THE STRUCTURE OF CALENDULOSIDES C AND D FROM THE ROOTS OF Calendula officinalis

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We have previously [1, 2] reported the structure of calendulosides A and B. In the present paper we give the results of the isolation of two more polar glycosides – calenduloside C (I) and calenduloside B (II) – and of the determination of their structures. Substances (I) and (II) were obtained by chromatographing the total glycosides from the roots of C. officinalis L. on a column of silica gel. When compounds (I) and (II) were subjected to complete acid hydrolysis, D-glucose and D-galactose were identified in ratios of 2:1 and 3:1, respectively, and oleanolic acid was also found. The formation of methyl oleanolate on the hydrolysis of the product of the esterification of (I) with diazomethane and of a permethylate of (I) showed that compound (I) has a single carbohydrate chain attached to the hydroxy group of the genin. This was confirmed by the fact that on alkaline hydrolysis (I) remained unchanged. When the product of the treatment of (II) with diazomethane and the permethylate (II) were hydrolyzed, oleanolic acid was identified. The alkaline hydrolysis of (II) gave (I), identical with that isolated from the roots, and D-glucose. Consequently, calenduloside D has two carbohydrate chains one of which, as on calenduloside C, is attached to the hydroxyl and the other (D-glucose) to the carboxy group of the genin.

On partial hydrolyxis with Kiliani's mixture, (I) decomposed into oleanolic acid, D-glucose, D-galactose, and a monoside (III) identical with the 3-O-β-D-glucopyranoside of oleic acid obtained previously [1], a bioside (IV) containing D-glucose, and another, less polar, bioside (V), which hydrolyzed to form D-glucose and D-galactose. The hydrolysis of (I) with 10% HCl gave (IV) and D-galactose. On treatment with Kiliani's mixture, calenduloside D gave oleanolic acid, (I), (III), (IV), D-glucose, and D-galactose.

When compound (I) and (II) were subjected to acetolysis followed by deacetylation [3], PC and TLC showed the presence not of lactose, as in the case of calenduloside A, but of D-glucose and D-galactose. On the basis of these facts it may be assumed that there is a 1→4 bond between the D-glucose and D-galactose.

When compounds (I), (II), and (V) were subjected to periodate oxidation, the D-glucose was unaffected, while the periodate oxidation of (IV) led to the destruction of both D-glucose molecules. Consequently, there is a 1→3 bond between the D-glucose and the D-galactose and a 1→2 or a 1→6 bond between the two D-glucose molecules [4].

TABLE 1. Results of a Calculation of the Configurations of the Glycosidic Centers [7]

Substance	M	$[\alpha]_D$	$[M]_D$	ΔC	Form of the bond
		deg			
Calenduloside D	1132,3	+16	+181,2	−137,9	β
Calenduloside C	997,2	+32	+319,1	+ 57,7	β
The bioside (IV)	817	+32	+261,4	− 57,9	β
The monoside (III)	618,9	+55,7	+329,3	—	—
Methyl α-D-glucopyranoside [8]	194,2	+149	+289,4	—	—
Methyl β-D-glucopyranoside [8]		−25	− 48,6	—	—
Methyl α-D-galactopyranoside [8]		+161	+312,7	—	—
Metnyl β-D-galactopyranoside [8]		+ 5	+ 9,7	—	—

