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Evidence for an Immonium Ion Intermediate in Pyrrolizidine Alkaloid Biosynthesis

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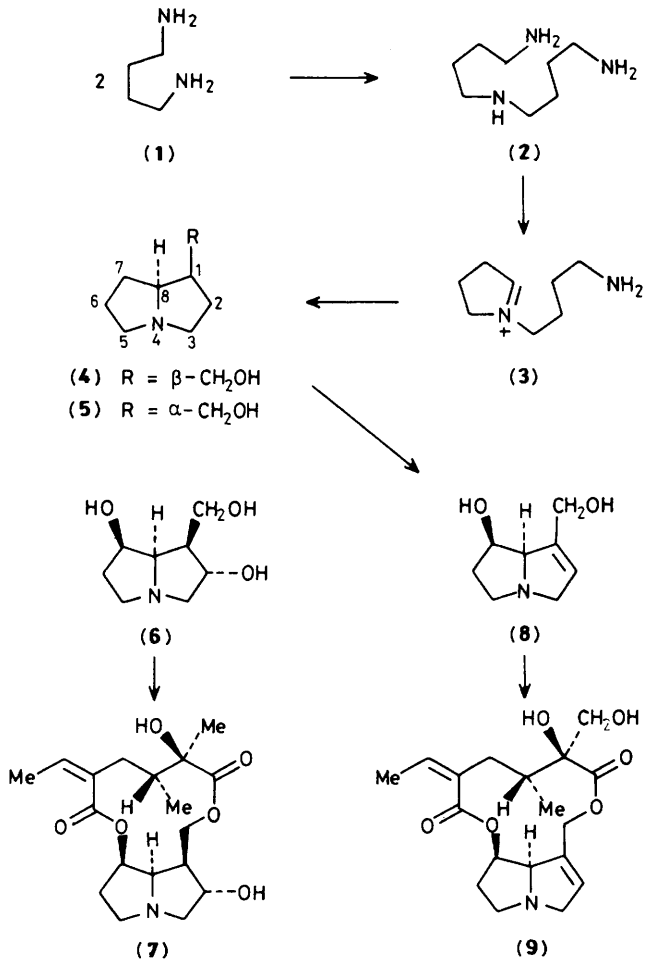
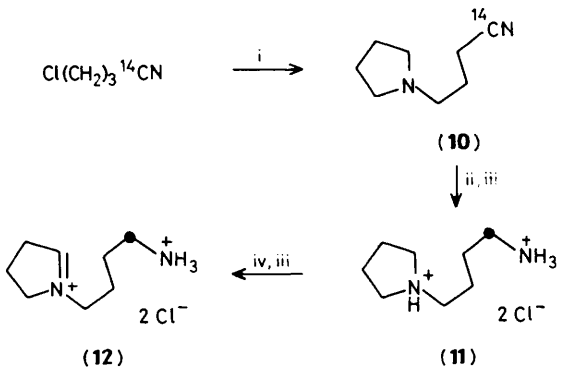
The immonium ion, *N*-(4-aminobutyl)-1,2-didehydropyrrolidinium (**3**), has been shown to be involved in the biosynthetic pathways to the pyrrolizidine bases rosmarinecine (**6**) and retronecine (**8**) by radioisotopic labelling studies and by an intermediate trapping experiment.

Biosynthesis of the pyrrolizidine bases rosmarinecine (**6**)[1] and retronecine (**8**)[2] is known to proceed from two molecules of putrescine (**1**) *via* homospermidine (**2**) (Scheme 1). However, the pathways must diverge later because 1β-hydroxymethyl-8α-pyrrolizidine (**4**) (isoretronecanol) is incorporated efficiently into rosmarinecine (**6**),[3] whereas the 1α-epimer (**5**) (trachelanthamidine) is a much better precursor for retronecine (**8**).[3,4] No intermediates have been identified in the pathways between homospermidine (**2**) and the 1-hydroxymethylpyrrolizidines (**4**) and (**5**), but synthesis of trachelanthamidine (**5**) was achieved from homospermidine using enzymes and physiological conditions.[5] Thus, treatment of homospermidine (**2**) with the diamine oxidase from pea seedlings followed by reduction with a coupled dehydrogenase gave (±)-trachelanthamidine (**5**). From this result, it was suggested that the action of the diamine oxidase on homospermidine yields an aldehyde ($CH_2NH_2 \rightarrow CHO$), which cyclises to the immonium ion (**3**). Further oxidation of the remaining primary amine, completion of a Mannich reaction, and a reduction step would lead to the 1-hydroxymethylpyrrolizidine (**5**). We now show that the immonium ion (**3**) is indeed an intermediate in pyrrolizidine alkaloid biosynthesis, and that oxidation of the primary amino groups in homospermidine therefore takes place in two discrete steps. The synthesis of the ^{14}C-labelled immonium ion (**12**) is shown in Scheme 2. The mesylate of 3-chloropropanol was treated with $Na^{14}CN$ to give [1-^{14}C]-4-chlorobutanenitrile, which underwent nucleophilic displacement with pyrrolidine to yield the pyrrolidinenitrile (**10**). Reduction of the nitrile followed by acidification gave the salt (**11**). Finally, oxidation with mercury(II) acetate afforded the immonium salt (**12**), $\nu_{max.}$ 1688 cm^{-1}. It should be noted that this oxidation process on *N*-alkylpyrrolidines is reported to lead to formation of an endocyclic double bond.[6] Nevertheless it was felt desirable to establish the position of the double bond. A sample of the immonium salt (**12**) was reduced with sodium cyanoborodeuteride to give the monodeuteriated saturated salt. A 200 MHz ^1H n.m.r. spectrum of the unlabelled salt (**11**) taken in D_2O displayed a four-proton multiplet at δ 2.95 ascribed to the four equivalent protons on the pyrrolidine ring on carbons adjacent to nitrogen. The monodeuteriated salt showed only a three-proton multiplet at δ 2.95. This is consistent with the assignment of an endocyclic double bond to the immonium salt (**12**).

