Pyrrolizidine Alkaloid Biosynthesis. Synthesis of *N*-([4-^{14}C]-4-Aminobutyl)-1,2-didehydropyrrolidinium and its Incorporation into Different Pyrrolizidine Bases (Necines)[1]

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N-([4-^{14}C]-4-Aminobutyl)-1,2-didehydropyrrolidinium chloride hydrochloride **19** was prepared from the mesylate of 3-chloropropanol by displacement of the mesylate with sodium [^{14}C]cyanide, nucleophilic substitution of the chloro group by pyrrolidine, reduction of the nitrile and oxidation of the amine with mercuric acetate. This material was fed together with [2,3-^{3}H]putrescine dihydrochloride to *Senecio isatideus* which produces retrorsine **6**, *Senecio pleistocephalus* which makes rosmarinine **11**, *Cynoglossum officinale* which contains echinatine **8** and *Cynoglossum australe* which produces cynaustraline **13** and cynaustine **15**. Comparison of the ^{3}H/^{14}C ratios of the precursor mixtures with the alkaloids obtained after the feeding experiments had been carried out showed that the ^{14}C-labelled immonium ion **19** is an efficient precursor for all the different base portions (necines) of the five pyrrolizidine alkaloids. The presence of the immonium ion **3** in *Senecio pleistocephalus* was established by an intermediate trapping experiment. Thus the immonium ion **3** is involved in the biosynthetic pathways to a range of necines, including retronecine **5**, heliotridine **7**, rosmarinecine **10**, (+)-isoretronecanol **12** and (+)-supinidine **14**.

Pyrrolizidine alkaloids are present in many species belonging to different plant families including *Senecio* (Asteraceae) and *Cynoglossum* (Boraginaceae).[2] Many of these alkaloids are hepatotoxic.[3] For this biosynthetic work, four species were used: *Senecio isatideus* which produces retrorsine **6**; *Senecio pleistocephalus* which makes rosmarinine **11**;[4] *Cynoglossum officinale* which contains echinatine **8**;[5] and *Cynoglossum australe* which produces cynaustraline **13** and cynaustine **15**.[6]

