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Stereochemistry of enzymic processes in the biosynthesis of pyrrolizidine alkaloids

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Abstract. The harmonization of biosynthetic pathways with organic reaction mechanisms has relied heavily on stereochemical studies. The field of biosynthesis of pyrrolizidine alkaloids exemplifies these connections through a wide range of common organic reactions including oxidation, condensation, and decarboxylation. Further, the applications of biogenetic concepts and enzyme-catalysed reactions to synthesis are illustrated. The results are exciting, not only for their intrinsic scientific interest, but because they point the way to using plant enzymes to recognise structurally modified biosynthetic intermediates and hence open routes to the synthesis of new compounds that would otherwise be difficult to obtain.
Key words. Pyrrolizidine alkaloid; biosynthesis; stereochemistry; retronecine; rosmarinecine; diamine oxidase.

Introduction

Pyrrolizidine alkaloids are important natural products because of their widespread occurrence (e.g. ragworts are *Senecio* spp., family Asteraceae) and because many are hepatotoxic[26, 30]. Many deaths to grazing animals have resulted from ingestion of pyrrolizidine alkaloids. The most toxic pyrrolizidine alkaloids are those that contain a 1,2-unsaturated pyrrolizidine diol (necine) such as retronecine (**1**). This is usually found joined to a dicarboxylic acid (necic acid) to form a macrocyclic dilactone as in retrorsine (**2**), present in *S. isatideus*. The alkaloids exhibit their major toxic effect in the liver, where they are oxidised to the corresponding pyrroles, which are bifunctional alkylating agents[18]. Alkaloids such as rosmarinine (**3**) from *S. pleistocephalus*, which contains the base portion rosmarinecine (**4**), are not hepatotoxic. Pyrrolizidine alkaloids are also implicated in human liver disease when they are eaten by accident when foodstuffs are contaminated, or by design when used as herbal remedies. A

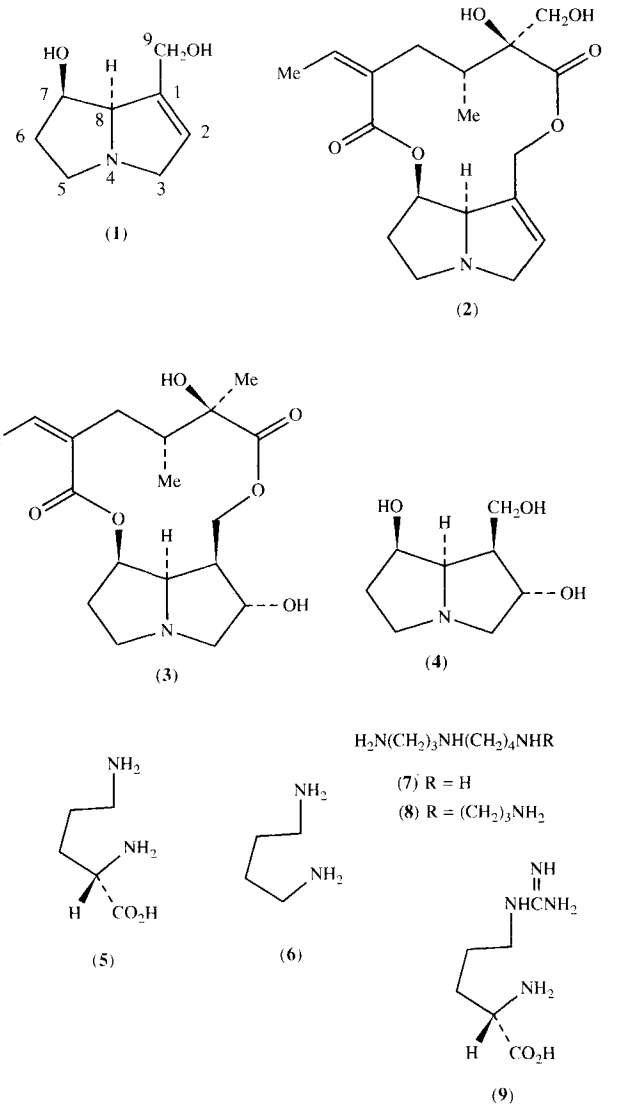
number of plants containing pyrrolizidine alkaloids are consumed by insects, and the alkaloids are stored for defensive purposes. Some butterflies go even further and convert the necine portions of the alkaloids into volatile ketones which act as pheromones[30]. We have been intrigued for many years by the ways in which a diverse range of pyrrolizidine alkaloids with different structures and stereochemistry are produced from simple amino acid precursors.

