FLAVONONE BIOSIDES OF ACINOS THYMOIDES

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Plants of the family Labiatae have been studied mainly for their alkaloids [1] or their essential oils [2, 3], while the polyphenolic compounds in the plants of this family have been studied comparatively little [4].

In the present paper we give the results of an investigation of the flavonoids of Acinos thymoides Moensch. (family Labiatae). When the combined flavonoid compounds were separated, we obtained two individual components, substances A and B (Table 1).

Table 1

Physicochemical Properties of the Biosides of Acinos thymoides and Their Derivatives

Substance	mp, °C	$[\alpha]_D$, deg	Mol. wt.	Composition	$R_f \times 100$ in systems	
					1	2
Bioside A	210—211	—81.7	594	$C_{28}H_{34}O_{14}$	75—76	30—32
Bioside B	210—212	—80.0	594	$C_{28}H_{34}O_{14}$	78—80	32—34
Poncirin [5]	210—211	—90.0	594	$C_{28}H_{34}O_{14}$	—	—
Monoside (I)	196—198	—72.0	448	$C_{22}H_{24}O_{10}$	42—45	48—50
Monoside (II)	197—199	—69.0	448	$C_{22}H_{24}O_{10}$	42—45	48—50
Isosakuranin [5]	190—201	—73.0	448	$C_{22}H_{24}O_{10}$	—	—
Aglycone of biosides A and B	188—190	0.0	286	$C_{16}H_{14}O_5$	29—30	90—92
Isosakuranetin [5]	188—190	0.0	286	$C_{16}H_{14}O_5$	—	—

On comparing the properties of substances A and B, it can be seen that they have identical molecular weights and similar melting points and optical rotations but differ somewhat in their chromatographic mobilities and in their solubilities. Both substances give bright red pigments in the cyanidin reaction and the reaction with sodium borohydride and hydrochloric acid [6]. The red pigment obtained in the cyanidin reaction is insoluble in octanol [7]. Both substances reduce Fehling's solution only after acid hydrolysis. All these results show that the compounds under investigation are glycosides of a flavonone nature.

To confirm the glycosidic nature of the substances, we subjected them to acid hydrolysis, and among the decomposition products we found D-glucose, L-rhamnose, and an aglycone. The aglycone content of both glycosides was 48-50%, which may characterize them as biosides. The stepwise acid hydrolysis of glycosides A and B gave monoglycosides (I) and (II), which contained a D-glucosyl substituent readily split off by emulsin (cf. Table 1).

In order to establish the position of the free phenol groups in glycosides A and B, the monoglycosides (I) and (II) and the aglycone, we carried out a spectroscopic investigation in the UV region using ionizing and complex-forming reagents [8, 9] (Table 2).

In analyzing the spectral data, it may be noted that in the glycoside there is no bathochromic displacement of the maximum of the long-wave band in the presence of sodium acetate, while in the aglycone this shift is equal to 10 mμ, which fixes the free phenolic group in the aglycone in position 7. The bathochromic displacement of the maximum in band I in the complex-forming reaction with aluminum chloride (50-55 mμ) in all the compounds investigated shows the presence of a free 5-hydroxyl group. This is also confirmed by the bathochromic shifts of the maxima of the long-wave band by 32-35 mμ under the influence of sodium ethoxide.

Consequently, the aglycone contains hydroxyl groups in positions 5 and 7 while the glycosides contain only one, in position 5, from which it can be concluded that the D-glycosidic substituent is in position 7.

The IR spectra of the compounds studied show, in addition to the frequencies of the γ-pyrone carbonyl group (1640-1645 cm^{-1}) a band at 832 cm^{-1} due to 1-4 substitution in the B phenyl ring of flavonoids [10]. On this basis, we assume that the B ring is substituted only in position 4'. To characterize this substituent, we carried out the alkaline decomposition of the aglycone, and in the degradation products found phloroglucinol and anisic acid, which shows the presence of a methoxyl group in the lateral phenyl ring. Bands at 2960 and 2840 cm^{-1} in the IR spectrum of the aglycone serve as confirmation of the methoxyl substituent [11].

The results obtained enable the aglycone of glycosides A and B to be characterized as 5, 7-dihydroxy-4'-methoxy-flavonone, or isosakuranetin [12], and the glycosides themselves as 7-biosides of isosakuranetin in which D-glucose is attached to the aglycone directly and L-rhamnose is attached to the D-glucose.