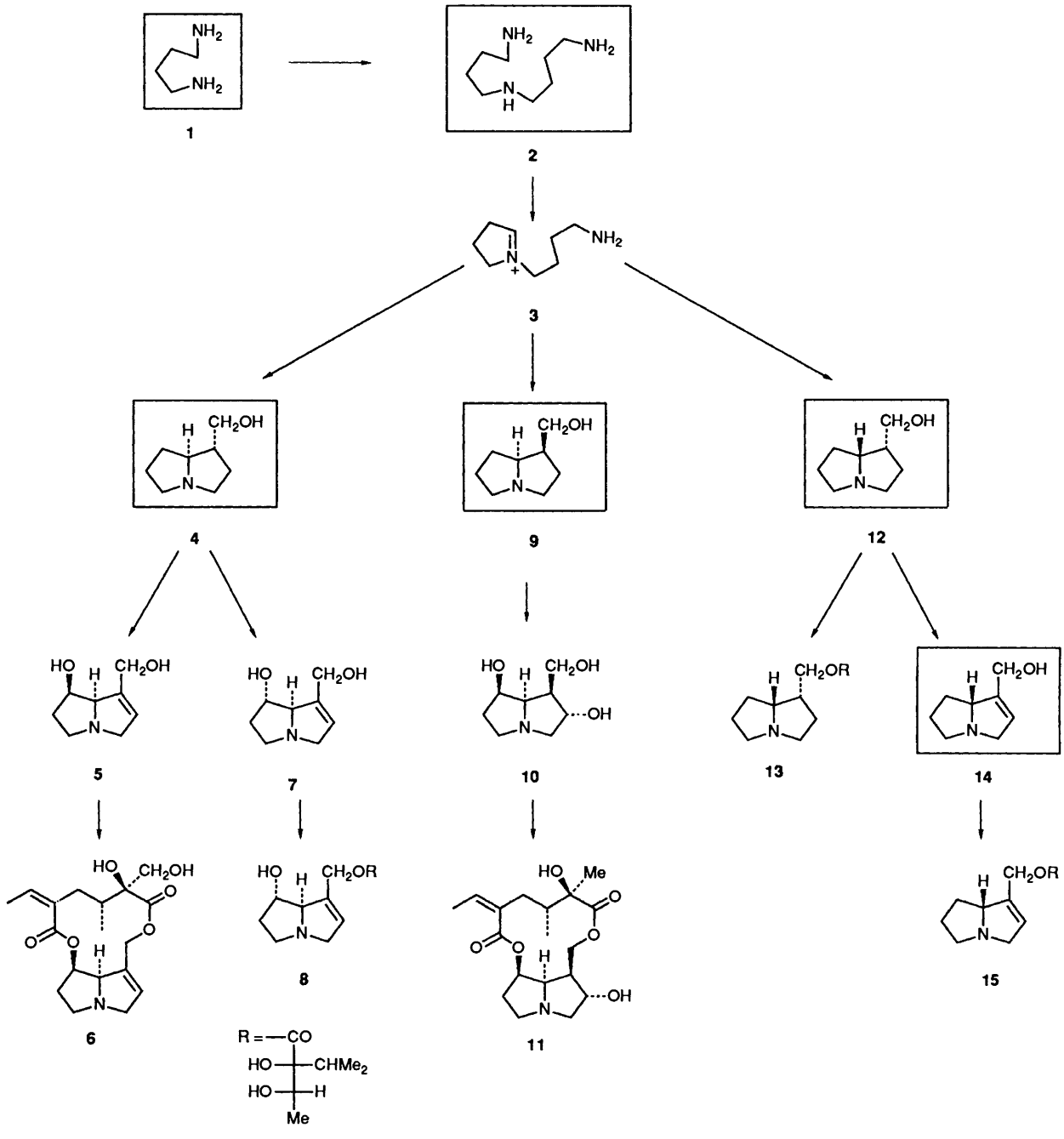
The biosynthetic pathways to the different necines contained in these alkaloids are shown in Scheme 1. Known intermediates are placed in boxes.[7] These pathways have been established as a result of feeding experiments with ^{3}H, ^{14}C and ^{13}C-labelled precursors. The necines are formed from putrescine **1**[8] *via* homospermidine **2**.[9] Homospermidine has been converted into trachelanthamidine **4** using diamine oxidase isolated from pea seedlings followed by reduction involving a coupled dehydrogenase.[10] An explanation for this result is that oxidation of one terminal group of homospermidine **2** leads to an immonium ion **3**, which can undergo further oxidation to an aldehyde, followed by cyclization to give 1-formylpyrrolizidine. A reduction step would then afford trachelanthamidine **4**. Alternative cyclization modes of the immonium ion **3** under enzyme control in the plants could lead to the necines (−)-isoretronecanol **9** and its enantiomer **12**. The facile synthesis of trachelanthamidine **4** did indicate that the 1-hydroxymethylpyrrolizidines should be tested as intermediates of the more complex necines. Accordingly, ^{3}H-labelled samples of trachelanthamidine[11,12] and isoretronecanol[12] were prepared as racemates. (±)-Trachelanthamidine was an efficient precursor for the retronecine **5** component of retrorsine **6** in *S. isatideus*, and the heliotridine **7** part of echinatine **8** in *C. officinale*. On the other hand, (±)-isoretronecanol was incorporated well into the rosmarinecine **10** portion of rosmarinine **11** in *S. pleistocephalus*,[12] and into the isoretronecanol **12** part of cynaustraline **13** and the supinidine **14** portion of cynaustine **15** in *C. australe*.[6]

The formation of all these necine bases from homospermidine in the plant species examined did suggest that the immonium ion **3** should be tested as a key intermediate in the biosynthetic pathways before they diverge to produce the different necines.