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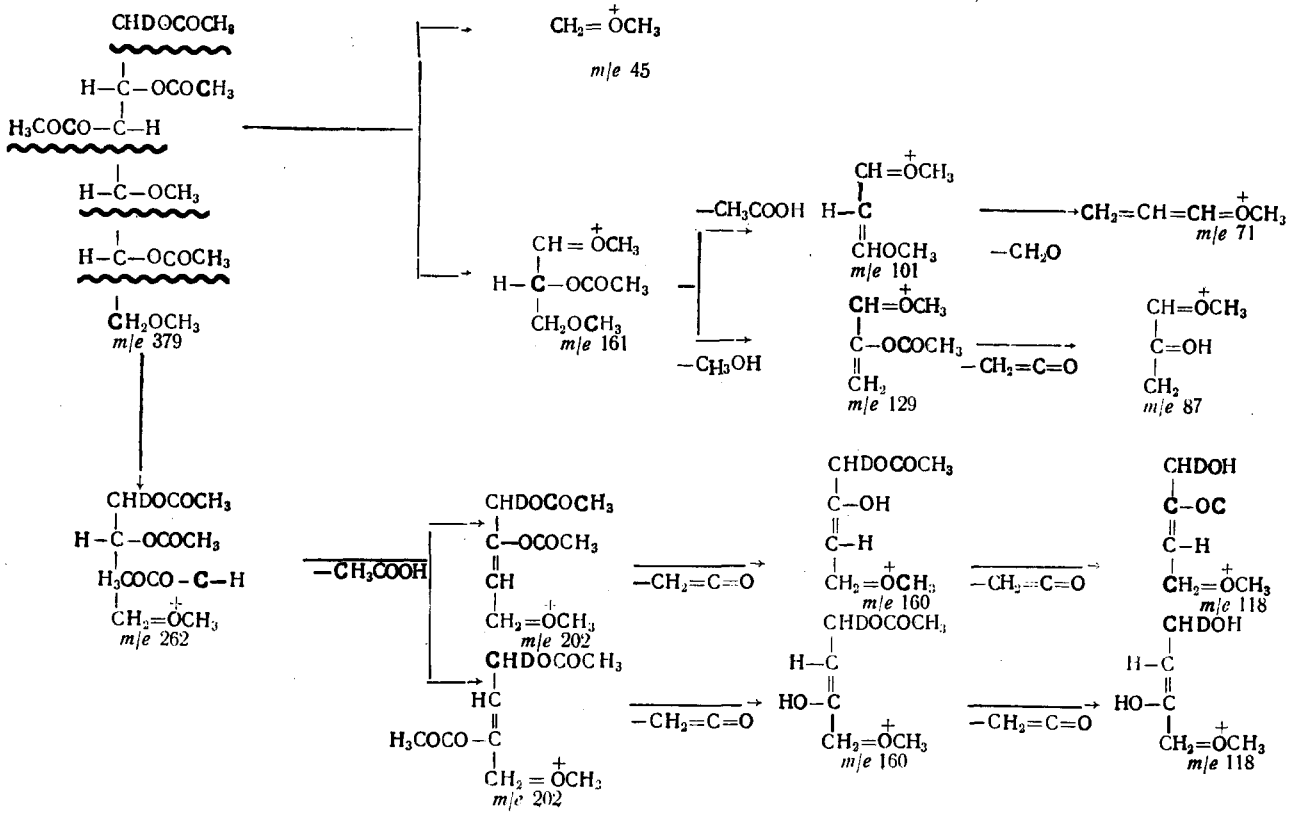
Further information on the structure of the carbohydrate chain was obtained by the methylation of (I), (II), (IV), and (V) by Hakomori's method [5]. The completeness of methylation was checked by IR spectroscopy. The products of hydrolysis of the methyl ester of (I) was shown by PC, TLC, and GLC to contain 2,3,4,6-tetra-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-galactopyranose in a ratio of 1 : 1, and also 4,6-di-O-methyl-D-glucopyranose [a sample of 4,6-di-O-methyl-D-glucopyranose was kindly given to us by R. Tschesche (GFR)].

To confirm the positions of the methyl groups, the dimethyl glucose was reduced with $NaBD_4$.

The mass spectrum of the acetate of the sorbitol obtained had the peak of the molecular ion with m/e 379 in low intensity. The detachment of a methoxymethyl group from the molecular ion formed an ion with m/e 334, and the detachment of a $CHDOCOCH_3$ group an ion with m/e 305.

The presence of these peaks and of a strong peak with m/e 45 shows the terminal position of another methyl group, i.e., its position at C_6 of the glucose residue. The feeble peaks with m/e 118 and 189, and also also the strong peaks with m/e 262 and 161, show that the remaining methyl group cannot occupy the corresponding position at C_2 or C_3 in the dimethylglucose residue. Thus, the second methyl group must occupy the position at C_4 in the dimethylglucose residue. This group of peaks and the ratio of the intensities corresponds to literature information for 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl-D-sorbitol [6].

