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SHORT COMMUNICATION

BETANIDIN GLUCOSYLATION IN *OPUNTIA DILLENII**†

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Key Word Index—*Opuntia dillenii*; Cactaceae; betacyanins biosynthesis; betanidin glucosylation.

Abstract—In fruits of *Opuntia dillenii* ^{14}C-labelled betanidin was incorporated into betanin (5-*O*-β-D-glucoside of betanidin). This result shows that glucosylation occurs late in the biosynthesis of the pigment and is possibly the last step.

INTRODUCTION

TO DATE, only limited information is available regarding the biogenesis of betacyanins,[1–4] glycosides of betanidin(I) and its C-15 diastereoisomer isobetanidin, and no work at all dealing with the glycosylation process has been reported. This investigation was undertaken in order to ascertain whether in the biosynthesis of betanin(II) (5-*O*-β-D-glucoside of betanidin) in *Opuntia dillenii* Haw. (Cactaceae) glucosylation is an early or late step.

RESULTS

Preparation of Betanidin-10-^{14}C

Betanidin-10-^{14}C was prepared by saturating with SO_2 an aqueous solution of 'cold' betanin and concentrating the bleached solution under reduced pressure. Addition of ^{14}C carboxyl-labelled *RS*-cyclodopa(5,6-dihydroxy-2,3-dihydroindole-2-carboxylic acid) resulted in the formation of a radioactive violet pigment, which was purified by chromatography to constant specific activity. Since preliminary experiments showed that in the course of this exchange reaction the C-15 carbon atom undergoes a complete racemisation, it follows that the product obtained (for sake of brevity designated as 'betanidin-10-^{14}C') is a mixture of equimolecular amounts of four optical isomers, i.e. betanidin (2*S*-15*S*), *enantio*-betanidin (2*R*-15*R*), isobetanidin (2*S*-15*R*) and *enantio*-isobetanidin (2*R*-15*S*). Of these, only betanidin and isobetanidin occur in Nature both free and in form of glycosides.[5]

* Part XV in the series "Pigments of Centrospermae". For part XIV see *Phytochem.* **10,** 3133 (1971).
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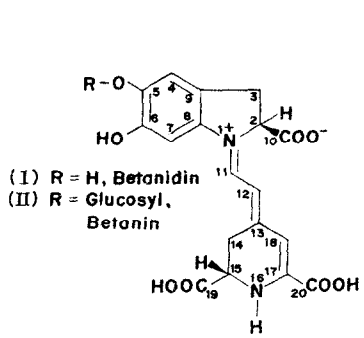
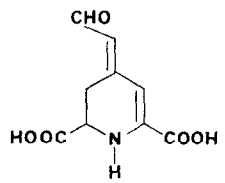
[1] L. HÖRAMMER, H. WAGNER and W. FRITZSCHE, *Biochem. Z.* **399,** 398 (1964).
[2] L. MINALE, M. PIATTELLI and R. A. NICOLAUS, *Phytochem.* **4,** 593 (1965).
[3] A. S. GARAY and G. H. N. TOWERS, *Can. J. Bot.* **44,** 231 (1966).
[4] H. E. MILLER, H. RÖSLER, A. WOHLPART, H. WYLER, M. E. WILCOX, H. FROHOFER, T. J. MABRY and A. S. DREIDING, *Helv. Chim. Acta* **51,** 1470 (1968).
[5] M. PIATTELLI and L. MINALE, *Phytochem.* **3,** 547 (1964).

Incorporation of 'Betanidin-10-^{14}C' into Betanin

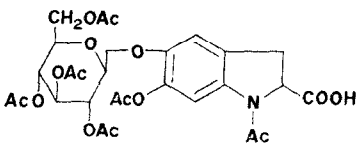
The above isomeric mixture was administered to fruits of *O. dillenii*. At the end of the incubation period, betanin was isolated by chromatography on strongly acid ion-exchange resin and purified to radiopurity by chromatography on polyamide and recrystallization from water. The incorporation of 'betanidin-10-^{14}C' into betanin* was 1·1% (mean value of five experiments). It must be stressed that the radioactive compound administered is an equimolecular mixture of four isomers, only one of which (betanidin, 2*S*-15*S*) is probably metabolized. In fact, circumstantial evidence[5,6] favours the hypothesis that isobetanidin derivatives, which co-occur with the corresponding betanidin derivatives, are not synthesized by the plants but are formed by spontaneous epimerization. On the basis of the utilization of one optical isomer, the incorporation would be 4·4%, a value much greater than the incorporation of L-tyrosine-2-^{14}C administered by the same method to *O. dillenii* fruits (0·35%).



