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ISOLATION AND CHARACTERIZATION OF EUGLOBALS FROM *EUCALYPTUS GLOBULUS* LABILL. BY PREPARATIVE REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The isolation of eleven novel acetogenin mevalonates, which have strong granulation-inhibiting activity, from *Eucalyptus globulus* Labill. is reported. These eleven compounds, which have closely related structures, were successfully isolated with a preparative reversed-phase column using acetonitrile or methanol as the eluent.

INTRODUCTION

In research on natural products the first essential step is the isolation of pure compounds from an extract. However, this is a difficult and tedious process when the mixture is complex and attempts often fail when the mixture is composed of compounds with closely related structures.

Recently we reported[1] on the determination of the structure of a novel granulation-inhibiting agent, euglobal-III, which was isolated from buds and leaves of *Eucalyptus globulus* Labill. We further investigated other active principles of this plant, which showed remarkable activity in the fertile egg method[2]. The results obtained exemplified the efficacy of high-performance liquid chromatography (HPLC) with a reversed-phase column for isolating these compounds with closely related structures.

This paper describes the isolation and characterization of euglobal-III and ten other potent granulation-inhibiting principles.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used for the ana-

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lytical separations. This consisted of a 6000A solvent delivery unit, a UV spectrophotometer (280 nm) and a U6K universal liquid chromatograph injector. A DuPont (Wilmington, DE, U.S.A.) Zorbax-ODS (250 × 4.6 mm) column was used and the recorder was a Hitachi (Tokyo, Japan) Model 056.

For conventional liquid chromatography, a Merck Lobar LiChroprep RP-8 column (310 × 25 mm) was used and the mini-micro pump was a Kyowa Seimitsu (Tokyo, Japan) Model KHD-94. An LKB (Stockholm, Sweden) Uvicord II (254 nm) detector–recorder and a Toyo Kagaku Sangyo (Osaka, Japan) Model SF-160K fraction collector were used.

Preparative separation was carried out on a Toyo Soda (Tokyo, Japan) automatic preparative liquid chromatograph, Model HLC-827, with a built-in UV spectrophotometer (254 nm), recorder and fraction collector. TSK-LS410KG reversed-phase columns (500 and 300 × 21.5 mm) were used for the isolation.

All melting points were determined with a Yanaco MP instrument and the spectrometers used were a Perkin-Elmer 450 for UV spectra, a Hitachi 260-10 for IR spectra, a Varian XL-100 for PMR spectra, a JEOL JMS-01SC for mass spectra and a Perkin-Elmer 141 for optical rotation.

Materials

The plant material was collected in October 1979 in Kyoto and Osaka, Japan.

n-Hexane, acetonitrile, methyl acetate, ethanol and acetic acid were of special grade and methanol was of the grade for liquid chromatographic use, all from Wako (Osaka, Japan). Kieselgel 60 (Merck, Darmstadt, G.F.R.) was used as the stationary phase support in preparative liquid chromatography.

RESULTS

Extraction of active principles

Dried buds (about 1 kg) were soaked in 5 l of *n*-hexane overnight at room temperature to remove the waxy substance from the surface. After filtration, the buds were dried and crushed, then soaked in 10 l of *n*-hexane for 24 h at room temperature and the active principles were extracted. The *n*-hexane extract was filtered and evaporated to dryness to afford 80 g of residue.

Through a column (280 × 85 mm) of Kieselgel 60 using *n*-hexane as eluent, the essential oil was eluted first followed by the active principles, which were collected in fractions 18–21 (a total of 12 l). Evaporation of the solvent *in vacuo* gave a pale yellow oil (4.3 g).

High-performance liquid chromatography (HPLC)

The oil obtained above gave six spots on reversed-phase thin-layer chromatography (TLC) with methanol, which suggested that it was a mixture of several components. A high-performance liquid chromatogram of the oil [dissolved in methyl acetate–methanol (1:1) and eluted with methanol–acetic acid–water (100:5:3) at a flow-rate of 1.7 ml/min] is shown in Fig. 1. More than ten components were detected on the chromatogram and the strong peaks were designated as euglobal-Ia, -Ib, -Ic, -IIa, -IIb, -IIc, -III, -IV, -V, -VI, -VII, -VIII and -IX, respectively, in order of elution.

