Pyrrolizidine Alkaloid Biosynthesis. Incorporation of ^{14}C-Labelled Precursors into Retronecine [1]

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The biosynthesis of the retronecine (3) portion of the pyrrolizidine alkaloid retrorsine (1) present in *Senecio isatideus* plants has been studied with the aid of ^{14}C-labelled precursors. Putrescine (5), spermidine (6), and spermine (7), are the most efficient precursors of the pyrrolizidine ring. Degradations of labelled retronecine samples indicated that two molecules of each precursor are utilised, and that from these two molecules, a symmetrical intermediate of the type C_4–N–C_4 is formed and converted into retronecine.

RETRONECINE (3) is the most common base portion (necine) found in pyrrolizidine alkaloids.[2] It is formed together with isatinecic acid (2) on alkaline hydrolysis of retrorsine (1), the major pyrrolizidine alkaloid in *Senecio isatideus* plants (Scheme 1).[3] According to biosynthetic experiments using radiolabelled compounds, ornithine,[4-6] arginine,[7] and 1,4-diaminobutane (putrescine),[5] are specific precursors for retronecine. Prior to this work, the only published degradation of retronecine was oxidation with osmium tetroxide and sodium periodate to give formaldehyde (C-9) (Scheme 1).[5] Thus degradation of retronecine (9) derived biosynthetically from [5-^{14}C]- and [2-^{14}C]-ornithine (4), and [1,4-^{14}C]putrescine (5), showed that in each case one quarter of the total necine radioactivity was at C-9 of retronecine (Scheme 2).[5] Further progress in establishing the biosynthetic route to retronecine has been hindered by the lack of degradative pathways for retronecine to locate the positions of the remaining labelled atoms. It is evident, however, that C-2 and C-5 of ornithine become equivalent (*via* putrescine) before forming ring B of retronecine, and therefore one quarter of the total retronecine radioactivity should be at C-3 in each of these experiments. If the second molecule of ornithine also passes through a symmetrical intermediate to form ring A of retronecine, then the remaining 50% of the radioactivity should be split equally between C-5 and C-8 of retronecine. To test this theory, we have degraded retronecine to yield a fragment from ring A. A range of ^{14}C-labelled precursors have been tested as precursors for retronecine, and their partial labelling patterns have been established.

RESULTS AND DISCUSSION

At the start of this work, a careful study was made of the various methods available for administering ^{14}C-labelled precursors to *Senecio isatideus* plants to produce labelled retrorsine (1). The best incorporations were obtained by absorption of sterile aqueous solutions of the precursors (as their hydrochlorides) directly into the xylems of the growing plants through stem punctures. This technique gave incorporation values considerably higher than those obtained by more widely used methods such as hydroponics,[5-7] and cut stems.[4] The range of ^{14}C-labelled precursors tested by the direct absorption method is shown in Table 1. [2,3-$^{14}C_2$]-Putrescine was prepared by treatment of 1,2-dibromo[1,2-$^{14}C_2$]ethane with potassium cyanide, followed by reduction with borane in tetrahydrofuran. *N*-(3-amino[3-^{14}C]propyl)-1,4-diaminobutane (12) was synthesised as shown in Scheme 3. Coupling of *N*-protected 4-aminobutanoic acid and 2-bromoethylamine by the mixed anhydride method gave the *N*-protected bromoamide (10). The ^{14}C-labelled nitrile (11) was formed by treatment of (10) with potassium[^{14}C]cyanide, and hydrogenolysis and reduction steps yielded spermidine (12) labelled with ^{14}C in the C_3-portion.

