OPTIMIZATION OF THE METHOD OF ISOLATING (25R)-SPIROST-5-ENE-3β,17α-DIOL (PENNOGENIN) FROM PLANT MATERIAL

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The method for the acid hydrolysis of pennogenin glycosides with the aim of isolating the native aglycon has been optimized. The highest yield of pennogenin (49.5% of its amount in the plant material) is given by hydrolysis with 1 N H_2SO_4 in 96% ethanol at 100°C for 3 h.

The results of an investigation of the synthetic possibilities of pennogenin [1, 2] demand a further study of its transformation into physiologically active steroids. In view of this, the question has arisen of optimizing the method for isolating it from plant material. We have investigated one of the most important stages of the isolation of aglycons — acid hydrolysis — with the aim of determining the conditions for the optimum yield of the desired product — pennogenin. The presence in pennogenin of a 17α-hydroxyspiroketal fragment leads to its increased acid lability — in comparison with other steroids of the spirostan series. Among the artefacts from the hydrolysis of pennogenin glycosides [3-5] the main one is kryptogenin. In preparative work, a 2 N solution of sulfuric acid in 50% ethanol with boiling for 2 h is most frequently used for hydrolysis [5]; a method of hydrolysis in a two-phase system has also been proposed [6]. However, the dependence of the yields of pennogenin on the conditions of acid hydrolysis were not investigated in the studies mentioned.

As the source of pennogenin we have used the hypogeal part (below, roots) of *Polygonatum stenophyllum* Maxim. [7]. Among the products of the hydrolysis of the total glycosides extracted from the roots with ethanol, the main aglycons are pennogenin and kryptogenin [8]. The amount of the other two aglycons that have been isolated and identified — β-sitosterol and diosgenin — is relatively small [9]. Since the question of the presence in *P. stenophyllum* of kryptogenin glycosides has not yet been answered, we arbitrarily assumed that the whole of the kryptogenin isolated consisted of an artefact of the acid-catalyzed transformation of pennogenin. In view of this, it also appeared of interest to follow its formation in the process of acid hydrolysis.

In the performance of the investigation on the quantitative level, a GLC procedure for determining pennogenin and kryptogenin was first developed. A study of the chromatographic behavior of the steroids showed that their acetates and TMS ethers were stable in a chromatographic column with the stationary liquid phase (SLP) OV-1, which was confirmed by the formation of single symmetrical peaks for each compound, and also by the agreement of the mass spectra taken with the direct introduction of the sample into the ion source of the instrument and via the chromatographic column. In the case of the diacetate and the di-TMS ether of kryptogenin, partial adsorption on the column due to the presence of two free carbonyl groups in its molecule was observed. This restricted the lower limit of linearity of the response of the detector. An attempt to improve the chromatographic properties of the di-TMS ether by the preparation of its dimethoxime proved inapplicable for the mixture of steroids under investigation. Under the conditions of the methoximation reaction of kryptogenin (10-fold excess of $CH_3ONH_2 \cdot HCl$ per carbonyl group, in pyridine, 100°C, 2 h), pennogenin underwent partial transformation into kryptogenin and its dimethoxime (TLC results), which made the unambiguous preparation of derivatives for GLC difficult. Consequently, in subsequent work we used the acetates with constant calibration factors of 1.16 and 1.30 in the 1.2-7.2 μg and 3.6-10.8 μg intervals for the acetates of pennogenin and of cryptogenin, respectively. Cholesterol acetate was used as internal standard. An ordinary GLC chromatogram of the steroid products of hydrolysis is given in Fig. 1.

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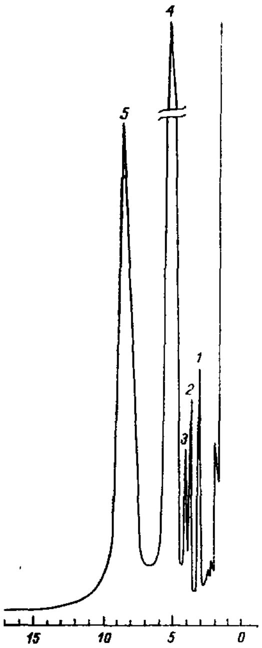

Fig. 1. Chromatogram (225-265°C, 5°C/min) of the products of the hydrolysis of an ethanolic extract of the roots of *Polygonatum stenophyllum*. Acetates of: 1) cholesterol; 2) diosgenin; 3) β-sitosterol; 4) pennogenin; 5) kryptogenin.

The investigation was performed on a sample of roots containing 6% of glycosides, calculated as the aglycon. This figure was taken for control purposes, and the results of the GLC analysis were calculated as percentages of it.

