ACYLATED C-GLYCOSIDES OF *Iris lactea*

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From the epigeal part of *Iris lactea* two acylated C-glycosylflavones not described in the literature have been isolated. On the basis of chemical transformations, and the results of UV, IR, and NMR spectroscopy, the following structures have been established for them: substance (I) — 5-hydroxy-4',7-dimethoxyflavone 6-C-[O-(2-acetyl-α-L-rhamnopyranosyl)-(1→2)-x-acetyl-β-D-glycopyranoside] (diacetylembinin); substance (II) — 5-hydroxy-4',7-dimethoxyflavone 6-C-[O(2-acetyl-α-L-rhamnopyranosyl)-(1→2)-D-glycopyranoside] (acetylembinin). The existence of rotamers for 6-C-glucoflavones has been established for the first time.

In an investigation of an ethyl acetate fraction of an ethanolic extract from the epigeal part of *Iris lactea* Pall. (*I. ensata* auc. non Thunb.)* we previously isolated four phenolcarboxylic acids and two dimethylapigenin C-glycosides [4-6]. One of them was characterized as embidin or 4',7-dimethylapigenin 6-C-[O-α-L-rhamnosyl-(1→2)-β-D-glucopyranoside] [7]. The second compound (substance (I)) was assigned to the acylated derivatives of embinin, but the nature of the acid and the position of its attachment to the carbohydrate component were not established [6].

Subsequently, when this fraction was chromatographed on a column of silica gel and eluted with chloroform containing 7% of methanol, another C-glycoside [substance (II)] was isolated.

Both compounds, (I), and (II), gave a negative Bryant test, which showed their glycosidic nature [8]. On paper chromatograms in UV light they appeared in the form of brown spots which had a yellow-green fluorescence in ammonia vapor. A free hydroxy group was detected in position 5 and substitution in position 4' and 7 was found from the results of UV spectra obtained with diagnostic reagents [9].

The IR spectra of both substances contained absorption bands of functional groups characteristic for flavonoids, and also bands at 3110 and 845 cm^{-1} relating to methoxy groups. In addition to this, substance (I) had a strong absorption band at 1782 cm^{-1} and substance (II) one at 1740 cm^{-1} showing the presence of ester groupings,

When they were heated with dilute acids, both substances formed an intermediate product (III), and L-rhamnose was detected in the hydrolysate. An investigation of the intermediate substance (III) by UV spectroscopy showed that the splitting out of the rhamnose had not led to the liberation of a hydroxy group of the aglycone, and on hydrolysis with dilute acids it underwent isomerization with the formation of a new compound (IV) having a lower R_f value although no sugar and aglycone were detected after this treatment. This behavior is characteristic for 6-C-glycosylflavones [10].

From its chromatographic behavior, melting point, and UV spectra, intermediate substance (III) was identified as embigenin — 4',7-dimethylapigenin 6-C-β-D-glucopyranoside. When (III)

*The species that we studied is taken in the Flora of the USSR [1] and a monograph on the *Iris* genus by G. I. Rodionenko [2] as *Iris ensata* Thunb. However, V. I. Grubov in "Critical Observations on the Taxonomy and Nomenclature of Some Species of the Genus *Iris* in the USSR Flora" [3] restored to priority binomial given to a Mongolian-Siberian salt marsh iris by P. S. Pallas in 1776 as *Iris lactea*. In papers that we have published previously, the information related to a species we assume in V. I. Grubov's interpretation to be *iris lactea* Pall. (*I. ensata* auc. non. Thunb.). The botanical affinity of the species was determined by Yu. N. Gorbunov of the All-Union Institute of Medicinal Plants and by G. I. Rodionenko of the Komarov Botanical Institute of the Academy of Sciences of the USSR.