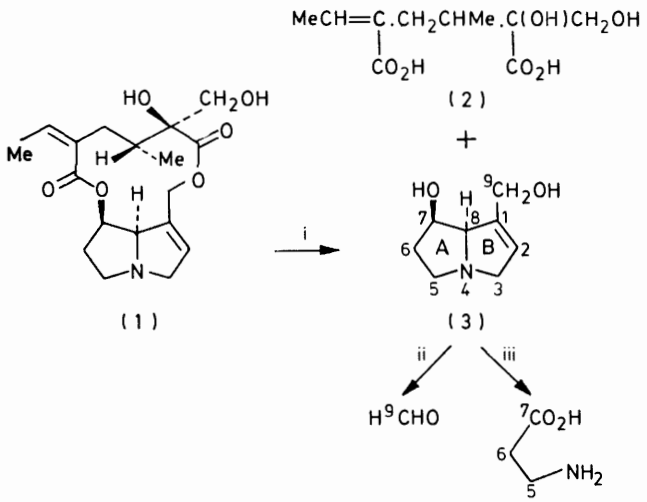
For the feeding experiments, a double-labelling technique was employed. Precursors were fed together with L-[5-^{3}H]arginine as a reference compound to give an initial ^{3}H/^{14}C ratio of 5.0. The figures for this ^{3}H/^{14}C ratio in the isolated retrorsine (Table 1) then provide a good measure of the relative efficiency of each ^{14}C-

TABLE 1

Incorporation of precursors into retrorsine (1) in *Senecio isatideus* plants

Expt.	Precursor	% Incorporation	^{3}H/^{14}C Ratio	% Radioactivity in (3)	% Radioactivity in (2)
1	L-[U-^{14}C]Proline	0.04	77	68	30
2	4-Amino[U-^{14}C]butanoic acid	0.04	37	52	48
3	L-[U-^{14}C]Glutamic acid	0.12	10	48	58
4	L-[U-^{14}C]Arginine	0.46	5.5	99	6
5	DL-[2-^{14}C]Ornithine	0.29	6.7	96	3
6	DL-[5-^{14}C]Ornithine	0.25	6.2	97	5
7	[1,4-$^{14}C_2$]Putrescine (5)	1.6	0.60	94	1
8	[2,3-$^{14}C_2$]Putrescine	0.7	0.98	95	2
9	*NN'*-Bis(3-aminopropyl)-1,4-diamino[1,4-$^{14}C_2$]butane (7)	5.2	0.73	103	1
10	*N*-(3-Aminopropyl)-1,4-diamino[1,4-$^{14}C_2$]butane (6)	2.0	0.80	95	1
11	*N*-(3-Amino[3-^{14}C]propyl)-1,4-diaminobutane (12)	0.02	66	41	57

labelled compound as a precursor for retrorsine. The best precursors were found to be putrescine, and the previously untested polyamines *N*-(3-aminopropyl)-1,4-diaminobutane (spermidine) (6) and *NN'*-bis(3-aminopropyl)-1,4-diaminobutane (spermine) (7). It should be noted that spermidine only functions as an efficient precursor for retrorsine when labelled with ^{14}C in the C_4 portion (*cf.* Expts. 10 and 11, Table 1). Putrescine, spermidine, and spermine, are at least five times more



SCHEME 1 *Reagents:* i, $Ba(OH)_2$; ii, OsO_4–$NaIO_4$; iii, CrO_3–H_2SO_4

efficient than ornithine or arginine as precursors for retrorsine, in agreement with the suggestion that putrescine follows these compounds in the biosynthetic pathway.[8] Furthermore, from hydrolysis of retrorsine to retronecine (3) and isatinecic acid (2) and measurement of the radioactivity of each fragment (Table 1), it is clear that ornithine, arginine, putrescine, spermidine (the C_4 portion) and spermine specifically provide the necine portion of the alkaloid. The poorer precursors, glutamate, 4-aminobutanoate, L-proline, and the C_3 portion of spermidine (Expt. 11, Table 1), are incorporated non-specifically into both acid and base portions.

