Pyrrolizidine Alkaloids. Stereochemistry of the Enzymic Processes Involved in the Biosynthesis of Rosmarinecine.

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The mode of incorporation of ^{2}H-labelled putrescines into the rosmarinecine (**14**) portion of the pyrrolizidine alkaloid rosmarinine (**15**) in *Senecio pleistocephalus* plants has been established by ^{2}H n.m.r. spectroscopy. The use of [1,1,4,4-$^2H_4$] putrescine (**16**) dihydrochloride produced rosmarinine (**18**) with ^{2}H labels mainly at C-3α, C-3β, and C-9*pro-S*. Rosmarinine (**22**) derived from [1,1-$^2H_2$]putrescine (**19**) dihydrochloride showed the same ^{2}H n.m.r. spectroscopic signals plus others for C-5α, C-5β, and C-8. These labelling patterns are interpreted by the intermediacy of homospermidine (**6**) in the biosynthetic pathway and by the existence of ^{2}H isotope effects. Feeding experiments with (*R*)-[1-^{2}H]-(**23**) and (*S*)-[1-^{2}H]putrescine (**26**) dihydrochloride gave a sample of rosmarinine (**25**) with ^{2}H present at C-3β, C-5α, C-8α, and C-9*pro-S* from the former precursor, and a sample (**28**) with ^{2}H present at C-3α and C-5β from the latter precursor. These patterns are consistent with oxidation of three of the four amino groups involved in the conversion of two molecules of putrescine into 1β-formyl-8α-pyrrolizidine (**12**) with stereospecific loss of the *pro-S* hydrogens. Reduction of the 1-formylpyrrolizidine (**12**) to isoretronecanol (**13**) takes place by attack of a hydride donor on the C-*re* face of the aldehyde group. Use of [2,2,3,3-$^2H_4$]putrescine (**29**) dihydrochloride gave rosmarinine (**30**) labelled with ^{2}H at C-1α, C-2β, C-6α, C-6β, and C-7α. Feeding experiments with (*R*)-[2-^{2}H]- (**31**) and (*S*)-[2-^{2}H]-putrescine (**33**) dihydrochloride gave rosmarinine (**32**) labelled with ^{2}H at C-2β and C-6α from the former, and rosmarinine (**34**) with ^{2}H present at C-1α, C-6β, and C-7α from the latter precursor. Formation of the pyrrolizidine ring involves stereospecific removal of the *pro-R* hydrogen and retention of the *pro-S* hydrogen on the carbon destined to become C-1 of rosmarinecine, and hydroxylation at C-2 and C-7 of isoretronecanol (**13**) therefore proceeds with retention of configuration to form rosmarinecine (**14**).