Determination of intermediates in the biosynthesis of necines

Before the stereochemical subtleties of the biosynthetic pathway can be considered, the intermediates must be identified as closely as possible.

A major research effort has been devoted by us to the elucidation of the biosynthetic pathways to the base portions of pyrrolizidine alkaloids[31]. The necic acids are known to be derived from the common amino acids, valine, leucine, isoleucine, and threonine[31]. At the start of our biosynthetic programme, it was believed that the base portion of most pyrrolizidine alkaloids, (+)-retronecine (**1**), was formed from ornithine (**5**) via 1,4-diaminobutane (**6**) (putrescine). We began our work by using radioactive precursors to confirm that ornithine and putrescine are the main building blocks used in the construction of (+)-retronecine. We also showed that spermidine (**7**) and spermine (**8**) are efficient precursors of retronecine in *Senecio isatideus* plants. These precursors probably function as good sources of putrescine[32]. Retronecine was also shown to be derived from the L-isomers of ornithine and arginine (**9**). This was achieved by making ^{14}C-labelled samples of D-, L-, and DL-ornithine and arginine. These were fed to *S. isatideus* plants together with L-[5-^{3}H]arginine as a reference standard, and the relative retention of ^{3}H and ^{14}C was measured in each experiment. The D-isomers were not incorporated into retronecine[34].

The approach to solving biosynthetic problems using radioactive precursors has limited usefulness, because of the difficulty of establishing the location of all the labelled atoms in the metabolite by degradation. In our work, initially only one carbon atom of retronecine (**1**) (C−9) could be removed by degradation. Subsequently, a three carbon fragment (C−5+6+7) was isolated and identified as β-alanine after chromic acid oxidation of retronecine (**1**)[33]. Since we were the first workers to obtain large total incorporations of radioactive precursors into retronecine by a new feeding technique, we were able to initiate the use of ^{13}C-labelled precursors in pyrrolizidine alkaloid biosynthesis. The first complete labelling pattern in retronecine was established by the powerful technique of ^{13}C NMR spectroscopy after feeding [1-^{13}C]putrescine to *S. isatideus* plants. Four of the eight carbon atoms in retronecine were equally labelled with ^{13}C, and the labelling pattern was consistent with



combination of two putrescine units to form retronecine, probably via a later symmetrical C_4-N-C_4 intermediate[9,11]. A refinement of this use of ^{13}C-labelled precursors which is particularly useful when ^{13}C enrichments are low is to use $^{13}C-{}^{13}C$ doubly labelled precursors. Labelling patterns can then be determined by observation of $^{13}C-{}^{13}C$ doublets in the ^{13}C NMR spectra of the labelled natural products. Accordingly, [2,3-$^{13}C_2$]-putrescine was synthesized and used to obtain a distinctive labelling pattern in retronecine[9,11]. A particularly useful precursor in this regard is [1,2-$^{13}C_2$]putrescine. This was prepared by me from [1,2-$^{13}C_2$]-1,2-dibromoethane in five steps, and used to produce ^{13}C-labelled retronecine. All eight ^{13}C NMR spectroscopic signals for retronecine were flanked by doublets, and four pairs of coupling constants were observed[28].