Results and Discussion

The ^{14}C-labelled immonium ion **19** was prepared as outlined in Scheme 2. 3-Chloropropanol was mesylated and the product was treated with sodium [^{14}C]cyanide to afford [1-^{14}C]-4-chlorobutanenitrile **16**. Nucleophilic displacement of the chloro group by pyrrolidine yielded the pyrrolidinenitrile **17**. The nitrile group was reduced and the product was acidified to give the diamine salt **18**. Formation of the desired ^{14}C-labelled immonium ion **19** was achieved by oxidation of the pyrrolidinium salt **18** with mercuric acetate. Although there is literature precedent for the formation of an endocyclic double bond in this type of oxidation process,[13] it was considered necessary to provide evidence for the position of the double bond in the immonium ion **19**. Accordingly, a portion of the unlabelled material **3** was reduced with sodium cyanoborodeuteride to give a monodeuteriated product **20** (Scheme 3). A ^{1}H NMR spectrum of this material showed that it contained a three proton multiplet at δ 2.95 instead of the four proton multiplet observed for the equivalent protons on the pyrrolidine ring on the carbons adjacent to nitrogen in the unlabelled material. This establishes the endocyclic nature of the double bond in the ^{14}C-labelled immonium ion **19**.

In order to carry out the feeding experiments with the immonium ion **19**, a double labelling technique was employed.[4] Each sample of the immonium ion was fed together with [2,3-^{3}H]putrescine dihydrochloride as a reference and the ^{3}H/^{14}C ratios for each precursor mixture were measured (Table 1). The mixtures of isotopically labelled species were fed to *S. isatideus* and *C. officinale* by direct absorption of sterile solutions into the xylems through stem punctures,[4] and to *S. pleistocephalus* and flowering stems of *C. australe* by the wick method. All feedings were carried out on alternate days over a five day period, and after a further ten days the alkaloids were isolated from the plants. Retrorsine **6** from *S. isatideus*,[4] and rosmarinine **11** from *S. pleistocephalus*,[5] were recrystallized to constant specific activity, whereas echinatine **8** from *C. officinale* was obtained as a gum after column chromatography.[12] Cynaustraline **13** and cynaustine **15** from *C. australe* were separated by repeated column chromatography and also obtained as gums.[6]



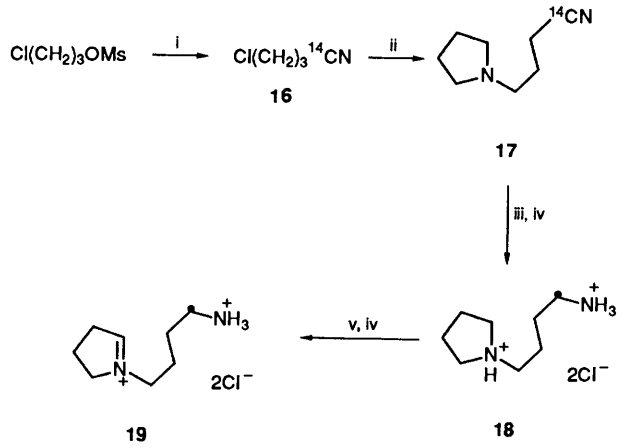
Scheme 1

Table 1 Incorporation of immonium ion **19** into pyrrolizidine alkaloids

Experiment	1	2	3	4	
Plant species	*S.p.*[a]	*S.i.*[b]	*C.o.*[c]	*C.a.*[d]	
Number of plants	1	1	8	8	
$^3H/^{14}C$ ratio (start)	5.1	12.3	2.4	2.0	
Alkaloid isolated	(11)	(6)	(8)	(13)	(15)
$^3H/^{14}C$ ratio (finish)	2.9	9.8	3.9	2.9	3.0
^{14}C Specific Incorporation	6.5	4.5	0.8	0.05	0.03
Necine isolated	(10)	(5)	(7)	(12)	(14)
$^3H/^{14}C$ ratio	2.8	9.9	4.0	2.7	3.0

