Chemistry of Natural Compounds, Vol. 30, No. 6, 1994

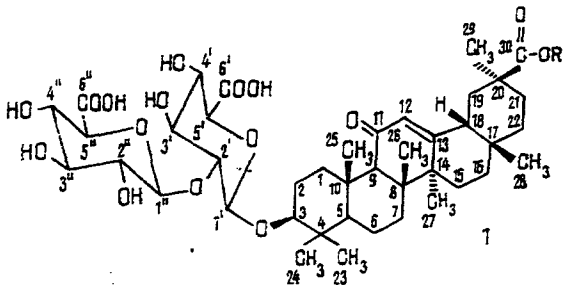
SOME PROPERTIES OF GLYCYRRHIZIC ACID

G. G. Zapesochnaya, E. N. Zvonkova,
V. A. Kurkin, E. V. Kazakova, L. N. Pervykh,
B. I. Sheichenko, and V. A. Bykov

UDC 547.915:543.422.23

The chemical and spectral properties of crystalline specimens of glycyrrhizic acid and its monoammonium salt have been studied. On the basis of ^1H and ^{13}C NMR results, diagnostic parameters of the 18β/18α-epimers have been revealed, and features of the structure of the carbohydrate moiety of these compounds are discussed.

Glycyrrhizic acid (**1**) belongs to the class of triterpenoids and is a bioside, the 3-O-[2′-(O-β-*D*-glucuronopyranosyl)-β-D-glucuronopyranoside] of 18β-glycyrrhetic acid (3β-hydroxy-11-oxo-18β,20β-olean-12-en-30-oic acid).



Sources of glycyrrhizic acid are the roots of common licorice *Glycyrrhiza glabra* L, and of Urals licorice *Glycyrrhiza uralensis* Fisch., family Fabaceae.

In the form of its ammonium salt (**2**) (Glitsiram), glycyrrhizic acid is widely used in medicine in cases of bronchial asthma and a number of other diseases.

Glitsiram (glycyrrhizin) and licorice and extracts of it are described in practically all pharmacopeias. In addition, glycyrrhizin is a well-known sweetening agent, and in the USA it is approved for use as GRAS (generally recognized as safe) [1].

In the course of development of variants of a waste-free technology for the manufacture of preparations from common licorice roots we have additionally studied some properties of glycyrrhizic acid and its monoammonium salt. It is known that the majority of catalogs, pharmacopeias, publications, and patents describe only the properties of monoammonium glycyrrhizate even though acid conditions are used in the course of processing the raw material — licorice roots and rhizomes — and the industrial process includes the isolation of technical glycyrrhizic acid. In individual cases preparations of highly purified glycyrrhizic acid, obtained either via salts or with the aid of preparative HPLC, have also been described [2, 3].

In the course of appropriate experiments, we have established that glycyrrhizic acid (GA), unlike Glitsiram, is unstable on heating, and when, on working with it, attempts are made to employ prolonged evaporation of its solutions, drying by the action of heat, and recrystallization with heating it decomposes, giving numerous solvolysis products and humic substances. In these circumstances, GA, being a glycoside, apparently decomposes autocatalytically.

In view of this, any technology for the production of GA must be conducted under gentle conditions with few stages. For this reason, such an expedient as the freeze-drying of aqueous and aqueous-alcoholic solutions of GA recommends itself.

All-Russian Scientific Research Institute of Medicinal and Aromatic Plants, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 772-780, November-December, 1994. Original article submitted June 13, 1994.

