Phytochemistry, 1967, Vol. 6, pp. 69 to 75. Pergamon Press Ltd. Printed in England

THE STRUCTURE OF TRITERPENIC GLYCOSIDES FROM THE FLOWERS OF *CALENDULA OFFICINALIS* L.

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(*Received* 1 *June* 1966)

Abstract—From the flowers of *Calendula officinalis* L., five glycosides of oleanolic acid were isolated and their structures determined as: 3-glucuronide; 3-(galactosyl-glucuronide); 3-(galactosyl-glucuronide); 17-glucoside; 3-(galactosyl-(glucosyl)-glucuronide); and 3-(galactosyl-(glucosyl)-glucuronide); 17-glucoside.

ALTHOUGH oleanolic acid, both free or bound in the form of glycosides, has been found in a great number of plants, very little has been done to elucidate the structure of the carbohydrate component of oleanolic acid glycosides. The glycosides known up till now contain hexoses, pentoses, methylpentoses and uronic acids, the molar ratio of sugars to oleanolic acid ranging from 2 to 10. In recent years methods have been elaborated for the isolation of pure glycosides of oleanolic acid from plants and the structures of some of these compounds have been determined.[1–3]

Winterstein and Stein in 1931 found that oleanolic acid, bound in the form of glycosides, occurs in the flowers of *Calendula officinalis*.[4] The carbohydrate component of a fraction of oleanolic acid glycosides from this plant after a partial alkaline hydrolysis was later found to be composed of glucose, galactose and uronic acid.[5]

The aim of the present work was to determine the structure of pure, individual glycosides isolated from the flowers of *C. officinalis*.

RESULTS AND DISCUSSION

The method of isolation previously used for obtaining oleanolic acid glycosides from *C. officinalis* flowers, based on repeated precipitation with cold acetone from a methanol extract[4, 6] did not remove the considerable amounts of other types of terpenoid glycosides present.[7] These compounds possess similar R_f values on thin-layer and paper chromatograms and give a positive reaction with $SbCl_3$. Complete separation of oleanolic acid glycosides from other terpenoic glycosides was achieved by precipitating the former at pH 6·2 by lead acetate.

Chromatographic analysis of the fraction obtained when the lead precipitate was

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[5] Z. KASPRZYKÓWNA and B. BUŁHAK, *4th Intern. Congr. Biochem.*, Vien (1958), Abstr. of Commun., p. 8.
[6] Z. KASPRZYKÓWNA, *Prace Głównego Inst. Chemii Przemysł.* **1**, 39 (1951).
[7] Z. KASPRZYK, M. FONBERG, E. POLUS, G. RACZYŃSKI and A. RAFALSKI, *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **13**, 77 (1965).

decomposed with H_2S, using TLC in three solvent systems (Table 1), showed the presence of five compounds (*A–F*) giving a violet-red coloration with dilute sulphuric acid. *Compound F* was present in much smaller amounts than the other four substances.

The fraction was separated into three bands by descending preparative paper chromatography in solvent V (Table 1). Band 1 contained only *glycoside A*, band 2—a mixture of *glycosides B* and *C*, and band 3 *glycoside D*. Band 2 was further separated in the solvent VI. Chromatography of the isolated *glycosides A* and *B* using this system, and final rechromatography of all compounds in solvent VII gave the glycosides free from flavonoid and carbohydrate impurities. The glycosides obtained, amounting to 20 per cent of the initial fraction, were used for further studies. Because of the small amount of *glycoside F* present, it was not isolated from the crude glycoside fraction.

TABLE 1. R_f VALUES OF OLEANOLIC ACID GLYCOSIDES FROM *C. officinalis* ON THIN-LAYER(TLC) AND PAPER(PC)CHROMATOGRAMS IN ASCENDING TECHNIQUE

Solvent system*		$R_f \times 100$ of glycosides					
		A	*B*	*C*	*D*	*E*	*F*
I *n*-Propanol	80	14	18	25	34	34	39
14% ammonia	20						
II. Chloroform	61	16	22	22	28	24	33
Methanol	32						
Water	7						
III. Ethyl acetate	3	14	19	19	28	28	42
Acetic acid	1						
Water	3						
V. Ethyl acetate	3	4	9	9	16	16	23
Pyridine	1						
Water	3						
VI. *n*-Butanol	4	15	14	27	29	29	51
Ethanol	1						
7% ammonia	1						
VII. Ethyl acetate	2	31	40	38	46	45	54
Pyridine	1						
Water	2						

* I–III on TLC, V–VII on paper.