[a] *S.p.* is *S. pleistocephalus.* [b] *S.i.* is *S. isatideus.* [c] *C.o.* is *Cynoglossum officinale.* [d] *C.a.* is *C. australe.*

Comparison of the $^3H/^{14}C$ ratios in the individual alkaloids with those at the start of each experiment (Table 1) provides a good measure of the relative efficiency of the ^{14}C-labelled immonium ion **19** as a precursor for each alkaloid relative to putrescine. The highest ^{14}C specific incorporation was observed for rosmarinine **11** after feeding the immonium ion **19** to *S. pleistocephalus* and the $^3H/^{14}C$ ratio decreased from 5.1 to 2.9. Hydrolysis of rosmarinine to yield rosmarinecine **10** gave material with essentially the same ^{14}C specific incorporation and $^3H/^{14}C$ ratio (Table 1) showing that all the radioactivity is located in the base portion. A high ^{14}C specific incorporation was also obtained for retrorsine after feeding the immonium ion **19** to *S. isatideus*, and the $^3H/^{14}C$ ratio decreased from 12.3 to 9.8. Alkaline hydrolysis of retrorsine afforded retronecine **5** with nearly the same ^{14}C specific incorporation and $^3H/^{14}C$ ratio. These results indicate that the immonium ion **19** is a more efficient precursor for rosmarinecine and retronecine than is putrescine. Feeding of the immonium ion to the two *Cynoglossum* species gave alkaloids with lower ^{14}C specific

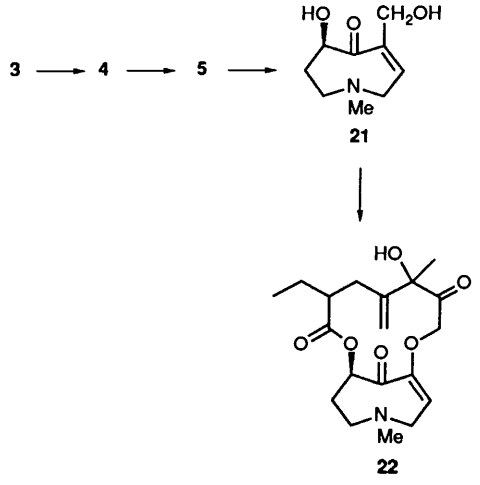


Scheme 2 *Reagents:* i, $Na^{14}CN$, DMSO, 90 °C, 2 h; ii, pyrrolidine, Na_2CO_3, KI, 103 °C, 18 h; iii, PtO_2, AcOH; iv, HCl; v, $Hg(OAc)_2$, 120 °C, 4 h; H_2S

Scheme 3

incorporations, although it should be noted that we have consistently observed lower incorporations of precursors into *Cynoglossum* compared with *Senecio* species. In the case of *C. australe*, the very low ^{14}C specific incorporation is partly due to the relatively large amount of alkaloids isolated and the consequent high dilution. The total incorporation of ^{14}C was 1% into cynaustraline **13** and 0.7% into cynaustine **15**. Nevertheless, comparison of the $^3H/^{14}C$ ratios in the alkaloids isolated from the *Cynoglossum* species with those of the precursor mixture indicated that the immonium ion **19** is incorporated with about 2/3 of the efficiency of putrescine. Alkaline hydrolysis of the three alkaloids gave the necines heliotridine **7**, (+)-isoretronecanol **12**, and (+)-supinidine **14** with virtually unchanged $^3H/^{14}C$ ratios. We conclude that the immonium ion **19** is a reasonable precursor for these three bases. We have previously shown that the immonium ion **19** is a good precursor for the secopyrrolizidine otonecine **21** derived from the alkaloid emiline **22** in *Emilia flammea* plants (Scheme 4).[14]

