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PYRROLIZIDINE ALKALOID BIOSYNTHESIS: DERIVATION OF RETRONECINE FROM L-ARGININE AND L-ORNITHINE

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Key Word Index—*Senecio isatideus*; Compositae; biosynthesis; retrorsine; retronecine; arginine; ornithine.

Abstract—The relative retention of ^{3}H and ^{14}C on incorporation of D-, L- and DL-isomers of [^{14}C]arginine and [^{14}C]ornithine into retrorsine using L-[5-^{3}H]arginine as an internal standard has been measured. The retronecine portion of the pyrrolizidine alkaloid retrorsine, present in *Senecio isatideus* plants, is shown to be derived from L-arginine and L-ornithine.

INTRODUCTION

Retronecine (**4**) is the most common of the necine bases found in pyrrolizidine alkaloids [1–3]. The biosynthesis of retronecine has been studied using precursors labelled with ^{14}C [4–8] and with ^{13}C [10–12]. These experiments support the original theory of Robinson [13] that the pyrrolizidine nucleus is biosynthesized from two C_4 units which are derived from arginine (**1**) or ornithine (**2**) via putrescine (1,4-diaminobutane). In experiments with the ^{14}C-labelled amino acids arginine and ornithine, DL-racemates have often been used, particularly with ornithine, with the assumption that the L-enantiomer is the actual precursor of the pyrrolizidine nucleus. We present evidence that retronecine (**4**) is derived from L-arginine and L-ornithine, with little or no contribution from the corresponding D-isomers.

RESULTS AND DISCUSSION

Arginine and ornithine labelled with ^{14}C are commercially available only in L- and DL-forms. It was, therefore, necessary to obtain ^{14}C-labelled samples of D-arginine and D-ornithine to test their involvement in retronecine biosynthesis. D-[5-^{14}C]Arginine was obtained by treating the racemic material with L-arginine decarboxylase which converts L-arginine into agmatine. The D-[5-^{14}C]arginine was separated from [1-^{14}C]agmatine by TLC on cellulose. D-[5-^{14}C]Ornithine was obtained in an analogous manner by treatment of DL-[5-^{14}C]ornithine with L-ornithine decarboxylase and separation of the unreacted D-[5-^{14}C]ornithine from [1,4-^{14}C]putrescine.

Parallel feeds of each amino acid labelled with ^{14}C in D-, L- and DL-forms were carried out by absorption of sterile aqueous solutions into *S. isatideus* plants through stem punctures [8, 9]. After one week, the major pyrrolizidine alkaloid present in these plants, retrorsine (**3**), was isolated and purified [8, 9]. The incorporation figures for retrorsine (Tables 1 and 2) suggest that both D-arginine and D-ornithine are very poor precursors for retrorsine compared with the corresponding L-isomers. However, it has long been recognized that incorporation figures on their own are not sufficient to establish the relative efficiencies of compounds as precursors in biosynthetic pathways [7, 14]. Therefore, in anticipation of this a double-labelling technique was employed. In each experiment with ^{14}C-labelled amino acids L-[5-^{3}H]arginine was added to provide an internal standard and give an initial $^{3}H/^{14}C$ ratio of *ca* 5. The values obtained for this $^{3}H/^{14}C$ ratio in the isolated retrorsine then provide a much better measure of the relative efficiency of each ^{14}C-labelled compound as a precursor for retrorsine. (The probable loss of some ^{3}H from L-[5-^{3}H]arginine in its conversion into retrorsine should be a constant factor in each experiment, and does not invalidate comparison of the $^{3}H/^{14}C$ ratios in each set of experiments.) With both arginine (Table 1) and ornithine (Table 2) the $^{3}H/^{14}C$ ratios with DL-material are almost double those of the L-isomers, indicating that nearly all of

Table 1. Relative retention of ^{3}H and ^{14}C on incorporation of [^{14}C]arginine isomers into retrorsine using L-[5-^{3}H]arginine as int. standard