 0009-3130/94/3006-0720$12.50 ©1995 Plenum Publishing Corporation

TABLE 1. Details of the ^1H NMR Spectra of Glycyrrhizic Acid (1) and Ammonium Glycyrrhizate (2) in Comparison with Literature Figures for Related Compounds [6, 9] (δ, ppm (J, Hz), TMS; s — singlet, d — doublet)

Compound	Characteristic protons of the terpenoid skeleton					Anomeric protons		Methyl groups, H-23, -24, -25, -26, -27, -28, -30; singlets
	H-1c d	H-3 dd	H-9 s	H-12 s	H-18 dd	H-1' d	H-1 d	
Glycyrrhizic acid (1) C_5D_5N, 200 MHz 20 C	3.02	3.37	2.43	5.96	2.52	5.04 7.36 Hz	5.52 7.00 Hz	0.75, 1.01, 1.16, 1.22, 1.34, 1.37, 1.41
Glycyrrhizic acid (1) C_5D_5N, 200 MHz 80 C	3.05 13.5 Hz	3.42 3.8 Hz	2.47	5.94	2.55 3.8Hz 13.5 Hz	5.00 7.10 Hz	5.33 7.34 Hz	0.84, 1.12, 1.21, 1.26, 1.36, 1.42, 1.47
Ammonium glycyrrhizate (2) C_5D_5N, 200 MHz; 20 C	3.04	3.39	2.44	6.00	2.53	5.07 7.28 Hz	5.64 7.00 Hz	0.80, 1.04, 1.16, 1.22, 1.33, 1.40, 1.43
Ammonium glycyrrhizate (2) C_5D_5N, 200 MHz 70 C	3.00 12.7 Hz	3.40 3.8 Hz 11.7 Hz	2.44	5.93	2.51 3.8 Hz 13.5 Hz	4.95 7.13 Hz	5.34 7.33 Hz	0.82, 1.06, 1.16, 1.20, 1.34, 1.37, 1.42
Ammonium glycyrrhizate (2) CD_3OD, 200 MHz 20 C	2.68 12.7 Hz		2.46	5.58		4.50 6.90 Hz	4.65 7.34 Hz	0.83, 0.84, 1.05, 1.13, 1.13, 1.16, 1.42
Glycyrrhizin 30-stearyl ester (3) $CDCl_3$, CD_3OD, 400 MHz [6]				5.63		4.50 7.2 Hz	4.61 7.2 Hz	0.83, 0.84, 1.05, 1.13, 1.13, 1.19, 1.40
18β-Glycyrrhetic acid (5) C_5D_5N, 300 MHz [9]	3.10 13.5 Hz	4.72	2.48	5.95	2.52 13.6 Hz	–	–	0.80, 0.93, 0.93, 1.10, 1.26, 1.30, 1.43
18α-Glycyrrhetic acid (6) C_5D_5N, 300 MHz [9]	2.99	4.72	2.36	5.76	2.34	–	–	0.72, 0.93, 0.93, 1.11, 1.32, 1.33, 1.43

In the presence of mineral acids it is all the more necessary to take account of the above-mentioned points — i.e., briefness of action and absence of heating. We have therefore tested the use of ion-exchange resins [4, 5]. This gave good results, but the use of ion-exchangers is possible only with large volumes of dilute aqueous solution of GA, which makes it necessary to combine this stage of the industrial process with freeze-drying.

We have been inclined to the following technological sequence in the production of Glitsiram: extraction of the GA from an enriched dry extract of licorice root with acidified alcohol, precipitation of the GA salt with ammonia, purification of the monoammonium salt with acetic acid, and recrystallization from aqueous alcohol. The whole process, including drying, is carried out at room temperature; the only exception is the heating of the monoammonium salt during recrystallization. This method permits an increase in yield of the desired product having constants agreeing with those given in the literature.

To study the authenticity and quality of the Glitsiram obtained, we made use of TLC, HPLC, UV spectroscopy, and NMR. The ^1H and ^{13}C NMR spectra of various specimens of glycyrrhizic acid and its monoammonium salt were studied. For these purposes we obtained crystalline glycyrrhizic acid with mp 228-230°C (decomp.), UV spectrum: λ 249 nm, ε 11800.