The aglycone of all the compounds examined was identified as oleanic acid by means of a complete acid hydrolysis of the glycosides followed by TLC in solvent IV. Paper chromatography of the sugar components of the hydrolysates showed that *glycosides A*, *B* and *C* contain glucose, galactose and glucuronic acid, whereas *glycoside D* contains only galactose and glucuronic acid. Quantitative analysis, using a colorimetric method for the determination of oleanolic acid as a complex with $FeCl_3$,[8] showed that *glycoside A* contains four, *glycosides B* and *C* three, and *glycoside D* two molecules of sugar. Densitometric comparison of the chromatograms of the sugar components in the hydrolysates of *glycosides A* and *B* showed that the ratio of glucose to galactose in *glycoside A* is 2:1 and in *glycoside B* is 1:1. The

[8] M. FONBERG and Z. KASPRZYK, *Chem. Anal., Warsaw* **10**, 1181 (1965).

results (Table 2) allow us to conclude that the molar ratio of oleanolic acid: glucose: galactose: glucuronic acid is 1:2:1:1 for *glycoside A*, 1:1:1:1 for *glycosides B* and *C* and 1:0:1:1 for *glycoside D*.

In order to determine where the sugar is linked to the aglycone (C-3 or C-17) all the glycosides were treated with diazomethane followed by acid hydrolysis.[1] Thin-layer chromatography in solvent IV showed that *glycosides A* and *C* yielded free oleanolic acid (R_f 0·43), whereas *glycosides B* and *D* gave the methyl ester (R_f 0·65) thus showing that the carboxyl group is free only in the two latter compounds. Application of alkaline hydrolysis (15% KOH) led to the same conclusion. Only *glycosides A* and *C* were hydrolysed out of four compounds tested. It was also found that under these conditions *glycoside A* is converted into *B*, and *glycoside C* into *D*.

TABLE 2. PROPERTIES OF OLEANOLIC ACID GLYCOSIDES FROM *C. officinalis*

Glycoside	% Oleanolic acid	Sugar components per mol. oleanolic acid	Aglycone after hydrolysis of methylated glycosides
A	42	2 glucose, 1 galactose, 1 glucuronic acid	Oleanolic acid
B	54	1 glucose, 1 galactose, 1 glucuronic acid	Me ester
C	51	1 glucose, 1 galactose, 1 glucuronic acid	Oleanolic acid
D	62	1 galactose, 1 glucuronic acid	Me ester
E	56	1 glucose, 1 glucuronic acid	Me ester
F	70	1 glucuronic acid	Me ester

Comparison of these results with the carbohydrate composition of individual glycosides indicates that the carboxyl group of oleanolic acid in *glycosides A* and *C* is linked to a sugar which is split during alkaline hydrolysis to yield *glycosides B* and *D*. From the differences in the analysis (Table 2) it is apparent that the carboxyl groups of *glycosides A* and *C* are linked to one molecule of glucose.

Hydrolysis with 2·5% sulphuric acid at 100° for 3 hr caused degradation of *glycosides A*, *B*, *C* and *D*, but only small amounts of free oleanolic acid were found in the hydrolysates. Thin-layer chromatography in solvents I, II and III showed, that all four glycosides tested yielded another glycoside, which was less hydrophylic. This glycoside has an R_f value identical with that of *compound F*, which was shown to be present in small amounts in the starting material (crude glycoside fraction). *Glycosides A* and *B* yielded, in addition, another intermediate hydrolysis product with R_f values similar to *glycoside D* (except in solvent II). This compound, *E*, was not observed in the crude fraction. The intermediate products (*E*

and *F*) were prepared in greater quantities by partial acid hydrolysis of the crude glycoside fraction followed by chromatography in system VII.

Chromatographic identification of the carbohydrates after complete acid hydrolysis showed, that *glycoside E* contains glucose and glucuronic acid, but *glycoside F* glucuronic acid only.

