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ISOLATION, STRUCTURE AND ABSOLUTE CONFIGURATION OF INDICAXANTHIN

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Abstract—Indicaxanthin, $C_{14}H_{14}O_6N_2$, has been isolated from *Opuntia ficus-indica* fruits, and its structure and absolute configuration (II) determined by NMR spectrum and degradative studies. This compound is the only known pigment belonging to the class of betaxanthins, which are yellow pigments occurring in the *Centrospermae*.

THE plants belonging to the order *Centrospermae* elaborate yellow and red-violet pigments which are easily differentiated from the flavonoid compounds, both because they react negatively to the Erdmann test and because they migrate as anions even at pH's as low as 2·4.[1] Up till now investigation has centred on the red-violet pigments (betacyanins) and it has been concluded that they possibly all derive from betanidin or its diastereoisomer isobetanidin.[2,3] The yellow pigments, first known as flavocyanins and later called betaxanthins, have received much less attention, since they are more difficult to isolate and because of their great sensitivity to chemical reagents.[4]

Recently a betaxanthin was isolated from *Opuntia ficus-indica* fruits.[5] As the compound could not be crystallized it was only partially characterized and shown to be an amphoteric substance containing a polymethylene cyanine group. Starting from the same plant material we have now isolated in crystalline form a betaxanthin, which we have called indicaxanthin. Aqueous extracts of orange-yellow fruits of *Opuntia ficus-indica*, adjusted to pH 3, were passed onto a column cooled to 5° of a strongly acid resin. Under these conditions betaxanthins and betacyanins are non-ionically bound to the resin. They were then eluted with water and separated by chromatography on polyamide.[6] The fraction containing the main betaxanthin was further purified by resin treatment yielding an orange crystalline compound, which decomposes at 160–162° ($[\alpha]_D^{20}$ +394°; Cotton effect positive). Analysis suggested the molecular formula $C_{14}H_{18}O_8N_2$. No sugar was obtained by acid hydrolysis. The IR spectrum (Fig. 1) offers little information; the UV spectrum (Fig. 2) measured for an aqueous solution shows three bands at 485 mμ (log ε 4·63), 305 mμ (log ε 3·19) and 260 mμ (log ε 3·73).

[1] For a summary of these compounds see A. S. Dreiding, *Recent Developments in the Chemistry of Natural Phenolic Compounds*, p. 194. Pergamon Press, New York (1961).

[2] H. Wyler and A. S. Dreiding, *Experientia*, **17**, 23 (1961).

[3] M. Piattelli and L. Minale, *Phytochemistry* (in press).

[4] The information on betaxanthin distribution obtained by Reznik on the basis of paper chromatography and paper electrophoresis (H. Reznik, *Z. Botan.* **43**, 499 (1955); *Planta* **49**, 406 (1957)) must be revised, taking into account the extreme instability of these pigments.

[5] M. Piattelli and L. Minale, *Rend. Ac. Sci. fis. mat.* **30**, 23 (1963).

[6] Extraction and chromatographic separation of the pigments were carried out at 5° in order to minimize their decomposition.

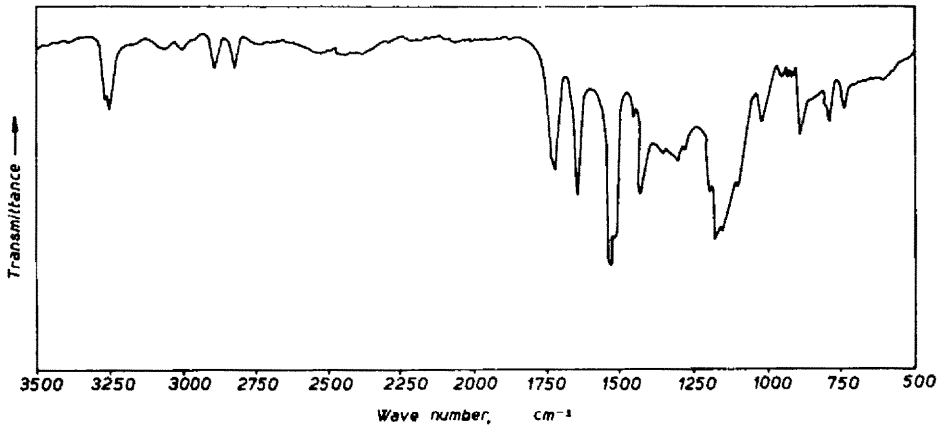


FIG. 1. IR spectrum of indicaxanthin.