(I) R = H, Betanidin
(II) R = Glucosyl, Betanin



(III) Betalamic acid



(IV) Hexaacetate of cyclodopa glucoside

The labelled betanin isolated after administration of radioactive 'betanidin' carried essentially all its activity in the dihydroindole moiety. This was proved by the following degradation experiments. Enzymatic hydrolysis with β-glucosidase gave betanidin with the same specific activity, while alkaline treatment of this radioactive betanidin yielded betalamic acid (III) free of ^{14}C. Finally, hydrolysis of labelled betanin with SO_2 gave cyclodopa glucoside, which was isolated as the hexaacetate (IV) and found to have a specific activity very similar to that of betanin.

DISCUSSION

Although degradation experiments to determine the absolute location of the label in radioactive betanin were not undertaken, it is clear from the above results that essentially all the activity of the pigment is localized in the dihydroindole moiety of the aglycone and this strongly suggests that no randomization occurred during the feeding experiment and that betanidin was specifically incorporated into betanin. Therefore, this indicates that glucosylation occurs towards the end of biosynthesis and is possibly the last step in the biosynthetic sequence.

EXPERIMENTAL

Plant material. Half-ripened fruits of *Opuntia dillenii* were used. Each fruit (av. wt. 40 g) contained *ca.* 20 mg of betanin-isobetanin and 3 mg of betanidin–isobetanidin.

Synthesis of ^{14}C *carboxyl-labelled* RS-*cyclodopa.* Essentially the method of Wyler *et al.*[7] was followed to prepare 0·12 mM of labelled *O,O,N*-triacetyl *RS*-cyclodopa methyl ester (spec. activity 1·74 × 10^9 dpm/mM)

* Total activity in betanin divided by total activity fed, ×100.

[6] M. PIATTELLI, M. GIUDICI DE NICOLA and V. CASTROGIOVANNI, *Phytochem.* **8**, 731 (1969).
[7] H. WYLER and J. CHIOVINI, *Helv. Chim. Acta* **51**, 1476 (1968).

starting from 0·25 mM of ^{14}C carboxyl-labelled DL-DOPA. Hydrolysis of the compound (10·5 mg) with 20% HCl under N_2 for 12 hr at 80° and evaporation to dryness under reduced pressure gave ^{14}C carboxyl-labelled cyclodopa (6·15 mg) which was used immediately without further purification.

Synthesis of 'Betanidin-10-^{14}C'. A solution of betanin (30 mg) in water (20 ml) was saturated with SO_2 and after 3 hr the bleached solution was concentrated to a volume of 6 ml. Racemic ^{14}C carboxyl-labelled cyclodopa (6·15 mg) in H_2O (1 ml) was added to the concentrate (under N_2) and the pH adjusted to 7·8. After 5 hr the reaction mixture was acidified to pH 4·5 and betanidin was separated from betanin by column chromatography on polyamide (2·5% citric acid in 50% aq. MeOH as the eluent) and purified by chromatography on Dowex 50. Yield 2·06 mg, spec. activity $1·73 \times 10^9$ dpm/mM. When an aliquot of the ^{14}C-labelled compound was mixed with non-radioactive betanidin and co-crystallized there was, allowed for dilution, no significant alteration in the specific radioactivity. In a parallel experiment carried out omitting the addition of cyclodopa, an approximately equimolecular mixture of betanin and isobetanin (separated by high voltage electrophoresis pH 4·5 and determined spectrophotometrically) was recovered thus showing that in the conditions adopted for the synthesis of 'betanidin-10-^{14}C' the C-15 carbon atom undergoes total racemization. The radioactive pigment is therefore a mixture of equimolecular amounts of four optical isomers.

Administration of 'betanidin-10-^{14}C' and isolation of ^{14}C-labelled betanin. In a typical experiment 'betanidin-10-^{14}C' (2 mg) was dissolved in the minimum amount of 0·05 M phosphate buffer (pH 6·8) and this solution was injected by means of a syringe into three fruits of *O. dillenii*. At the end of the incubation period (12 hr) the fruits were homogenized with MeOH and the homogenate clarified by centrifuging. The supernatant, containing 60 mg of betanin as determined spectrophotometrically, was concentrated under reduced pressure to remove MeOH and betanin was isolated by polyamide column chromatography followed by chromatography on Dowex 50. Yield 40 mg, spec. activity $9·33 \times 10^5$ dpm/mM. Recrystallization from water gave 16 mg of betanin with essentially the same activity ($9·29 \times 10^5$ dpm/mM); incorporation based on the utilization of one optical isomer 4·4% (in the same experimental conditions incorporation of L-tyrosine-2-^{14}C into betanin was 0·35%).