Experiment	1	2	3
Arginine precursor	D-[5-^{14}C]	L-[U-^{14}C]	DL-[5-^{14}C]
^{14}C activity (μCi)	22.0	50	50
$^{3}H/^{14}C$ ratio fed	4.45	4.98	4.93
% incorporation of ^{14}C in retrorsine (**3**)	0.001	0.49	0.31
$^{3}H/^{14}C$ ratio in retrorsine (**3**)	437	3.7*	7.2
% ^{14}C activity in retronecine (**4**)	—	95	97
% ^{14}C activity in isatinecic acid (**5**)	—	3	4
% ^{3}H activity in retronecine (**4**)	96	96	93
% ^{3}H activity in isatinecic acid (**5**)	4	1	3

* The $^{3}H/^{14}C$ ratio was multiplied by a factor of 4/6 to correct for the probable incorporation of only four of the six carbon atoms of arginine.

Table 2. Relative retention of ^3H and ^{14}C on incorporation of [^{14}C]ornithine isomers into retrorsine using L-[5-^3H]arginine as int. standard

Experiment	1	2	3
Ornithine precursor	D-[5-^{14}C]	L-[U-^{14}C]	DL-[5-^{14}C]
^{14}C activity (μCi)	21.3	50	50
$^3H/^{14}C$ ratio fed	4.63	5.08	5.11
% incorporation of ^{14}C in retrorsine (3)	0.002	0.52	0.38
$^3H/^{14}C$ ratio in retrorsine (3)	392	3.3*	6.4
% ^{14}C activity in retronecine (4)	—	93	98
% ^{14}C activity in isatinecic acid (5)	—	5	4
% ^3H activity in retronecine (4)	101	92	94
% ^3H activity in isatinecic acid (5)	2	5	2

* The $^3H/^{14}C$ ratio was multiplied by a factor of 4/5 to correct for the probable incorporation of only four of the five carbon atoms of ornithine.

the ^{14}C-incorporation in these experiments can be ascribed to utilization of the L-isomers. Furthermore, with both D-labelled amino acids, the $^3H/^{14}C$ ratios are very large, indicating that less than 1% of the ^{14}C-labelled D-amino acids is converted into retrorsine compared with the corresponding L-isomer. Alternatively, if it is assumed that the initial enzymic processes operating on L-arginine and L-ornithine to form retrorsine are stereospecific, then there must be less than 1% of enantiomeric impurity in the D-amino acids used in these experiments.

It has been shown previously that ornithine and arginine are specific precursors for the base portion of retrorsine [7, 8]. This was confirmed by hydrolysis of the labelled retrorsine obtained in each experiment to retronecine (4) and isatinecic acid (5) (Fig. 1), and measurement of the radioactivity in each fragment (Tables 1 and 2). The radioactivity from both ^{14}C and ^3H was almost entirely (> 92%) confined to the base portion. (There was insufficient activity to measure the ^{14}C distribution in the experiments with both D-amino acids.)

These results clearly indicate that D-arginine and D-ornithine are not normal precursors for retronecine biosynthesis.

EXPERIMENTAL

General. All radiochemicals were purchased from the Radiochemical Centre, Amersham, or the Commissariat a l'Énergie Atomique, France, except for D-[5-^{14}C]arginine and D-[5-^{14}C]ornithine which were prepared as described below. Activities of ^{14}C and ^3H were measured with a Philips liquid scintillation counter using toluene–MeOH solns. Sufficient counts were accumulated to give a s.e. of less than 1% for each determination. A Panax RTLS-1A was used for the radioscanning of TLC plates.

D-[5-^{14}C]*Arginine.* An aq. soln of DL-[5-^{14}C]arginine HCl (50 μCi, 1 ml) was added to a soln of L-arginine decarboxylase [EC 4.1.1.19, type II from *E. coli*, 10 units, Sigma (London)] in Pi buffer (0.2 M, pH 5.2, 4 ml). The mixture was shaken gently at 37°.