The next requirement was to carry out an intermediate trapping experiment to demonstrate the presence of the



Scheme 4

immonium ion **3** in one of the plant species used in this work. [1,4-^{14}C]Putrescine dihydrochloride was fed to one *S. pleistocephalus* plant and after one day the plant was harvested and extracted with methanol. A sample of the unlabelled immonium salt was added to the methanol extract followed by sodium borohydride. The *N*-phenylthiourea derivative of *N*-(4-aminobutyl)pyrrolidine was prepared[15] and recrystallized to constant specific radioactivity. This derivative contained 0.4% of the radioactivity administered to the plant. Furthermore the derivative contained only one radioactive band on TLC coincident with the authentic unlabelled derivative. As an added check on the involvement of the immonium ion in the biosynthetic pathway, a similar methanol extract obtained after feeding [1,4-^{14}C]putrescine dihydrochloride to *S. pleistocephalus* was treated with sodium [^3H]borohydride, then the *N*-phenylthiourea derivative was prepared as before and crystallized to constant specific radioactivity. This derivative contained a similar amount of ^{14}C as before and about 5% of the ^3H was incorporated into the derivative, demonstrating that the immonium ion **3** is present in *S. pleistocephalus*.

We then decided to investigate the status of *N*-(4-aminobutyl)pyrrolidine as an intermediate in the biosynthetic pathway to rosmarinecine **10**. A sample of ^{14}C-labelled material **18** available from the synthesis of the immonium ion (Scheme 2) was mixed with [1,4-^3H]putrescine dihydrochloride to give a $^3H/^{14}C$ ratio of 1.5 and this precursor mixture was fed to one *S. pleistocephalus* plant as before. A ^{14}C specific incorporation of 2.1% was observed in the isolated rosmarinine **11** with a $^3H/^{14}C$ ratio of 2.5. This result shows that *N*-(4-aminobutyl)pyrrolidine is a reasonable precursor for rosmarinine, although it is only incorporated to about 1/3 of the efficiency of the immonium ion **19**. It was therefore essential to carry out another trapping experiment. [1,4-^{14}C]Putrescine dihydrochloride was fed to one *S. pleistocephalus* plant and after one day a sample of unlabelled *N*-(4-aminobutyl)pyrrolidine was added to the methanol extract of the plant. The *N*-phenylthiourea derivative was prepared as before and crystallized to constant specific radioactivity. This derivative contained less than 0.017% of the ^{14}C radioactivity fed. The most likely explanation for these results is that *N*-(4-aminobutyl)pyrrolidine is not actually on the biosynthetic pathway to rosmarinecine but it can be oxidized to the immonium ion **3** by enzymes present in *S. pleistocephalus*.

Experimental

General.—M.p.s were measured with a Kofler hot-stage apparatus and are uncorrected. Organic solutions were dried with anhydrous $MgSO_4$, and solvents were removed under reduced pressure below 40 °C. NMR spectra were obtained for solutions in D_2O unless otherwise stated at 90 MHz or on a Bruker WP200-SY spectrometer operating at 200 MHz for ^1H and 50 MHz for ^{13}C, *J* values are given in Hz. Radiochemicals were purchased from Amersham International. Radioactivity was measured with a Philips PW 4700 Liquid Scintillation Counter using toluene–methanol solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Radioactive samples (except echinatine, cynaustraline and cynaustine) were recrystallized to constant specific activity and they were counted in duplicate. Thin layer chromatography was carried out on silica gel 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.[16] A Panax thin-layer scanner RTLS-1A was used for the radioscanning of TLC plates.