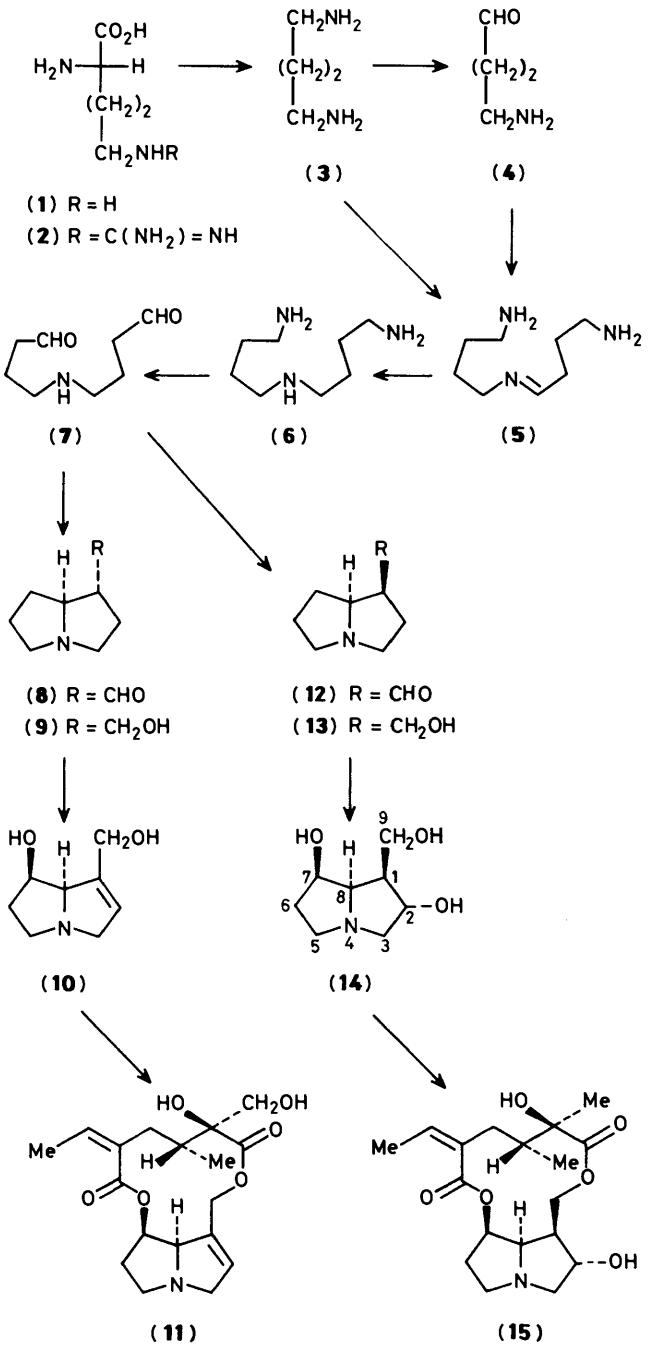
Pyrrolizidine alkaloids have a wide distribution in a number of plant families.[1] Most of the biosynthetic studies on the base portions (necines) of these alkaloids have been carried out on the most common necine, retronecine (**10**). Feeding experiments with radioactive precursors indicated that retronecine is formed from two molecules of L-ornithine (**1**)[2-6] or L-arginine (**2**)[4-6] *via* putrescine (**3**),[3,5] although only partial labelling patterns were obtained by degradation of the radioactive samples.[5,7] This problem was overcome by feeding ^{13}C-labelled putrescines to *Senecio isatideus* plants, and by determining complete labelling patterns in retrorsine (**11**) using ^{13}C n.m.r. spectroscopy.[8] These results confirmed that retronecine (**10**) is formed from two molecules of putrescine (**3**) and suggested that a later intermediate with C_{2v} symmetry, homospermidine (**6**), is involved in the biosynthetic pathway.[7,9] The intermediacy of homospermidine was supported by feeding experiments,[10] and by conversion of homospermidine into (±)-trachelanthamidine (**9**) using diamine oxidase, and dehydrogenase enzymes and physiological conditions.[11] Recent experiments with ^{13}C-labelled precursors have demonstrated that rosmarinecine (**14**), the necine component of rosmarinine (**15**), isolated from *Senecio pleistocephalus* plants, is also biosynthesized from two molecules of putrescine (**3**) *via* homospermidine (**6**).[12] The facile formation of (±)-trachelanthamidine (**9**) from homospermidine using enzymes suggested that 1-hydroxymethylpyrrolizidines might be intermediates in the pathways to the more complex necines. It was found that indeed (±)-trachelanthamidine is an efficient precursor for retronecine (**10**),[13,14] and that the diastereoisomeric (±)-isoretronecanol (**13**) is incorporated efficiently and specifically into rosmarinecine (**14**).[13]