An analysis of the molecular rotations of the biosides and their monosides (Table 3) shows that both sugars have the pyranose form and that the D-glucose is attached to the aglycone by a β-glycosidic link and the L-rhamnose to the D-glucose by an α-glycosidic link [13].

Table 2

Spectral Characteristics of the Flavonone Biosides and Their Derivatives

Solutions in reagents	Absorption bands	Bioside A		Bioside B		Monoside (I)–(II)		Aglycone	
		λ_{max}, mμ	Δλ	λ	Δλ	λ	Δλ	λ	Δλ
10^{-4} M solution in anhydrous ethanol	I	325	—	330	—	328	—	325	—
	II	282	—	280	—	280	—	288	—
		225						230	
The same + sodium acetate	I	325	0	330	0	328	0	335	10
	II	282	0	280	0	280	0	295	7
The same + sodium ethoxide	I	360	35	365	35	360	32	370	45
	II	284	2	285	5	282	2	290	2
The same + aluminum chloride	I	380	55	385	55	385	57	375	50
	II	225	--	—	--	--	—	230	—

The β-glucosidic link of the D-glucose to the aglycone is also confirmed by the enzymatic hydrolysis of the monosides (I) and (II) by emulsin.

Table 3

Comparative Analysis of the Molecular Rotations of the Glycosides

Glycoside	$[M]_D$	K_f	$[M]_D \cdot K_f$	ΔC	K_m	$\Delta C \cdot K_m$
Bioside A	—485.0	0.67	—325.0	—141.0	0.74	—104.0
Monoside (I)	—323.0	0.57	—184.0	—	—	—
Bioside B	—475.0	0.67	—318.0	—142.0	0.74	—105.0
Monoside (II)	—309.0	0.57	—176.0	—	—	—
Phenyl β-D-glucopyranoside	—182.0	1.00	—182.0	—	—	—
Methyl α-L-rhamnopyranoside	—110.0	—	—	—	1.00	—110.0

The results of polarimetric analysis are substantiated by the spectroscopic method used in the IR region, where the glucosidic substituent was investigated with the exclusion of the spectrum of the aglycone from the spectra of the monoglycosides and the L-rhamnosidic component with the exclusion of the spectra of the monoglucosides from the spectra of the biosides [13] (Table 4).

The results obtained show that biosides A and B do not differ either in the position of the carbohydrate substituents in the aglycone, or in the form of the sugars, or in the configuration of the glycosidic links. However, in the stepwise acid hydrolysis of the biosides to the monosides it was found that bioside B is hydrolyzed considerably more rapidly than bioside A (2% HCl at 98° C: 15–20 min for A and 2 hr for B). This difference may probably be explained by the particular arrangement of the bond between the sugars. A 1–6 bond is contradicted by the results of the enzymatic hydrolysis of biosides A and B by "rhamnodiastase," which hydrolyzed rutinose to rutin in a control experiment carried out in parallel [14, 15]. We must assume that the L-rhamnose occupies the 2-, 3-, or 4-hydroxyl group in the D-glucose.

To solve this problem, we carried out the alkaline hydrolysis and the periodate oxidation of the biosides under investigation. Chromatographic analysis of the products of alkaline hydrolysis showed that both glycosides were decomposed. But different substances were found in the degradation products: bioside B formed phloroglucinol and anisic acid, and bioside A formed anisic acid and a bioside of phloroacetophenone. The appearance of this bioside on alkaline hydrolysis could occur only if the 2-hydroxyl group in the D-glucose were substituted by the L-rhamnose [16, 17]. On periodate oxidation of the biosides with subsequent hydrolysis, no D-glucose was found among the hydrolysis products,

which excludes a 1–3 bond between the sugars. When the aldehydes of the hydrolyzate were further oxidized with nitric acid, tartaric acid was isolated only from bioside B, which shows the presence of a substituent at the 4-hydroxyl group in the D-glucose part of bioside B [18].