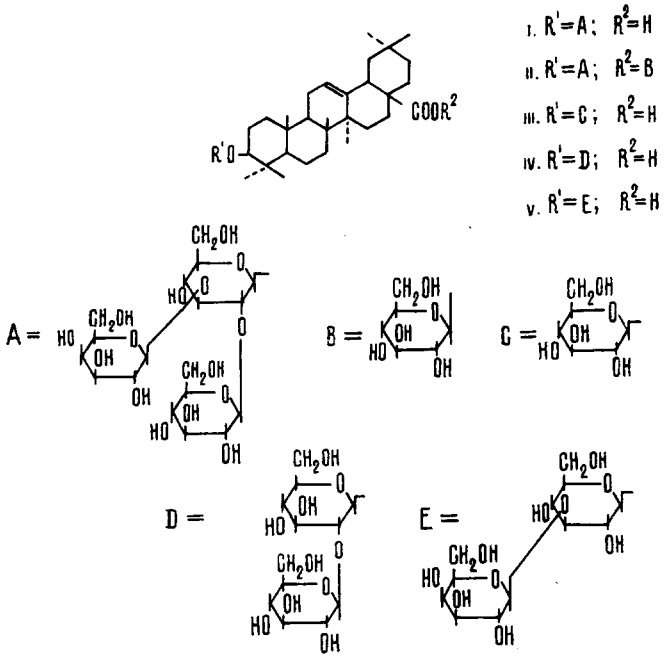


When the permethylate of (IV) was subjected to methanolysis, methyl 3,4,6-tri-O-methyl-D-glucopyranoside and methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside were identified by GLC, and the methanolysis of the permethylate of (V) gave methyl 2,4,6-tri-O-methyl-D-glucopyranoside and methyl 2,3,4,6-tetra-O-methyl-D-galactopyranoside in a ratio of 1 : 1.

On hydrolysis of the permethylate of (II), PC, TLC, and GLC showed the presence of 2,3,4,6-tetra-O-methyl-D-glucopyranose, 2,3,4,6-tetra-O-methyl-D-galactopyranose, and 4,6-di-O-methyl-D-glucopyranose in a ratio of 2 : 1 : 1.

Thus, in calendulosides C and D the hydroxy group of the genin is substituted by D-glucose which forms a center of branching of the carbohydrate chain. D-glucose is attached to the hydroxy group at C_2 and D-galactose to that at C_3.

Consequently, the structures of (I) and (II) can be represented as:



EXPERIMENTAL METHOD

Chromatography was performed on type KSK silica gel, M [slow] paper of the Volodarskii Leningrad mill, and the following solvent systems: 1) chloroform–methanol–water (61 : 32 : 7); 2) n-propanol–14% ammonia (8 : 2); 3) butan-1-ol–pyridine–water (6 : 4 : 3); 4) benzene–butan-1-ol–pyridine–water (1 : 5 : 3 : 3); 5) ethyl acetate–n-propanol–water (2 : 7 : 1); 6) chloroform–methanol–water (25 : 10 : 5); 7) chloroform–ethyl acetate (4 : 1); 8) chloroform–ethyl acetate (1 : 2); 9) butan-1-ol–ethanol–water (5 : 1 : 4); 10) benzene–acetone (2 : 1); 11) benzene–acetone–water (5 : 5 : 1); and 12) chloroform–acetone (10 : 1).

The sugars were revealed with aniline phthalate and with a mixture of aniline, diphenylamine, and phosphoric acid, and the glucosides and their derivatives with 20% sulfuric acid.

The IR spectra were taken on a UR-10 spectrophotometer (paraffin oil) and the UV spectra on a Hitachi recording spectrophotometer.

Gas–liquid chromatography (GLC) was performed on a Varian model 2740 instrument (10% of Carbowax 20 M with programming of the temperature to 140/2° C and on neopentyl glycol succinate with programming of the temperature to 150/4° C). The mass spectra were obtained on a Varian model CH-6 mass spectrometer.

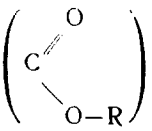
Before analysis, the substances were recrystallized from methanol. The analyses of all the compounds corresponded to the calculated figures. The melting points were determined on a Kofler block and the specific rotations in methanol (with the avoidance of heating) on an AI-EPL polarimeter.

Isolation of the Calendulosides. The combined glycosides (30 g) were chromatographed on a column containing 800 g of silica gel in system 1. The elution process was monitored by the TLC method on silica gel in the same system. Fractions with similar compositions were combined and evaporated. This gave 4.5 g of a mixture of calendulosides C and D and 9.4 g of a mixture of calendulosides D and E, the rechromatography of which in systems 1 and 2 yielded 3.6 g of (I) and 4.6 g of (II). These calendulosides, after purification with activated carbon, were recrystallized from methanol. Calenduloside C (I) had mp 226-228° C, $[\alpha]_D^{27}$ +32° (c 0.5). IR spectrum, cm^{-1}: 1700 (COOH) and 3450 (OH).