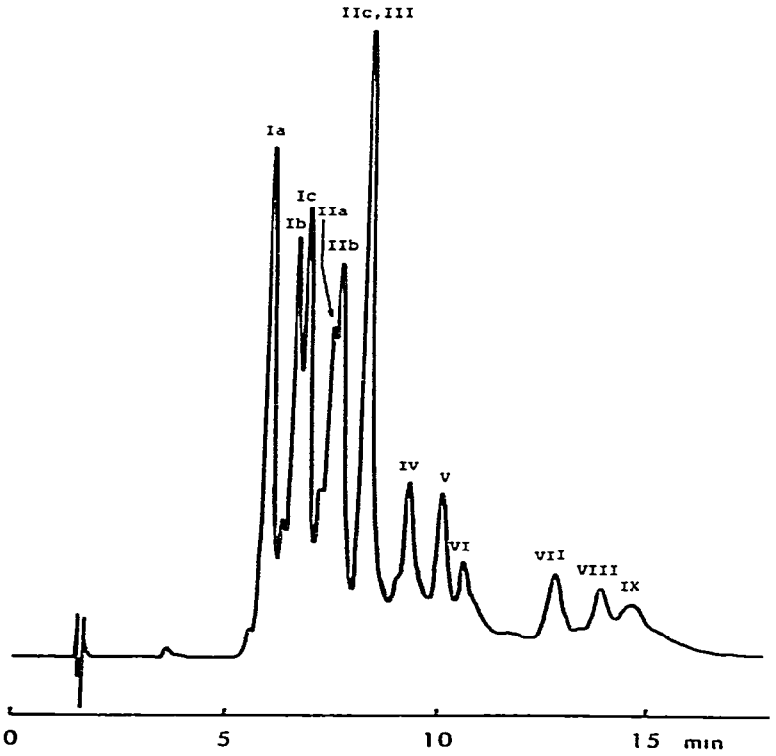

Fig 1. High-performance liquid chromatogram of fraction 18–21. Column, Zorbax-ODS (250 × 4 6 mm), mobile phase, methanol–acetic acid–water (100·5·3); flow-rate, 1 7 ml/min; sample, 20 μg of fraction 18–21 in 5 μl of methyl acetate–methanol (1 1); detector, UV (280 nm), 0 05 a u.f s

Rough separation

For the rough separation, the oil (50 mg) was dissolved in 1 ml of methyl acetate and applied to a conventional preparative column. Acetonitrile was used as the eluent (2.4 ml/min) and 5-ml portions of eluate was collected with a fraction collector. Fig. 2 shows the chromatogram obtained. Two main fractions eluting from 200 to 290 ml (fraction X) and from 310 to 600 ml (fraction Y) were collected.

Repetition of this process for 50-mg portions of the oil afforded 1 g of fraction X and 1.5 g of fraction Y. HPLC of the fractions showed that euglobal-Ia, -Ib, -Ic, -IIa, -IIb and -IIc were contained in fraction X and euglobal-III, -IV, -V, -VI, -VII, -VIII and -IX in fraction Y. The time required for one fractionation process was about 4.5 h.

Isolation of the components

Fraction X (1 g) was dissolved in 50 ml of methyl acetate–acetonitrile (1:1) and portions containing 30–50 mg of the oil were successively separated by automatic preparative liquid chromatography using acetonitrile as the eluent. The flow-rate was adjusted to 14.9 ml/min and a run required about 1 h. An example of the chromatograms is shown in Fig. 3. Five fractions (360–380, 385–410, 435–450, 455–480 and 490–510 ml) were collected and named fractions A, B, C, D and E, respectively. On