The potentiometric titration in aqueous solution with standard alkali shows the presence of two strongly acid groups ($pK_a = 3{\cdot}3$), but when indicaxanthin is heated in boiling quinoline, 2·7 moles CO_2 are evolved. These facts establish that indicaxanthin possesses three carboxyl groups, one of which is neutralized internally by a basic group.

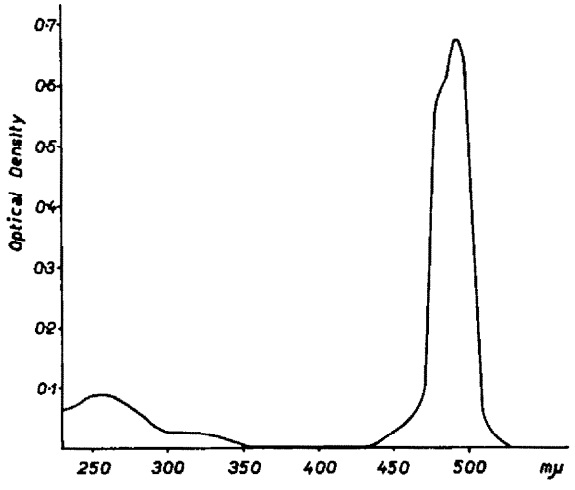
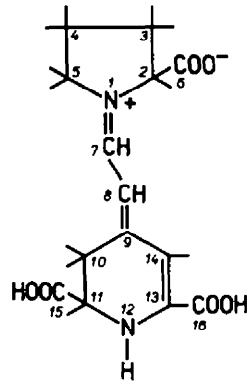


FIG. 2. UV spectrum of indicaxanthin.

This led to the hypothesis that the compound is closely related to betanidin. The NMR spectrum (Fig. 3) supports this view and suggests the gross structure I. The NMR spectrum, when compared with those of betanidin[7] and proline,[8] may be interpreted as follows: the doublet at 1·35 τ (J 12) must arise from the proton situated on C—7. The singlet 3·33 τ and the doublet 3·65 τ (J 12) are due to the olefinic protons at C—14 and C—8 respectively. Furthermore, the multiplets at 4·95 τ and 5·29 τ

[7] T. J. Mabry, H. Wyler, G. Sassu, M. Mercier, I. Parikh and A. S. Dreiding, *Helv. Chim. Acta* **45**, 640 (1962).

[8] NMR spectrum of proline shows peaks at 5·28τ (H at C—2), 6·30τ (2H at C—5) and 7·58τ (4H at C—3 and C—4).



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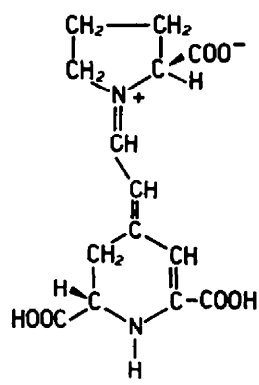

II

arise from the protons at C—2 and C—11 respectively. The signal centred at 6·00 τ corresponds to protons attached to a carbon atom (C—5) linked to a positive nitrogen atom. The two protons at C—10 are characterized by a broad multiplet (6·55 τ). The complex band centred at 7·48 τ indicates methylene protons (four) at C—3 and C—4.

In accordance with formula I, indicaxanthin yields proline and 4-methylpyridine-2,6-dicarboxylic acid by fusion with alkali in the absence of oxygen.