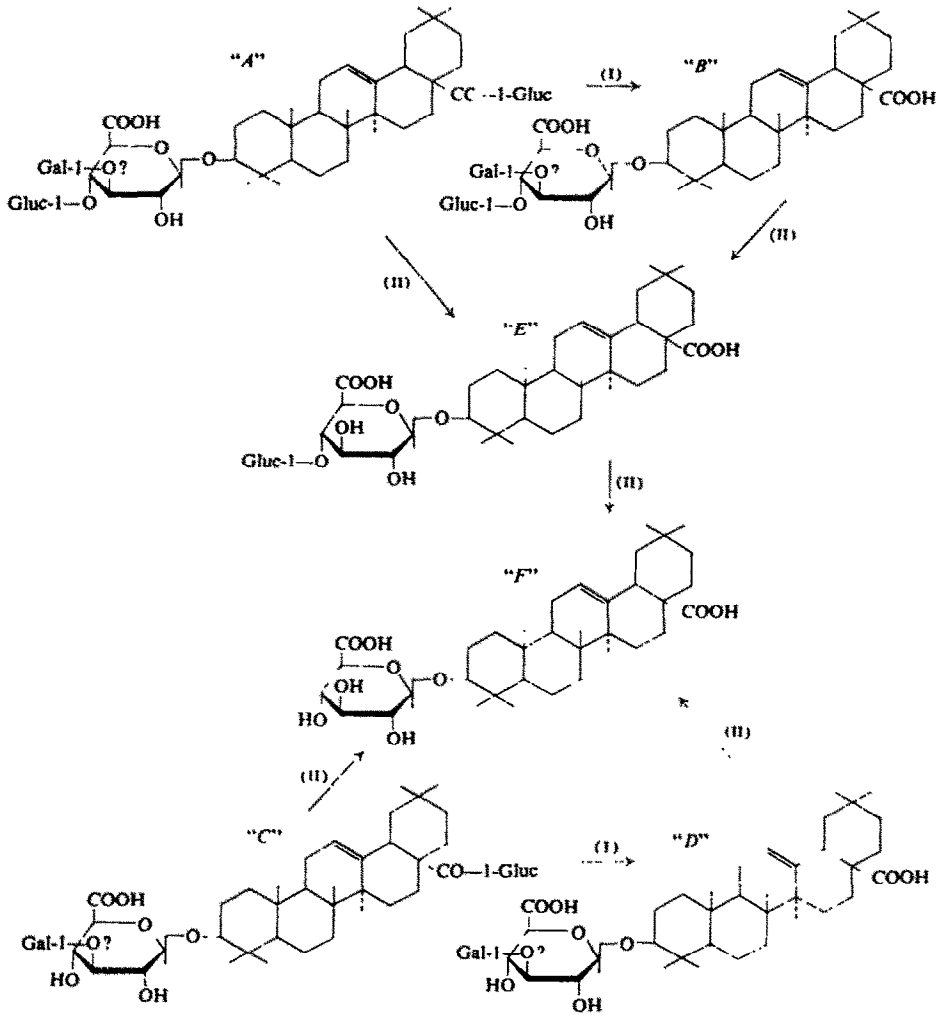

FIG. 1. I—ALKALINE HYDROLYSIS (15% KOH, 100°, 75 MIN) II—PARTIAL ACID HYDROLYSIS (2·5% H_2SO_4, 100°, 3·5 HR) Gluc—GLUCOSE, Gal—GALACTOSE.

Glycoside F, after desalting on a cation exchange resin, was recrystallized from diluted ethanol, m.p. 197–199°, $[\alpha]_D^{18} + 18{\cdot}5° \pm 3°$ ($c = 0{\cdot}5$ in ethanol). *Glycoside F* was methylated with diazomethane yielding methylation product, m.p. 201–208° $[\alpha]_D^{18} + 10{\cdot}5° \pm 2°$ ($c = 0{\cdot}5$ in chloroform).

It can be assumed on the basis of results obtained, including elementary analysis, that *glycoside F*, which occurs in small amounts in *C. officinalis* flowers, is identical with the

glucuronide of oleanolic acid formed as a result of incomplete hydrolysis of all other glycosides.

