Phytochemistry 1974, Vol 13 pp 2771 to 2775 Pergamon Press Printed in England

BIOGENESIS OF BETALAMIC ACID

CHRISTINA CHANG, LINDA KIMLER* and TOM J MABRY

The Cell Research Institute and Department of Botany, The University of Texas at Austin, 78712, U S A

(*Received* 27 *April* 1974)

Key Word Index—*Portulaca grandiflora*, Portulacaceae, betalamic acid, betanin, betalains, indicaxanthin, D,L-dopa, decarboxylation of betanin, ORD

Abstract—When D,L-dopa-[1-^{14}C] and -[2-^{14}C] was fed to yellow flower buds of *Portulaca grandiflora* betalamic-[^{14}C] acid was obtained The labeled betalamic acid was converted to ^{14}C-labeled betanin in order to obtain a stable substance which could be recrystallized to a radio-pure sample Decarboxylation of the radiopure betanin obtained from the sequence using dopa-[1-^{14}C] indicated that the ^{14}C-carboxyl group of dopa corresponded to a ^{14}C-carboxyl group in betanin and hence in betalamic acid The shape of the ORD curve for the naturally occurring betalamic acid was the same as that recorded for a sample of [S]-betalamic acid derived by degradation of betanin These data support the hypothesis that betalamic acid is formed *in vivo* by an oxidative cleavage of L-dopa and that it is an intermediate in the biogenesis of other betalains from dopa

INTRODUCTION

BETALAMIC acid has long been considered to be the key biogenetic precursor of the dihydropyridine moiety in all betalains,[1] the red and yellow pigments which are unique in Angiosperms to the Centrospermae. It has also recently been detected as a naturally occurring pigment in plants which synthesize betalains.[2] In addition, betalamic acid has been shown to be an intermediate in the *in vitro* interconversion of yellow betaxanthins and red betacyanins.[2b] An isomer of betalmic acid, the yellow muscaflavin, along with other new betalamic acid-derived pigments (the orange musca-aurin-**1** and -**2**, the red brown muscarubin and the violet muscapurpurin) have recently been reported from the mushroom, *Amanita muscaria*.[3]

Since it has been previously shown that L-dopa serves as a precursor for the dihydropyridine moiety in betalains,[4,5] it was of interest to determine if dopa gives rise to betalamic acid in Centrospermae plants. *Portulaca grandiflora* was selected for the investigation because most of the yellow pigment in the flower petals of this plant is betalamic acid [2] Since procedures for both the recrystallization to a radiopure sample[4] and for determining the position of the label are well established for betanin,[4-7] the ^{14}C-labeled betalamic acid isolated from the plant material was converted into ^{14}C-labeled betanin for purification and decarboxylation experiments.

* Utica College of Syracuse University, Division of Science and Mathematics, Burrstone Road, Utica, New York 13502

[1] (a) MABRY, T J (1964) in *Taxonomic Biochemistry and Serology* (LEONE, C A, ed) p 247, Ronald, New York, (b) WYLER, H WILCOX, M E and DREIDING, A S (1965) *Helv Chim Acta* **48**, 361

[2] (a) KIMLER, L, LARSON, R A, MESSENGER, L, MOORE, J B and MABRY, T J (1971) *Chem Commun* 1329, (b) MABRY, T J, KIMLER, L and LARSON, R A (1972) *Hoppe Seyler Physio Chem* **353**, 2

[3] DOPP, H and MUSSO, H (1973) *Chem Ber* **106**, 3473

[4] MULLER, H E, ROSLER, H, WOHLPART, A, WYLER, H, WILCOX, M E, FROHOFER, H, MABRY, T J and DREIDING, A S (1968) *Helv Chim Acta* **51**, 470

[5] MINALE, L, PIATTELLI, M and NICOLAUS, R A (1965) *Phytochemistry* **4**, 593

[6] FISCHER, N and DREIDING, A S (1972) *Helv Chim Acta* **55**, 649

[7] IMPELLIZZERI, G and PIATTELLI, M (1972) *Phytochemistry* **11**, 2499

RESULTS AND DISCUSSION

Biogenesis of betalamic acid from dopa

It has been previously established that the dihydropyridine moiety in betalains is derived by an *in vivo* extra-diol oxidative cleavage of [S]-dopa (Fig. 1)[6,7] Here we repor