We first studied hydrolysis in a two-phase system: benzene—aqueous sulfuric acid (1:2) [6]. Here pronounced resinification of the hydrolysis products wss observed. At the same time, no complete cleavage of the glycosidic bonds was achieved because of the insolubility in the aqueous medium of the weakly polar glycosides and of the progenins formed as a result of the hydrolysis and detected in TLC. This circumstance is also mentioned in [10]. Here the yields of pennogenin and kryptogenin amounted to 26 and 10%, respectively. A variation in the concentration of the acid and also in the time of hydrolysis did not increase the yields of the products mentioned.

To raise the solubility of the glycosides and progenins we used as the reaction medium aqueous solutions of methanol (single-phase system). In the experiments the concentration of sulfuric acid, the amount of ethanol in the reaction mixture, and the time of hydrolysis were varied; the temperature of hydrolysis (100°C) and the ratio of the amounts of material and of the acid (1:100, g/ml) were kept constant in all cases. The results obtained (means of three repetitions) are given in Table 1, from which it can be seen that, under otherwise identical conditions, with an increase in the amount of ethanol in the reaction mixture the yield of the main products rose.

The maximum yield of desired product pennogenin (49.5%) and also of kryptogenin (41.8%) was achieved by the use of 1 N sulfuric acid in 96% ethanol with a hydrolysis time of 3 h. The total yield of these products then amounted to 91.8% on the amount of pennogenin in the sample of roots. An increase in the concentration of acid to 1.5 N appreciably lowered the yield of the main products. The use of acid of still higher concentrations (2 N and 3 N) led to pronounced resinification of the hydrolysis products, as was shown by the increase in the amount of secondary products of steroid nature (TLC) which were recorded by GLC as multiple peaks poorly separated from the pennogenin and kryptogenin peaks, which made the quantitative determination of the latter impossible.

Experiments using 5 g of the material showed the possibility of halving the volume of ethanol in the reaction mixture while retaining the other optimized parameters of hydrolysis with no lowering of the yield of pennogenin which is important for preparative work.

When the 1 N H_2SO_4 was replaced by hydrochloric acid under the optimum conditions of hydrolysis, the yield of pennogenin was only 5%. The main products under these conditions were kryptogenin and compound (1), which, after isolation with the aid of column chromatography on SiO_2, was identified by its physicochemical characteristics and ^1H NMR spectrum as 26-chloro-26-deoxykryptogenin [8]. The identification of (1) was also confirmed by the ^{13}C NMR spectra of this compound and of its acetate (2), recorded for the first time. The assignment of the signals in the spectra of (1) and (2) was made on the basis of a comparison of these spectra with those of kryptogenin, and its diacetate.

^{13}C NMR spectrum of (1) ($CDCl_3$, δ, ppm): 37.1 – C(1), 31.7 – C(2), 71.7 – C(3), 42.3 – C(4), 120.9 – C(6), 141.2 – C(5), 31.8 – C(7), 31.1 – C(8), 49.9 – C (9), 36.7 – C(10), 20.7 – C(11), 39.6 – C(12), 41.8 – C(13), 51.4 – C(14), 38.8 – C(15), 217.9 – C(16), 66.4 – C(17), 15.4 – C(18), 19.4 – C(19) 43.5 – C(20), 13.0 – C(21), 213.8 – C(22), 37.3 – C(23), 27.6 – C(24), 34.8 – C(25), 50.9 – C(26).

EXPERIMENTAL

A sample of the roots of *Polygonatum stenophyllum* collected in the full budding phase in the Shkotovo region of the Maritime Territory was used. The roots were ground in a ball mill to a grain size of 5-10 μm and were dried to constant weight. The analytical indices calculated on the dry matter were as follows: 51.5% of extractive substances extractable by boiling 70% ethanol; 6% of pennogenin according to the results of spectrophotometry using the reaction with 72% sulfuric acid catalyzed by Fe(III) ions [5]; 0.12% of diosgenin; and 0.05% of β-sitosterol, according to GLC results after acid hydrolysis.

Hydrolysis in a Single-Phase System. The dry residue from the ethanolic extract of 0.1 g of material was treated with 10 ml of 0.5, 1.0, 1.5, and 2.0 N solution of H_2SO_4 in 50, 70, and 96% ethanol, and the mixtures were heated at the boil for 2, 3, and 5 h. Each hydrolysate was diluted with water (20 ml) and extracted with diethyl ether (3 × 3 ml). The combined ethereal extracts were washed with water, with 0.5% $NaHCO_3$ solution, and again with water and were dried with anhydrous Na_2SO_4, and the volume of the extract was made up accurately to 10 ml with ether. An aliquot part (2.5 ml) of this solution was treated with 0.05 ml of cholesterol in 0.5 ml of ether and the mixture was evaporated to dryness. Dry pyrdine (0.1 ml) and freshly distilled acetic anhydride (0.1 ml) were added to the residue and this mixture was left at room temperature for 12 h. The reagents were carefully driven off by the passage of a current of air, the residue was dissolved in $CHCl_3$ (0.25 ml), and 3-4 μl was introduced into the GLC column.