The ^1H NMR spectra of GA and Glitsiram were recorded at 200 MHz on a Gemini-200 spectrometer for solutions in deuteropyridine, and proved to be identical (see Fig. 1 and Table 1). It must be mentioned that it was possible to obtain well resolved spectra only for dilute solutions at 70-80°C. We used the standard programs of the Gemini spectrometer for experiments on line narrowing, especially for detecting the signals of the anomeric protons, which were masked by the water signal.

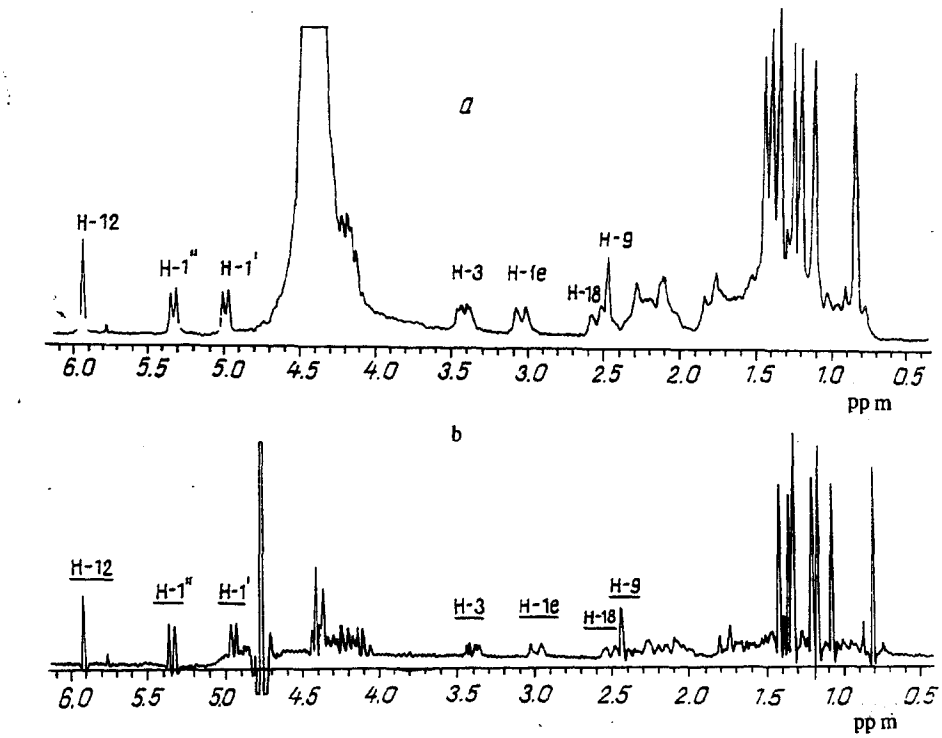


Fig. 1. ^{1}H NMR spectra of glycyrrhizic acid (*a*) and of monoammonium glycyrrhizate (*b*) (C_5D_5N, 200 MHz, 80°C, TMS).

No impurity signals were detected in the PMR spectrum, while the signals of anomeric protons in the form of well-resolved doublets with coupling constants greater than 7 Hz (δ 5.33 and 5.00) confirmed the β-configurations of the glycosidic bonds and the $^4C_1$-conformation of both *D*-glucuronic acid fragments in the pyranose form.

The spectrum (see Fig. 1) well shows the singlet signals of seven methyl groups (0.8-1.5 ppm), those of the proton at C-9 (δ 2.47) and of a proton at a double bond, H-12 (δ 5.94), and also a doublet of doublets for the proton at C-3 (3.42 ppm, 3.8 and 11.7 Hz) and a doublet of the equatorial proton H-1_e at 3.05 ppm (J = 13.5 Hz). A doublet of doublets for H-18 is located at 2.55 ppm (J = 3.8 and 13.5 Hz). The assignment of the other overlapping proton signals is extremely problematical even with the use of two-dimensional homonuclear HH COSY and heteronuclear CH spectroscopies with the aid of which an assignment has been made previously of the ^{1}H and ^{13}C signals for the steoisomeric pair of 18α/18β-glycyrrhetic acid acetates [9].