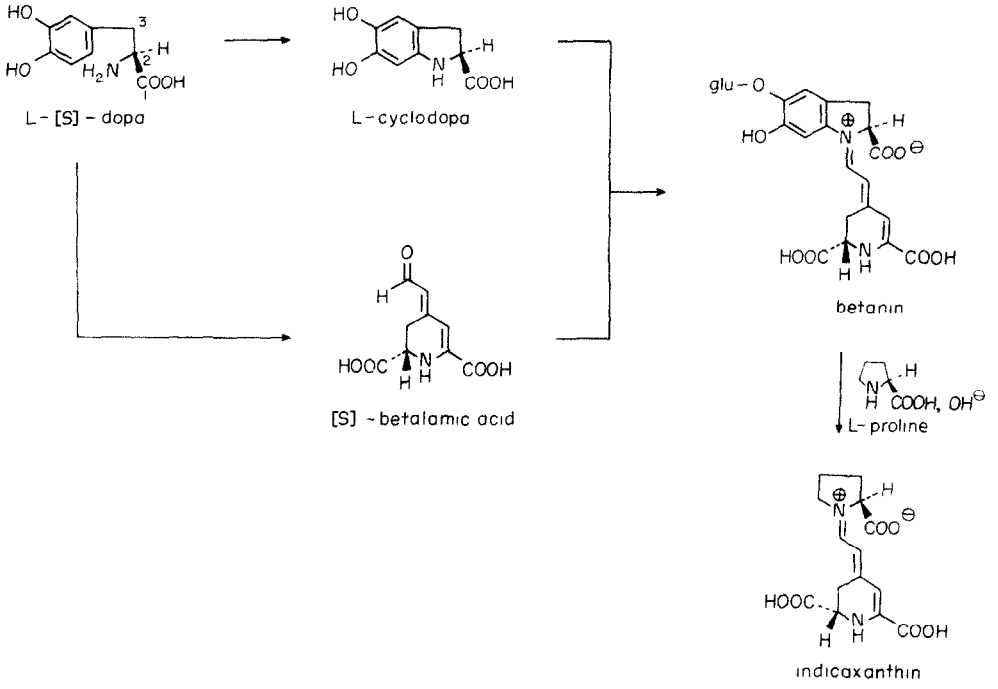

FIG. 1. BIOGENESIS OF BETANIN FROM L-DOPA AND CONVERSION OF BETANIN TO INDICAXANTHIN.

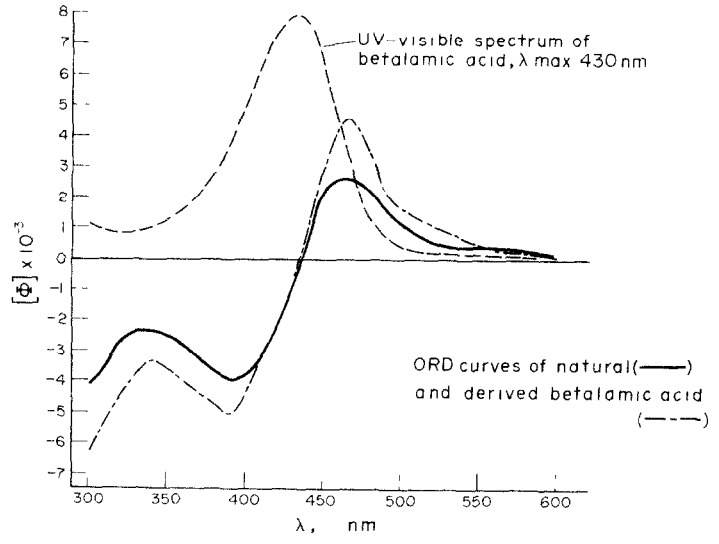

FIG. 2. ORD CURVES FOR BETALAMIC ACID ISOLATED FROM *Portulaca grandiflora* AND FROM THE ALKALINE HYDROLYSATE OF BETANIN

that D,L-dopa-[1-^{14}C] and -[2-^{14}C] give labeled betalamic acid and that the naturally occurring betalamic acid has the same configuration, namely [S], as has L-dopa (Fig. 2). The flower buds of *P. grandiflora* which were allowed to take up D,L-dopa-[1-^{14}C] and -[2-^{14}C] were extracted after 48 hr to yield, after workup, ^{14}C-labeled betalamic acid.