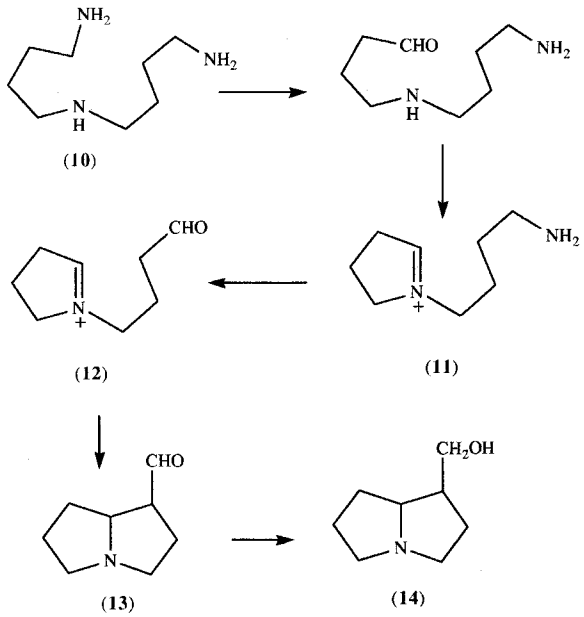
Feeding experiments with ^{13}C-labelled precursors had provided circumstantial evidence for the existence of a later intermediate with C_{2v} symmetry in the biosynthetic pathway to retronecine (**1**). More definitive evidence on

this point was obtained by the use of [$^{13}C-^{15}N$]-doubly labelled putrescine, which was prepared from $K^{13}C^{15}N$ and *N*-protected 1-amino-3-bromopropane. This material was incorporated efficiently into retronecine in *S. isatideus* plants. The ^{13}C NMR spectrum of retronecine showed two pairs of doublets, attributed to the presence of two species labelled with $^{13}C-^{15}N$, arising from a C_4-N-C_4 symmetrical intermediate in the pathway[10,11]. A similar finding was reported simultaneously[3,4].

We proceeded further to identify this later intermediate as *N*-(4-aminobutyl)-1,4-diaminobutane (homospermidine) (**10**). This was first prepared labelled with ^{14}C at the terminal carbons, and the [1,9-^{14}C]-homospermidine was incorporated specifically into retronecine. The limited degradations possible indicated that it was incorporated intact[10]. A complementary labelling pattern was obtained by synthesizing and feeding [4,6-^{14}C]homospermidine, and degrading the labelled retronecine. Homospermidine was also shown to be present in *S. isatideus* plants by an intermediate trapping experiment[12]. Proof that homospermidine is incorporated intact into retronecine was obtained by preparing [1,9-$^{13}C_2$]homospermidine and using it to obtain ^{13}C-labelled retronecine. A geminal coupling constant of 6 Hz was observed between the two ^{13}C-labelled atoms in retronecine[21]. Similar experiments with ^{13}C-labelled putrescines and homospermidine were carried out on another pyrrolizidine alkaloid, rosmarinine (**3**), in *S. pleistocephalus* plants. The biosynthesis of the base portion of rosmarinine, rosmarinecine (**4**), was shown to proceed from putrescine via homospermidine. Of particular note were the extraordinarily high specific incorporations (up to 50%) obtained for the incorporation of [1-^{13}C]-putrescine into rosmarinecine (**4**)[6].

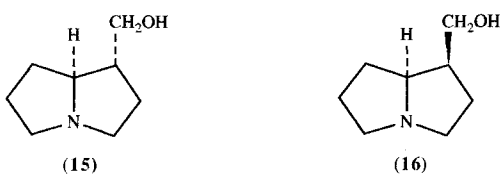
Further support for homospermidine (**10**) as a key intermediate in pyrrolizidine alkaloid biosynthesis was obtained by carrying out a biogenetically patterned synthesis of a simple pyrrolizidine base (**14**) using enzymes and physiological conditions. Homospermidine was incubated with the diamine oxidase from pea seedlings for six days. The triamine was oxidised to a dialdehyde in equilibrium with the iminium ion (**12**) which underwent non-enzymic cyclisation to give 1-formylpyrrolizidine (**13**). Reduction of the aldehyde to the corresponding alcohol (**14**) was achieved with a coupled dehydrogenase system in an overall yield of 22%. The facility of these transformations does suggest that similar reactions are involved in the biosynthesis of pyrrolizidine alkaloids[27]. The development of this exciting result to use readily available, isolated enzymes to carry out further reactions that are difficult to achieve by standard organic chemistry is feasible and is now under way.