Found: mol. wt. 990.4, 980.5 (spectrophotometrically) [9]. $C_{48}H_{78}O_{18} \cdot 3H_2O$. Calculated: mol. wt. 997.2.

After drying by azeotropic distillation with toluene the substance had the composition $C_{48}H_{78}O_{18} \cdot 2H_2O$. The melting point of the acetate of (I), $C_{68}H_{98}O_{28}$, was 183-185° C, $[\alpha]_D^{30}$ + 26.7° (c 0.3).

Calenduloside D (II) also formed a crystalline hydrate with mp 245-247° C, $[\alpha]_D^{27}$ + 16° (c 0.5). IR spec-

trum, cm^{-1}: 1760  and 3450 (OH).

Found: mol. wt. 1153, 1130 (spectrophotometrically) [9]. $C_{54}H_{88}O_{23} \cdot 1.5\ H_2O$. Calculated: mol. wt. 1132.3.

Melting point of the acetate of (II), $C_{82}H_{116}O_{37}$: 156-158° C, $[\alpha]_D^{30}$ 0° (c 0.4).

Identification of the Aglycone and of the Monosaccharides. A. Compound (I) (0.96 g) was hydrolyzed with 40 ml of a mixture of methanol and 10% HCl (1 : 1) in a tube (100° C, 3 h). The methanol was distilled off from the reaction mixture and the residue was filtered off and washed with water to neutrality. Yield 0.45 g, mp 306-308° C. From its chromatographic mobility, a mixed melting point, and its IR spectrum, the substance was identical with an authentic sample of oleanolic acid.

The filtrate was neutralized with Dowex-3 (OH^- form), and evaporated to dryness to give 0.42 g of a mixture of sugars. D-Glucose and D-galactose were identified by PC (systems 3 and 4) and TLC (system 5). Chromatography in a column containing 20 g of silica gel in system 5 yielded 0.26 g of D-glucose with $[\alpha]_D^{20}$ + 50° (c 1; water) and 0.11 g of D-galactose with $[\alpha]_D^{20}$ + 78° (c 1; water). Literature data for D-glucose – $[\alpha]_D^{20}$ + 52.7° (water) [10] and for D-galactose – $[\alpha]_D^{20}$ + 80° (c 1; water) [11].

B. Compound (II) (0.5 g) was hydrolyzed under the same conditions. This gave 0.2 g of oleanolic acid, 0.17 g of D-glucose, and 0.05 g of D-galactose.

Partial Acid Hydrolysis. A. 1) A solution of 1 g of (I) in 30 ml of methanol and 30 ml of Kiliani's mixture [concentrated $HCl-CH_3COOH-H_2O$ (10 : 35 : 55)] was boiled for 3 h. Then 50 ml of water was added to the reaction mixture and the precipitate was filtered off. Yield 0.9 g. On chromatography on a column containing 50 g of silica gel successively in systems 6 and 1, 0.02 g of oleanolic acid and 0.1 g of a monoside (III), mp 240-242° C, $[\alpha]_D^{20}$ + 55° (c 0.5), identical with the 3-O-β-D-glucopyranoside of oleanolic acid [1] were obtained. On hydrolysis with 10% HCl, D-glucose (systems 3, 4, and 6) and 0.15 g of the bioside (IV) with mp 272-275° C, $[\alpha]_D^{20}$ + 32° (c 0.5), were identified in the hydrolyzate.

Found: mol. wt. 789.3, 844.4 (spectrophotometrically) [9]. $C_{42}H_{68}O_{13} \cdot 2H_2O$. Calculated: mol. wt. 817.

On hydrolysis, D-glucose and 0.03 g of an amorphous bioside (V) with mp 268-278° C were obtained; the hydrolysis of the latter yielded D-glucose and D-galactose.

2) A solution of 0.5 g of (I) in 45 ml of a mixture of methanol and 10% HCl (1 : 2) was boiled for 7 h. The reaction product was treated as described above. After chromatography on a column containing 25 g of silica gel, 0.1 g of the bioside (V) with mp 273-275° C, chromatographically identical with the (IV) isolated previously, and 0.31 g of the initial glycoside (I) were obtained. D-galactose was found in the hydrolyzate.