TABLE 1 LABELING EXPERIMENTS (**1, 2** AND **3**) FOR INCORPORATING D,L-DOPA-[1-^{14}C] AND -[2-^{14}C] INTO BETALAMIC ACID IN *Portulaca grandiflora*

Radioactive precursor	(1) D,L-Dopa-[1-^{14}C]	(2) D,L-Dopa-[1-^{14}C]	(3) D,L-Dopa-[2-^{14}C]
(a) specific activity	3.2 mCi/mmol	53 mCi/mmol	9.3 mCi/mmol
(b) amount fed to flower buds	9 μCi (2.0 × 10^7 dpm) to 20 buds	30 μCi (6.6 × 10^7 dpm) to 48 buds	9 μCi (2.0 × 10^7 dpm) to 15 buds
Betalamic acid from Ecteola column			
(a) amount (by UV, ϵ 25000)	0.093 mg	0.63 mg	0.096 mg
(b) radioactivity	99871 dpm	736427 dpm	64345 dpm
Betanin and exchange reaction			
(a) amount betanin (by UV at 538 nm, ϵ 60500) employed	155 mg	262 mg	171 mg
(b) amount of betanin after exchange reaction and purification over Dowex and Ecteola columns	81.6 mg	139.8 mg	80.3 mg
(c) amount (by UV) and specific activity* of betanin after 4th recrystallization	8.6 mg (302 dpm/mg)	42.1 mg (903 dpm/mg)	15.9 mg (194 dpm/mg)
Per cent radioactivity in CO_2 obtained by decarboxylation at 200–230°†	91	85	—
Per cent radioactivity in CO_2 obtained by total combustion at 1000° of decarboxylated sample†	9	15	—
Indicaxanthin-[^{14}C] obtained from betanin-[^{14}C]			
(a) amount (by UV) and specific activity (in dpm/mmol) of betanin-[^{14}C]	5.7 mg (166000)	5.5 mg (496700)	6.0 mg (95000)
(b) yield (by UV at 483 nm, ϵ 42700‡) and specific activity (in dpm/mmol) indicaxanthin-[^{14}C]	3.2 mg (143800)	2.3 mg (387000)	3.0 mg (59300)
yield (by UV at 483 nm, ϵ 62000‡ and specific activity (in dpm/mmol) indicaxanthin-[^{14}C]	2.1 mg (208600)	1.6 mg (560000)	2.1 mg (85400)

* The specific activity remained almost constant after the 2nd recrystallization

† Determined by H. Frohofer, Microlaboratory, Organic Chemistry Institute University of Zurich, Switzerland

‡ The ϵ-value of indicaxanthin was reported by Piattelli and co-workers in 1964[10] to be 42700, however, Wyler and co-workers later reported 62000 for indicaxanthin[1b] and 51000 for isoindicaxanthin.[8] Both investigators recently in private communications to T. J. M. supported their published data. The radioactivity results determined here suggest that the true ϵ-value for indicaxanthin may be intermediate between the two published values.

[10] PIATTELLI, M., MINALE, L. and PROTA, G. (1964) *Tetrahedron* **20**, 2325

Because betalamic acid is difficult to crystallize and purify, the labeled samples were converted to betanin, a substance for which procedures are available for recrystallization to a radiopure sample (Table 1) and which can be readily decarboxylated to determine the position of the label.[4-7]

The decarboxylation at 200–230° of the betanin-[^{14}C] obtained from the betalamic-[^{14}C] acid (from DL-dopa-[1-^{14}C]) established that 85–90% of the radioactivity was associated with carboxyl groups of betanin, therefore, one of the carboxyl groups in betalamic acid must have contained the label which had been present in the carboxyl group of dopa. Although it was difficult to calculate the amount of incorporation of dopa into betalamic acid because of decomposition problems with most of the compounds involved in the study, the data suggested that the per cent incorporation was 1–2% or more for all experiments. Since all the betanin-[^{14}C] samples from both DL-dopa-[1-^{14}C] and -[2-^{14}C] gave indicaxanthin-[^{14}C] with similar specific activities (Table 1), it follows that the radioactivity is associated with the dihydropyridine moiety in betanin. These results establish that dopa is a precursor for betalamic acid and support the hypothesis that betalamic acid is the intermediate that leads to all other betalains.

Stereochemistry of betalamic acid from Portulaca grandiflora

ORD curves for both the naturally occurring betalamic acid obtained from *P. grandiflora* flower buds and the betalamic acid obtained by alkaline hydrolysis of betanin exhibited positive Cotton effects with an inversion point at around 430 nm, which is the maximum visible absorption wavelength of betalamic acid. Since the absolute configuration at C_{15} in betanidin is known to be [S],[8] the chiral center in the betalamic acid present in the flower buds of *P. grandiflora* can also be assigned an *S*-configuration, the same as in L-dopa. Therefore, this finding in combination with the labeling results indicates that L-dopa is the precursor of [S]-betalamic acid.

EXPERIMENTAL

Plant material. Yellow *Portulaca grandiflora* flower buds.