The enzymic conversion of homospermidine into the pyrrolizidine alcohol (**14**) did suggest that the iminium ion (**11**) was worthy of consideration as an intermediate in the biosynthetic pathway. Accordingly ^{14}C-labelled



iminium ion (**11**) was prepared and fed to both *S. isatideus* and *S. pleistocephalus* along with ^3H-labelled putrecine as a reference. In alkaloids from both plants high ^{14}C specific incorporations were observed and the $^3H/^{14}C$ ratios decreased, indicating that the iminium ion (**11**) is involved in the biosynthetic pathways to retronecine (**1**) and rosmarinecine (**4**)[8].

The ready formation of 1-hydroxymethylpyrrolizidine (**14**) from homospermidine (**10**) did suggest that it might be an intermediate in the biosynthetic pathway to the more complex pyrrolizidine bases. Accordingly, ^3H-labelled samples of the two diastereoisomeric 1-hydroxymethyl-pyrrolizidines were prepared, and it was shown that the *exo*-alcohol (**15**) is an efficient precursor for retronecine (**1**) in *S. isatideus*[14,16]. A similar result was obtained for the retronecine portion of riddelliine[17,20]. We made the further intriguing discovery that the *endo*-alcohol (**16**) is a much more efficient precursor than the *exo*-alcohol for the base portion, rosmarinecine, of rosmarinine (**3**) in *S. pleistocephalus* plants[14,16].



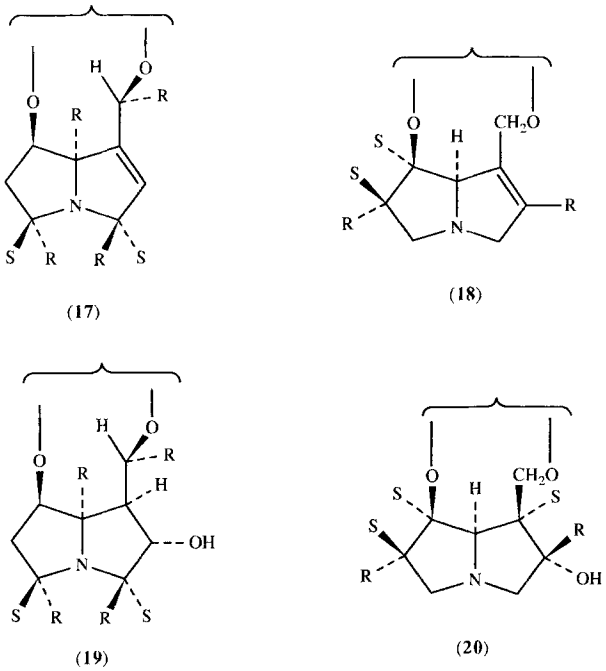
The identification of many of the intermediates in the biosynthetic pathways to retronecine and rosmarinecine and the efficiency of their conversion into these pyrrolizidine bases should permit the preparation of new analogues of pyrrolizidine alkaloids by feeding structurally modified biosynthetic intermediates to plants that produce these alkaloids. The plants should carry out the

sequence of transformations necessary to make these alkaloid analogues. Such analogues would be difficult to synthesize and may have useful biological activity. This application of molecular recognition has significant industrial potential.

Determination of the stereochemistry of the enzymic processes in the biosynthesis of necines

We have also carried out a major programme which seeks to define the stereochemistry of all the enzymic operations involved in the biosynthesis of different types of necines. The first step in the biosynthesis of retronecine (**1**) from L-ornithine (**5**) or L-arginine (**9**) is a decarboxylation process. I was able to demonstrate that the decarboxylation of L-ornithine, catalysed by L-ornithine decarboxylase, and that of L-arginine, catalysed by L-arginine decarboxylase, both take place with retention of configuration. Two stereospecifically ^{3}H-labelled putrescine samples were prepared by the action of the decarboxylase enzymes on L-ornithine and L-arginine (after hydrolysis of the intermediate agmatine). These samples were assayed by incubation with diamine oxidase isolated from pea seedlings, which is known to remove the *pro-S* hydrogen from putrescine. Retention of ^{3}H label was observed in both samples of putrescine, proving that they possessed the *R*-configuration, and that enzymic decarboxylation of both ornithine and arginine proceeds stereospecifically with retention of configuration[29] (see also following paper). Similar results using different methods have been reported by two other groups[19,25].

