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THE STRUCTURE OF GLYCOSIDES OF OLEANOLIC ACID ISOLATED FROM THE ROOTS OF *CALENDULA OFFICINALIS*

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Abstract—A new series of glycosides of oleanolic acid was discovered in the roots of old *Calendula officinalis* plants. These compounds derivatives of 3-glucoside of oleanolic acid are different, from those previously isolated from the flowers of this plant, derivatives of 3-glucuronoside of oleanolic acid. The sugar components of 8 representatives of the new series are glucose and galactose in following ratios: in glucoside I, 1:0; in II, 1:1; in III, 1:2; in IV, 2:1; in V, 3:1; in VI, 3:2; in VII, 4:1; in VIII, 4:1. The sugars in I–VII were attached only in position 3 of oleanolic acid, but in VIII one glucose molecule was joined to 28-carboxyl of oleanolic acid. I was identified as 3-monoglucoside and II as 3-(4′-galactosyl)-glucoside of oleanolic acid. Besides these compounds, 6′-methyl ester of 3-glucuronoside of oleanolic acid was found in *Calendula* roots.

INTRODUCTION

IT HAS been shown in previous work,[1] that flowers of *Calendula officinalis* contain a series of five glycosides of oleanolic acid, namely, 3-monoglucuronoside (glycoside F), 3-(3′-galactosyl)-glucuronoside (D), 3-(3′-galactosyl)glucuronoside, 28-glucoside (C), 3-(3′-galactosyl,4′-glucosyl)-glucuronoside (B), and 3-(3′-galactosyl,4′-glucosyl)-glucuronoside, 28-glucoside (A). The same compounds were found in green parts and roots of the plant in variable quantities, depending on the organ and the period of growth.[2] At the post flowering period it was observed in roots that oleanolic acid glycosides with different chromatographic properties were found.

In 1969 Zinkevich *et al.*[3] isolated from the roots of *C. officinalis* eight glycosides of oleanolic acid and found that one of them is a diglycoside with one molecule of glucose and one molecule of galactose attached in position 3 of oleanolic acid.

The aim of the present work was to isolate and to determine the structure of these glycosides.

RESULTS AND DISCUSSION

From ethyl ether extract of the dry *Calendula* roots, collected in late autumn, free oleanolic acid was isolated as the sodium salt. It was the first time that we identified free oleanolic acid in *Calendula*. Our previous investigations carried out on different organs of this plant at different periods of growth[4] indicated that oleanolic acid is present only in the form of glycosides.

From the methanol extract of defatted roots, a fraction of glycosides was obtained and separated on the column with silica gel, yielding nine glycosides with unknown structure

[1] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* **6**, 69 (1967).
[2] Z. KASPRZYK, M. FONBERG, P. CHOMCZYNSKI and M. KONARSKA, *FEBS Abstracts*, 25 (1967).
[3] L. P. ZINKEVICH, E. P. LIBIZOV and N. J. BANKOWSKI, *Khim. Prir. Soedin.* **5**, 58 (1969).
[4] Z. KASPRZYK and M. FONBERG-BROCZEK, *Physiol. Plantarum* **20**, 321 (1967).

and small quantity of the glycosides typical for flowers. Glycosides were purified by TLC in different solvent systems to yield homogeneous compounds.

