43 %), 46.20 (1.02 %), 49.48 (23.37 %), 49.90 (1.42 %), 50.97 (1.46 %), 52.43 (6.23 %), 52.76 (1.06 %), 53.02 (2.14 %), 54.14 (0.44 %), 54.45 (18.68 %), 55.13 (6.20 %), 55.59 (3.91 %), 59.12 (1.05 %). mins respectively.

2.7.2 Purification and properties of Mixture 1

Mixture 1 was obtained by hexane extract and eluted by solvent 70% CHCl_3 -hexane to give fraction No. 96-103 by silica gel column chromatography. The compound obtained was recrystallized from hot hexane several times to give colorless needle-like crystals 50 mg. (0.5×10^{-3} % wt. by wt.) with a m.p. of 129-131 °C.

The R_f value of this compound was 0.71 using 10% MeOH-CHCl₃, SiO₂. It was soluble in chloroform, dichloromethane, acetone, and hot hexane.

ν_{\max} (KBr, cm⁻¹) 3617-3177 (br,m), 2961,2936,2868(s), 1638(w), 1464(m), 1381(m), 1057(m), 959(w), 801(w). δ_H (200 MHz,CDCl₃) 0.68-2.26 (m), 3.50(1H,m), 5.08 (1H,m), 5.35(2H,d) ppm. δ_C (50.26 MHz, CDCl₃) 11.9-56.9, 71.8, 121.7, 129.3, 138.3, 140.7 ppm. m/z (EI) 414.0, 412.0, 410, 396.0, 381.0, 329.0, 303.0, 273.0, 255.0. GLC analysis column OV-1, column temperature 255 °C, injection temperature 290 °C, N₂ flow rate 50 ml/min, Detector FID.

GLC analysis indicated two peaks on the chromatogram at retention times shown in Table 9.

Table 9 The retention times of various peak from the gas chromatogram of mixture 1 compared with standard steroids.

standard steroid	retention time (mins)	% area
campesterol	-	-
stigmasterol	18.58	19.54
β -sitosterol	21.16	16.89
Mixture 1	-	-
	18.84	9.47
	21.35	11.56

2.7.3 Purification and properties of Mixture 2

Mixture 2 was a solid in brown oil in fractions No194-210 eluted by 100% CHCl_3 and fractions No. 211-222, 225-237 eluted by 2% MeOH-CHCl_3 from acetone extract. Rechromatography on SiO_2 eluted with to 70% EtOAc-hexane afford solid in brown oil in fraction No. 146-158 . After recrystallization from hot ethanol several times, a white amorphous solid, 30 mg (0.3×10^{-3} % wt.by wt.) with m.p. of 273-275 °C was obtained. The R_f value of this compound was 0.59 (20% MeOH-CHCl_3 , SiO_2). It was soluble in hot ethanol, methanol, dimethylsulfoxide (DMSO) and insoluble in hexane, chloroform, dichloromethane and ethyl acetate.

ν_{max} (KBr, cm^{-1}) 3600-3200(m), 2934,2867(s), 1653(w), 1463(m), 1370(m), 1072,1026(s), 887(w), 797,776(w). δ_{H} (200 MHz, CDCl_3) 0.64-2.42(m), 2.72-3.30 (m), 3.36 m), 4.20(d), 4.45(t), 5.09(m), 5.30(m) ppm. δ_{C} (50.26 MHz, CDCl_3) 11.6-56.1, 61.0, 70.0, 73.4, 76.7, 76.9, 100.7, 121.2, 128.5, 138.1, 140.4 ppm. m/z (EI) 414.0, 412.0, 400, 396.0, 381.0, 329.0, 303.0, 273.0, 255.0.

2.7.4 Purification and properties of Compound 3

Compound 3 was obtained from the hexane extract by elution with 20% CHCl_3 -hexane in fraction No.19-22 as a yellow oil from silica gel column and was converted into compound 3A after an attempt to purify by preparative GLC to afford a colorless oil (trace amount) in fraction I (retention time 18.5 min), after which each fraction was analyzed by GC/MS.