The feasibility of studying the stereochemistry of the enzymic processes in retronecine biosynthesis was demonstrated by carrying out feeding experiments with ^{2}H-labelled putrescines on *S. isatideus*, followed by determination of the labelling patterns in retrorsine by ^{2}H n.m.r. spectroscopy.[15] The key stereochemical deductions made from the labelling patterns after feeding (*R*)-[1-^{2}H]- and (*S*)-[1-^{2}H]-putrescine dihydrochloride are as follows.[16,17] The oxidation of putrescine (**3**) to 4-aminobutanal (**4**) takes place with loss of the *pro-S* hydrogen. After coupling of the aldehyde (**4**) and amine (**3**), reduction of the imine (**5**) occurs by attack of a hydride donor on the C-*si* face of the imine to give homospermidine (**6**). Two further oxidation steps each take place with loss of *pro-S* hydrogens to give the dialdehyde (**7**). Mannich cyclisation leads to the 1-formylpyrrolizidine (**8**), and reduction to trachelanthamidine (**9**) takes place by addition of a hydride equivalent on the C-*re*-face of the carbonyl group. The ^{2}H labelling patterns in retrorsine formed from (*R*)-[2-^{2}H]- and (*S*)-[2-^{2}H]-putrescine dihydrochloride indicated that hydroxylation at C-7 of the necine proceeds with retention of configuration, while formation of the 1,2-double bond on retronecine (**10**) involves removal of the *pro-S* hydrogen and retention of the *pro-R* hydrogen at C-2.[18]

Because of the divergence of the biosynthetic pathways to retronecine (**10**) and rosmarinecine (**14**) at the 1-hydroxymethylpyrrolizidine stage, it was considered necessary to establish the stereochemical details of the pathway to rosmarinecine. An additional incentive was the lack of a 1,2-double bond in rosmarinecine, which would facilitate the study of the stereochemistry of pyrrolizidine ring formation, involving the fate of hydrogens at C-1, and of hydroxylation at C-2 of rosmarinecine (**14**).

Results and Discussion

All of the proton signals in rosmarinine (**15**) were assigned from the 200 MHz ^{1}H n.m.r. spectrum taken in deuteriochloroform



Scheme 1.

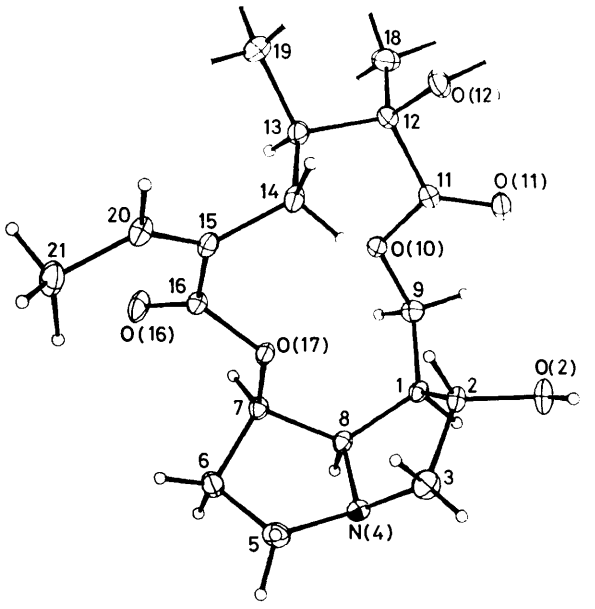


Figure 1. *X*-Ray crystal structure of rosmarinine (**15**)[23]

