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The Non-volatile Acids of Succulent Plants Exhibiting a Marked Diurnal Oscillation in their Acid Content

I. On the Detection of Piscidic Acid in *Agave americana* L.

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Piscidic acid has been detected in the leaves of *Agave americana* L. The employed procedure can be summarized as follows: The acid-mixture, isolated over the lead salts from fresh leaves of the plant, was converted into the corresponding methyl- and ethylesters, and the ester-mixtures fractionated *in vacuo*. From the individual fractions the hydrazides and benzylidene hydrazides were prepared and examined. In this way a crystalline hydrazide ($C_{11}H_{16}O_5N_4$; m.p. 185–187°C) that corresponds to the hydrazide of piscidic acid, was isolated from the highest boiling fractions, and from this hydrazide the corresponding benzylidene hydrazide ($C_{25}H_{25}O_5N_4$; m.p. 236–238°C) was prepared. From the highest boiling fractions of the methyl ester mixture a crystalline ester ($C_{13}H_{16}O_7$; m.p. 126–127°C) which corresponds to the methyl ester of piscidic acid was isolated and from this an acetyl derivative (m.p. 84°C) was prepared.

From the properties of the four above mentioned derivatives and from the I.R. spectra of the methyl ester, it was concluded that the acid in question is piscidic acid. So far this compound has been found only in two other plants: *Piscidia erythrina* and *Narcissus poeticus*.

The diurnal oscillation in the acid content of certain succulent plants has been known and studied for about 80 years.[1] One would therefore expect that the organic acids involved in this process would be quite familiar.

A closer look into the problem seems to indicate that this assumption is far from the truth: An investigation carried out in this laboratory some years ago on the organic acids of certain crassulaceous plants revealed that the so-called "crassulacean malic acid" consists mainly of D-isocitric acid.[2]

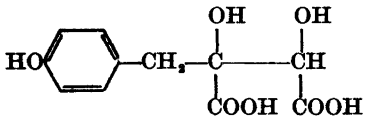
Encouraged by the above finding, we investigated the non-volatile acids from the crude drug Euphorbium, derived from the African stem succulent *Euphorbia resinifera* Berg, since it was believed that this plant also contained a malic acid with unusual properties. The investigation led to the isolation of a lactone acid,[3] which on closer investigation turned out to be a new dilactone acid, which was named phorbic acid.[4] Non-published work seems to

indicate that phorbic acid is the predominant organic acid in succulent plants of the genus *Euphorbia*, and that it exists, in negligible amounts, also in the non succulent species of the same genus.

Being a dihydroxy-tri-carboxylic acid, there is reason to believe that phorbic acid too plays an important role in the diurnal acid metabolism of succulents belonging to the genus *Euphorbia*.

The fact that both isocitric and phorbic acid had for such a long time escaped the attention of the scientists, led us to assume that other succulents too might contain unrecorded organic acids that could be found by employing the same or similar methods as those which led to the detection of isocitric and phorbic acids. We therefore decided to undertake a systematic investigation of the available succulents from different families and genera with the purpose of looking for unrecorded organic acids that might be involved in the diurnal acid metabolism of the plants in question. An investigation along these lines led to the detection of piscidic acid in *Agave americana*.

Piscidic acid was first isolated in 1901 by Freer and Clover [5] from *Piscidia erythrina* L. In 1954 Smeby, Zbinovsky, Burris and Strong [6] from the bulbs of *Narcissus poeticus* L. isolated an acid that in 1955 was found to be identical with piscidic acid.[7] The structure of the acid was elucidated by Bridge, Coleman and Robertson [8] and by Buckle, McGookin and Robertson.[9] The work of the last mentioned authors has shown that piscidic acid is *p*-hydroxybenzyl-tartaric acid:



Piscidic acid represents a unique structure amongst those aromatic acids which have so far been detected in plants, as the predominating acids in this category are derived either from benzoic or cinnamomic acid.[10]

It is interesting to notice that piscidic acid has been found in two plant groups (*Leguminosae* and *Liliaceae*) which stand remote from each other in the natural plant system. This finding might serve as an example of convergence or parallel development of unrelated species,[11] or it might indicate that the acid is widely distributed within the vegetable kingdom.

The procedure which led to the detection of piscidic acid in *Agave americana* was as follows: The non-volatile acids, isolated over the lead salts from the fresh plant, were esterified with diazoethane and the ester-mixture fractionated *in vacuo*. In the lower boiling fractions malic and citric acid were detected by means of their hydrazides and benzylidene hydrazides.