Thus, on the basis of the chemical and spectroscopic investigation, bioside A can be characterized as isosakuranetin 7-(β-D-glucopyranosyl-2-α-L-rhamnopyranoside) and bioside B as isosakuranetin 7-(β-D-glucopyranosyl-4-α-L-rhamnopyranoside). Consequently, from the herb Acinos thymoides we have isolated two isomeric 7-rhamnoglucosides of isosakuranetin, one of which might be identified with poncirin, which has been isolated from species of Citrus [19]. However, some authors [17, 20, 21] ascribe to poncirin the structure of bioside A and Nakabayashi [22] states that this glycoside has the structure of bioside B. The experimental results of these investigations do not leave doubt as to their reliability. Consequently, the assumption arises that these authors [20–22] had different biosides of isosakuranetin. In this case, neither of the poncirins can be characterized as a neohesperidoside, since neohesperidose, which was first isolated from neohesperidin by Zemplen and Tettamonti [23] has been described as O-4-β-L-rhamnopyranosyl-D-glucopyranose. When the results of the investigation carried out by Nakabayashi [22], Horowitz, et al., [20], and ourselves are taken into account, it may be concluded that in each case the plants studied * contain pairs of poncirins, naringins, neohesperidins, and other rhamnoglucosides with, respectively, 1–2 and 1–4 arrangements of bonds between the sugars. In view of this, we have found it desirable to retain the name poncirin for bioside A having the 1–2 arrangement of the bond between the sugars and to propose the name of acinoside for bioside B.

Table 4

Spectroscopic Investigation of the Structure of The Carbohydrate Substituents in the IR Region

Carbohydrate moiety of the glycoside	Vibrations (frequency, cm^{-1})			
	anomers		rings and C–O groups	
	α	β	Pyranose	furanose
D-Glucosyl (I)	—	893	1090, 1050, 1025	—
L-Rhamnosyl A	840	—	1085, 1055, 1020	—
D-glucosyl (II)	—	895	1085, 1045, 1027	—
L-Rhamnosyl B	843	—	1088, 1057, 1022	—

Experimental

The chromatographic analysis of the flavonoids and sugars was carried out on paper of type Goznak-85 in the following systems: 1) 15% acetic acid, 2) butan-1-ol–acetic acid–water (4:1:2), 3) ethyl acetate–benzene–acetic acid–formamide (73.5:24.5:2:1), and 4) pyridine–butan-1-ol–water (4:6:3). The spectroscopic investigation in the UV region was carried out on a SF-4 spectrophotometer and in the IR region on a UR-10 spectrophotometer. The optical activity was measured on an SPU-E spectropolarimeter.

Separation of the total flavonoids. The total flavonoids obtained from the herb Acinos thymoides as described previously [24] were dissolved in boiling methanol and left for crystallization. On cooling, the solution deposited white needle-like crystals of poncirin, which after repeated crystallization from ethanol had mp 210°–211° C, [α] –81.7° (c 1.0; dimethylformamide).

Found, %: C 56.62, 56.45; H 5.80, 5.76. Calculated for $C_{28}H_{34}O_{14}$, %: C 56.56; H 5.72.

The mother liquor after the crystallization of the poncirin contained mainly acinoside. To free this from impurities, the mother liquor was evaporated to dryness and the residue was dissolved in a small amount of acetic acid. The acetic acid solution was diluted with water to a 15% concentration of the acid and was transferred to a column of cellulose powder. Elution was carried out with 15% acetic acid. The fractions containing acinoside were combined and left to crystallize with slow evaporation of the solvent at 20°–22° C. The white needle-like crystals of acinoside which were deposited had, after recrystallization, mp 210°–212° C, $[\alpha]_D$ – 80.0° (c 0.5; dimethylformamide).

Found, %: C 56.70, 56.34; H 5.66, 5.70. Calculated for $C_{28}H_{34}O_{14}$, % : C 56.56; H 5.72.

Acid hydrolysis of poncirin and acinoside. The two biosides were hydrolyzed separately (in 1.0-g samples), with

* Poncirus trifoliata (L.) Raf. (trifoliate orange), Citrus paradisi L. (grapefruit), and other Citrus species.

5% hydrochloric acid in 50% alcohol at 100° C for 4 hr. The hydrolyzates were evaporated until the alcohol had been eliminated completely, and the aglycones were extracted with ether. The ethereal extracts were washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness. This yielded 0.489 g of the aglycone from poncirin and 0.501 g from acinoside. The aglycones of poncirin and acinoside proved to be identical, and after crystallization from alcohol had mp 188°–190° C and 188°–189.5° C, respectively.

Found for isosakuranetin from poncirin, %: C 67.20, 67.08; H 4.73, 4.90; OCH_3 14.35, 14.42. Calculated for $C_{16}H_{14}O_5$, %: C 67.13; H 4.89; OCH_3 14.33.