with the aid of selective ^1H decoupling and nuclear Overhauser enhancement (n.O.e.) experiments using the NEOMULT.AU programme.[19] These assignments were confirmed by homonuclear (^1H) and heteronuclear (^{13}C-^1H) chemical-shift correlation spectroscopy, and corroborated and amplified previous work.[20] It was particularly important to be certain of the assignment for the prochiral hydrogens at C-9 of rosmarinine (**15**). There is a chemical-shift difference of 0.8 p.p.m. between these two diastereotopic protons. From previous ^1H n.m.r. spectroscopic studies[21] and *X*-ray crystallographic data[22] on retrorsine (**11**), it is believed that the conformation of the alkaloid is similar in organic solution and the solid state, and that the 9-H *pro-S* is deshielded as a consequence of being in the plane both of the adjacent carbonyl and double bond. The chemical-shift difference between the protons at C-9 is *ca.* 1.4 p.p.m. *X*-Ray crystallographic data on rosmarinine (**15**) suggests that a similar situation exists (Figure 1).[23] The deshielding effect is less in this case because of the absence of the double bond in rosmarinine, but the 9-H *pro-S* is assigned as the more deshielded of the prochiral hydrogens at δ 4.9. Additional support for this assignment and for the similarity of the conformation of rosmarinine in the solid state and chloroform solution was obtained from ^1H n.m.r. data. Irradiation at δ 2.48 (1-H) produced n.O.e. effects on the signals for H-9 *pro-S* of rosmarinine at δ 4.88 (*ca.* 8%) and for H-9 *pro-R* at 4.09 (*ca.* 5%). The coupling constants $J_{1,9pro\text{-}R}$ = 1.2 Hz and $J_{1,9pro\text{-}S}$ = 5.5 Hz indicate dihedral angles of *ca.* 70° and 40°, respectively, between these prochiral hydrogens and the 1-proton. The assignments of the protons on the necine component of rosmarinine were confirmed by n.O.e. experiments. For example, irradiation at δ 2.90 (3β-H) led to observation of an n.O.e. for 2β-H at δ 4.25 (*ca.* 5%), and for 5β-H at δ 2.59 (*ca.* 6%).

Seven different ^2H-labelled putrescines were prepared for this study of the stereochemistry of the enzymic processes involved in rosmarinine biosynthesis. Samples of [1,1,4,4-$^2H_4$]- (**16**) and [2,2,3,3-$^2H_4$]-putrescine (**29**) dihydrochloride were made from succinonitrile as described.[21] The dihydrochloride of [1,1-$^2H_2$]putrescine (**19**) was prepared by catalytic reduction of 4-phthalimidobutyronitrile under an atmosphere of deuterium gas, followed by acid hydrolysis of the product. Enzymatic decarboxylation of L-ornithine in ^2H_2O and of [2-^2H]-DL-ornithine in H_2O using L-ornithine decarboxylase yielded (*R*)-[1-^2H]-(**23**) and (*S*)-[1-^2H]-putrescine (**26**), respectively.[24] The enantiomeric (*R*)-[2-^2H]- (**31**) and (*S*)-[2-^2H]-putrescine (**33**) dihydrochlorides were prepared from (*S*)- and (*R*)-aspartic acids, respectively.[25] The ^2H content of each precursor was estimated from ^1H and ^2H n.m.r. and mass spectral data.

Each ^2H-labelled precursor was mixed with [1,4-^{14}C]putrescine dihydrochloride (10 μCi) and portions of each precursor were administered to well established *Senecio pleistocephalus* plants (one plant per experiment) by the wick method—one tenth of each sample was added per day for 10 days. Ten days later, the plants were harvested and rosmarinine (**15**) was extracted and recrystallised to constant specific radioactivity.

Table. Incorporation of ^2H-labelled putrescines into rosmarinine in *Senecio pleistocephalus* plants

Expt.	Precursor	Quantity fed (mg)	Amount of rosmarinine (**15**) isolated (mg)	% ^{14}C Specific incorporation in rosmarinine (**15**)[a]	% ^2H Specific incorporation in rosmarinine (**15**) per C_4 unit
1	(**16**)	100	400	5.1	3.6
2	(**19**)	100	238	6.3	5.5
3	(**23**)	100	157	9.2	9.1
4	(**26**)	50	234	3.7	2.5
5	(**29**)	100	194	9.1	7.1
6	(**31**)	100	130	4.9	5.1
7	(**33**)	100	282	6.6	6.5

[a] Specific ^{14}C incorporation per C_4 unit is calculated from [(Molar activity of product $\times 0.5$)/(Molar activity of precursor)] $\times$ 100%.