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TABLE 1. NMR Spectra of Embinin and Its Derivatives

Protons	Chemical shifts of the signals* of the protons, ppm, multiplicities, and spin-spin coupling constants				
	embinin, CCl_4, TMS ether	acetylembinin, pyridine, 90°C	diacetyl embinin, CCl_4, TMS ether	Diacetylembinin, pyridine, 100°C	product of the acid hydrolysis of embinin, TMS ether
2′, 6′	d, 7,72, 2H, J=8,0	d, 7,66, 2H, J=8,0	d, 7,79, 2H J=10,0	d, 7,82, 2H J=8,0	d, 7.82, 2H, J=10,0
3′, 5′	d, 6,90, 2H J=8,0	d, 6,86, 2H, J=10,0	d, 6,94, 2H, J=8,0	d, 7,00, 2H J=10,0	d, 6,97, 2H, J=8,0
3	s, 6,30, 1H	s, 6,60, 1H	s, 6,37, 1H	s, 6,72, 1H	s, 6,50, 1H
8	s, 6,42, 1H	s, 6,75, 1H	s, 6,50, 1H	s, 6,75, 1H	s, 6,37, 1H
H-1 of glucose	d, 4,75, 1H, J=9,0	d, 5,26, 1H J=10,0	d, 4,81 1H J=10,0	d, 5,40, 1H J=10,0	d, 4,70, 1H J=10,0
H-1 of rhamnose	d, 4,90 1H, J=4,5	d, 5,68, 1H J=4,0	d, 5,02, 1H J=3,0	d, 5,94, 1H, J=2 5	
OCH_3	s, 3,84, 3H s, 3,82, 3H	s, 3,68, 3H s, 3,60, 3H	s, 3,94, 3H s, 3,87, 3H	s, 3,83, 3H s, 3,79, 3H	s, 3,96, 3H s , 3,94, 3H
CH_3CO	—	s, 1,68, 3H	s, 2,01, 3H s, 1,62, 3H	s, 1,91, 3H s, 1,81, 3H	—
CH_3 of rhamnose	d, 0,73, 3H, J=6,0	d, 0,75, 3H, J=6,0	d, 0,60, 3H, J=6,0	d, 1,03, 3H J=6,0	

*Relative to the signal of TMS.

was subjected to acid hydrolysis by Kiliani's method [11] and also to oxidation by ferric chloride, apigenin and D-glucose, with a trace of D-arabinose, were obtained, which is also characteristic for embigenin.

Substances (I) and (II) were subjected to mild alkaline hydrolysis, which yielded embigenin. Acetic acid was detected in the hydrolysates with the aid of the hydroxamic test and the formation of the diethylammonium salt [12-15].

When compounds (I) and (II) and embigenin were acetylated with heating for 4 hours, one and the same product was formed with mp 215°C, a mixture of which with the full acetate of embigenin gave no depression of the melting point. The same products of alkaline hydrolysis were characteristic for substances (I) and (II) — embinin and acetic acid; however, these substances had different mobilities in system 2. The Rf value of substance (I) was 0.85 and that of substance (II) 0.82, and they also had different melting points: (I) 144-146°C; (II) 171-173°C.

In the NMR spectra taken at 90°C (pyridine) substance (I) showed two sharp singlets at 1.91 and 1.81 ppm, and substance (II) one singlet in the 1.68-ppm region (Table 1).

The acetyl group in (II) and one of the two in (I) was present in position 2 of the rhamnose residue, as followed from the small value of the spin-spin coupling constant of the geminal proton with the vicinal proton. The large value of the constants for the second geminal proton permitted the assumption that the acetyl group was present in position 3 or 4 of the glucose residue [16].

In spite of the fact that they were chromatographically individual substances, more signals were observed in the NMR spectra of embinin and substances (I) and (II) than were to be expected from the structural formulas of these compounds. In view of this, we assumed that the flavonoids investigated existed in two stable states. In this case, it may be ex-

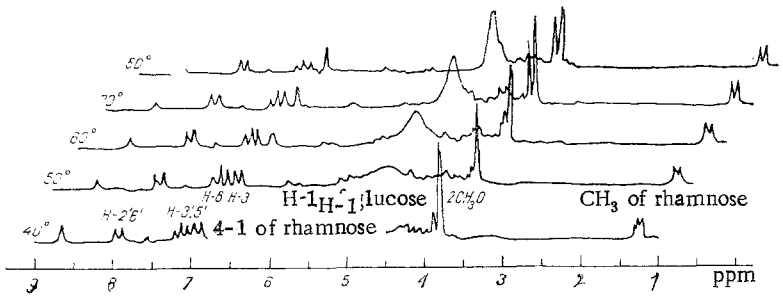


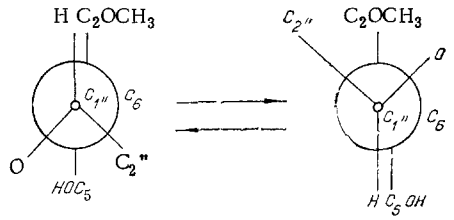
Fig. 1. Spectra of embinin at various temperatures.

pected that with a rise in the temperature the signals corresponding to one and the same protons of the molecules in the two states would fuse.