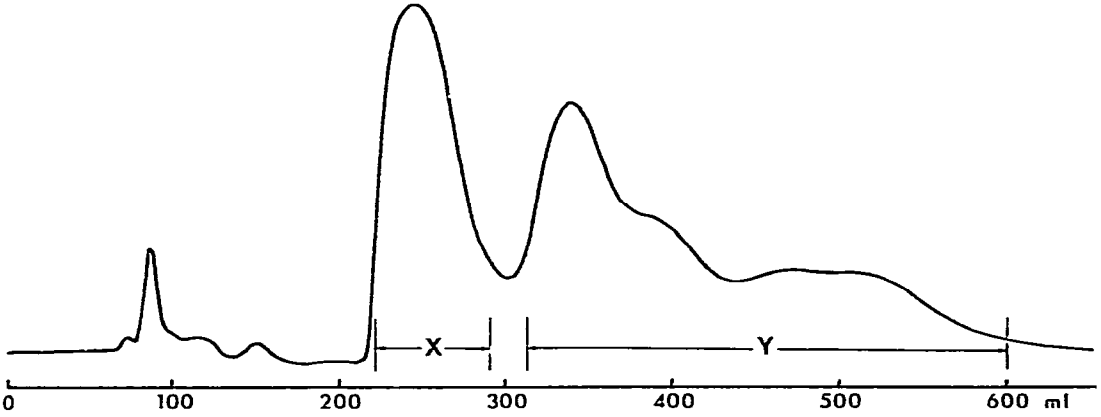

Fig. 2. Rough separation of fraction 18–21. Column, LiChroprep RP-8 (310 × 25 mm); mobile phase, acetonitrile; flow-rate, 2.4 ml/min; sample, 50 mg of fraction 18–21 in 1 ml of methyl acetate–acetonitrile (1:1); detector, UV (254 nm)

repetition of the process and evaporation of the solvent, about 100 mg each of fractions A, B, C and E and 200 mg of fraction D were obtained.

HPLC examination revealed that the purity of fractions A, B, C and E was more than 80% but fraction D consisted of two components. However, PMR spectra

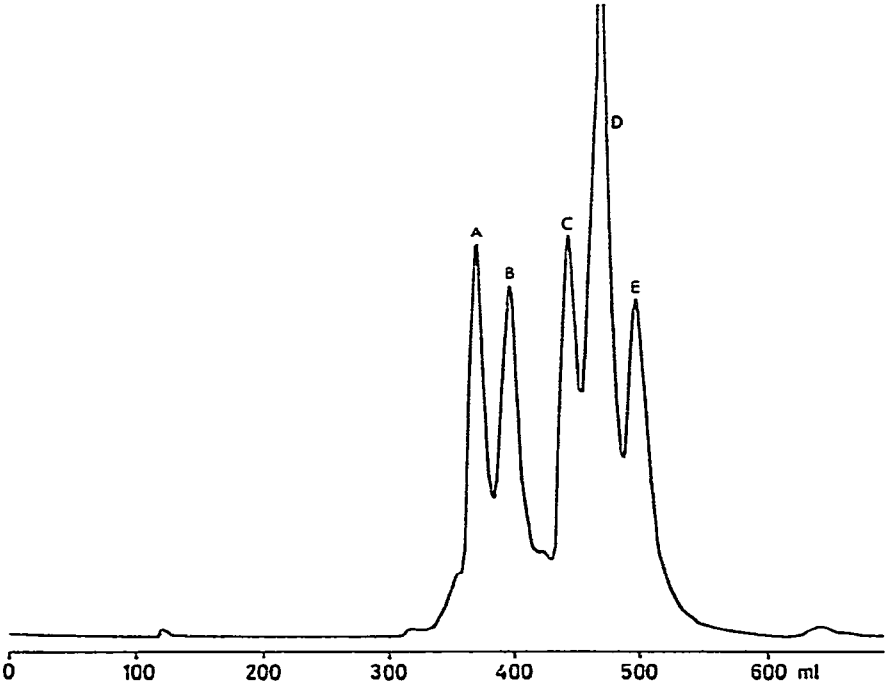

Fig. 3. Preparative liquid chromatogram of fraction X. Peaks: A = euglobal-Ia$_1$ and -Ia$_2$; B = euglobal-Ib; C = euglobal-Ic; D = euglobal-IIb and -IIc; E = euglobal-IIa. Column, TSK-LS410KG (500 × 21.5 mm); mobile phase, acetonitrile; flow-rate, 14.9 ml/min; sample, 30–50 mg of fraction X in 2 ml of methyl acetate–acetonitrile (1:1); detector, UV (254 nm), 1.28 a.u.f.s.