The function of the C_4 portions of spermine and spermidine in retronecine biosynthesis is not yet clear. These polyamines are known to produce 1-pyrrolines on oxidation with the polyamine oxidases present in higher plants, or they may be metabolised to putrescine.[9]

The partial labelling patterns for the retronecine samples specifically labelled with ^{14}C were established as follows. Oxidation of retronecine with osmium tetroxide and sodium periodate gave formaldehyde, isolated as its dimedone derivative, corresponding to C-9 of retronecine (Scheme 1). Thus, from degradations of samples

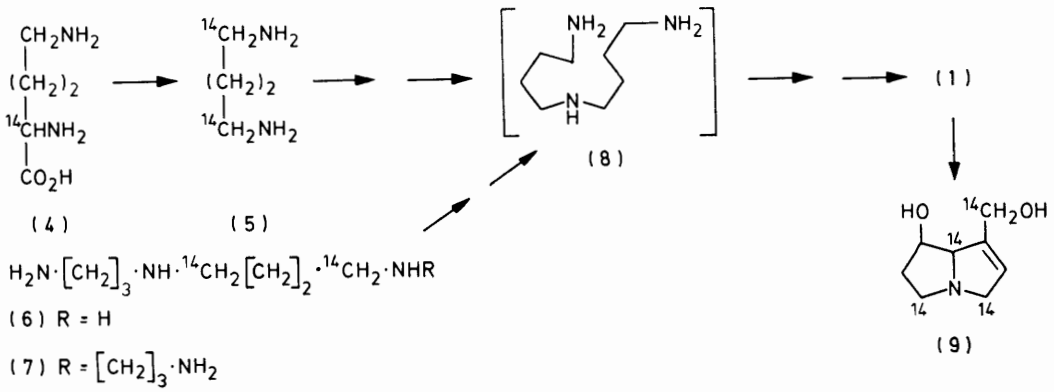
TABLE 2

Distribution of ^{14}C-radioactivity in retronecine (3).

Expt.	% Radioactivity at C-9	% Radioactivity at C-(5 + 6 + 7)
5	24	24
6	25	22
7	24	22
8	1	47
9	23	23
10	22	24

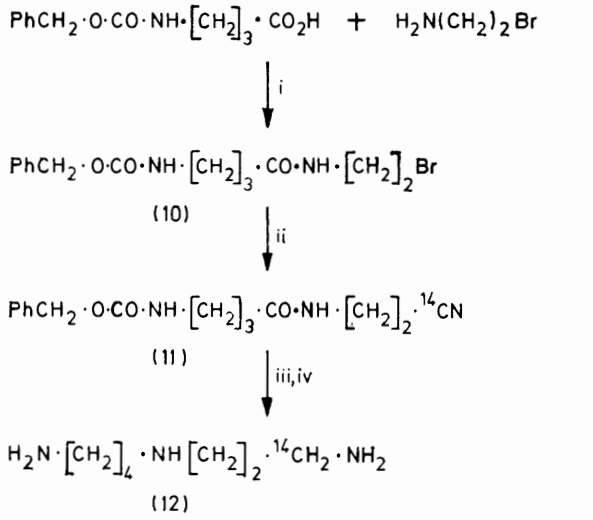
of retronecine labelled with [2-^{14}C]- and [5-^{14}C]ornithine, [1,4-$^{14}C_2$]putrescine, and spermidine and spermine labelled [1,4-$^{14}C_2$] in the C_4-portion (Expts. 5—7 and 9—10, Table 2), 22—25% of the total necine activity was shown to be at C-9. With [2,3-$^{14}C_2$]putrescine (experiment 8), the complementary pattern of labelling was observed with less than 1% of the necine radioactivity at C-9. These results are consistent with the passage of all these precursors through a symmetrical C_4 intermediate (*e.g.* putrescine) to form ring B of retronecine (3).

Attention was then directed towards the identification of a degradation fragment from ring A of retronecine. A large number of possible degradation pathways for retronecine, 1-hydroxymethyl-7-hydroxypyrrolizidine (retronecanol), and their *O*-acyl derivatives were investigated. Hofmann degradations, oxidations with potassium permanganate, ozonolyses, and treatment with cyanogen bromide of these compounds gave no identifiable products. Eventually it was found that modified Kuhn–Roth oxidation [10] of retronecine gave small but reproducible yields of β-alanine (Scheme 1) which was isolated as its derivative with 1-fluoro-2,4-dinitrobenzene. This corresponds to C-(5 + 6 + 7) from ring A of retronecine (3). This degradation was then carried out on samples of



SCHEME 2

retronecine specifically labelled with ^{14}C. Thus, in experiments 5—7 and 9—10, 22—24% of the total necine activity is located at C-(5 + 6 + 7). Again, in experiment 8, the complementary pattern of labelling was observed for [2,3-$^{14}C_2$]putrescine and 47% of the total radioactivity is at these three carbon atoms. These results demonstrate that C-2 and C-5 of ornithine become equivalent in the formation of ring A of retronecine (3). Furthermore, the finding that in all the experiments (5—10) the radioactivity is equally distributed between rings A and B of retronecine does suggest that a later, symmetrical C_4–N–C_4 intermediate, such as (8), derived from two molecules of putrescine, is involved in retronecine biosynthesis.