Thus, the results of our investigations, like the literature information for 30-stearyl ester of glycyrrhizin [6], point to the structure of glycyrrhizic acid (**1**) shown above.

It must be mentioned that the structure of glycyrrhizic acid was proposed in 1950 [7], and, although it had been shown as early as 1956 [8] that both glucuronic acid residues had the β-configuration, many authors still give the erroneous α-configuration for the bond of the biose with the aglycon (this includes the Fluka and Aldrich catalogues, the Merck Index, and the VFS [Provisional Pharmaceutical Standard] 42-419-75 "Glitsiram").

With the aid of proton NMR spectra it has become possible to choose between the 18α- and 18β-isomers of glycyrrhetic acid (the aglycon of glycyrrhizic acid), and experiments on such differentiation on the basis of ^{13}C NMR spectra have also been described in the literature [9, 10].

The results of our work enable us to recommend as diagnostic for the GA bioside the singlet signals of the H-12 and H-9 protons, which are readily identified in the spectra and are therefore the most informative in differentiating 18α- and 18β-glycyrrhizic acids. The H-18 and H-22 signals are also fairly informative; however, for the 18α- and 18β-isomers these signals are located in the 2.18-2.48 region and overlap one another (Table 1), only the singlet H-9 signal standing out.

In the investigations of proton spectra we based ourselves on literature results obtained for the acetates of 18α- and 18β-glycyrrhetic acids, and also onthe results of an examination of corresponding regions of the PMR spectra of a whole series of specimens of glycyrrhizic acidand Glitsiram. In the PMR spectrum ofthe 18β-isomer the H-12 and H-9 singlet signals were

TABLE 2. Comparison of the 13C NMR Chemical Shifts of Glycyrrhizic Acid and Glitsiram with 18β-Glycyrrhetic Acid and its Acetate (δ, ppm, TMS)

Carbon atom	Glycyrrhizic acid (1), deuteropyridine, 50 MHz, 80°C	Monoammonium glycyrrhizate (2), deuteropyridine, 50 MHz, 70°C	18β-Glycyrrhetic acid (4), $CDCl_3$—DMFA, 22.5 MHz [10]	18β-Glycyrrhetic acid acetate (5), deuteropyridine, 75 MHz [9]
1	39.79	39.83	39.11	39.00
2	27.06	27.09	27.12	23.90
3	89.53	89.84	78.44	80.47
4	40.17	40.21	39.11	38.35
5	55.74	55.71	54.93	55.07
6	17.87	17.87	17.47	17.61
7	33.19	33.22	32.75	32.72
8	43.87	43.79	43.20	43.46
9	62.38	62.38	61.77	61.91
10	37.48	37.48	37.07	37.32
11	200.02	200.04	200.43	199.41
12	128.84	128.85	128.40	128.56
13	170.07	170.10	169.49	169.91
14	45.83	45.85	45.45	45.49
15	26.91	26.87	26.40	26.58
16	26.91	26.87	26.40	26.76
17	32.39	32.40	31.85	32.10
18	49.00	49.03	48.17	48.65
19	41.96	41.99	40.86	41.61
20	44.37	44.39	43.79	44.04
21	31.82	31.86	30.90	31.50
22	38.66	38.68	37.70	38.21
23	28.37	28.33	28.06	28.12
24	17.08	17.12	15.61	16.97
25	16.96	16.91	16.36	16.64
26	19.07	19.08	18.68	18.75
27	23.78	23.81	23.39	23.52
28	28.93	28.97	28.43	28.68
29	28.93	28.97	28.53	28.68
30	179.52	179.55	181.53	179.08
1′	105.24	105.15	–	–
2′	84.54	82.65	–	
3′	77.53	77.15	–	
4′	73.20	73.56	–	
5′	77.92	78.00	–	
6′	172.34	173.87	–	