of the fractions suggested that fraction A was a mixture of two components whereas fractions B, C and E were pure, as concluded from the HPLC. Re-chromatography of fraction A on the same column using a slightly different solvent (acetonitrile–water, 95:5) gave two peaks [fraction A-1 (505–545 ml) and fraction A-2 (560–580 ml)] and finally about 20 mg of A-1 and 60 mg of A-2 were obtained. Similar procedures with fraction D using methanol–water (100:1) afforded about 60 mg of fraction D-1 (240–290 ml) and 50 mg of D-2 (305–340 ml). Each re-chromatographed fraction, A-1, A-2, D-1 and D-2, was ascertained to be pure from its PMR spectrum.

By HPLC under the conditions described above, fractions A-1 and A-2, B, C, D-1, D-2 and E were identified as euglobal-Ia, -Ib, -Ic, -IIb, -IIc and -IIa, respectively. Fractions A-1 and A-2, which had identical retention times in this analysis but were separated using acetonitrile–water (95:5) as the eluent, gave definite PMR spectra. They were designated as euglobal-Ia_1 and -Ia_2, respectively.

Fraction Y was similarly treated by automatic preparative liquid chromatography, giving the chromatogram shown in Fig. 4. Four main peaks eluting at 490–520, 600–635, 660–690 and 710–745 ml were collected (fractions F, G, H and I, respectively).

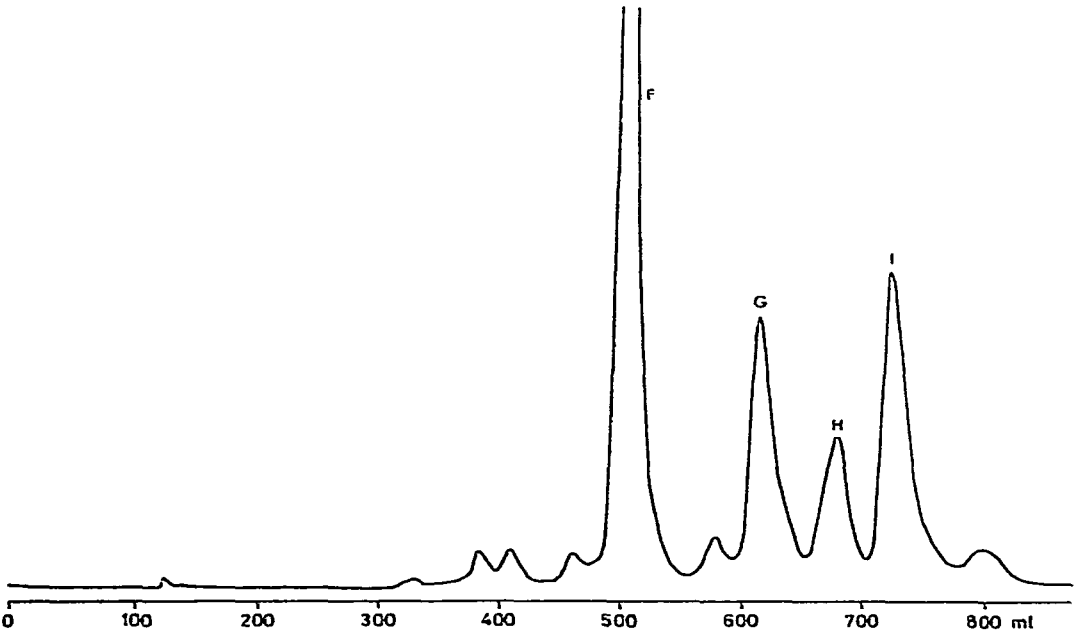

Fig. 4. Preparative liquid chromatogram of fraction Y Peaks: F = euglobal-III; G = euglobal-IV; H = euglobal-VII and -IX; I = euglobal-V and -VIII. Conditions as in Fig. 3.