SCHEME 3 *Reagents:* i, PhCOCl; ii, K^{14}CN; iii, H_2–Pd/C; iv, BH_3–THF

Complete labelling patterns for retronecine in agreement with the above results have recently been established by Khan and Robins by ^{13}C n.m.r. spectroscopy on retronecine after feeding ^{13}C-labelled putrescines to *Senecio isatideus* plants.[11]

EXPERIMENTAL

General.— M.p.s were measured with a Kofler hot-stage apparatus. Organic solutions were dried with anhydrous $MgSO_4$, and solvents were evaporated off under reduced pressure below 40 °C. N.m.r. spectra were run for solutions in deuteriochloroform unless otherwise stated with tetramethylsilane as internal standard. Mass spectra were obtained with an A.E.I. MS 12 spectrometer. Thin layer chromatography was carried out on silica G plates of 0.25 mm thickness developed with chloroform–methanol–conc. ammonia (85 : 14 : 1), and the bases were located by oxidation with *o*-chloranil, followed by treatment with Ehrlich's reagent.[12]

Radiochemical Methods.—All radiochemicals were purchased from the Radiochemical Centre, Amersham, or the New England Nuclear Corporation, except for 1,4-diamino-[2,3-$^{14}C_2$]butane and *N*-(3-amino[3-^{14}C]propyl)-1,4-diaminobutane, which were prepared as described below. Activities of ^{14}C and ^{3}H were measured with a Philips liquid scintillation analyser using toluene–methanol solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination.

Radioactive samples were normally recrystallised to constant specific activity and counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for the radioscanning of t.l.c. plates.

1,4-*Diamino*[2,3-$^{14}C_2$]*butane Dihydrochloride.*—A solution of potassium cyanide (195 mg, 3 mmol) in water (1 ml) was added to 1,2-dibromo[1,2-$^{14}C_2$]ethane (188 mg, 1 mmol, 100 μCi) in ethanol (20 ml), and the mixture was heated at reflux for 5 h. The cooled solution was concentrated to near dryness, and the residue was taken up in water (10 ml) and extracted repeatedly with chloroform. The chloroform extracts were dried, filtered, and concentrated to an oil. Purification was effected by column chromatography on silica gel and elution with benzene–methanol (200 : 1). Solid 1,2-dicyano[1,2-$^{14}C_2$]ethane was crystallised from benzene (yield 42 mg, 52%; 93.1 μCi mmol^{-1}), m.p. 54—55 °C (lit.,[13] 57 °C), $\nu_{max.}$ (KBr) 2 250 cm^{-1}; δ(CD_3OD) 2.88 p.p.m. (s); *m/z* 80, 53, and 50 (Found for unlabelled sample: C, 59.9; H, 5.2; N, 34.7. Calc. for $C_4H_4N_2$: C, 60.0; H, 5.0; N, 35.0%). 1M-Borane in tetrahydrofuran (6 ml) was added to a solution of 1,2-dicyano[1,2-$^{14}C_2$]ethane (40 mg, 0.5 mmol) in dry tetrahydrofuran (5 ml), and the solution was heated at reflux for 16 h. Dry ethanol (10 ml) was added to the cooled solution, followed after 1 h by passage of dry HCl gas. The solid dihydrochloride of 1,4-diamino-[2,3-$^{14}C_2$]butane was filtered off and recrystallised to constant specific activity from aqueous ethanol (yield 49 mg, 61%; 94.9 μCi mmol^{-1}), m.p. >290 °C. Radioscanning of a t.l.c. cellulose-coated plate developed in propan-2-ol–conc. ammonia (7 : 3) showed one radioactive band coincident with authentic unlabelled putrescine dihydrochloride.