Hydrolysis in a Two-Phase System [6]. The dry residue of the ethanolic extract from 0.1 g of material was treated with a 3 N aqueous solution of sulfuric acid (10 ml) and with benzene (5 ml) and the mixture was heated in the boiling water bath for 5 h. The benzene layer was separated off and the hydrolysate was extracted with ether (3 × 3 ml). The combined benzene layer and ethereal extract were worked up and analyzed as described above.

The GLC analysis of the hydrolysis products was carried out on a Tsvet-104 chromatograph with a FID and a packed column (0.3 × 200 cm) containing 1.5% of the SLP OV-1 on Chromaton N-super (0.25-0.315 mm) at 225-265°C (5°C/min) with a rate of flow of helium of 70 ml/min.

TABLE 1. Yields of Pennogenin and Kryptogenin as Functions of the Conditions of Acid Hydrolysis (in % on the amount of pennogenin in the sample)

Hydrolysis conditions			Found, %		
H_2SO_4, N	ethanol, %	time, h	pennogenin	kryptogenin	sum
1,0	50	3	12,5	20,6	33,1
1,0	70	3	14,2	27,7	41,9
1,0	96	3	49,5	41,8	91,8
0,5	96	3	35,5	19,8	55,1
1,5	96	3	39,5	31,0	70,5
1,0	96	2	41,7	29,7	71,4
1.0	96	5	49.0	33.0	82.0

Mass spectra were taken on a LKB-9000 instrument with direct introduction of the sample into the ion source and via a chromatographic column (0.3 × 200 cm) containing 1.5% of SE-30 on Chromaton N-super (0.250-0.315 mm) at 245°C, and ^{1}H and ^{13}C NMR spectra ($CDCl_3$) on a Bruker-250 WM instrument at working frequencies of 250 and 62.6 MHz, respectively. The accuracy of the measurement of the CSs was better than 0.1 ppm in the ^{13}C NMR spectra and better than 0.01 ppm in the ^{1}H spectra.

Analytical TLC was carried out on Silufol plates in the hexane—acetone (7:3) system. Preparative chromatography was performed on plates coated with KSK silica gel (200-215 μm) in hexane containing increasing proportions of acetone up to a ratio of 1:1.

SUMMARY

The method for the acid hydrolysis of pennogenin glycosides with the aim of isolating the native aglycon has been optimized. The highest yield of pennogenin (49.5% of its amount in the plant raw material) is obtained by the use of 1 N H_2SO_4 in 96% ethanol (3 h, 100°C). The remainder of it undergoes acid-catalyzed transformation into kryptogenin (41.8%) and other by-products. The replacement of sulfuric acid by hydrochloric acid of the same concentration led to the (~95%) conversion of pennogenin into kryptogenin and its 26-chloro-26-deoxy analog.

LITERATURE CITED

1. G. B. Elyakov, T. V. Ilyukhina, A. V. Kamernitskii, et al., Izv. Akad. Nauk SSSR, Ser. Khim., No. 5, 1150 (1977).
2. G. B. Elyakov, T. V. Ilyukhina et al., Izv. Akad. Nauk SSSR, Ser. Khim., No. 5, 1164 (1979).
3. R. E. Marker and J. Lopes, J. Am. Chem. Soc., 69, No. 1, 2386 (1947).
4. T. Nohara, K. Miyahara, and T. Kawasaki, Chem. Pharm. Bull., 22, No. 8, 1772 (1974).
5. K. Nakano, Y. Kashiwada, and T. Nohara et al., Yakugaku Zasshi, 102, 1031 (1982).
6. L. I. Strigina, E. V. Kol'chuk, G. B. Elyakov, and P. G. Gorovoi, USSR Inventor's Certificate No. 475852; Byull. Izobret., No. 27, 186 (1976).
7. L. I Glebko, L. I. Berezhevskaya, Zh. I. Ul'kina, L. I. Strigina, and P. G. Gorovoi, Rast. Resur., No. 1, 76 (1986).
8. L. I. Strigina, E. V. Kol'chuk, and E. N. Pilipenko, Khim. Prir. Soedin., 121 (1977).
9. L. I. Glebko, Zh. I. Ul'kina, L. I. Berezhevskaya, S. A. Zinova, L. I. Strigina, and L. G. Gorovoi, Khim. Prir. Soedin., 841 (1985).
10. L. I. Strigina, Khim. Prir. Soedin., 653 (1983).