Kochetkov *et al.*[1–3] isolated three glycosides of oleanolic acid from a methanol extract of *Aralia manschurica* roots, called aralosides A, B[2] and C.[3] A compound with the structure of a 3-β-D-glucuronide of oleanolic acid was isolated from hydrolysates of aralosides in the form of its dimethyl ester.

Comparison of the physical properties of the dimethyl ester of *glycoside F* isolated from *C. officinalis* with those of the dimethyl ester of oleanolic acid 3-β-D-glucuronide obtained by Kochetkov *et al.* (m.p. 200–205°, $[\alpha]_D^{20}$ + 11·5° to + 13·8° in chloroform) showed that these are identical compounds.

The structures of the oleanolic acid glycosides isolated from calendula flowers and that of the aralosides is substantially the same. Oleanolic acid 3-β-D-glucuronide is a basic unit of all these compounds. In *glycosides A* and *C* from calendula (as in all aralosides) the carboxyl group of oleanolic acid is linked to a molecule of glucose. The differences concern mainly those sugars which are attached to the glucuronic acid molecule. Araloside C and *glycoside A* from calendula show great similarities. Kochetkov *et al.* established, on the basis of analysis of the methyl derivatives of the carbohydrates after total hydrolysis of methylated araloside C, that in araloside C galactose and xylose are bound to glucuronic acid at positions 3 and 4 respectively. Mild acid hydrolysis of this glycoside yielded the 3-β-D-glucuronide of oleanolic acid together with a diglycoside of oleanolic acid containing glucuronic acid and xylose.[3] Oleanolic acid 3-β-D-glucuronide as well as a diglycoside containing glucose and glucuronic acid (*glycoside E*) are formed from both *glycosides A* and *B* from calendula under similar conditions. Moreover the resulting diglycoside (*E*) when subjected to mild acid hydrolysis is only partially degraded to the monoglucuronide (*F*) and free oleanolic acid, whereas the diglycoside *D*, under these conditions, is completely hydrolysed to the monoglucuronide and the free acid. Since the *glycosides E* and *D* differ only in the aldohexose moiety (*glycoside E* contains glucose and *glycoside D* galactose), one can assume, that in *glycosides A*, *B* and *E* the molecule of glucose is bound to glucuronic acid at position 4, whereas the molecule of galactose in *glycosides A*, *B*, *C* and *D* is at position 3 (or 2).

Figure 1 shows the structures of oleanolic acid glycosides from *C. officinalis* flowers and their interrelationships.

MATERIAL AND METHODS

Isolation of Crude Oleanolic Acid Glycosides

500 g of dry lingulate flowers of *Calendula officinalis* was extracted with ethyl ether and then with methanol. The methanol extract was concentrated in a flash evaporator to 350 ml, and after addition of 150 ml of water a fraction of crude glycosides was precipitated with lead acetate at pH 6·2. The washed, dried precipitate was extracted with 3 × 250 ml portions of boiling methanol for 15 min. The extracts were discarded and the residue decomposed with H_2S in 500 ml of 80% methanol. The yield of a crude fraction of oleanolic acid glycosides was 27·5 g.

Thin-layer Chromatography

Thin-layer chromatography was performed on silica gel coated plates (Kieselgel G nach Stahl, Merck) in the solvent systems shown in Table 1. Chromatograms were sprayed with

10% H_2SO_4 and heated for 5 min at 140° revealing the oleanolic acid glycosides as violet–red spots.

Chromatography of oleanolic acid and its methyl ester was carried out in the solvent system IV, chloroform:methanol (95:5). The chromatograms were heated for 5 min at 105° and then immediately sprayed with 20% $SbCl_3$ solution in chloroform containing 1% thionyl chloride.

Preparative Paper Chromatography

The separation of a crude glycoside fraction (150 mg) was carried out on Whatman 3 paper for 48 hr in the solvent system V. (Table 1). The bands were detected by spraying a side strip with 20% SbCl in 1% methanolic solution of HCl and heating during 3 min at 100°. Glycosides, separated into three main fractions, were eluted with hot methanol, and rechromatographed for 24 hr in solvent VI; the compounds (50 mg) were finally rechromatographed in solvent VII (Table 1). The glycosides isolated, amounting to about 20% of the original fraction, were almost colourless and amorphous.