B. Compound (II) (0.8 g) in 50 ml of methanol was hydrolyzed with 100 ml of Kiliani's mixture (100° C, 5 h), and by the treatment described 0.05 g of oleanolic acid, 0.07 g of the monoside (III) with mp 240-242° C, 0.08 g of the bioside (IV) with mp 273-275° C, and 0.48 g of calenduloside C with mp 224-226° C, $[\alpha]_D^{25}$ + 30° (c 0.5), were isolated. In its chromatographic behaviour and IR spectrum, the last-mentioned product was identical with the (I) isolated from the roots.

Alkaline Hydrolysis. A. Compound (I) (0.15 g) was hydrolyzed with 15 ml of a mixture of methanol and 5% KOH (1 : 1) (100° C, 6 h). The solution was neutralized with Dowex 50 W 100/200 (H^+ form). This gave unchanged calenduloside C.

B. The hydrolysis of 0.1 g of (II) was performed similarly. This gave 0.07 g of a glycoside identical in chromatographic behavior and physicochemical constants with (I). D-Glucose was found in the hydrolyzate by PC and TLC.

Acetolysis. A. A mixture of 0.1 g of (I) in 5 ml of acetic anhydride and 5 ml of 2% H_2SO_4 solution in acetic anhydride was left at room temperature for 48 h. Then the mixture was diluted with 25 ml of water, neutralized with Na_2CO_3, and extracted with chloroform. The residue after the evaporation of the extract was dissolved in 5 ml of absolute methanol, and gaseous ammonia was passed through the solution for 30 min. D-glucose and D-galactose were identified by PC and TLC.

B. The acetolysis of 0.1 g of (II) as described above led to the detection of D-glucose and D-galactose.

Treatment with Diazomethane. A. A solution of 0.05 g of (I) in 5 ml of methanol was methylated with 2.5 ml of an ethereal solution of diazomethane. The mixture was left at room temperature for 24 h. Then the solvent was distilled off and the residue was hydrolyzed with 2.5 ml of 10% HCl in a sealed tube (100° C, 5 h). After the mixture had been cooled, methyl oleanolate was filtered off (mp 198-200° C). It was identical with an authentic sample (TLC in system 7).

B. Compound (II) (0.05 g) was treated with diazomethane as described above, and after hydrolysis oleanolic acid was identified with a marker.

Periodate Oxidation. A. A solution of 0.05 g of (I) in 50 ml of methanol was treated with 25 ml of acetate buffer (pH 3.6) and 0.2 g of $NaIO_4$, and the mixture was left in the dark at +6° C for three days. Then a few drops of ethylene glycol was added and the mixture was filtered. The filtrate was evaporated to dryness, the residue was hydrolyzed with 2 ml of 10% HCl (100° C, 5 h), and oleanolic acid and D-glucose were identified.

B. A solution of 0.1 g of (II) in 70 ml of water was treated with 0.25 g of $NaIO_4$ and, as described, D-glucose was found in the reaction product.

C. When 0.03 g of (IV) was oxidized with 0.08 g of $NaIO_4$ in 10 ml of methanol, no D-glucose was found.

D. When 0.03 g of (V) was subjected to periodate oxidation as described above, D-glucose was found.

Methylation. A. To a solution of 0.4 g of (I) in 8 ml of dimethyl sulfoxide were added 0.4 g of sodium hydride and 2.5 ml of methyl iodide. The reaction mixture was stirred at room temperature for 14 h and was then concentrated in vacuum. The residue was diluted with water and extracted with chloroform. The extracts were washed with $Na_2S_2O_3$ solution and with water and were evaporated to dryness. Chromatography on a column of silica gel (2 × 40 cm) in system 8 yielded 0.42 g of the methyl ester of (I), $C_{59}H_{100}O_{18}$, with mp 176-178° C, $[\alpha]_D^{20}$ + 42.9 (c 0.5).

B. The methylation of 0.5 g of (II) as described above yielded 0.58 g of the methyl ester (II), $C_{68}H_{116}O_{23}$, mp 211-213° C, $[\alpha]_D^{20}$ +30° (c 0.5).

C. The methylation of 40 mg of (IV) and chromatography on a column containing 10 g of silica gel in system 12 yielded 30 g of the amorphous methyl ester (IV) with mp 101-103° C, $[\alpha]_D^{20}$ + 16.5° (c 0.52).