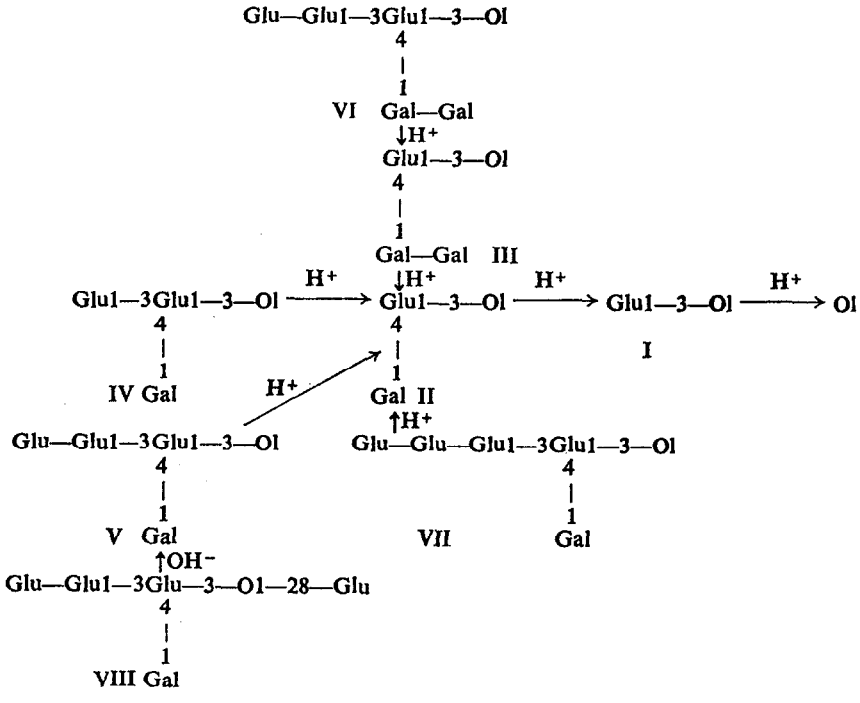
Only one of the new glycoside (G) yielded, after acid hydrolysis, oleanolic acid and glucuronic acid and, after alkaline hydrolysis, a product identical by chromatographic criteria with the 3-monoglucuronoside of oleanolic acid (glycoside F). Methylation of glycoside G with diazomethane yielded product identical with the methylation product of glycoside F. Elementary analysis indicated the structure of the monomethyl ester of the monoglucuronoside of oleanolic acid, but the fact that the product of the acid hydrolysis of glycoside G was oleanolic acid and not its methyl ester indicated that it is the carboxyl group of glucuronic acid which is methylated in G. Therefore this compound is the 6′-*O*-methyl-3-glucuronoside of oleanolic acid.

The remaining eight glycosides did not contain glucuronic acid. Glucose and galactose were identified as their sugar components after acid hydrolysis. Densitometric determination of the molar ratios of glucose to galactose after chromatographic separation of the hydrolysis products of eight glycosides were as follows: in I, 1:0; in II, 1:1; in III, 1:2; in IV, 2:1; in V, 3:1; in VI, 3:2; in VII, 4:1 and in VIII, 4:1. Glycosides VII and VIII with different positions on chromatograms have the same molar ratio of glucose to galactose 4:1. The acid hydrolysis of the glycosides methylated with diazomethane yielded as the product of the aglycone moiety of glycosides I–VII methyl ester of oleanolic acid and of glycoside VIII–free oleanolic acid. This result indicates that in glycosides I–VII the carboxyl group of oleanolic acid is free and the sugar components are bound in position 3. Alkaline hydrolysis of VIII yielded V, which shows that in VIII sugars are attached to both functional groups of oleanolic acid, glucose being bound with carboxyl group in position 28.

Action of 1 % HCl on individual glycosides resulted, in addition to the small amount of aglycone, in the formation of the products of the incomplete glycoside hydrolysis. VI was transformed to III, II and I, glycosides IV, V and VII to II and I, glycoside VIII as it was shown to V and then to II and I.

It was concluded from the position of glycosides I and II that I can be a monoglycoside and II a diglycoside. They differ from the previously isolated derivatives of 3-glucuronosides of oleanolic acid only by the presence of glucose in the place of glucuronic acid. The structures of I and II were proved in the following way. The samples of the glycosides I, II, F and D were methylated with dioazomethane and then subjected to the reduction with $LiAlH_2$. In these conditions the methyl ester of oleanolic acid was reduced to erythrodiol and methyl ester of glucuronic acid to glucose. As it was expected a 3-monoglucoside of erythrodiol was obtained from glycosides I and F. The identity of glycosides and of their hydrolysis products (i.e. erythrodiol and sugars) was proved by chromatography. The chromatographic identity of the reduction products of the glycosides II and D indicated that in both galactose could be bound in position 3′ of the glucose molecule but greater stability of II in the conditions of mild acid hydrolysis indicates that in II galactose is bound rather in position 4′ and hence it is a 3-(4′-galactosyl)-glucoside.

The data presented above allow us to propose a scheme of the structural relationship among the glycosides I–VIII, which is given in Fig. 1. The easy splitting of all glucose molecules, except those bound to the aglycone in position 3, with 1 % HCl (IV, V and VII to II and VI to III) indicates that they are most probably linked by 1–3 (or 2) bonds, which are more sensitive to mild acid hydrolysis than those in position 4. The same can be true for the second galactose molecule present in III and VI which is split by the weak acid after glucose but before galactose bound in position 4. The last steps of weak acid hydrolysis are the



Ol, oleanolic acid; Glu, glucose; Gal, galactose.