With a view to elucidate the absolute configuration of the asymmetric centre at C—2, indicaxanthin was subjected to acid degradation, since the proline obtained by alkali fusion was largely racemized. The isolation of L-proline permits the unequivocal

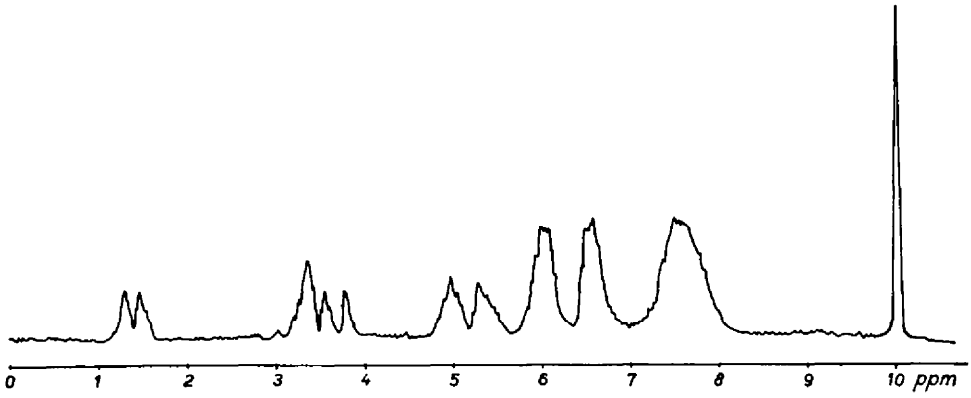

FIG. 3. NMR spectrum of indicaxanthin in CF_3COOH, in ppm with tetramethylsilane taken at τ = 10·0 ppm.

stereochemical assignment to C—2 (S-configuration). To ascertain the stereochemistry at C—11, indicaxanthin was oxidized with hydrogen peroxide. Under these conditions, the dihydropyridine moiety yields, besides pyridine-2,4,6-tricarboxylic acid which is the main degradation product, a number of ninhydrin-positive substances, among which aspartic acid was identified and isolated chromatographically pure. This

material, which could not be crystallized on account of the minimal quantities obtained, had a specific rotation in agreement with that of L-aspartic acid; moreover, it was unaffected by D-amino acid oxidase. These results support the conclusion that the asymmetric centre at C—11 must also have the S-configuration; indicaxanthin, therefore, can be represented by the absolute configuration II.

EXPERIMENTAL

All m.ps are uncorrected. IR spectra were taken on a Beckmann IR 9 instrument in KBr pellets and UV spectra on an Unicam SP 500 spectrophotometer. N.M.R. spectra were recorded in CF_3COOH immediately after preparation of the solutions (15% w/v), with tetramethylsilane as internal reference, on a Varian Associates A-60 spectrometer. Chromatograms were carried out on Whatman no. 1 paper (descending technique). Whatman 3 MM paper, washed with 2% HCl, was used for preparative chromatograms. The solvent systems used, prepared on a vol/vol basis, are as follows: BAW, n-butanol–acetic acid–water (60:15:25); EAW, ethanol–33% ammonia–water (80:4:16); IW, isopropanol–water (80:20); PW, phenol–water (80:20). Electrophoretograms were run on Whatman no. 1 paper for about 1 hr at 16 V/cm in a horizontal apparatus in the following electrolytes: A, phosphate buffer 0·05 M (pH = 6·8); B, acetate buffer 0·05 M (pH = 5). As spraying reagents the following were used: ninhydrin (0·2% solution in acetone), isatin (0·2% solution in acetone) and $FeSO_4$ (5% aq solution).