Temperature changes confirmed these hypotheses. Figure 1 shows the spectra of embinin obtained at various temperatures. Up to a temperature of 60°C is a region of slow exchange, in the 60-80°C interval a region of intermediate exchange, and above 80°C a region of rapid exchange.

The two states are due to the steric interaction of the sugar moiety with the substituents in positions 5 and 7, as is shown by the splitting of the signals of the methoxy group and of the sugar residue. In these circumstances the H_1 proton of the glucose residue differs in its orientation, being turned in the direction of the methoxy group in one case, and the direction of the hydroxy group in the other case. The states due to the departure of the substituents from the plane of the aromatic ring in different directions from this plane should not give separate signals because of the symmetry of the molecule.

Making use of Newman projections along the C_1''—C_6 bond, a fragment of the molecule of rotational isomers can be represented in the following way:



From the form of the signals of the acetyl groups of acetylembinin at different temperatures (Fig. 2) we determined the energy barrier (E_a) between the states. The calculations of the form of the signals were performed by means of a general formula for exchange processes [17]. In this formula the measured parameter is

$$\tau = \frac{\tau_A \cdot \tau_B}{\tau_A + \tau_B},$$

where τ_A and τ_B are the lifetimes in states A and B, and the parameter

$$\Delta P = \frac{\tau_A \cdot \tau_B}{\tau_A - \tau_B}$$

was selected in such a way that the observed and calculated curves describing the form of the signals coincided. The parameter τ_2 was determined from the half-width of the signal (1.5 Hz) at 20°C when broadening due to exchange processes is small. The error in the determination of the temperature amounted to ±1°C.

The activation energy E_a of the transitions was determined from the values of τ found (Table 2) using the Arrhenius equation [18] by the method of least squares.

TABLE 2. Parameters τ and ΔP at Various Temperatures

Index	Experiment No.					
	1	2	3	4	5	6
τ, c	0,200	0,092	0 050	0,034	0,022	0,016
T. °C	323	333	338	343	348	353
ΔP	0,081	0,075	0,058	0,060	—	—

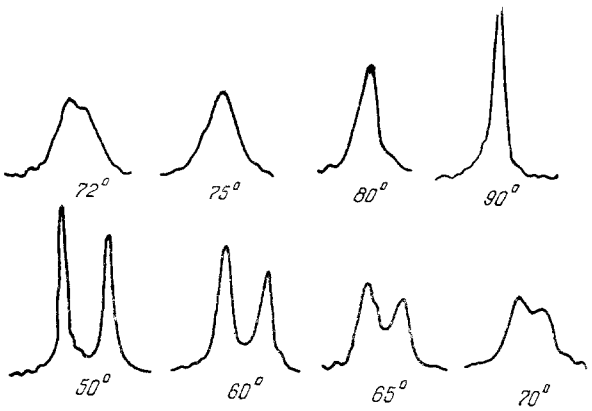


Fig. 2. Signals of the acetyl group of acetylembinin at various temperatures.

At a value of the linear concentration coefficients $r^2 = 1.00$, the energy barrier proved to be 19.6 kcal/mole. The change in the standard enthalpy ΔH^0 in the temperature interval of 50-70°C was determined from the formula

$$\ln \frac{K(T_2)}{K(T_1)} = \frac{\Delta H^0}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$

where $K = (1 + \Delta P)/(1 - \Delta P)$ is the equilibrium constant, equal to 0,3 kcal/mole.

The calculations of the shape of the signals and the activation energy were carried out on a HP-67 programmed microcalculator for which the corresponding program were drawn up.

It must be mentioned that a number of studies, have been devoted to the investigation of embinin, but the presence of rotamers of embinin has never been reported.

In the first publications of Japanese authors [19, 20] it was stated that in the embinin molecule the sugar residue was a dirhamnoglucosyl residue. It must be assumed that the error of the Japanese authors was connected with the presence of two rotamers.