[1-^{14}C]-4-*Chlorobutanenitrile* **16**.—Sodium cyanide (588 mg,

12.0 mmol) and sodium [^{14}C]cyanide (500 μCi) were dissolved in dry DMSO (40 cm^3) at 90 °C under a dry argon atmosphere. 3-Chloropropan-1-yl methanesulphonate (2.0 g, 11.6 mmol) was added with stirring, and heating was continued at 85–90 °C for 2 h, then the mixture was allowed to cool to room temperature overnight. The mixture was diluted with diethyl ether (100 cm^3) and water was added (50 cm^3). The layers were separated and the aqueous fraction was extracted with further diethyl ether (75 cm^3). The organic extracts were combined, washed with water (4 × 100 cm^3) and brine (2 × 100 cm^3), dried, filtered, and concentrated to give a yellow oil. To remove traces of DMSO and starting material, the oil was dissolved in diethyl ether (20 cm^3) and passed through a short silica column (10 g). The column was eluted with diethyl ether (50 cm^3) and the combined organic solutions were concentrated to give [1-^{14}C]-4-chlorobutanenitrile **16** as a pale yellow oil (725 mg, 58%; 28.3 μCi mmol^{-1}). All physical properties were identical to authentic, unlabelled material,[17] δ_H(90 MHz; $CDCl_3$) 2.15 (2 H, m), 2.55 (2 H, t, *J* 7) and 3.65 (2 H, t, *J* 7).

N-([4-^{14}C]-3-*Cyanopropyl*)*pyrrolidine* **17**.—A solution of the 4-chlorobutanenitrile **16** (688 mg, 6.65 mmol) in dry butanol (3 cm^3) was added to a stirred mixture of pyrrolidine (667 mm^3, 8.0 mmol), anhydrous sodium carbonate (707 mg, 6.65 mmol) and potassium iodide (184 mg, 1.11 mmol), and was stirred for 18 h. The solution was cooled and filtered, and the solid residue was washed well with diethyl ether (50 cm^3). The washings and filtrate were combined and extracted with hydrochloric acid (4 mol dm^{-3}; 3 × 50 cm^3). The aqueous extracts were washed with diethyl ether (2 × 100 cm^3), basified with sodium carbonate and extracted with diethyl ether (3 × 100 cm^3). The ethereal extracts were dried, filtered, and concentrated to give an oil which was distilled (403 mg, 44%; 30.8 μCi mmol^{-1}); b.p. 55 °C at 0.2 mmHg. All physical properties were identical with authentic unlabelled material,[18] ν_{max}($CHCl_3$)/cm^{-1} 2260 (CN); δ_H(90 MHz) ($CDCl_3$) 1.76 (6 H, m) and 2.49 (8 H, m).

N-([4-^{14}C]-4-*Aminobutyl*)*pyrrolidinium Dihydrochloride* **18**.—Adam's catalyst [platinum(IV) oxide] (30 mg, 15% w/w) was added to the ^{14}C-labelled nitrile **17** (200 mg, 1.45 mmol) in glacial acetic acid (5 cm^3), and the mixture was hydrogenated at atmospheric pressure for 18 h. The catalyst was removed by filtering through Celite and concentrated hydrochloric acid (8 cm^3) was added to the filtrate. The solution was concentrated under reduced pressure to give a yellow solid. The dihydrochloride **18** was obtained by slow recrystallization from aqueous ethanol (95%); 280 mg (90%, 29.7 μCi mmol^{-1}); m.p. 256–258 °C; ν_{max}(KBr disc)/cm^{-1} 3000; δ_H(90 MHz) 1.62 (4 H, m, 2′- and 3′-H_2), 1.90 (4 H, m, 3- and 4-H_2), 2.95 (4 H, m, 2- and 5-H_2), 3.05 (2 H, m, 4′-H_2) and 3.55 (2 H, m, 1′-H_2); δ_C 23.6, 23.7, 25.0 (C-3 and -4, -2′ and -3′), 47.9 (C-4′), 55.1 (C-1′) and 55.2 (C-2 and -5). The phenylamino(thiocarbonyl) derivative had m.p. 189 °C; *m*/*z* 277 (M^+, 7%) (Found: M^+, 277.1609. $C_{15}H_{23}N_3S$ requires *M*, 277.1612.

