COLOR REACTIONS OF HEDERAGENIN AND ITS GLYCOSIDES

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The spectrophotometric reactions of triterpene compounds with concentrated sulfuric acid, a mixture of sulfuric acid and ethanol, and a mixture of sulfuric and acetic acids has been studied with hederagenin and its glycosides — caulosides A, C, and D, isolated from the Far Eastern plant *Caulophyllum robustum* Maxim — as examples. Conditions for performing the reaction which exclude the influence of the carbohydrate components of the glycosides from the determination of hederagenin have been suggested. The reaction with a mixture of sulfuric acid and ethanol has been used to determine the amounts of caulosides A and C in a total preparation after their separation by the TLC method. The relative error of the determination did not exceed 5 rel.%.

In recent years, triterpene glycosides and, in particular, hederagenin glycosides, have attracted attention as compounds possessing many valuable types of physiological activity. However, information on the methods for their quantitative determination in plant raw material and drug preparations is extremely limited.

At the present time, the determination of each glycoside in the presence of other is done photometrically after chromatographic separation, for example, in a thin layer of sorbent. Consequently, the choice and study of the photometric reaction are of great importance. It must be sufficiently sensitive to ensure the possibility of determining small amounts of glycosides after their separation and should be specific in relation to the aglycone, since a set of pure glycosides for the plotting of calibration graphs is not always available.

We have studied a number of color reactions of hederagenin and a group of its glycosides — caulosides isolated from the Far Eastern plant *Caulophyllum robustum* Maxim [1].

In spite of the voluminous literature on the color reaction of steroids and terpenoids, it did not appear possible to obtain a clear idea of the behavior of hederagenin and its glycosides in these reactions without an experimental check. Consequently, we have tried a large number of known reagents, of which the best proved to be concentrated sulfuric acid and its mixtures with acetic acid and with ethanol.

It had previously been established that the adsorption spectra of hederagenin glycosides — leontosides — in concentrated sulfuric acid have two characteristic maxima, at 310-

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320 and 450 nm — the latter of which is characterized by a constancy of its intensity on passing from hederagenin to its glycosides [2]. However, no metrological characteristics of the reaction are given in the paper cited.

The absorption spectra of hederagenin and the caulosides in concentrated sulfuric acid were identical and had the same two maxima at 310 and 405 nm. In addition, one scarcely distinguishable maximum was observed at 530 nm the intensity of which depended on small variations in the concentration of the acid. A slow increase in the intensity of absorption at 405 nm with an increase in the time of the reaction was also observed. Raising the temperature accelerated this process. For hederagenin, the optical density of a solution, A, reached its maximum value at 70°C in 30 min. Under these conditions the molar absorption coefficient E was 19,450. However, the influence of the sugar component of the glycosides on A_{405} for hederagenin was considerable. Milder conditions were necessary in order to ensure the specificity of the reaction. As was found, at 60°C and 10 min the influence of the sugars was practically absent, and the value of E_{405} of the chromophore formed from hederagenin was still fairly high. The results of the measurements are given in Table 1.

It appeared to us that the specificity of the reaction could be increased still further if the determination were carried out in the longer-wave region of the spectrum. With this aim we studied the behavior of hederagenin and caulosides in reactions with sulfuric acid diluted with glacial acetic acid or with ethanol. Regardless of which "diluent" was added to the sulfuric acid, well-defined stable maxima appeared in the absorption spectra both of hederagenin and of the caulosides. Their intensity depended primarily on the ratio of sulfuric acid and "diluent," and also on the temperature and time of the reaction. It was found that the rise in A_{530} with the addition of the diluent was accompanied by a simultaneous fall in A_{405}. An increase in the proportion of diluent in the reaction mixture above 7 minutes led to a decrease of both A_{530} and A_{405}, as far as the complete disappearance of the coloration. These changes can be seen in Fig. 1.

The optimum ratio of acid and diluent at which A_{530} reaches its greatest value is 1:1 (v/v) in the case of mixtures of sulfuric and acetic acids and 1:0.7 (v/v) for sulfuric acid and ethanol. For these mixtures we also selected those reactions conditions at which the sugar components of the glycosides do not affect A_{530}: for the mixture of sulfuric and acetic acid these are 60°C and 25 min, and for the mixture of sulfuric acid and ethanol 60°C and 30 min. Calibration graphs for the reaction considered were linear in the range of 0.001-0.03 μmole of hederagenin/ml.