N-(3-*Amino*[3-^{14}C]*propyl*)-1,4-*diaminobutane* (12).—A solution of *N*-benzyloxycarbonyl-4-aminobutanoic acid[14] (474 mg, 2 mmol) and triethylamine (202 mg, 2 mmol) in dry dichloromethane (5 ml) was cooled to −5 °C and benzoyl chloride (282 mg, 2 mmol) was added slowly with stirring. After 1.5 h, a pre-cooled solution of 2-bromoethylamine hydrobromide (410 mg, 2 mmol) and triethylamine (202 mg, 2 mmol) in dichloromethane (5 ml) was added and the mixture was allowed to warm to 5 °C. After 18 h, the organic solution was washed successively with dilute HCl, water, dilute aqueous $NaHCO_3$, and water. The organic layer was dried, filtered, and concentrated to give a white solid. Crystallisation from ethyl acetate gave 4-[(*benzyloxycarbonyl*)*amino*]-N-(2-*bromoethyl*)*butanamide* (10) (341 mg, 49%), m.p. 95—96 °C, $\nu_{max.}$ (KBr) 3 370, 3 280, 1 710, 1 660, and 1 550 cm^{-1}; δ ($CDCl_3$) 1.85 (2 H, m), 2.2 (2 H, m), 3.5 (6 H, m), 5.02 (2 H, s), 5.2 and 6.5 (2 H, each br s, NH), and 7.28 (5 H, s); *m/z* 344, 342, and 262 (Found: C, 48.7; H, 5.6; Br, 23.7; N, 8.0. $C_{14}H_{19}BrN_2O_3$ requires C, 49.0; H, 5.6; Br, 23.3; N, 8.2%). Finely ground potassium [^{14}C]cyanide (49 mg, 0.75 mmol; 1 mCi) was added to a solution of the bromoamide (10) (172 mg, 0.5 mmol) and 18-crown-6 (70 mg) in dry HMPA (1 ml). The mixture was heated for 1 h at 50 °C, and then poured into water (10 ml); the aqueous solution was then extracted with ethyl acetate (3 × 10 ml). The combined organic extracts were washed with water (6 × 2 ml), dried, filtered, and concentrated to a thick oil of 4-[(*benzyloxycarbonyl*)-*amino*]-N-(2-[^{14}C]*cyanoethyl*)*butanamide* (11), (133 mg, 92%; 309 μCi mmol^{-1}), $\nu_{max.}$ ($CHCl_3$) 2 245 cm^{-1}; δ ($CDCl_3$) 1.8 (2 H, m), 2.2 (2 H, m), 3.2 (2 H, m), 3.5—4.2 (4 H, m), 5.0

(2 H, s), 5.5 (1 H, br s), and 7.25 (5 H, s); *m/z* 289 and 262 (Found for unlabelled sample: M^+ 289.3351. $C_{15}H_{19}N_3O_3$ requires *M* 289.3349). The nitrile (11) (131 mg, 0.45 mmol) in methanol (10 ml) was hydrogenolysed for 1 h at room temperature in the presence of 10% Pd–C (15 mg). The product (76 mg) obtained by filtration and removal of the solvent was used directly in the next step. A solution of 1M-borane in tetrahydrofuran (8 ml) was added to the product of the previous reaction in dry tetrahydrofuran (15 ml), and the solution was heated at reflux for 18 h. Dry ethanol (5 ml) was added to the cooled solution, and after 1 h, dry HCl gas was passed through the solution. The solid dihydrochloride of *N*-(3-amino[3-^{14}C]propyl)-1,4-diaminobutane (12) was filtered off and recrystallised to constant specific activity from aqueous ethanol (yield 59 mg, 47%; 305 μCi mmol^{-1}), m.p. 255—256 °C (lit.,[15] 256—258 °C; undepressed mixed m.p.). Radioscans of a t.l.c. cellulose-coated plate developed in propan-2-ol–conc. ammonia (7 : 3) indicated one radioactive band coincident with authentic unlabelled spermidine trihydrochloride.