Repetition of the process using a total of 1.5 g of fraction Y yielded fractions F (600 mg), G (200 mg), H (100 mg) and I (300 mg). Fractions F and G were determined to be composed of a more than 90% pure single component both by HPLC and PMR. Fractions H and I, found to be mixtures of two components by HPLC, were re-chromatographed in a manner similar to that used for the separation of fraction D. The process gave fraction H-1 (50 mg) at 305–335 ml and H-2 (30 mg) at 345–380 ml,

and I-1 (150 mg) at 255–275 ml and I-2 (50 mg) at 305–330 ml. Fractions H-1 and I-1 were pure by HPLC and PMR but fractions H-2 and I-2 were slightly contaminated with unresolved H and I, respectively. By using HPLC, the fractions were identified as follows: F = euglobal-III, G = euglobal-IV, H-1 = euglobal-VII, H-2 = euglobal-IX, I-1 = euglobal-V and I-2 = euglobal-VIII.

These fractions were crystallized from ethanol. Fractions B, E and F afforded colourless needles of euglobal-Ib, -IIa and -III, respectively, and fractions C, G and I-1 crystallized as colourless prisms of euglobal-Ic, -IV and -V, respectively. The other fractions have not yet been obtained in crystal form. The physico-chemical properties of each compound are shown in Table I. Also shown are the results of the elemental analyses of the crystallized fraction. For euglobal-Ic and -III, high-resolution mass spectroscopic data were obtained and are listed in Tables II and III.

DISCUSSION

From the *n*-hexane extract of buds of *Eucalyptus globulus* Labill., eleven components, active in the fertile egg method, were isolated in pure form by repeated application of reversed-phase liquid chromatography. The physico-chemical data in

TABLE I
PHYSICO-CHEMICAL PROPERTIES OF EUGLOBALS

Property	*Ia*$_1$	*Ia*$_2$	*Ib*	*Ic*	*IIa*
Appearance	Oil	Oil	Colourless needles	Colourless prisms	Colourless prisms
Melting point* (°C)			119–121	108–110	130–132
Mass spectrum**	386 (M^+), 329, 251, 204, 195 (base), 136, 93	386 (M^+), 329, 251, 204, 195 (base), 136, 93	386 (M^+), 329, 251, 204, 195 (base), 136, 93	386 (M^+), 329, 251, 204, 195 (base), 136. 93	386 (M^+), 329, 251, 204, 195 (base), 136, 93
Elemental analysis·					
Found (%)			C 71 57, H 7 71	C 71 56, H 7 63	C 71 37, H 7 82
Calculated (%)			$C_{23}H_{30}O_5$. C 71.48, H 7.82	$C_{23}H_{30}O_5$. C 71.48, H 7.82	$C_{23}H_{30}O_5$. C 71.48, H 7.82
UV:					
λ_{max}^{EtOH} (nm)	277 (ε = 32,000) 340 (ε = 3700)	277 (ε = 32,000) 340 (ε = 3600)	277 (ε = 36,000) 340 (ε = 4300)	277 (ε = 38,000) 340 (ε = 4100)	277 (ε = 38,000) 340 (ε = 4100)
IR.					
$\nu_{max}^{liq\ film}$ (cm^{-1})	3300, 2950, 1620, 1430, 1300, 1170	3350, 2950, 1630, 1440, 1300, 1180			
ν_{max}^{KBr} (cm^{-1})			3400, 2950, 1630, 1430, 1300, 1170	3350, 2950, 1630, 1440, 1300, 1170	3420, 2950, 1620, 1440, 1280, 1180
$[\alpha]_D^{20}$ (c = 1, $CHCl_3$)	−216.7°	+31.8°	−1.94°	−3.12°	+9.24°

* All melting points were determined on a hot-block and are reported uncorrected.
** Conditions: ionization, electron impact; ionization energy, 70 eV; ionization current, 200 μA.