For flavonoid glycosides the existence of the molecule in two states has been detected in the case of C-glycosides with carbohydrate substituents in position 8 [21]. However, the authors concerned observed that the effect disappears if there is a hydroxy group present in the ortho position to the sugar substituent or when a carbohydrate substituent is present in position 6. To all appearance, for the compound with an acetyl group in position 7 described by the authors the molecule is present predominantly in one of the two possible states. For the 8-C-glucosides the region of slow exchange extends to 0°C, while for compounds of the embinin series the corresponding temperature is 60°C.

EXPERIMENTAL

Melting points were determined on a Kofler block. The spectra characteristics were obtained on a CF-16 instrument (UV) and UR-20 instrument using a mull in paraffin oil (IR); Varian HA-100D, pyridine, 0 — TMS, ppm (NMR); specific rotations were measured on a Perkin-Elmer 241 instrument. Silica gel L 100/160 μm and 5/40 μm were used as sorbent. Chromatographic

monitoring was carried out by PC in the following systems: 1) 15% acetic acid; 2) 1-butanol—acetic acid—water (4:1:2); 3) 30% acetic acid; 4) ethanol—amonia (9:1); 5) 1-butanol—diethylamine—water (100:1:15). TLC was performed on Silufol plates in the chloroform—methanol (9:1) system.

Isolation of the Flavonoids. The comminuted air-dry raw material (2 kg) was extracted several times with 50% ethanol at room temperature. The ethanolic extracts were combined and concentrated in vacuum at 35-40°C to 2/3 of their original volume, and the combined flavonoids were repeatedly treated with ethyl acetate. The concentrated ethyl acetate extracts were deposited on a column of silica gel. Chloroform and chloroform with increasing concentrations of methanol were used as eluents. The elution of the column with 4% methanol in chloroform yielded substance (I), and when the concentration of methanol was increased to 7% substance (II) was obtained. Compound (I) was purified by rechromatography on a column of silica gel (with chloroform containing 3.5% of methanol as the eluent).

Acid Hydrolysis with 5% Sulfuric Acid. A mixture of 100 mg of substance (I) or (II) with 5 ml of 5% sulfuric acid was heated on the boiling water bath under reflux for 2.5 h. In both cases, hydrolysis product (III) was obtained.

Substance (I) (diacetylembinin). mp 144-146°C (70% ethanol), R_f 0.83 (system 1) and 0.85 (system 2). $[\alpha]_D^{20}$ +89 (c 0.1 ethanol). UV spectrum, nm: $\lambda_{max}^{C_2H_5OH}$ 331, 274; +NaOAc 332, 273; +NaOAc + H_3BO_3 330, 274; +$AlCl_3$ 352, 282; +$AlCl_3$ + HCl 345, 282; +NaOEt 302, 380. IR spectrum, cm^{-1}: 1640 (C=O), 1520, 1335, 1260, 1220 (C=C), 1782 (ester bond); 3400-3800 (—OH).

Substance (II) (acetylembinin). mp 171-173°C (70% ethanol); R_f 0.33 (system 1) and 0.82 (system 2). $[\alpha]_D^{20}$ −75.11° (c 0.1; ethanol). UV spectrum, nm: $\lambda_{max}^{C_2H_5OH}$ 330, 273 +NaOAc 331, 274; +NaOAc + H_3BO_3 331, 274; +$AlCl_3$ 352, 282; +$AlCl_3$ + HCl 347, 282; +NaOEt 302, 280. IR spectrum, cm^{-1}: 1660 (C=O), 1520, 1310, 1250, 1210 (C=C), 1740 (ester bond), 3200-3400 (—OH).

Substance (III) (embigenin). mp 243-244°C, $[\alpha]_D^{20}$ −29.41° (c 0.1; ethanol); R_f 0.65 (system 1) and 0.81 (system 2), 0.83 (system 3). UV spectrum, nm: $\lambda_{max}^{C_2H_5OH}$ 322, 377) NaOAc 330, 275; +$AlCl_3$ 345, 285; +NaOAc + H_3BO_3 330, 274; +$AlCl_3$ 352, 282) +$AlCl_3$ + HCl 345, 282) +NaOEt 302, 380. IR spectrum, cm^{-1}: 1640 (C=O), 1520, 1335, 1260, 1220 (C=C). 1782 (esterbond); 3400-3800 (—OH).