The molar absorption coefficients obtained under these conditions for hederagenin, the caulosides, and the monosaccharides most frequently found in the composition of hederagenin

TABLE 1. Molar Absorption Coefficients of Free and Glycoside-Bound Hederagenin, and Also of Some Monosaccharides, in the Reactions with Sulfuric Acid and Mixtures of It with Acetic Acid and with Ethanol

Compound	E, liter/(mole·cm)		
	sulfuric acid (conc.)	sulfuric acid–ethanol (1:0.7)	sulfuric acid–acetic acid (1:1)
Hederagenin	14262±122	17625±141	12225±174
Caulosides			
A. Hed-3-O-arabinose	14069±161	17650±107	12200±336
C. Hed-3-O-arabinose–glucose	14095±160	17430±225	12228±231
D. Hed<3-O-arabinose, 28-O-glucose–glucose–rhamnose	14076±314	17780±110	12260±203
D-Glucose	131	60	75
L-Arabinose	65	62	88
L-Rhamnose	149	33	42
D-Glucuronic acid	5,0	6,0	2,5

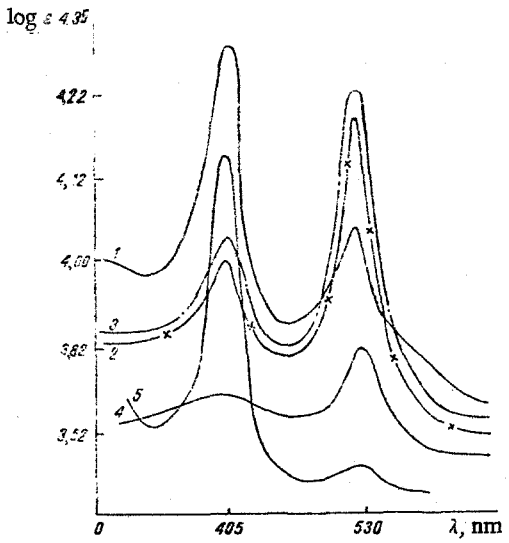

Fig. 1. Changes in the maxima at 405 and 530 nm of the absorption spectrum of hederagenin as functions of the ratio of sulfuric acid and ethanol in the reaction mixture. (Concentration of hederagenin 0.03 mole/ml, time of the reaction 30 min at 60°C): 1) 1:0.3; 2) 1:0.5; 3) 1:0.7; 4) 1:1; 5) spectrum of hederagenin in concentrated sulfuric acid (60°C, 10 min).

glycosides are given in Table 1, from which it can be seen that all three reactions considered, under the conditions specific for hederagenin, belong to the group of reactions of medium sensitivity. Even less sensitive is the reaction with sulfuric acid and vanillin recently proposed for determining triterpene glycosides [3]. In this case, the values of E_{459} and E_{530} for hederagenin amount to only 3000. Furthermore, the high blank value considerably worsens the reproducibility of their results of a determination.

Thus, of the reactions considered, that with sulfuric acid and ethanol proved to be preferable for the determination of hederagenin glycosides: it possesses a greater sensitivity, a greater specificity in relation to the aglycone, and a better reproducibility of the results. We have used this reaction to determine glycosides A and C in a combined preparation of them after separation in a thin layer of silica gel. The determination procedures is described in the Experimental part. The relative error of the determination for each glycoside did not exceed 5 rel.%.

EXPERIMENTAL

Materials. Hederagenin, mp 326-328°C, $[\alpha]_D^{20}$ + 81°. Caulosides A, C, and D were identified by thin-layer chromatography in the chloroform–ethanol (3:2) and chloroform–methanol (2:1) systems, and als- from their melting points: for cauloside A, 225-228°C; C 248-250°C; D, 235-237°C. The results of elementary analysis corresponded to the calculated figures. Type KSK silica gel washed with methanol, two-hour fraction.