Feeding Method.—*Senecio isatideus* plants were propagated by stem cuttings and grown in a standard compost. Two six-month-old plants were used for each feeding experiment. L-[5-^{3}H]Arginine was added to each ^{14}C-labelled precursor as a reference to give a ^{3}H/^{14}C ratio of 5.0. Droplets of a sterile aqueous solution of each precursor (as its hydrochloride) were placed on the stems of the plants. Stem punctures were made through the droplets with a sterile needle. The solution was absorbed quickly into the xylems of the plants. The precursors tested are listed in Table 1.

Extraction of Retrorsine (1).—One week after administration of the precursor, the plants were removed from the compost and the roots were washed well with water. The plants were macerated repeatedly with methanol in a Waring blender and the methanolic suspension was subjected to ultrasonic vibrations until the filtered methanolic extracts were colourless. The methanolic extracts were concentrated and the residue taken up in 1M-H_2SO_4 (25 ml). The acidic solution was washed with $CHCl_3$ (5 × 25 ml), and then stirred with zinc dust for 90 min to reduce *N*-oxides. The mixture was filtered through Celite, and the filtrate was basified strongly with conc. ammonia and extracted with chloroform (6 × 50 ml). The chloroform extracts were dried, filtered, and concentrated to afford almost pure retrorsine (1). The yield of retrorsine varied between 0.5 and 2% based on the dry weight of plant material. Retrosine was sublimed at 200 °C and 0.5 mmHg and recrystallised to constant specific activity from acetone, m.p. 216—217 °C (lit.,[2a] 216—216.5 °C). In each experiment, radioscans of t.l.c. plates showed only one radioactive band, coincident with authentic unlabelled retrorsine at R_F 0.35. In most experiments, inactive retrorsine (100—300 mg) was added to the labelled material and the mixture was recrystallised to constant specific activity.

Hydrolysis of Retrorsine (1).—This was carried out as described for the hydrolysis of senecionine.[16] Isatinecic acid (2) was recrystallised from ethyl acetate–light petroleum (b.p. 60—80 °C) as white needles (130 mg, 65%), m.p. 147—149 °C (lit.,[17] 148 °C). Retronecine (3) was sublimed at 90 °C and 1 mmHg and recrystallised from acetone–light petroleum (b.p. 60—80 °C) to give white needles (85 mg, 62%), m.p. 118—120 °C (lit.,[5] 119—120 °C).

Degradation for C-9 *of Retronecine* (3).—This was carried out as previously described.[5] Retronecine (25 mg, 0.16 mmol) yielded formaldehyde dimedone (5—10 mg, 10—20%), which was recrystallised to constant specific activity from aqueous ethanol, m.p. 189—190 °C (undepressed on mixing with authentic formaldehyde dimedone).

Degradation for C-(5 + 6 + 7) *of Retronecine* (3).—Retronecine (50 mg, 0.32 mmol) was dissolved in hot aqueous H_2SO_4 (6M; 2 ml) and CrO_3 (250 mg) in water (2 ml) was added in several portions. The mixture was heated under reflux for 24 h. The cooled solution was worked up as described [10] and treated with a methanolic solution of 1-fluoro-2,4-dinitrobenzene to give a mixture of solid yellow dinitrophenyl amino-acids which were separated by preparative t.l.c. (1-mm thick plates) developed with benzene–pyridine–acetic acid (80 : 20 : 2). The major yellow band had R_F value 0.65—0.70 identical with that of an authentic sample of *N*-2,4-dinitrophenyl-β-alanine. This band was eluted with methanol, and the methanolic extracts were concentrated to a yellow solid. This residue was dissolved in water (1 ml), acidified to pH 2 with dilute HCl, and the mixture was extracted with ether (4 × 5 ml). The ether extracts were dried, filtered, and evaporated to dryness. Recrystallisation to constant specific activity from aqueous ethanol gave yellow needles of *N*-2,4-dinitrophenyl-β-alanine, yield 8.4 mg (10%), m.p. 144—146 °C (m.p. of authentic material [10] 145—146 °C; mixed m.p. undepressed.

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