Isolation of indicaxanthin. Mature orange-yellow fruits (5 kg) of *Opuntia ficus-indica* Mill. were macerated in 500-g batches in a blender with icy water (7 l.). The homogenate was filtered through several layers of cheese-cloth and the residue was re-extracted with icy water (3 l.). The combined extracts, adjusted to pH 3 by addition of N HCl and clarified by centrifuging, were passed onto a column of Dowex 50W-X2 (H^+ form, 30 × 5 cm) kept at 5°; the column was thoroughly washed with 0·1% HCl (5 l.). The betaxanthins and the betacyanins, non-ionically absorbed by the resin, were then eluted with water. The eluant (8 l.) was concentrated under red. press. at 30° (bath temp) to a volume of about 50 ml. The concentrated solution was applied on top of a 5 × 35 cm powdered polyamide column, cooled at 5°. Two minor yellow pigments were removed by washing with water (1·5 l.); subsequent development with 0·004M phosphate buffer (pH 6·8) effected the separation between the main betaxanthin (indicaxanthin) and the violet pigment (betanin). The indicaxanthin fraction, adjusted to pH 3 with N HCl, was further purified by resin treatment as above. The eluate, concentrated under red. press. at 30° to a small volume (about 10 ml), was allowed to stand overnight at 4°. The crystals formed were collected, washed with a little cold water and dried in a vacuum desiccator over P_2O_5. (Yield 100–110 mg; $[\alpha]_D^{20}$ +394° (c = 1, in phosphate buffer 0·1M at pH 7)). (Found: C, 53·98; H, 5·39; N, 8·85. $C_{14}H_{16}O_6N_2$ requires: C,54·54; H, 5·23; N, 9·09%). Indicaxanthin decomposes at 160–162°.

Potentiometric titration of indicaxanthin. Indicaxanthin (15–20 mg) was dissolved in water (40 ml) and the resulting solution was titrated with standard KOH aq (0·05N) in a 3-necked ground-joint flask equipped with calomel and glass electrodes, while H_2 was passed through the solution. Titration curves showed indicaxanthin to be a strong acid (pK_a = 3·3); a single break was observed and two moles of base were required for neutralization.

Thermic decarboxylation of indicaxanthin. A suspension of indicaxanthin (29·2 mg) in quinoline (5 ml) was heated at 235–240° (bath temp) for 1·5 hr in a slow current of N_2. The amount of CO_2 evolved was measured by absorbing the gas in 0·02N $Ba(OH)_2$ aq and titrating (phenolphthalein) the excess of base with 0·05N oxalic acid aq. (Found: CO_2, 39·1. $C_{14}H_{16}O_6N_2$ requires: 3 CO_2, 42·8%).

NaOH *fusion of indicaxanthin.* Indicaxanthin (150 mg) was added to a boiling mixture of NaOH (1·5 g) and water (0·75 ml) under N_2. After cooling, the mixture was taken up in water (25 ml) and the resulting solution was freed from alkali by passing through a column of Amberlite IRC-50 (H^+ form); the eluate was continuously extracted with ether. In the aqueous solution proline was identified by paper chromatography in the solvent systems BAW, EAW and IW (isatin as spray reagent). The ether extract was concentrated under red. press. to a residue which was dissolved in 0·1M ammonia (0·5 ml). The solution was streaked on thick paper (Whatman 3 MM) which was then developed in EAW. The band R_f 0·42, located by spraying with $FeSO_4$ a vertical strip cut from the side of the sheet, was cut out and eluted with water. The eluate, acidified with conc. HCl, was extracted with

ether. The ether extract was taken to dryness under red. press. and the residue, recrystallized from water, furnished a product m.p. 244–245° dec (39 mg). Its R_f values in BAW and EAW and its electrophoretic mobilities in electrolytes A and B were the same as those of an authentic sample of 4-methylpyridine-2,6-dicarboxylic acid. The m.p. of the methyl ester (129–130°), obtained by reaction with diazomethane, was not depressed on admixture with an authentic sample of methyl 4-methyl-pyridine-2,6-dicarboxylate.