Found for isosakuranetin from acinoside, %: C 67.15, 67.18; H 4.85, 5.02; OCH_3 14.17, 14.30. Calculated for $C_{16}H_{14}O_5$, %: C 67.13; H 4.89; OCH_3 14.33.

The acid hydrolyzate was neutralized by means of the ion-exchanger Amberlite IR-45 (OH^-). After evaporation to small bulk, it was analyzed for its sugar content in systems 2 and 4. Equal amounts of D-glucose and L-rhamnose were found.

Alkaline degradation of the aglycones of poncirin and acinoside. A 0.05-g sample of the isosakuranetin from each of the two glycosides was dissolved in molten caustic potash (2 g). The melt was rapidly cooled, dissolved in water, and neutralized with 20% hydrochloric acid to pH 4–5. The degradation products were extracted with ether. The ethereal extracts were evaporated to dryness and the residues, dissolved in 0.2 ml of alcohol, were used for paper-chromatographic analysis in systems 2 and 3. In both cases, the degradation products included phloroglucinol and anisic acid.

Stepwise acid hydrolysis of poncirin and acinoside. The glycosides (0.5-g samples in each case) were hydrolyzed with 2% hydrochloric acid in 50% ethanol with heating on a water bath. The hydrolysis was monitored by means of paper chromatography (system 1) every 5 min for 1 hr and then every 30 min for 4 hr. Half hydrolysis of acinoside was found after 15 min and of poncirin after 2 hr. To isolate the intermediates, the hydrolysis was stopped when half the amount of the initial glycoside had been decomposed. The substances formed were separated on Kapron powder columns with elution by water and dilute alcohols. Both alcohols gave, in addition to isosakuranetin and the initial compounds, the monoglycosides (I) and (II), which proved to be identical.

The isosakuranin from poncirin had mp 196°–198° C, $[\alpha]_D$ −72.0° (c 0.1; dimethylformamide).

Found, %: C 59.10, 58.83; H 5.42, 5.38. Calculated for $C_{22}H_{24}O_{10}$, %: C 58.92; H 5.35.

The isosakuranin from acinoside had mp 197°–199° C, $[\alpha]_D$ −69.0° (c 0.05; dimethylformamide).

Found, %: C 58.77, 59.01; H 5.27, 5.32. Calculated for $C_{22}H_{24}O_{10}$, %: C 58.92; H 5.35.

The acid hydrolysis of the monoglycosides mentioned gave isosakuranetin (63–65% of the weight of the glycoside) and D-glucose. The enzymatic degradation of isosakuranin with emulsin also led to the formation of isosakuranetin and D-glucose.

Fermentation of poncirin and acinoside with rhamnodiastase. The biosides (0.01-g samples) were dissolved in small amounts of 50% alcohol and the solutions were diluted with water until they showed a slight opalescence. Then, in each case, a solution of rhamnodiastase in water (0.01 g in 10 ml) was added and the mixture was left for fermentation at 30° C for 24 hr. A control experiment with rutin was carried out in parallel. The progress of the hydrolysis was analyzed by paper chromatography in system 1 every 6 hr, samples of 1–2 ml being taken with careful stirring of the reaction mixture. Before the solutions were deposited on the chromatograms, the enzymes were precipitated with alcohol and by heating to the boil. Rutin was hydrolyzed to the aglycone and rutinose, while poncirin and acinoside were unchanged.

Alkaline degradation of poncirin and acinoside. A sample (0.5 g) of each of the biosides was dissolved in 10 ml of 30% aqueous caustic potash and the solution was boiled on a water bath for 3 hr, the reaction being carried out in an atmosphere of nitrogen. The cooled solutions were neutralized with 20% hydrochloric acid to pH 6.5–7.0. The precipitates which were deposited were filtered off and washed with cold water.

Only phloroglucinol and anisic acid were found in the precipitate from acinoside, while anisic acid and a new substance of glycosidic nature were found in the products of the degradation of poncirin. Recrystallization of the degradation product of poncirin from water gave white needle-like crystals of a chromatographically homogeneous substance with mp 162°–164° C, $[\alpha]_D$ −90.0° (c 0.01; dimethylformamide). The acid hydrolysis of the substance yielded D-glucose, L-rhamnose, and phloroacetophenone, which were identified by chromatography using authentic samples.