Introduction of the ^{14}C-labelled immonium ion (**12**) together with [1,4-^3H]putrescine dihydrochloride (^3H : ^{14}C)



Scheme 1



Scheme 2. *Reagents and conditions*: i, pyrrolidine, Na_2CO_3, KI, 103 °C, 18 h; ii, PtO_2, AcOH, iii, HCl; iv, $Hg(OAc)_2$, 120 °C, 4 h; H_2S.

ratio 5.1) into one well established *Senecio pleistocephalus* plant was carried out as described previously.[1] Rosmarinine (**7**) was extracted and recrystallised to constant specific activity (^{14}C specific incorporation 6.5%, $^{3}H:^{14}C$ ratio 2.9). Alkaline hydrolysis of rosmarinine (**7**) gave rosmarinecine (**6**) with almost the same specific activity and $^{3}H:^{14}C$ ratio (2.8). A similar feeding experiment ($^{3}H:^{14}C$ ratio 12.3) was carried out on one *Senecio isatideus* plant. Retrorsine (**9**) was isolated[2] and recrystallised to constant specific activity (^{14}C specific incorporation 4.5%, $^{3}H:^{14}C$ ratio 9.8). Similar specific radioactivity and $^{3}H:^{14}C$ ratio (9.9) were observed after alkaline hydrolysis of retrorsine (**9**) to retronecine (**8**). These data show that the immonium salt (**12**) is a very efficient precursor for rosmarinecine (**6**) and retronecine (**8**), and that it is incorporated better into these necines than putrescine (**1**).

Moreover, the immonium ion (**3**) was shown to be present in *Senecio pleistocephalus* by an intermediate trapping experiment. A sample of [1,4-^{14}C]putrescine dihydrochloride was fed to one plant, and after one day the plant was macerated in methanol. Inactive immonium salt (**12**) was added to the filtered extract, followed by sodium borohydride. After one day, phenyl isothiocyanate was added to the mixture and the *N*-phenylthiourea derivative of the diamine (**11**) was isolated and purified as described for other polyamines.[7] This derivative, m.p. 189 °C, was recrystallised to constant specific activity, and it contained 0.4% of the original radioactivity fed. A radioscan of the isolated derivative, run on silica gel G and developed with CH_2Cl_2–MeCN (9:1), showed one radioactive band at R_F 0.65 coincident with the authentic unlabelled derivative of the diamine (**11**).

Finally, we needed to ascertain the status of *N*-(4-aminobutyl)pyrrolidine as an intermediate in the biosynthetic pathway to rosmarinecine (**6**). The ^{14}C-labelled material (**11**) together with [1,4-^{3}H]putrescine dihydrochloride ($^{3}H:^{14}C$ ratio 1.5) was fed to *S. pleistocephalus* as before. Rosmarinine (**7**) was isolated and recrystallised to afford material with a ^{14}C specific incorporation of 2.1% ($^{3}H:^{14}C$ ratio 2.5). This result shows that the saturated salt (**11**) is incorporated less efficiently (*ca.* 1/3) into rosmarinecine than the immonium ion (**12**). However, when an intermediate trapping experiment was carried out with inactive *N*-(4-aminobutyl)pyrrolidine after feeding [1,4-^{14}C]putrescine dihydrochloride to *S. pleistocephalus*, the *N*-phenylthiourea derivative of the diamine (**11**) contained <0.017% of the ^{14}C radioactivity fed.

These results are convincing evidence that the immonium ion (**3**) is a normal precursor of rosmarinecine (**6**) and retronecine (**8**). However, they also suggest that in *Senecio* species, the immonium ion (**3**) and the corresponding pyrrolidine [as (**11**)] may be interconverted in an enzymatic process. The pyrrolidine [as (**11**)] cannot therefore be entirely excluded as a precursor that might arise from homospermidine (**2**) by a route not passing through the immonium ion (**3**).

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