N-([4-^{14}C]-4-*Aminobutyl*)-1,2-*didehydropyrrolidinium Chloride Hydrochloride* **19**.—To mercury(II) acetate (1.18 g, 3.7 mmol) in 5% aqueous acetic acid (6 cm^3) was added the dihydrochloride **18** (200 mg, 0.9 mmol), and the mixture was heated at reflux for 4 h. The solution was cooled, and the mercury(I) acetate was filtered off. The filtrate was saturated with hydrogen sulphide to remove excess of mercury(II) ions as black mercury sulphide. A pale yellow filtrate was obtained which was made alkaline by the addition of 40% aqueous sodium hydroxide (4 cm^3), and then extracted with chloroform (3 × 20 cm^3). The chloroform extracts were dried and filtered. Addition of dry HCl gas gave the immonium salt **19** (102 mg, 51%; 28.8 μCi mmol^{-1}), m.p. 215–216 °C; ν_{max}(Nujol)/cm^{-1} 3000, 2025 and 1688; δ_H(90 MHz) 1.60–2.05 (6 H, m, 4-, 2′- and 3′-H_2), 2.21 (2 H, m, 3-H_2), 2.90–3.20 (4 H, complex, 5- and 4′-H_2), 3.95 (2 H, m, 1′-H_2) and 8.65 (1 H, br s, 2-H); δ_C 20.0, 24.1 and 24.4 (C-4, -2′ and -3′), 39.5 (C-3), 47.5 (C-4′), 53.9 and 59.6 (C-5 and -1′) and 182.2 (C-2) (Found: C, 44.9; H, 8.5; N, 12.95. $C_8H_8N_2Cl_2$ requires C, 45.06; H, 8.51; N, 13.18%).

A sample of the product (24 mg, 0.11 mmol) was dissolved in deuteriomethanol (2 cm^3) and stirred with sodium cyanoborodeuteride (15 mg, 0.22 mmol) for 4 h at room temperature. Excess of cyanoborodeuteride was destroyed by slow addition of 10% aqueous sodium hydroxide (*ca.* 2 cm^3). The resulting solution was extracted with chloroform (4 × 5 cm^3). The chloroform extracts were dried, filtered, and concentrated to give an oil. The oil was taken up in concentrated hydrochloric acid and then evaporated to dryness to give [2-^{2}H]-*N*-(4-aminobutyl)pyrrolidinium dihydrochloride **20** (17 mg, 71%); δ_H 1.65 (4 H, m, 2′- and 3′-H_2), 1.95 (4 H, m, 3- and 4-H_2), 2.90 (3 H, m, 2-H and 5-H_2), 3.15 (2 H, m, 4′-H_2) and 3.50 (2 H, m, 1′-H_2).

Feeding Methods.—*Senecio isatideus* and *S. pleistocephalus* plants were propagated by stem cuttings, whereas *Cynoglossum officinale* (Suttons seeds) and *C. australe* were grown from seed. All plants were grown in a standard compost in a greenhouse. A sample of [2,3-^{3}H]putrescine dihydrochloride was added to the ^{14}C-labelled immonium ion **19** and the initial ^{3}H/^{14}C ratio was measured (Table 1). The precursor mixture was dissolved in sterile water and fed on alternate days for 5 d to the plants. Ten days later the plants were harvested and alkaloids isolated as described.[4]

Retrorsine **6** was recrystallized to constant specific radioactivity from acetone, m.p. 216–217 °C (lit.,[4] 216–217 °C), R_F 0.35.

Rosmarinine **11** was recrystallized to constant specific radioactivity from CH_2Cl_2–acetone, m.p. 201–202 °C (lit.,[19] 202–204 °C), R_F 0.30.