TABLE 2. (Continued)

Carbon atom	Glycyrrhizic acid (1), deuteropyridine, 50 MHz, 80°C	Monoammonium glycyrrhizate (2), deuteropyridine, 50 MHz, 70°C	18β-Glycyrrhetic acid (4), $CDCl_3$—DMFA, 22.5 MHz [10]	18β-Glycyrrhetic acid acetate (5), deuteropyridine, 75 MHz [9]
1'''	106.92	105.94	-	-
2'''	76.92	76.69	-	-
3'''	77.79	77.46	-	-
4'''	73.46	73.74	-	-
5'''	78.50	78.25	-	-
6'''	172.69	174.07	-	-

located at 5.95 and 2.48 ppm, while for the 18α- isomer they were at 5.76 and 2.36 ppm, respectively (solution in deuteropyridine, see Table 1) [9].

The chemical shift of the H-12 singlet (δ 5.96 in deuteropyridine) characterizes the substances studied (**1**) and (**2**) as 18β-epimers. However, in some specimens an additional singlet was observed at δ 5.76 with an intensity of about 15% of that of the signals at δ 5.96.

In the purest samples of Glitsiram (HPLC monitoring) there was no trace whatever of the signal at 5.76 ppm (and an HPLC chromatogram had no peak with a RT of 18 min), which is readily detected in GA specimens containing 15% of a component with a RT of 18 min on HPLC chromatograms (obviously 18α-glycyrrhizic acid).

We must mention the good agreement of the percentage content of the peak having an RT of 18 min with the percentage ratio of the intensities of the H-12 singlet signals (δ 5.76; δ 5.96) in the specimens of glycyrrhizic acid and Glitsiram that were studied.

Thus, the region of H-12 singlet signals (5.96 and 5.76 ppm) may serve not only for distiguishing the 18α- and 18β-isomers but also for estimating their relative purities.

The signals of the terpenoid protons of glycyrrhizic acid and Glitsiram practically coincided with the analogous signals of 18β-glycyrrhetic acid acetate (Table 1), only the chemical shifts of the protons at C-3 being different. In the compounds studied in which the 3-OH group was glycosylated, this signal was located at about 3.4 ppm, while in each of the acetates mentioned (**5**, **6**) the hydroxyl at C-3 was esterified by acetic acid and the gem-acyl H-3 proton had a chemical shift of 4.72 ppm.

So far as concerns the HPLC analysis of samples of glycyrrhizic acid and Glitsiram, when acid and neutral mobile phases were used the substances exhibited instability, and solvolysis products, absent from the sample to be analyzed, were formed as impurities, which makes it difficult to use this method to determine the purity of preparations and their principles.

In the structural analysis of compounds of this group, ^{13}C NMR spectroscopy is fairly informative. A number of authors [9-13] have investigated and interpreted the ^{13}C NMR spectra of several pairs of derivatives of 18α/18β-glycyrrhetic and liquiritic acids and have shown that the configuration at C-18 can readily be determined from the chemical shifts (CSs) of several characteristic carbon atoms (C-12, C-13, C-18, C-28) the positions of the signals of which are due to the type of linkage of rings *D/E* and do not depend on the substituents at C-3 and C-20.

There were almost no differences in the ^{13}C NMR spectra of the terpenoid moieties of our specimens of Glitsiram and glycyrrhizic acid from those of the 18β-aglycon (the acid and its acetate), although appreciable differences in the chemical shifts of a whole series of carbon atoms of the 18β- and 18α-epimers are known [9].

Table 2 gives details of the ^{13}C NMR spectrm of glycyrrhizic acid and its monoammonium salt and also literature figures for 18β-glycyrrhetic acid and its acetate [9, 10]. The assignment of the signals of the triterpene part was made by comparison with the above-mentioned aglycon, the chemical shifts of these skeletal carbon atoms of glycyrrhizic acid and of

18β-glycyrrhetic acid being very close, with appreciable differences observed only for C-3 (89.53 and 78.44 ppm, respectively), which is connected with the O-glycosylation of this atom.