Preparation of Monoglycoside F and Diglycoside E

A crude glycoside fraction (5 g) was heated at 100° with 300 ml of 2·5% aqueous H_2SO_4 for 3·5 hr. The precipitate which formed was filtered, and extracted three times with 200-ml portions of boiling ethyl acetate for 15 min. The combined extracts were concentrated to dryness and the residue (2·4 g) chromatographed twice on Whatman 3 paper in system VII. Pure glucosides E and F were obtained in a yield of 5 and 24% of the chromatographed material respectively. To remove inorganic impurities, monoglycoside F was dissolved in 50% ethanol and passed through a column with Zeo Carb 225 (H^+ form). The substance was then recrystallized from aqueous ethanol, m.p. 197–199° (decomp.), $[\alpha]_D^{18} + 18{\cdot}5° \pm 3°$ ($c = 0{\cdot}5$ in ethanol). (Found: C, 68·32; H, 8·95. Calc. for $C_{36}H_{56}O_9$: C, 68·30; H, 8·91%.)

Preparation of Methyl Ester of Glycoside F

Glycoside F (100 mg) was methylated with diazomethane during 48 hr at 2°. After removal of solvent the residue was crystallized from 70% ethanol yielding 70 mg of ester, m.p. 201–208°, $[\alpha]_D^{18} + 10{\cdot}5° \pm 2°$ ($c = 0{\cdot}5$ in chloroform). (Found: C, 69·40; H, 9·22. Calc. for $C_{38}H_{60}O_9$: C, 69·05; H, 9·15%.)

Partial Acid Hydrolysis

Samples (15 mg) of all glycosides were heated with 1 ml of 2·5% H_2SO_4 in sealed tubes for 3·5 hr at 100°. Hydrolysates were extracted with n-butanol and the extracts were subjected to TLC in the solvent systems I, II and III.

Alkaline Hydrolysis

The glycosides (15 mg) were heated on a water bath with 5 ml of 15% KOH for 75 min. After acidification with HCl to pH 5 the products extracted with n-butanol and the extract subjected to TLC in systems I, II and III and to paper chromatography in system VII.

Complete Acid Hydrolysis

Samples (25 mg) of all glycosides were heated in sealed tubes at 100° for 4 hr with 2 ml of a mixture consisting of HCl:acetic acid:water (10:35:55). The precipitate was filtered,

dissolved in ethyl ether and then chromatographed on silica gel in solvent IV. The filtrates were desalted by passing through a column of Amberlite IR-45 (OH^- form) and Amberlite IR-120 (H^+ form). The effluents were concentrated to about 0·5 ml and used for identification of the sugars.

Chromatographic Identification of Sugars

Sugars were identified by means of ascending chromatography using standard sugars on Whatman 1 paper in the solvent systems n-butanol:benzene:pyridine:water (5:1:3:3), and ethyl acetate:pyridine:water (2:1:2). Chromatograms were developed twice during 24 hr, and sugars were detected with aniline phthalate. The ratio of glucose to galactose in the hydrolysates of glycosides A and B was determined densitometrically using Chromatograph Joyce, Loebl Ltd., Apparatus (England).

Quantitative Determination of Oleanolic Acid

A methanolic solution (1·0 ml), containing 200–400 μg of the glycoside, was taken to dryness and 3 ml of the reagent was added (50·4 mg anhydrous $FeCl_3$ in 1 ml of water made up to 100 ml with acetic acid distilled over CrO_3) followed by 1·5 ml of conc. H_2SO_4. The glass-stoppered tubes were heated for 1 hr at 100°, and the absorbancy of the complex formed measured at 445 nm on a Carl Zeiss spectrophotometer VSU-1.[8]

Methylation of Glycosides and Hydrolysis of Methyl Derivatives

Samples of glycosides (20 mg) were dissolved in 5 ml of methanol, an excess of diazomethane solution in ether was added and the mixture was left at 2° for 24 hr. More diazomethane was added and the whole allowed to stand for further 24 hr at 2°. The residue, after removal of solvent, was hydrolysed as before and examined for the presence of oleanolic acid or its methyl ester on TLC in solvent IV.