Echinatine **8** was purified by column chromatography on basic alumina eluted with CH_2Cl_2–$CHCl_3$ (3:1) to give the alkaloid as a gum, R_F 0.28.[12]

Cynaustraline **13** (R_F 0.32) and cynaustine **15** (R_F 0.35) were separated by repeated column chromatography on basic alumina by eluting with CH_2Cl_2, then addition of up to 1% MeOH.[6]

Radioscans of TLC plates run after each feeding experiment showed radioactive bands for each alkaloid. Purified alkaloids showed one radioactive band coincident with authentic unlabelled alkaloid.

Hydrolysis of Alkaloids.—This was carried out for all 5 alkaloids with barium hydroxide as described for the hydrolysis of senecionine.[20]

Retronecine **5** was crystallized from acetone–light petroleum (b.p. 60–80 °C), m.p. 118–119 °C (lit.,[4] 118–120 °C).

Rosmarinecine **10** was crystallized from acetone–light petroleum (b.p. 60–80 °C) m.p. 171–172 °C (lit.,[19] 171–172 °C).

(+)-Heliotridine **7** was crystallized from acetone, m.p. 115–117 °C (lit.,[20] 115–116 °C).

(+)-Isoretronecanol **12** and (+)-supinidine **14** were both obtained as gums.[6]

Intermediate Trapping Experiments.—(*a*) A solution of [1,4-^{14}C]putrescine dihydrochloride (25 μCi) was fed to one well-established *S. pleistocephalus* plant by the wick method. After 24 h, the plant was blended in methanol (100 cm^3). Inactive immonium salt **19** (40 mg) and sodium borohydride (71 mg) were added to the methanolic extract (100 cm^3) and the mixture was stirred for 24 h. Isothiocyanatobenzene (1 cm^3) was added to the solution, which was stirred for a further 3 d at room

temperature. Brine (100 cm^3) was added and the resultant solution was extracted with dichloromethane (4 × 100 cm^3). The organic extracts were dried, filtered, and concentrated to give a mixture of derivatized polyamines. A radioscan of a chromatogram of the mixture developed in CH_2Cl_2–MeCN (9:1) indicated two major bands at R_F 0.30 and 0.65. These bands correspond to the phenylamino(thiocarbonyl) derivatives of putrescine[9] and *N*-(4-aminobutyl)pyrrolidine respectively. The band with R_F 0.65 was removed, and the derivative was recrystallized to constant specific radioactivity.

(*b*) The previous experiment was repeated except that unlabelled *N*-(4-aminobutyl)pyrrolidine dihydrochloride (28 mg), sodium [^{3}H]borohydride (200 μCi), and inactive sodium borohydride (70 mg) were added to the methanol extract. The mixture was stirred at room temperature overnight, then the phenylamino(thiocarbonyl) derivatives were prepared as before. The radioactive band corresponding to the phenylamino(thiocarbonyl) derivative of *N*-(4-aminobutyl)pyrrolidine was separated by preparative TLC and the derivative was recrystallized to constant specific radioactivity.

(*c*) *N*-(4-Aminobutyl)pyrrolidinium dihydrochloride (25 mg) and isothiocyanatobenzene (1 cm^3) were added to a third methanolic extract (100 cm^3) produced as in the previous two experiments. This solution was stirred for 3 d at room temperature. The reaction was worked up as described above to yield a crystalline residue. Autoradiography of a chromatogram of this material developed in CH_2Cl_2–MeCN (9:1), displayed one major band, R_F 0.31, corresponding to the phenylamino(thiocarbonyl) derivative of putrescine. The band which corresponded to the *N*-phenylamino(thiocarbonyl) derivative of the saturated dihydrochloride (as in **18**), was extracted and recrystallized until the radioactivity was too low to measure.

Acknowledgements

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