The highest boiling fraction yielded a nicely crystallizing hydrazide, $C_{11}H_{16}O_5N_4$, and a corresponding benzylidene hydrazide, $C_{25}H_{25}O_5N_4$. An I.R. spectrum in nujol showed hydroxygroup absorption and also carbonyl absorption at 1680 cm^{-1} and at 1640 cm^{-1}. The I.R. spectrum showed that the compound contained an aromatic nucleus, and a strong absorption at 835 cm^{-1} indicated two adjacent hydrogen atoms on the ring.

The ester fraction gave no reaction with 2,4-dinitrophenylhydrazine, no colour with ferric chloride and was not attacked by periodic acid.

An attempt to isolate the free acid from the hydrazide after hydrolysis of this compound with sodium hydroxide yielded only a resin-like product with no defined melting point. As all attempts to get the ethyl ester to crystallize also failed, the methyl esters were prepared from a second sample of the acid-mixture from the *Agave* plant by means of diazomethane, and the ester-mixture fractionated *in vacuo*. This time the crystalline trimethyl ester of citric acid was isolated from the medium fraction while from the highest boiling fraction, after dilution with ether, scraping and agitation of the mixture with a glass rod, a methyl ester, $C_{13}H_{16}O_7$, crystallized out. The ester could be converted into a hydrazide identical with the first isolated hydrazide, $C_{11}H_{16}O_5N_4$.

An I.R. spectrum of the ester in potassium bromide showed two ester-carbonyl absorption maxima (1745 cm^{-1} and 1721 cm^{-1}). A strong absorption band at 841 cm^{-1}, which indicates the presence of two adjacent ring hydrogen atoms, was also observed.

The empirical formula of the ester together with its melting point indicate that the isolated substance is the dimethylester of piscidic acid. This assumption also agrees with the fact that periodic acid does not attack this ester, and that the phenol group of piscidic acid, according to Bridge, Coleman and Robertson,[8] is not methylated when treated with diazomethane.

If the compounds $C_{13}H_{16}O_7$ and $C_{11}H_{16}O_5N_4$ are derivatives of piscidic acid, one should expect that the stretching frequency of the two carbonyl groups would be nearly the same. However, as the I.R. spectra of the two compounds were recorded in solid phase, there is reason to believe that intramolecular hydrogen bonds might interfere and lower one of the frequences. To test this possibility, an I.R. spectrum of the ester $C_{13}H_{16}O_7$ in chloroform was recorded, and also a corresponding spectrum of an acetyl derivative of $C_{13}H_{16}O_7$ in potassium bromide. In both cases a carbonyl absorption at 1742 cm^{-1} was observed.

The empirical formulas of the isolated compounds, the melting point of the methyl ester and its acetyl derivative, as well as the I.R. spectra make it reasonable to assume that *Agave americana* contains piscidic acid. This finding seems to be supported by the fact that *Agave americana* and *Narcissus poeticus* (from which plant also piscidic acid has been isolated) both belong to the same plant family.

EXPERIMENTAL

18 kg of fresh leaves of *Agave americana* (supplied by the botanical garden, University of Oslo), was passed through a chopping machine whereafter the material was macerated with 20 l of water for 24 h. After pressing and filtration the juice was precipitated with 1250 ml of saturated lead acetate solution, whereafter the lead salts were filtered off, washed with water, suspended in 3 l of water and decomposed with hydrogen sulphide. The filtrate from the lead sulphide precipitate was evaporated *in vacuo* at 10—11 mm Hg.

To remove possible traces of calcium salts and to dry the syrup-like remainder, this was treated with 500 ml of anhydrous ethanol, filtered and the filtrate again evaporated *in vacuo*. The procedure was repeated with 500 ml of anhydrous acetone. Yield: 100 g of a thick, brown and acid-tasting syrup.

Table 1.

Number of fraction	B.p. interval in °C	Yield in g	Appearance of fraction
1	80–115	5.4	Yellow liquid
2	115–150	2.3	Yellowish thick liquid
3	150–200	0.3	Brown syrup
4	200–235	5.2	Brown, glass like mass

25 g of the acid-mixture thus isolated was dissolved in 25 ml of anhydrous ethanol and esterified with diazoethane in excess at 0°C. The reaction mixture was put aside for 5 h at room temperature and then the organic solvents were removed by distillation. The residue was then fractionated *in vacuo* at about 10^{-1} mm Hg. The result of the fractionation is shown in Table 1.