So far as concerns the signals of the carbohydrate moiety, we made their assignment on the basis of a comparison with related flavonoid biosides [14]. As was to be expected, in the spectra of the bioside under investigation and its salt form the C-2′ signal had shifted downfield considerably in comparison with the glycosides, which confirmed the attachment of the terminal glucuronic acid residue to the hydroxyl at this carbon atom.

As mentioned above, in catalogs of pure substances an incorrect form of the carbohydrate moiety is given for glycyrrhizic acid and there are also variants in the position of attachment of the ammonium group. We have made an attempt to refine this aspect in the structure of monoammonium glycyrrhizate.

Earlier, the ammonium group was assigned to the C-30 carboxyl of glycyrrhetic acid on the basis of IR spectra [15]. However, an analysis that we have made of ^{13}C NMR spectra (region of resonance of the COOH group) has permitted the exclusion of the formation of the salt at the C-30 carboxyl, since the signal of this atom (179.5 ppm) undergoes no changes in monoammonium glycyrrhizate in comparison with the initial glycyrrhizic acid. At the same time, the roughly equal small changes in the chemical shifts of the C-6′′ and C-6′ signals (+1.53 and +1.38 ppm, respectively) (see Table 2) lead to the hypothesis of the simultaneous participation of the carboxy groups of the two glucuronic acid residues in salt formation and thereby raises doubt as to whether a mono- or a di-substituted salt is formed in the course of known industrial processes for obtaining Glitsiram.

Potentiometric titration of glycyrrhizic acid has provided evidence in favor of a mono- salt: a determination of the ionization constant showed the existance of a transition at pK_a 7.4 corresponding to the consumption of one equivalent of titrant (NH_4OH) and relating to the strongest acid group out of the three present in the molecule.

Elementary analysis for the nitrogen content also characterized the substance as monoammonium glycyrrhizate. In view of what has been said, it is obvious that the NH_4^+ cation is bound with the carboxy groups of the biosidic part, and the nature of this bond is in need of further investigation.

Thus, a study of chemical and spectral properties has permitted a critical interpretation of features of the technology for the production of glycyrrhizic acid and Glitsiram and has also led to the proposal of diagnostic parameters of the NMR spectra of the 18β/18α-epimers of these compounds. In view of the experimental and literature facts presented, it appears desirable to perform additional investigations to refine the structural problem connected with features of the salt-forming process.

EXPERIMENTAL

^1H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 spectrometer with working frequencies of 200 and 50 MHz, respectively. The solvents were C_5D_5N and CD_3OD (with TMS as external standard). Experiments on line narrowing and the recording of APT spectra were carried out by the standard Varian programs of the Gemini-200 spectrometer.

Glycyrrhizic Acid (1). A solution in distilled water (3000 ml) of 50 g of dry licorice root extract, freed from flavonoids and containing 31% of GA, was passed through a column containing 130 g of the cation-exchange resin KU-2-8 in the H^+ form. The filtrate was lyophilized. The residue after lyophilization was extracted with 260 ml of ethanol, the alcohol was evaporated off in vacuum (bath temperature below 40°C), and the residue (9.8 g) was crystallized from 15 ml of glacial acetic acid. The crystals were separated on a filter, washed with 10 ml of cooled ethanol, and dried.

Yield 2.6 g (17%). Colorless plates, $C_{42}H_{62}O_{16}\cdot 4H_2O$, mp 228-230°C (decomp.), $[\alpha]^{20}_{546}$ +58.0°, $[\alpha]^{20}_{D}$ +46.2° (c 1.5; EtOH). UV spectrum in ethanol: λ_{max} 249 nm, ε 11800; for the ^1H NMR spectrum see Fig. 1 and Table 1, and for the ^{13}CNMR spectrum see Table 2.