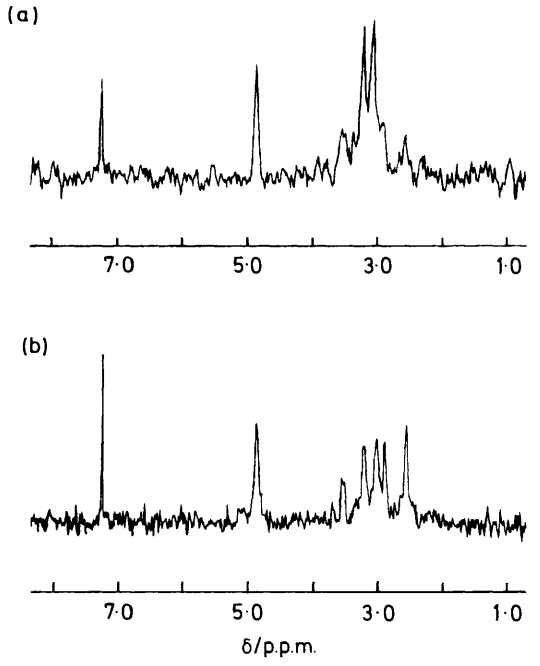
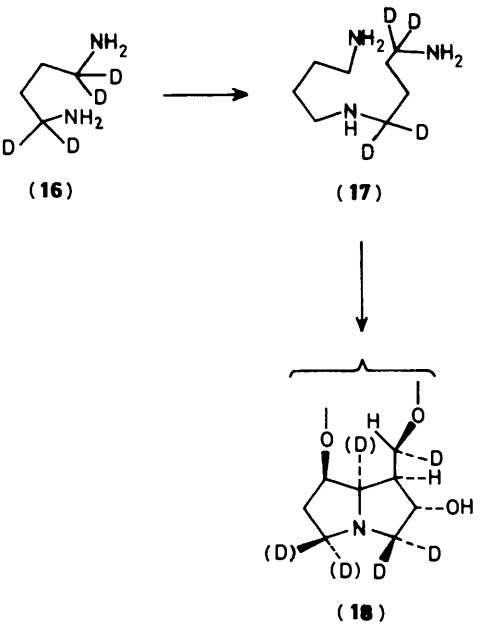


Figure 2. 55.28 MHz ^2H $\{^1H\}$ N.m.r. spectra of rosmarinine in $CHCl_3$ at 60 °C: (a) sample of rosmarinine (**18**) derived from [1,1,4,4-$^2H_4$]-putrescine (**16**); (b) sample of rosmarinine (**22**) derived from [1,1-$^2H_2$]-putrescine (**19**). The signal at δ 7.25 is natural abundance ^2H in $CHCl_3$



Scheme 2.

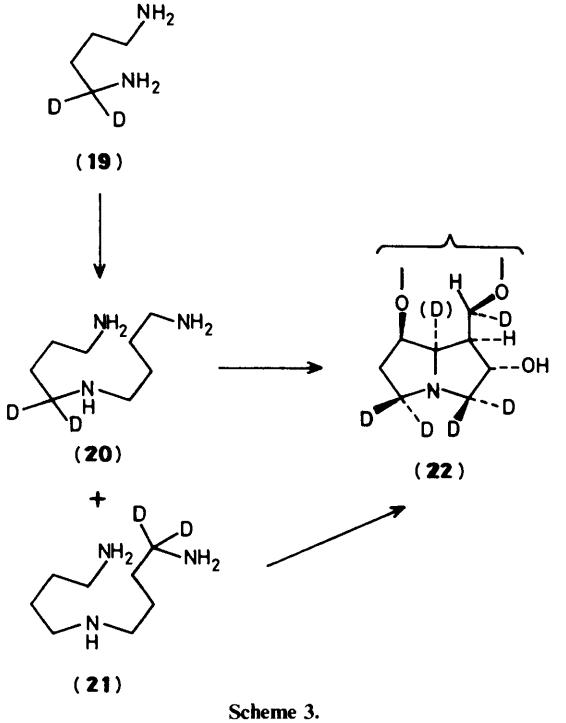
The ^{14}C specific incorporations per C_4 unit for each precursor are listed in the Table.