ν_{\max} (liquid film, cm^{-1}) 3082 (w), 2967,2929(s), 2860(m), 1642(m),1440,1375 (m), 1006(w), 890(s). δ_{H} (500 MHz, CDCl_3) 0.99 (3H,s), 1.43 (2H,m), 1.54 (2H, m), 1.99 (1H, m), 4.57,4.80 (2H, m), 4.9 (2H, m), 4.89,4.91 (2H, m), 5.80 (1H,dd, J = 10.0,18.0 Hz), 1.60 (2H, m), 1.9 (3H,m), 1.71 (3H, m), 1.93 (1H, m) ppm. δ_{C} (125.6 MHz, CDCl_3) 16.4 (q), 21.1 (q), 24.7 (q), 26.8 (t), 32.9 (t), 39.8 (s), 39.9 (t), 45.7 (d), 52.8 (d), 108.2 (d), 109.8 (d), 112.1 (d), 142.7 (s), 150.3 (d), 150.4(s) ppm.

2.7.5 Purification and properties of Compound 4

Compound 4 was a solid in brownish oil in fractions No.100-107 eluted by 60% CHCl_3 -hexane and fractions No. 108-125, 126-155 eluted by 80% CHCl_3 -hexane from the acetone extract by silica gel column chromatography. The obtained compound was recrystallized from hot methanol several times to give a yellow solid, 120 mg (1.2×10^{-3} % wt.by wt.) with a m.p. of 229-231 °C. The R_f value of this compound was 0.59 (5% MeOH- CH_2Cl_2 , SiO_2). It was soluble in hot chloroform, hot methanol, acetone, dimethylsulfoxide (DMSO) and insoluble in hexane.

ν_{\max} (KBr, cm^{-1}) 3500-3100(br,s), 2975,2839(w), 1659(s) 1587, 1508(s), 1336 (m), 1157,1114(m), 1092,1046(m), 838,804(w). δ_{H} (500 MHz, CDCl_3) 3.84(s), 6.32 (d), 6.67(d), 7.07(d), 7.66(dd), 7.71(d), 9.28 (s), 9.51(s), 12.41(s) ppm. δ_{C} (125.6 MHz, CDCl_3) 55.6(q), 56.0(q), 91.9(d), 97.5(d), 104.0(s), 111.8(d), 114.9(d), 119.8 (d), 123.4(s), 136.4(s), 146.2(s), 146.7(s), 149.5(s), 156.1(s), 160.4(s), 164.9(s), 176.1 (C=O) ppm. m/z (EI) M^+ 330, 315, 287, 259, 231,167, 151,135.

2.7.6 Purification and properties of Compound 5

Compound 5 was a yellow solid in brownish oil in fractions No.100-107 eluted by 60% CHCl₃-hexane and in fractions No. 108-125, 126-155 eluted by 80% CHCl₃-hexane from acetone extract. Rechromatography on SiO₂ using 50% CHCl₃-hexane as eluent afforded yellow needles in brown oil. After recrystallization from hot chloroform several times yellow needle crystals, 20 mg (0.2x10⁻³ % wt.by wt.) with a m.p. of 178-180 °C were obtained. The R_f value of this compound was 0.80 (5% MeOH-CH₂Cl₂, SiO₂). It was soluble in chloroform, acetone, methanol and insoluble in hexane.

ν_{\max} (KBr, cm⁻¹) 3400-3100,(s), 2966,2946(w), 1649(s), 1595,1503, 1466(s), 1353(w), 1257,1194(s), 1089(m), 879,833(m). δ_{H} (500 MHz, CDCl₃) 3.88, 3.89(s), 6.37(d), 6.48(d), 6.61(s), 7.03(dd), 8.16(dd), 11.73(s) ppm. δ_{C} (125.6 MHz, CDCl₃) 55.4 (q), 55.8(q), 92.2(d), 97.9(d), 103.9(s), 114.1(d), 123.2(s), 129.4(d), 135.7(s), 145.7(s), 156.8(s),160.8(s), 161.2(s), 165.7(s), 175.2 (C=O) ppm.
 m/z (EI) M⁺314, 313, 299, 285, 271, 243, 167, 135.

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