Glitsiram (Monoammonium Glycyrrhizate) (2). Dry licorice roots extract freed from flavonoids and containing 31% of GA (20 g) were treated three times with a 0.25% solution of H_2SO_4 in alcohol (ratio 1:10) with stirring for 30 min. The combined filtered solution was treated with 0.2 ml of 25% NH_3, and the precipitate that deposited was separated off, washed with alcohol, and dissolved in 20 ml of glacial acetic acid. The precipitate that again deposited was filtered off, well washed with alcohol, recrystallized from 85% alcohol and dried in the air.

Yield 2.0 g (30% of the GA present in the initial extract). Colorless prisms,$C_{42}H_{62}O_{16}\cdot NH_3\cdot 4H_2O$ (Found, % C 55.08, H 8.03, N 1.62; calculated, %: C 55.30, H 8.07, N 1.54), mp 220-222°C (decomp.). $[\alpha]^{20}_{546}$ +58.9°, $[\alpha]^{20}_{D}$ +46.9° (c 1.5; 50% EtOH); UV spectrum in ethanol: λ_{max} 252 nm, ε 10500; for the ^1H NMR spectrum see Table 1, and for the ^{13}C NMR spectrum Table 2.

REFERENCES

1. Martindale's Extra Pharmacopeia (28th ed.), Pharmaceutical Press, London (1982).
2. L. A. Baltina, N. G. Serdyuk, N. A. Somov, Yu. I. Murinov, and G. A. Tolstikov, in: The Study and Use of Licorice in the National Economy of the USSR [in Russian], Gylym, Alma-Ata (1991), p.140.
3. L. V. Krasnova, A. A. Berg, L. I. Afanas'ev, L. A. Baltina, and Yu. I. Murinov, ibid., p. 126.
4. G. A. Tolstikov, U. M. Dzhemilev, D. Ya. Soshnikov, A. G. Petunina, and A. Sh. Yukhanova, Method of Isolating Glycyrrhizic Acid [in Russian], USSR Inventors' Certificate 419521 (applied for: June 14, 1972).
5. I. Tosisuke, Japanese Patent 15875 (October 21, 1960); Ref. Zh. Khim., 10N222P (1963).
6. H. Tsuji, S. Osaka, and H. Kiwada, Chem. Pharm. Bull., **39**, No. 4, 1004 (1991).
7. B. Lythgoe and S. Trippet, J. Chem. Soc., 1983 (1950).
8. C. A. Marsch and G. A. Levy, Biochem. J., **63**, No. 1, 9 (1956).
9. L. M. Khalilov, E. V. Vasil'eva, A. A. Fatykhov, and G. A. Tolstikov, Khim. Prir. Soedin., 363 (1991).
10. G. A. Tolstikov, L. M. Khalilov, L. A. Baltina, R. M. Kondratenko, A. A. Panasenko, and E. V. Vasil'eva, Khim. Prir. Soedin., 645 (1985).
11. H. Duddeck, M. H. A. Elgamal, G. S. Ricca, B. Danieli, and G. Palmisano, Org. Magn. Reson., **11**, No. 3, 130 (1978).
12. G. S. Ricca, B. Danieli, G. Palmisano, H. Duddeck, and M. H. A. Elgamal, Org. Magn. Reson, **11**, No. 4, 163 (1978).
13. U. Wrzeciono, L. Zaprutko, J. Budzianowsky, H. Wojtowicz, and D. Dubowska, Magn. Reson. Chem., **25**, 223 (1987).
14. K. R. Markham and V. Mohan Chari, in: The Flavonoids: Advances in Research, J. B. Harborne and T. J. Mabry (eds.), Chapman and Hall, London (1982).
15. I. A. Murav'ev, V. D. Ponomarev, and É. K. Kravchenko, Khim. Prir. Soedin., 121 (1971).