The ^2H $\{^1H\}$ n.m.r. spectra of the ^2H-labelled samples of rosmarinine were obtained in chloroform, and some narrowing of the signals was observed at higher temperatures.[21] The spectra were therefore routinely taken at 60 °C in chloroform and compared with natural abundance spectra run under the same conditions. The sample of rosmarinine obtained after feeding [1,1,4,4-$^2H_4$]putrescine (**16**) dihydrochloride to *S. pleistocephalus* showed three main signals in the ^2H $\{^1H\}$ n.m.r. spectrum at δ 2.93, 3.08, and 4.88 [Figure 2(a)] corresponding to rosmarinine (**18**) labelled with ^2H at C-3β, C-3α, and C-9*pro-S*, respectively (Scheme 2). The three sites are labelled to about the same extent with enrichment factors * of 3.5 ± 0.3% ^2H. Signals due to ^2H at C-5β, C-5α, and C-8 may be present at δ 2.62, 3.28, and 3.58, respectively, with much lower enrichment factors. The specific incorporation of ^2H for the C_4 unit in which most of the ^2H appears (C-1, -2, -3, and -9 of rosmarinine) is 3.5 × 100/96 = 3.6% [where 96 atom % ^2H is the average content of $^2H_4$ species in the sample of the precursor (**16**)]. An analogous labelling pattern was observed for retrorsine (**11**) after feeding the $^2H_4$-labelled putrescine (**16**) to *S. isatideus* plants.[21] This was explained by the involvement of ^2H isotope effects during the biosynthetic pathway to retrorsine. The initial oxidation of [1,1,4,4-$^2H_4$]putrescine (**16**) to [1,4,4-$^2H_3$]-4-aminobutanal is likely to be subject to an intermolecular ^2H isotope effect [this effect on precursor (**16**) is known to be 1.26 when the oxidation is catalysed by hog kidney diamine oxidase[26]]. This will probably result in the formation of [1,1,4,4-$^2H_4$]homospermidine (**17**) as the major ^2H-labelled intermediate, by combination of [1,1,4,4-$^2H_4$]putrescine (**16**) with unlabelled 4-aminobutanal (**4**) already present in the plant. Since hog kidney diamine oxidase is known to have a four times greater preference for oxidation of the unlabelled primary amino group of [1,1-$^2H_2$]putrescine (**19**),[26] a considerable intramolecular ^2H isotope effect is expected for the next oxidation step. It is likely that the non-deuteriated end of homospermidine (**17**) will be preferentially converted into an aldehyde, leading to ros-

* The enrichment factor for a labelled site in rosmarinine (**15**) is calculated from (integral of unlabelled site in rosmarinine/concentration of rosmarinine)/(natural abundance integral of ^2H in $CHCl_3$ at δ 7.25/concentration of $CHCl_3$) × 0.0156%.

marinine (**18**) containing most of the ^2H at C-3 and C-9. (It should be noted that involvement of diamine oxidases in the biosynthetic pathways from putrescine to the necines has not yet been demonstrated. Nevertheless, the existence of ^2H isotope effects in these pathways is necessary to explain the observed labelling patterns).