Tables I–III indicate that euglobal-Ia$_1$, -Ia$_2$, -Ib, -Ic, -IIa, -IIb and -IIc have similar chemical structures and the same molecular formula of $C_{23}H_{30}O_5$, mol.wt. = 386. Euglobal-III, -IV, -V and -VII are also isomers of $C_{28}H_{38}O_5$, mol.wt. = 454. High-resolution mass spectra showed that euglobal-Ic and -III were fragmented into two parts. One part is common to both euglobal-Ic and -III, *m/z* 251 ($C_{13}H_{15}O_5$). The other parts differ by C_5H_8 between the two components and are *m/z* 136 ($C_{10}H_{16}$) for euglobal-Ic and *m/z* 204 ($C_{15}H_{24}$) for euglobal-III.

These results, together with the fact that various mono- and sesquiterpenes[3] occur in *Eucalyptus globulus*, suggested that the compounds with a molecular weight of 386 have structures constructed from an aromatic part ($C_{13}H_{15}O_5$) and a monoterpene moiety ($C_{10}H_{16}$), and the compounds with a molecular weight of 454 are constructed from the same aromatic part and a sesquiterpene moiety ($C_{15}H_{24}$). Differences among the compounds are attributed to the difference in the terpene moiety. The chemical structures of these compounds were determined as shown in Fig. 5, on the basis of physico-chemical data and X-ray crystallographic analysis. Detailed studies on the structures of these active principles will be described elsewhere.

Each of the eleven compounds possessed greater activity than indomethacin and comparable to that of berberine. Note that such a group of compounds has not

Ib	*IIc*	*III*	*IV*	*V*	*VII*
)il	Oil	Colourless needles 169–171	Colourless prisms 187–190	Colourless prisms 184–185	Oil
86 (M^+), 29 (base), 51, 195, 36, 93	386 (M^+), 343, 251, 203 (base), 195, 148, 136, 93	454 (M^+), 397, 251, 204, 203, 195 (base), 161	454 (M^+), 397, 251, 204, 195, 161, 121 (base)	454 (M^+), 397, 251, 204, 203 (base), 195, 163	454 (M^+), 397, 251, 204, 203 (base), 161, 121
		C 73.96, H 8 41 $C_{28}H_{38}O_5$· C 73.98, H 8.43	C 74.16, H 8.73 $C_{28}H_{38}O_5$· C 73 98, H 8.43	C 74 01, H 8 40 $C_{28}H_{38}O_5$. C 73 98, H 8 43	
277 (ε = 36,000) 340 (ε = 4500)	277 (ε = 35,000) 340 (ε = 3900)	276 (ε = 35,000) 340 (ε = 4500)	275 (ε = 34,600) 340 (ε = 4400)	275 (ε = 32,200) 340 (ε = 5000)	276 (ε = 36,000) 340 (ε = 3900)
3400, 2950, 1620, 1430, 1300, 1170	3400, 2950, 1620, 1430, 1290, 1180				3450, 2950, 1620, 1430, 1290, 1180
		3400, 2950, 1620, 1430, 1300, 1160	3450, 2950, 1630, 1440, 1310, 1170	3450, 2950, 1630, 1440, 1310, 1160	
+12.2°	−144.0°	+229°	+235°	−206°	−137°

TABLE II
ELEMENTAL ANALYSIS OF EUGLOBAL-IC
Conditions: ionization, electron impact; ionization energy, 70 eV; ionization current, 200 μA.

Mass observed	*Mass calculated*	*Error*	*Number of atoms in fragment*		
			C	*H*	*O*
93.0703	93.0704	−0.1	7	9	0
136.1253	136.1252	+0.1	10	16	0
161.1303	161.1330	−2.6	12	17	0
195.0288	195.0293	−0.4	9	7	5
196.0335	196.0371	−3.6	9	8	5
204.1872	204.1878	−0.6	15	24	0
251.0910	251.0919	−0.9	13	15	5
263.0932	263.0919	+1.2	14	15	5
273.0761	273.0763	−0.1	15	13	5
287.0887	287.0919	−3.2	16	15	5
329.1400	329.1389	+1.1	19	21	5
343.1556	343.1545	+1.1	20	23	5
386.2053	386.2093	−3.9	23	30	5