Degradation of radioactive betanin. (1) *Enzymatic hydrolysis of betanin.* ^{14}C-Betanin was hydrolyzed with β-glucosidase to give betanidin which after isolation by polyamide chromatography had spec. activity $9·36 \times 10^5$ dpm/mM. (2) *Betalamic acid from betanidin.* A slow stream of N_2 was passed for 3 hr, through a wash bottle containing 15% NH_3 solution, into a de-aeriated solution of betanidin (20 mg in 20 ml H_2O) obtained from the enzymatic hydrolysis of radioactive betanin. The alkaline reaction mixture, after removal of much of the NH_3 by a rapid stream of pure N_2, was chromatographed on Dowex 50 (H^+) using 1% HCOOH. A band λ_{max} (nm) 414 was collected and concentrated under reduced pressure. The concentrated solution was chromatographed on Whatman No. 3MM filter paper (MeOH) and the yellow band R_f 0·2 excised and eluted with water. The eluate was freeze-dried giving an orange residue of betalamic acid (1·89 mg), λ_{max} (nm) (ε) 434 (23 000) (H_2O). This compound, which reacted with excess cyclodopa (pH 3) to give betanidin in quantitative yield, was counted and its radioactivity found negligible. (3) *Cyclodopa 5-O-β-D-glucoside hexaacetate from betanin.* An aqueous solution of ^{14}C betanin (10 mg) was saturated with SO_2 and after 3 hr taken to a small volume under reduced pressure. The concentrated solution was chromatographed on Whatman No. 3MM filter paper using 50% aq. MeOH saturated with SO_2 as the eluent. A band R_f 0·75, located by a test strip spraying with diazotised 4-nitro-*o*-anisidine, was extracted with water. The extract, which reacted with betalamic acid to give betanin, was freeze-dried. This product gave by acetylation a compound which was purified to radiopurity by chromatography on silica gel ($CHCl_3$-MeOH, 7:3) and recrystallization from EtOAc. Yield 4 mg, m.p. 228–230°. Mass spectral fragmentation showed a parent peak at *m/e* 609 (1·5%) (M^+, $C_{27}H_{31}O_{15}N$) corresponding with the structure of the hexaacetate of cyclodopa glucoside (IV). The spectrum exhibited an important peak at *m/e* 331 (9·8) due to the tetraacetylglucoseoxonium ion, the usual series of ions derived from its further fragmentation by loss of acetic acid and ketene [271 (2·2), 211 (3·0), 169 (87·8), 127 (26·5) and 109 (60·6)],[8–11] and a peak at *m/e* 43 (100) due to the acetyl ion. Cleavage of the glycosidic linkage with hydrogen transfer to the aglycone moiety and charge retention on the aglycone gave the *m/e* 279 (2·3) ion, and successive stepwise loss of two ketene molecules the *m/e* 237 (12·9) and 195 (18·5) ions. In addition, when cleavage of the glycosidic linkage was preceded by loss of two ketene molecules, the *m/e* 567 (4·5) and 525 (1·1) ions were formed. Other peaks at *m/e* 194 (11·3) and 150 (22·7) can be attributed to fragmentation products of the *m/e* 195 ion by loss of one hydrogen and a carboxyl group. $[\alpha]_D^{20}$ — 78 (*c*, 1·6, HOAc); IR (KBr) 1750, 1675, 1495, 1420, 1375, 1255, 1230, 1180, 1073, 1049, 914 cm^{-1}; UV λ_{max}^{MeOH} (nm) (ε) 295 (9200), 258 (22 085); spec. activity $9·26 \times 10^5$ dpm/mM. Methylation with CH_2N_2 gave an ester, m.p. 170–71°, MS *m/e* (%): 623 (2·8) (M^+,$C_{28}H_{33}O_{15}N$), 581 (0·6), 539 (0·03),

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538 (0·07), 331 (30·9), 293 (16·9), 271 (4·9), 251 (36·6), 211 (2·8), 209 (22·5), 208 (23·9), 169 (100), 150 (14), 149 (5·6), 127 (10·5), 109 (33·8), 43 (70·4).

Measurements of radioactivity. Countings were performed with a scintillation spectrometer using Insta-gel (Packard) as the scintillation solution. Prior to counting, coloured samples were bleached with H_2O_2 in the presence of excess NaOH. All counts were corrected for background, quenching and instrument efficiency.