Labeling experiments with DL-*dopa-*[1-^{14}C] *and* -[2-^{14}C] *and the isolation of labeled betalamic acid.* An aq. (deoxygenated) soln containing DL-dopa-[1-^{14}C] was placed in spot plates and flower buds (1 cm stems) of *P. grandiflora* were arranged such that the stems dipped into the soln. the buds were allowed to take up the soln. for 2 days in an O_2-free atmosphere (a small amount of deoxygenated H_2O was constantly supplied to the spot plate). The stem and petal parts of the flowers were separately ground with a buffer soln containing 80% EtOH and 20% 0.1 M NH_4HCO_3. The yellow extract from about 20 flower petals was filtered through a bed of celite and then chromatographed on an Eeteola column (3 × 18 cm). A pH 7.8 0.1 M NH_4HCO_3 buffer was used to elute the dopa, cyclodopa, betaxanthin, betalamic acid and betacyanin fractions in this order. The betalamic acid fraction was detected by paper electrophoresis ($R_{Bet.}$ = 2 at pH 7.8) and identified by UV (λ_{max} at 430 nm).

Purification of the betalamic-[^{14}C] *acid by conversion to betanin-*[^{14}C]. About 150 mg of betanin (as a pyridine formate salt) was dissolved in 100 ml of H_2O. Conc. NH_4OH was added to the betanin solution under N_2 to bring the pH to 10. After about 10 min the soln turned brownish yellow, the fraction from the Eeteola column containing the betalamic-[^{14}C] acid was mixed with the pH 10 solution and then conc. HCO_2H was added immediately to bring the pH of the resultant soln back to 5. After storage at 0° overnight, the soln was acidified to pH 3 with aq. HCl and passed through a Dowex 50W-X2 cation exchange column (5 × 30 cm, in H$^+$ form). The betanin was retained by the column which was further washed with 100 ml of pH 3 aq. HCl. The betanin-[^{14}C] band was eluted with deionized H_2O and then freeze dried. The betanin-[^{14}C] material was recrystallized × 4 (from 1 M pyridine formate acidified with conc. HCl to pH 2) until a constant sp. act. was obtained (see Table 1).

Decarboxylation and combustion of the betanin-[^{14}C].[4-6] Decarboxylations were performed under high vacuum at 200–230°. The CO_2 which evolved was first passed through Ag wool to remove any halide and subsequently

[8] WILCOX, M. E., WYLER, H. and DREIDING, A. S. (1965) *Helv. chim. acta* **48**, 1134.

[9] FROHOFER, H. private communication.

through a dry ice-Me_2CO cooled trap to freeze out C_5H_5N and H_2O. The purified CO_2 was collected as a solid at liquid air temp and its radioactivity was measured. The residue remaining after the decarboxylation was heated to 1000° for total oxidation by the CuO The CO_2 evolved at 1000° was also purified and counted[4,9] (see Table 1)

Conversion of betanin-[^{14}C] to indicaxanthin-[^{14}C] Excess L-proline (about × 10 the amount of betanin-[^{14}C]) was added to an aq soln of betanin-[^{14}C] (about 6 mg in 10 ml H_2O) and the resultant soln deoxygenated Conc NH_4OH was added dropwise to raise the pH of the soln to 10 Alkaline hydrolysis was allowed to continue under N_2 for 30 min at 20° then the pH of the soln was adjusted to 6 with aq HCl and the soln kept at 0° for 16 hr The indicaxanthin-[^{14}C] obtained from the exchange reaction was purified by passing the soln through a Dowex 50W-X2 column using the procedure used for betanin

Radioactive counting A 0 5 ml aliquot, sampled at each experimental step, was dissolved in Hays' counting fluid containing 10% biosolve The colored samples were bleached with 0 025–0 05 ml of chlorox before the radioactivity was determined with a scintillation counter

ORD determination A soln of the naturally occurring unlabeled betalamic acid in 0 1 M NH_4HCO_3 was obtained from yellow *P grandiflora* flowers by the same procedures described above Betalamic acid was also obtained as a soln in 0 1 M NH_4HCO_3 from betanin by hydrolysis under N_2 with NH_4OH at pH 10 for 30 min followed by Ecteola column chromatography Measurements of both samples of betalamic acid (2 2 mg/l and 2·4 mg/l for the natural and derived samples, respectively, both values were calculated on the basis of UV at 430 nm, ϵ 25000) were carried out at 27° with 0 1 M NH_4HCO_3 as the reference soln

Acknowledgements—The work was supported by grants from the Robert A Welch Foundation (Grant F-130), the National Science Foundation (Grant GB 29576X) and the National Institutes of Health (Grant HD-04488)