FIG. 1. THE STRUCTURE OF GLYCOSIDES OF OLEANOLIC ACID ISOLATED FROM THE ROOTS OF *Calendula officinalis* AND THEIR TRANSFORMATIONS IN THE CONDITIONS OF WEAK ACID (H^+) AND ALKALINE (OH^-) HYDROLYSIS.

splitting of galactose in position 4′ and then of glucose in position 3. The transformation of VIII to V is due to the alkaline hydrolysis of a glucose molecule bound in position 28.

EXPERIMENTAL

Isolation of Oleanolic Acid

The roots of *Calendula* plants at the post-flowering stage were collected in November (1·39 kg). After drying at 40–60° and disintegration, the material was extracted in the Soxhlet apparatus with Et_2O. From the extract oleanolic acid was separated as an insoluble Na salt. After decomposition with HCl and crystallization from MeOH, 50 mg of oleanolic acid, methyl ester m.p. 196–198° (ref. 198–200°), was obtained.

Isolation of Glycosides

The defatted roots were extracted with MeOH. The extract was evaporated to dryness and the residue extracted severalfold with *n*-BuOH. The extract was washed with H_2O, dried (Na_2SO_4) and evaporated to dryness (6·2 g). 1·3 g of this fraction was separated on a chromatographic column containing 700 g of silica gel (Serva 200–300 mesh) in the mixture of $CHCl_3$–MeOH–H_2O (to saturation) with MeOH increasing from 0 to 35%, collecting 1 l. fractions. The fractions of a similar composition were pooled and further purified by means of TLC on a silica gel (Kiselgel nach Merck) in systems: I, $CHCl_3$–MeOH–H_2O (61:32:7); II, ethyl acetate–HOAc–H_2O (3:1:3); III, *n*–PrOH–NH_4OH 14% (8:2). The purified compounds were crystallized from MeOH–H_2O. Obtained were nine glycosides, three of them crystalline. Glycoside G m.p. 177°. (Found C, 68·43; H, 9·51. Required for $C_{37}H_{58}O_9$: C, 68·6; H, 9·0%.) Glycoside I m.p. 179; glycoside III m.p. 210°. Other glycosides were obtained in the form of amorphous white substances.

Methylation

Et_2O solution of CH_2N_2 was added in excess to the MeOH solution of glycosides and left for 24 hr at room temp., the solvent being removed by distillation.

Reduction of Oleanolic Acid Glycosides to Erythrodiol Derivatives

1·0 ml of a suspension of 50 mg of $AlLiH_4$ in Et_2O was added to 5 mg of samples of glycosides F, D, I and II dissolved in 5 ml of dioxane and the mixture was boiled for 4 hr. After decomposition of excess $AlLiH_4$ the reaction mixture was filtered and the solvents removed by distillation.

Hydrolysis

Total acid hydrolysis was carried out by means of N H_2SO_4 in a mixture of dioxane–water (1:3) during 3 hr at 100°.[5] Partial acid hydrolysis was conducted using 1% HCl in 80% MeOH during 1 hr at 100°.

Alkaline hydrolysis was performed by means of 10% aqueous NaOH during 1 hr at 100°.

Investigation of the Hydrolysis Products

Aglycones obtained as the result of total acid hydrolysis were extracted with ethyl ether and compared on TLC in $CHCl_3$–MeOH (95:5) with oleanolic acid, oleanolic acid methyl ester or erythrodiol.

The products of incomplete acid hydrolysis and of alkaline hydrolysis were extracted by means of *n*-BuOH and then chromatographed in systems I, II and III.

The residue left after extraction of aglycone containing carbohydrates was neutralized on a column with Amberlite IR-45 (OH^-). The sugars were separated by means of paper chromatography on Whatman No. 1 developing three times in a system benzene–*n*-BuOH–pyridine–H_2O (1:5:3:3). The spots were visualized by means of $AgNO_3$.[6] The molar ratio of glucose to galactose was determined by densitometric method[7] using a Joyce-Loebl Ltd Apparatus.

[5] B. GESTETNER, Y. BIRK and A. BONDI, *Phytochem.* **5**, 799 (1966).
[6] W. E. TRAVELYAN, D. P. PROCTER and J. S. HARRISON, *Nature* **166**, 444 (1950).
[7] E. F. MCFARREN, K. BRAND and H. R. RUTKOWSKI, *Anal. Chem.* **23**, 1146 (1951).