Periodate oxidation of poncirin and acinoside. A sample (0.1 g) of each of the biosides was dissolved in 20 ml of 50% ethanol with heating. The cooled solution was treated with 0.12 g of periodic acid and was left in a dark place at 18°–20° C for 8 hr. Then the reaction mixture was treated with a small amount of glycol to eliminate the excess of periodic acid and was neutralized with ion-exchangers in the OH^- and H^+ forms. The filtrate was evaporated to 5 ml,

and part of this solution (1 ml in each case) was hydrolyzed with 5% hydrochloric acid. The products were analyzed by paper chromatography (systems 2 and 4). D-Glucose was not found among the resulting oxidation products.

A second part of each of the evaporated solutions was mixed with 20% nitric acid (1 ml) and the mixture was evaporated with the addition of small amounts of water until oxides of nitrogen had been completely eliminated. The residues were dissolved in 0.1 ml of water and were analyzed by paper chromatography in systems 2 and 4. The products of the oxidation of acinoside were found to contain tartaric and oxalic acids, while the products of the oxidation of poncirin did not contain tartaric acid.

The modified method for the periodate–nitric acid oxidation of the biosides was proposed by A. V. Gorin [KhNIKhFI (Khar'kov Chemical and Pharmaceutical Scientific Research Institute)], and the IR spectra of the flavonoids were taken by I. P. Kovalev (KhNIKhFI).

Summary

It has been established by a chemical and spectroscopic study of the flavonoid biosides isolated from the hypogeal part of Acinos thymoides that bioside A is 5,7-dihydroxy-4'-methoxyflavan-7-one β-(D-glucopyranosyl-2-α-L-rhamnopyranoside), or poncirin, and bioside B is 5,7-dihydroxy-4'-methoxyflavan-7-one β-(D-glucopyranosyl-4-α-L-rhamnopyranoside), which we have called acinoside.

REFERENCES

1. A. P. Orekhov, Chemistry of the Alkaloids [in Russian], Moscow, 1955.
2. M. Kh. Goryaev, Essential Oils of the Flora of the USSR [in Russian], Alma-Ata, 1952.
3. E. Goldemeister and F. Hoffman, Die Atherische Öle, 3 Aufl., Bd. III-a, Leipzig, 1961.
4. F. M. Dean, Naturally Occuring Oxygen Ring Compounds, London, Butterworths, 1963.
5. A. Vystrcil, Rostlinne gylkosidy, Naclad, Č–S. A. Praha, 1955.
6. R. M. Horowitz, J. Org. Chem., 22, 1733, 1957.
7. E. T. Bryant, J. Am. Pharm. Assoc., 39, 481, 1950.
8. T. A. Geissman, The Chemistry of Flavonoid Compounds, Pergamon Press, New York, 107, 1962.
9. V. I. Litvinenko and N. P. Maksyutina, KhPS, 56, 1965.
10. L. Bellamy, The Infra-Red Spectra of Complex Molecules [Russian translation], Moscow, 1963.
11. A. R. Katrizky and N. A. Coats, J. Chem. Soc., 2062, 1959.
12. S. Hattori, Acta Phytochim., 4, 219, 1929.
13. I. P. Kovalev and V. I. Litvinenko, KhPS, 233, 1965.
14. H. Friedrich, Arch. Pharm., 295, 467, 1961.
15. H. Imaseki and T. Yamamoto, Arch. Biochem. Biophys., 92, 467, 1961.
16. C. M. McCloskey and G. H. Coleman, J. Org. Chem., 10, 184, 1945.
17. R. M. Horowitz and B. Gentili, Tetrah., 19, 773, 1963.
18. H. Janecke and W. Weisser, Pharmazie, 20, 580, 1965.
19. S. Hattori, M. Shimokoriyama, and M. Hasegawa, Acta Phytochim., 14, 1, 1944.
20. J. B. Harborne, Biochemistry of Phenolic Compounds, Acad. Press, London and New York, 545, 1964.
21. K. Christiansen and P. M. Boll, Planta Medica, 12, 77, 1964.
22. T. Nakabayashi, J. Agr. Chem. Soc. Japan, 35, 942, 1961.
23. G. Zemplen and A. K. Tettamonti, Ber., 71B, 2511, 1938.
24. T. A. Sergienko and L. S. Kazarnovskii, Aptechn. delo, 14, no. 2, 31, 1965.

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