Acid degradation of indicaxanthin. Indicaxanthin (200 mg) was refluxed for 15 min with 200 ml N HCl. The solution was evaporated to dryness *in vacuo*, and excess HCl removed by adding a small amount of water to the residue and evaporating to dryness 4 successive times. The residue was taken up in water (1 ml) and chromatographed on Whatman 3 MM paper using BAW as the developing solvent. The band R_f 0·35, located with isatin, was cut out and eluted with water. The eluate was concentrated to a syrup which, after being left for 24 hr over P_2O_5 in a desiccator and crystallized from anhydrous ethanol, yielded colourless needles (41 mg), m.p. 215–220° dec, $[\alpha]_D^{20}$ $-75 \pm 5°$ ($c = 2$ in water), picrate m.p. 153–154° undepressed on admixture with authentic picrate of L-proline.

Oxidation of indicaxanthin. A mixture of indicaxanthin (480 mg), methanol (15 ml), acetic acid (120 ml) and 36% H_2O_2 (5 ml) was kept at 35–40° for 24 hr with occasional stirring. The solution was heated for 30 min on the steam bath and then evaporated to dryness. The residue, dissolved in water (20 ml), was extracted with ether (60 ml in 3 portions). The combined extracts were dried (Na_2SO_4) and the solvent was distilled off, giving a residue which was subjected to paper chromatography (Whatman 3 MM) with EAW as the developing solvent. The band R_f 0·19, located with $FeSO_4$, was cut out and eluted with water. The eluate, acidified with N HCl, was extracted with ethyl acetate (50 ml in 3 portions). Evaporation of the solvent left a crystalline residue (50·5 mg) which was recrystallized from water giving 40·5 mg of a product m.p. 226–227° dec, I.R. spectrum identical to that of an authentic sample of pyridine-2,4,6-tricarboxylic acid. For additional confirmation on identity of this degradation product, it was converted by reaction with diazomethane into the corresponding methyl ester, whose m.p. (153·5–154°) was undepressed on admixture with an authentic sample of methyl pyridine-2,4,6-tricarboxylate. Chromatographic analysis of the degradation products insoluble in ether and soluble in water showed the presence of a number of ninhydrin-positive substances. All these compounds were formed in minute amounts; among them the two present in larger quantity were identified by paper chromatography as proline and aspartic acid respectively. By descending chromatography on Whatman 3 MM paper using the EAW system, the concentrate of the "amino acid fraction" was first separated in several zones one of which, with R_f value of 0·31, contained the aspartic acid. The water eluate of this zone was further subjected to paper chromatography with BAW and IW, in that order. After this purification the obtained amino acid was checked against an authentic sample of aspartic acid on one- and two-dimensional paper chromatograms (solvent systems: BAW, EAW, IW and PW alone and in combination), thin layer chromatograms on silica gel (solvent systems: chloroform–methanol–17% ammonia (40:40:20) and phenol–water (80:20) alone and in combination) and paper electrophoretogram (electolyte A). In every case, the isolated amino acid gave a single spot and corresponded to the reference aspartic acid on the same chromatogram or electrophoretogram. Owing to the scarcity of this degradation product (2·7 mg determined by quantitative paper chromatography) no crystallization attempt was made. Rotation measurements gave the following results: $[\alpha]_D^{60}$ $+25 \pm 5·5°$ ($c = 0·18$, 0·15N HCl) (authentic L-aspartic acid $[\alpha]_D^{60}$ $+25 \pm 5·5°$), $[\alpha]_{420}^{60}$ $+235 \pm 0·5°$ ($c = 0·18$, 0·15N HCl) (authentic L-aspartic acid $[\alpha]_{420}^{60}$ $+252 \pm 0·5°$). Part of the amino acid (1·3 mg), dissolved in 0·05M sodium pyrophosphate buffer (pH 8·3), was incubated at 37° with kidney D-amino acid oxidase (0·1 mg) for 24 hr. Paper chromatographic analysis of the digest and of an enzyme-free control solution showed no diminution of the ninhydrin-positive spot.

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