As the esters could not be brought to crystallization in the course of three weeks, the ester fractions were investigated according to the ester-hydrazide method: 0.5 g of the ester fractions was dissolved in 3 ml of anhydrous ethanol, the solution mixed with 0.5–1 ml hydrazine hydrate and put aside overnight for crystallization.

Fraction 1 gave a white, microcrystalline hydrazide, which, upon extraction with boiling ethanol, melted at 175–176°C and showed no melting point depression when mixed with pure malic acid dihydrazide.

Fraction 2 yielded a viscous reaction product, which, upon dissolution in water and treatment with benzaldehyde, gave citric acid tri-benzylidene hydrazide. The compound was identified by means of the melting point of the substance itself and by the melting point of the compound when mixed with pure citric acid tri-benzylidene hydrazide.

From *fraction 3* no crystalline product could be isolated. *Fraction 4* yielded long white needles, which upon crystallization from diluted ethanol melted at 185–187°C. Yield: 400 mg from 1 g ester-mixture. (Found: C 46.60; H 5.76; N 19.72; Ethoxyl 0.71. Calc. for $C_{11}H_{16}O_5N_4$: C 46.46; H 5.67; N 19.70; Ethoxyl 0.0).

The hydrazide was dissolved in water and mixed thoroughly with benzaldehyde, which was added gradually drop by drop. The white precipitate which separated, was filtered off, dried and extracted with boiling ether whereupon it was recrystallized twice from boiling ethanol. M.p. 236–238°C. (Found: C 65.12; H 5.44; N 12.18. Calc. for $C_{25}H_{25}O_5N_4$: C 65.06; H 5.46; N 12.14).

The methyl esters. 30 g of the ester-mixture (referred to under preparation of the ethyl esters) was esterified with diazomethane and fractionated in the same way as the corresponding ethyl esters. The results of the fractionations are shown in Table 2.

Table 2.

Number of fraction	B.p. interval in °C	Yield in g	Appearance of fraction
1	60–100	5.8	Yellow liquid
2	100–180	2.2	Brownish liquid
3	180–215	2.0	Brown syrup
4	215–235	4.2	Brown, glass like mass

From *fraction 2* citric acid trimethyl ester crystallized out after dilution with ethanol.

Fraction 4 was diluted with twice its volume of ether and put aside in the cold store with occasional stirring and scraping of the inner walls of the flask for three weeks. During that time big transparent crystals had been formed, which were filtered off and

recrystallized three times from a mixture of equal volumes of ethyl acetate and petrol ether. Yield: 50 mg. M.p. 126–127°C. (Found: C 54.82; H 5.41; Methoxyl 21.06. Calc. for $C_{13}H_{16}O_7$: C 54.91; H 5.67; Methoxyl 21.80).

When treated with hydrazine hydrate in the same way as the ethyl ester fractions, the methylester $C_{13}H_{16}O_7$ gave a crystalline hydrazide that melted at 185–187°C. When mixed with the hydrazide, $C_{11}H_{16}O_5N_4$ no melting point depression was observed and the I. R. spectrum of the hydrazide was identical with that of $C_{11}H_{16}O_5N_4$.

Preparation of the acetylderivative of the ester $C_{13}H_{16}O_7$. 100 mg of the ester $C_{13}H_{16}O_7$ was dissolved in 1 ml of acetic anhydride and 100 mg of anhydrous sodium acetate was added. The mixture was boiled under reflux for 30 min, and then it was cooled, mixed with 5 ml of water, neutralized with sodium bicarbonate and extracted three times with 10 ml of ether each time. The combined ether extracts were dried over anhydrous calcium chloride, filtered, and the ether distilled off. In the bottle there then remained a white, crystalline compound which melted at 84°C.

Recording of the I. R. spectra. The isolated substances were examined in a Beckman IR 5 A double beam spectrophotometer either in nujol mulls or in potassium bromide pellets. $C_{13}H_{16}O_7$ was also examined in chloroform that had been freed from the added ethanol in order to avoid interference of undesired hydrogen bonds in the recording of the I. R. spectrum. The ethanol (which is usually added to the chloroform as a preservative agent) was removed by passing the chloroform through a column of aluminium oxide (Brockman).

DISCUSSION

As will be seen from Table 1 piscidic acid represents about one half of the acids which accumulate in the tissue of *Agave americana*. In *Euphorbia resinifera*, phorbic acid represents the main part of the cumulate acids. It is a well established fact that there exists a close connection between the carbohydrates and the cumulate acids in the succulents that exhibit typical diurnal variations in the acid content. As the two acids were never considered during earlier investigations, there is a possibility that they might serve to throw new light on certain points connected with the diurnal acid variations in succulent plants.

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