Acid Hydrolysis by Kiliani's Method. A suspension of 50 mg of substance (III) in 4 ml of Kiliani's mixture (10 parts of hydrochloric acid, 3.5 parts of acetic, and 5.5 parts of water) was sealed into a tube and heated on the boiling water bath for 24 h. The hydrolysate was diluted with 20 ml of water and the aglycone was extracted with diethyl ether, the ether was distilled off, and the residue was chromatographed on paper in systems 1 and 2 in the presence of markers. This gave the aglycone with mp 348-350°C. It was identified as epigenin. The aqueous residue was neutralized to pH 7 with 10% caustic soda solution and evaporated to dryness. The residue was treated with methanol. On chromatography in the 1-butanol—pyridine—water (6:4:3) system and treatment with the aniline phthalate reagent, D-glucose with traces of D-arabinose was identified.

Oxidation with Ferric Chloride. A mixture of 50 mg of substance (III) and 200 mg of ferric chloride was heated on the boiling water bath for 6 h. Then the reaction mixture was cooled, diluted with a fivefold amount of water, and treated with 10% caustic soda solution. The solution (pH 8) was filtered, acidified with 10% hydrochloric solution to pH 7, and evaporated to dryness. D-Glucose with traces of D-arabinose was identified in the residue. On chromatography in systems 1 and 2 the aglycone apigenin was detected.

Alkaline Hydrolysis. A solution of 20 mg of substance (I) or (II) in 3 ml of 0.5% aqueous caustic soda was kept at room temperature for 1 h. The saponification reaction was followed by paper chromatography in system 1. Then the mixture was neutralized with 1% hydrochloric acid to pH 6 and, in both cases, the reaction mixture deposited a crystalline precipitate with mp 183-185°C (embinin).

Detection of Acetic Acid. A solution of 150 mg of substance (I) or (II) in 10 ml of 0.5% aqueous KOH was left at room temperature for 1 h. The solution was neutralized with 5% hydrochloric acid to pH 5 and was treated repeatedly with diethyl ether. The combined ethereal extracts were dried with calcined sodium sulfate and evaporated to small volume. The residue, which smelled of acetic acid (pH 1) was diluted with 2-3 drops of water, and diethylamine was added to give pH 10. The diethylammonium acetate formed was identified by paper chromatography with a marker of the authentic salt. The chromatograms were revealed with a 0.1% solution of bromothymol blue in ethanol. This lead to the appearance of lilac

spots with R_f 0.56 (system 2), 0.45 (system 4), 0.39 (system 5), which behavior was identical with that of a marker.

Hydroxylaminolysis of Substances (I) and (II). The appropriate flavonoid (50 mg) was placed in a test-tube, 3-4 drops of a saturated solution of hydroxylamine in methanol was added, and the reaction mixture was warmed slightly. Then 1 drop of a saturated methanolic solution of KOH was added and the tube was placed in the boiling water bath until its contents boiled. After cooling, it was neutralized and acidified with 1% HCl solution and chromatographed in system 1. In parallel, under the same conditions a control experiment with ethyl acetate was set up. When the chromatograms were revealed with a 1% solution of ferric chloride and ethanol, acethydroxamic acid was detected (R_f 0,86) in the form of a gray-lilac spot [11].

Acetylation. A solution of 70 mg of substance (I) or (II) in 0,5 ml of pyridine was treated with 1 ml of acetic anhydride and heated in the boiling water bath for 4 h. Then it was poured into cold water containing ice. Colorless crystals were obtained of the full acetates of substances (I) and (II); mp. 215°C. A mixture with embinin acetate gave no depression of the melting point.

CONCLUSIONS

1. Two acylated C-glycosylflavonoids have been isolated from the epigeal part of *Iris lactea* Pall.: substances (I) — 5-hydroxy-4'-7-dimethoxyflavone 6-C-[O-(2-acetyl-α-L-rhamnopyranrosyl)-(1→2)-x-acetyl-β-D-glucopyranoside] (diacetylembinin); and substance (II) — 5-hydroxy-4',7-dimethoxyflavone 6-C-[O-(2-acetyl-α-L-rhamnopyranosyl)-(1→2)-β-D-glucopyranoside] (acetylembinin).

2. The existence of rotamers of 6-C-glycosylflavones has been established for the first time.

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