Further work in this area required the preparation of a number of specifically deuteriated putrescines. The choice of deuterium was made so that labelling patterns in the alkaloids could be established by ^{2}H NMR spectroscopy, and the fate of the hydrogen atoms in the biosynthetic pathway could be monitored. To start this work, [1,4-$^2H_4$]- and [2,3-$^2H_4$]-putrescines were prepared and fed to *S. isatideus* plants to obtain ^{2}H-labelled retrorsine samples. The labelling patterns in the base portion of retrorsine were consistent with reduction of 1-formylpyrrolizidine to the corresponding alcohol (**15**) in the biosynthetic pathway by stereospecific addition of a hydride equivalent to the *re*-face of the carbonyl group[22,24]. This is the usual stereochemistry observed with coupled dehydrogenase enzyme systems[2]. Next, the enantiomeric [1-^{2}H]putrescines were prepared enzymically, making use of the stereospecificity of the decarboxylation process of ornithine decarboxylase on L-ornithine which I[29] and others[19,25] had established earlier. Labelling patterns established in retrorsine (**17**) after feeding (*R*)- and (*S*)-[1-^{2}H]putrescine showed that all the oxidation processes on the primary amino groups of homospermidine (**10**) occur with stereospecific loss of the *pro-S* hydrogens[23,24]. This is consistent with the known stereospecificity of diamine oxidases; the *pro-R* hydrogen is retained in the aldehyde[1]. A similar result was reported independently for the incorporation of the enantiomeric [1-^{2}H]putrescines into a mixture of pyrrolizidine alkaloids[5].



Incorporation of D-labelled putrescines into retrorsine (**2**) and rosmarinine (**3**). R and S denote D present after feeding (**R**)- and (**S**)-D-putrescines in separate experiments. The structures for retrorsine (**17**) and (**18**), and for rosmarinine (**19**) and (**20**), are composite representations of all D-labelled species present.

In order to complete the stereochemical picture, samples of (*R*)- and (*S*)-[2-^{2}H]putrescine were prepared chemically from the enantiomers of aspartic acid by modification of an unpublished route to (*R*)-[2-^{2}H]-succinic acid due to Arigoni. Feeding experiments with these putrescines on *S. isatideus* plants gave labelled retrorsine (**18**) which showed that the hydroxylation process at C-7 in retronecine biosynthesis takes place with retention of configuration, and formation of the 1,2-double bond in retronecine involves removal of the *pro-S* hydrogen and retention of the *pro-R* hydrogen at the carbon of the double bond in retronecine (**1**) that is derived from C-2 of putrescine[13,15]. Similar experiments with the enantiomeric [1-^{2}H]- and [2-^{2}H] putrescines have also been carried out to give labelled samples of rosmarinine (**19**) and (**20**), respectively, in *S. pleistocephalus*. The results show that both hydroxylation processes take place with retention of configuration[7]. The stereochemistry of the formation of the pyrrolizidine ring in rosmarinecine thus involves stereospecific retention of the *pro-S* hydrogen at C-1. The introduction of the 1,2-double bond in retronecine (**1**), which is crucial for the observed hepatotoxicity in the alkaloid, may occur by hydroxylation at C-2α and C-7 of the pyrrolizidine alcohol (**15**) with the usual retention of configuration, followed by *trans*-elimination of the elements of water. It should be noted that

formation of a 1,2-double bond in rosmarinecine (**4**) would require *cis*-elimination of the elements of water [7]. In the biosynthetic pathways to necines so far investigated, there are clearly many enzymic reactions which show expected stereospecificity e.g. decarboxylases, diamine oxidases, and hydroxylases. Families of enzymes, presumably genetically related, show strong correlations in mechanism and stereochemical course. However, superimposed on this background are the individual features of the pathways leading to either hepatotoxic necines such as retronecine (**1**) or the less toxic bases such as rosmarinecine (**4**). Here the unexpected can often be found. The determination of the stereochemistry of the enzymic processes in the pathways to these pyrrolizidine bases has prepared the way for a detailed study of the individual enzymes involved in pyrrolizidine alkaloid biosynthesis.

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