D. Similarly, the methylation of 30 mg of (V) yielded 30 mg of the permethylate of (V) with mp 82-84° C, $[\alpha]_D^{20}$ +15° (c 0.5).

Hydrolysis of the Methyl Ethers and Identification of the Methyl Glycosides. A. The permethylate of (I) (0.3 g) in 40 ml of methanol was boiled with 6 ml of concentrated HCl for 6 h, and then 18 ml of water was added and the mixture was boiled for another 3 h. After cooling, 0.12 g of methyl oleanolate was filtered off. The filtrate was neutralized with Dowex-3 (HCO_3^- form) and evaporated to dryness, giving 0.15 g of methyl glycosides. By PC (system 9), TLC (systems 10 and 11), and GLC, 2,3,4,6-tetra-O-methyl-D-glucopyranose, 2,3,4,6-tetra-O-methyl-D-galactopyranose, and 4,6-di-O-methyl-D-glucopyranose were identified (in the presence of markers). Chromatography on a column containing 40 g of silica gel in system 11 yielded 38 mg of 2,3,4,6-tetra-O-methyl-D-glucopyranose with $[\alpha]_D^{20}$ +80° (c 1; water) (literature data: $[\alpha]_D^{20}$ +79° (water) [12]); 12 mg of a mixture of 2,3,4,6-tetra-O-methyl-D-glucopyranose and 2,3,4,6-tetra-O-methyl-D-galactopyranose; 30 mg of 2,3,4,6-tetra-O-methyl-D-galactopyranose with $[\alpha]_D^{20}$ +105° (c 1; water) (literature data: $[\alpha]_D$ + 117.8° (water) [13]); and 27 mg of 4,6-di-O-methyl-D-glucopyranose with mp 160-162° C, $[\alpha]_D^{20}$ +65° (c 1; water) (literature data: mp 158-160° C (methanol–ethyl acetate), $[\alpha]_D^{20}$ + 63.7° (water) [14]).

4,6-Di-O-methyl-D-glucopyranose (13 mg) was dissolved in 2.5 ml of methanol, and 2 ml of water and about 3 mg of $NaBD_4$ were added and the mixture was left at +6° C for 10 h. Then it was neutralized with Dowex-3 (H^+ form) and evaporated to dryness with the addition of 5-ml portions of methanol four times. The residue was acetylated and was purified on a column of silica gel. The mass spectrum contained the following peaks: 43 (110), 45 (75), 71 (25), 74 (20), 85 (10), 86 (27), 87 (42), 88 (12), 98 (12), 99 (22), 101 (67), 111(5), 112 (10), 113 (5), 115 (7), 116 (7), 117 (7), 118 (5), 128 (37), 129 (100), 130 (13), 143 (7), 157 (6), 159 (4), 160 (7), 161 (82), 185 (3), 189 (5), 202 (7), 203 (3), 217 (1), 218 (1, 5), 220 (1), 232 (2), 243 (1), 245 (1), 261 (4), 262 (30), 305 (0.37), 320 (0.75), 334 (0.37), 379 (0.07%). These peaks and the ratio of their intensities correspond to literature information for 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl-D-sorbitol [6].

B. When 0.2 g of the permethylate of (II) was hydrolyzed, 2,3,4,6-tetra-O-methyl-D-galactopyranose, 2,3,4,6-tetra-O-methyl-D-glucopyranose, and 4,6-di-O-methyl-D-glucopyranose were identified (with markers) by PC, TLC, and GLC.

C. The permethylate of (IV) (25 mg) was hydrolyzed with 2.5 ml of 5% HCl in methanol in a sealed tube (100° C, 7 h). In the presence of markers, methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside and methyl 3,4,6-tri-O-methyl-D-glucopyranoside were identified by GLC.

D. After the hydrolysis of 25 mg of the permethylate of (V), as described above, methyl 2,3,4,6-tetra-O-methyl-D-galactopyranoside and methyl 2,4,6-tri-O-methyl-D-glucopyranoside were identified by GLC.

SUMMARY

The structure of new triterpene glycosides from the roots of potmarigold calendula (C. officinalis) has been established: oleanolic acid 3-{[galactopyranosido-(1→3)] [glucopyranosido-(1→2)]-β-D-glucopyranoside} (calenduloside C) and the 28-acyl-β-D-glucopyranoside of calenduloside C (calenduloside D).

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