TABLE III
ELEMENTAL ANALYSIS OF EUGLOBAL-III
Conditions as in Table II

Mass observed	*Mass calculated*	*Error*	*Number of atoms in fragment*		
			C	*H*	*O*
93.0709	93.0704	+0.5	7	9	0
105.0699	105.0704	−0.4	8	9	0
121.1003	121.1017	−1.3	9	13	0
136.1241	136.1252	−1.0	10	16	0
161.1327	161.1330	−0.2	12	17	0
195.0313	195.0293	+1.9	9	7	5
203.1818	203.1799	+1.8	15	23	0
204.1864	204.1878	−1.3	15	24	0
247.0613	247.0606	+0.6	13	11	5
251.0923	251.0919	+0.3	13	15	5
275.0888	275.0919	−3.0	15	15	5
303.1218	303.1232	−1.4	17	19	5
397.2024	397.2015	+0.9	24	29	5
454.2739	454.2719	+2.0	28	38	5

been isolated thus far from the genus *Eucalyptus* and from other natural sources. Also note that reversed-phase chromatographic techniques were found to be highly efficient for isolating these closely related compounds, for which normal-phase techniques were ineffective.

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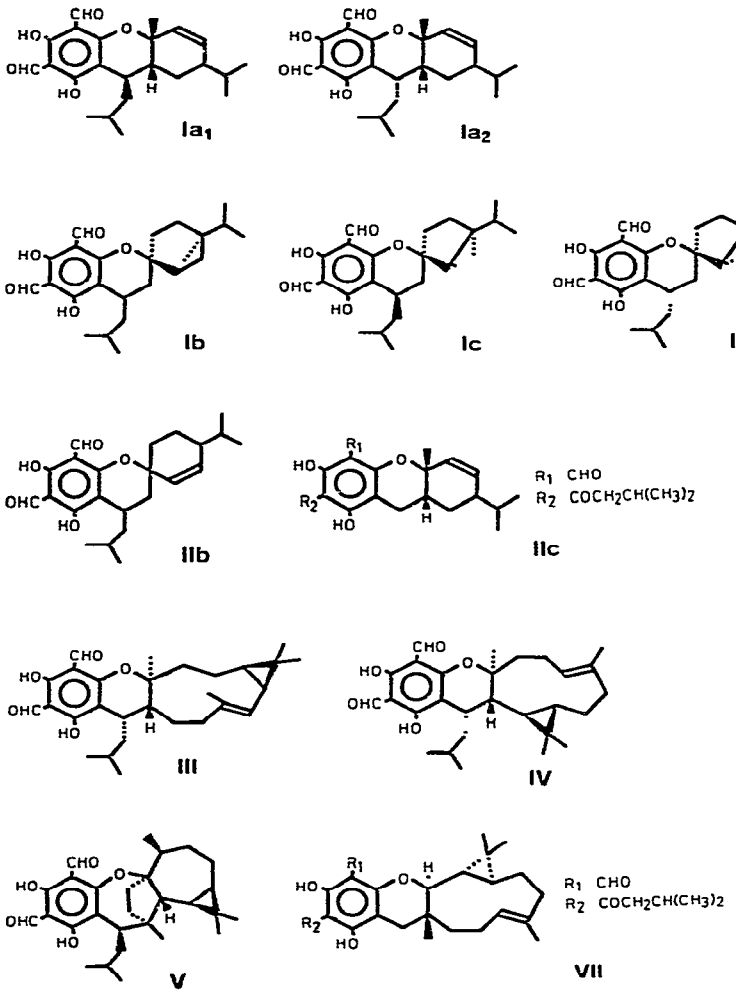

Fig. 5. Structures of euglobals isolated from buds of *Eucalyptus globulus* Labill.

this work. We also thank the staff of the Chemical Research Laboratories, Takeda Chemical Industries, Ltd., for their collaboration during various stages of this work.

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