Reagents. Sulfuric acid (conc.), redistilled, mp 336°C; glacial acetic acid freed from impurities by boiling with $KMnO_4$ and distillation over fused sodium acetate, bp 118°C; ethanol, dried by boiling over CaO for six hours and redistilled; methanol, redistilled.

Procedure for Performing the Color Reaction. To each of a number of test tubes containing a definite number of micrograms of hederagenin or of cauloside A, C, or D was added 2 ml of concentrated sulfuric acid or 2 ml of a mixture of sulfuric acid and ethanol or of sulfuric and acetic acids, the mixture was stirred, heated at a predetermined temperature for a predetermined time, and cooled in running water, and the optical density was measured against a blank sample in cells 1 cm thick on a VSU-2-P spectrophotometer (GDR) at 405 and 530 nm.

Procedure for Determining Caulosides A and C in a Combined Preparation. To construct a calibration curve, various amounts of a 1.2 mM methanolic solution of hederagenin corresponding to concentrations of 0.001-0.03 μmole/ml were deposited on glass plates (6 × 9 cm) each coated with 0.8 g of silica gel. For the deposition of the solutions a microsyringe of the "Hamilton" type with a capacity of 100 μl was used. The plates were chromatographed for 15 min in the chloroform–ethanol (3:2) system and were dried in the air, and the hederagenin was detected with iodine. After the elimination of the iodine, the spots observed were scraped off and each was transferred to a filter-paper thimble placed in another of dense platinum gauze. The thimbles were suspended from the tip of a reflux condenser and extraction was per-

formed with 2 ml of methanol in the Soxhlet manner for 2 h. Then the methanol was evaporated off, 2 ml of a mixture of sulfuric acid and ethanol was added to the test tube, and it was heated at 60°C for 30 min and was cooled, and the optical density was measured at 530 nm against the reaction mixture.

The blank experiment was carried out similarly using pure silica gel removed from an area of the plate corresponding to the area of the hederagenin spot.

To determine caulosides A and C in a combined preparation, 40 µl of a methanolic solution of the preparation with a concentration of 2 mg/ml was deposited on a plate together with a synthetic mixture of caulosides A and C for the clearer identification of the spots, and a pure band of silica gel was left for the blank experiment. The determination was carried out in the way described above.

The samples of caulosides C and D were presented by N. I. Uvarova and N. S. Chetyrina.

SUMMARY

Using hederagenin and its glycosides — caulosides A, C, and D isolated from the Far Eastern plant *Caulophyllum robustum* Maxim — as examples, the spectrometric reactions with concentrated sulfuric acid and mixtures of sulfuric acid with ethanol and with glacial acetic acid have been studied. Conditions have been proposed for performing the reaction which exclude the influence of the carbohydrate components of the glycosides on the determination of hederagenin. The reaction with a mixture of sulfuric acid and ethanol has been used to determine the amounts of caulosides A and C in a combined preparation after their separation by the TLC method. The relative error of the determination did not exceed 5 rel. %.

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MECHANISM OF THE REACTION OF TRITERPENOIDS WITH SULFURIC ACID

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The single maximum of triterpenoids in sulfuric acid solutions at 310 nm is due to the formation of a carbocation. Under the action of sulfuric acid, oleanolic acid undergoes lactonization at the COOH group and the Δ^{12} $\geq C{=}C\leq$ bond.

Brieskorn et al. [1, 2] have reported studies of the Lieberman—Burchard reaction. On the basis of the results of these investigations, the authors came to the conclusion that under the reaction of sulfuric acid triterpenoids undergo dehydration in ring A, after which the double bond formed migrates until conjugation arises in rings C and D. Considering ursolic acid (I), in particular, the authors suggested that because of dehydration followed by migration of the double bond ursadienecarboxylic acid (II) is formed. The formation of the suggested diene was judged on the basis of the fact that the reaction product after isolation had an absorption maximum in ethanolic solution at 246 nm (Scheme 1).

Thus, according to the proposed mechanism, dehydration in ring A leads to the appearance of a double bond in it and then, as a consequence of intramolecular rearrangement, migrations of two types takes place: the Δ^{12} double bond migrates into ring D and the double bond formed as a result of dehydration migrates from ring A to ring C and, in the final result, a conjugated system of double bonds — Δ^{11} and Δ^{13} — is formed. The absorption maximum of an

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