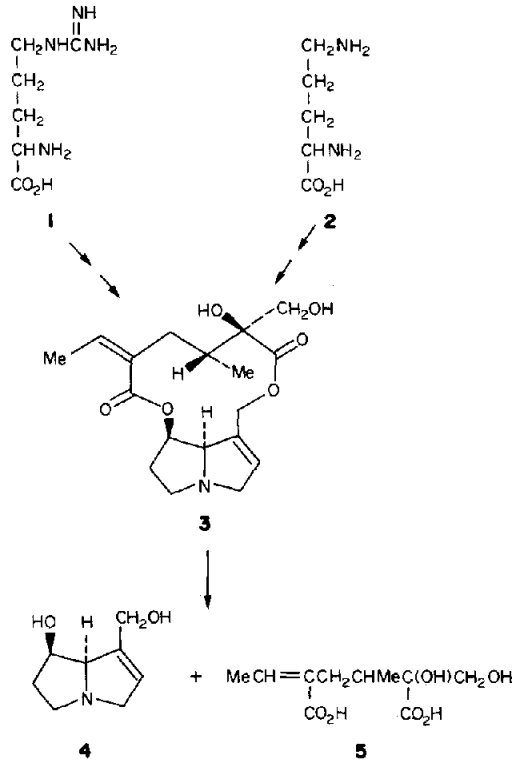

Fig. 1.

Radioscanning of a TLC cellulose-coated plate developed in *iso*-PrOH–conc. NH_3 (5:3) indicated that the original radioactive band at R_f 0.3, corresponding to arginine, gradually decreased with concomitant formation of a second radioactive band at R_f 0.4, corresponding to agmatine. After 1 hr, the amount of radioactivity associated with each band was nearly equal. Portions of unlabelled L-arginine HCl (5 × 0.5 mg) were then added to the incubation mixture at 1 hr intervals to ensure complete removal of the labelled L-arginine. Total incubation time was 8 hr. The mixture was then worked-up as described [14] to give D-[5-^{14}C]arginine (22.5 μCi). A portion of this sample (0.45 μCi) was incubated with L-arginine decarboxylase. No radioactivity at R_f 0.4 could be detected by radioscanning or autoradiography of TLC plates.

D-[5-^{14}C]*Ornithine.* An aq. soln of DL-[5-^{14}C]ornithine HCl (50 μCi, 1 ml) was added to a soln of L-ornithine decarboxylase [EC 4.1.1.17, from *E. coli*, 5 units, Sigma (London)] in Pi buffer (0.2 M, pH 5.0, 4 ml) and the mixture was agitated gently at 37°. Radioscanning (as above) indicated that the original radioactive band (ornithine, R_f 0.25) gradually decreased as a second radioactive band (putrescine, R_f 0.55) appeared. After 1 hr (equal radioactivity in each band), portions of unlabelled L-ornithine HCl (5 × 0.5 mg) were added at 1 hr intervals. After 8 hr incubation, the mixture was worked-up as described [14] to give D-[5-^{14}C]ornithine (21.8 μCi). A portion of this sample (0.44 μCi) was incubated with L-ornithine decarboxylase. No radioactivity at R_f 0.55 could be detected by radioscanning or autoradiography of TLC plates.

Feeding method. S. isatideus plants were propagated from stem cuttings and grown in a standard compost. Four 6-month-old plants were used for each expt. L-[5-^3H]Arginine was added to an aq. soln of each ^{14}C-labelled precursor to give an initial $^3H/^{14}C$ ratio of *ca* 5. A sterile aq. soln of each precursor was

introduced directly into the xylem of plants through stem punctures. All expts were carried out in parallel and the compounds tested are listed in Tables 1 and 2.

Isolation and purification of retrorsine (3). One week after administration of the precursor, the plants were harvested and retrorsine extracted as previously described [8, 9]. Retrorsine was obtained in 1–2% yield based on the dry wt of the plants. After dilution with inactive retrorsine, purification was effected by sublimation at 200° and 0.5 mm Hg, followed by recrystallization to constant sp. act. Retrorsine had mp 216–217°.

Hydrolysis of retrorsine (3). Retrorsine was hydrolysed as described for senecionine [15] to retronecine (4) and isatinecic acid (5) [8, 9].

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