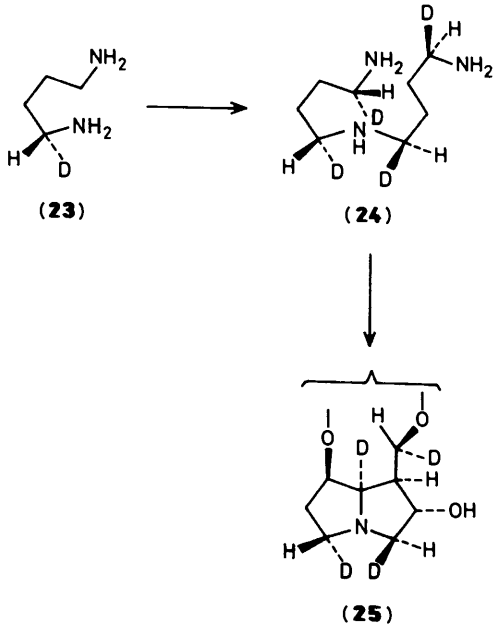
In order to produce more information about the presence of ^2H isotope effects in the biosynthetic pathway to rosmarinecine (**14**), [1,1-$^2H_2$]putrescine (**19**) dihydrochloride was fed to *S. pleistocephalus*. The ^2H {^1H} n.m.r. spectrum of rosmarinine [Figure 2(b)] showed five signals for ^2H with approximately equal enrichment factors of 2.6 ± 0.3% ^2H at δ 2.55 (5β-H), 2.90 (3β-H), 3.02 (3α-H), 3.23 (5α-H), and 4.88 (9-H*pro-S*). A smaller signal was observed at δ 3.56 for the 8-H (Scheme 3). The ^2H



Scheme 3.

specific incorporation per C_4 unit is therefore 2.6 × 2/95 = 5.5% [where 95/2 atom % ^2H is the average enrichment of $^2H_2$ species at the 1- and 4-positions of the putrescine (**19**)]. A possible explanation of the observed labelling patterns in rosmarinine (**22**) is provided by consideration of the two possible $^2H_2$-homospermidine species (**20**) and (**21**), which can be formed in the biosynthetic pathway. Utilisation of homospermidine (**20**) in the pathway will afford rosmarinine with equal ^2H labelling at C-3 and C-5, while homospermidine (**21**) is likely to be preferentially oxidised at the non-deuteriated primary amino group, leading to rosmarinine with more ^2H at C-9 than at C-8. The two most likely $^2H_4$-homospermidines which might also be formed in the biosynthetic pathway are [1,1,6,6-$^2H_4$]-and [1,1,9,9-$^2H_4$]-homospermidine. The participation of [1,1,6,6-$^2H_4$]homospermidine should lead to rosmarinine with most of the ^2H at C-5; while [1,1,9,9-$^2H_4$]homospermidine would be expected to be a considerably poorer substrate for the enzyme system. The labelling patterns in rosmarinine samples (**18**) and (**22**) can be explained by the existence of intermolecular and intramolecular ^2H isotope effects in the biosynthetic pathway. These labelling patterns are consistent with the sequential oxidation of the amino groups in homospermidine (**6**). Oxidation of the first primary amino group leads to an iminium ion which constitutes the left hand ring of the necine.

Attention was next directed towards the stereochemistry of the enzymic processes involving hydrogen atoms initially present at the 1- and 4-positions of putrescine. Accordingly, (*R*)-[1-^2H]- (**23**) and (*S*)-[1-^2H]-putrescine (**26**) dihydrochloride were prepared [24] and fed to *S. pleistocephalus* plants. Four main signals were visible in the ^2H {^1H} n.m.r. spectrum of rosmarinine obtained after feeding the (*R*)-isomer (**23**) [Figure 3(a)]. These signals occurred at δ 2.90, 3.24, 3.55, and 4.87, corresponding to rosmarinine (**25**) labelled with ^2H at C-3β, C-5α, C-8α, and C-9*pro-S*, respectively (Scheme 4)*. From the



Scheme 4.

nearly equal enrichment factors of 4.4 ± 0.4% ^2H for each labelled site, the specific incorporation of ^2H per C_4 unit is estimated to be 4.4 × 2/97 × 100 = 9.1% [where 97/2 atom % is the average content of $^2H_1$ species at the terminal carbons of the putrescine precursor (**23**)]. When (*S*)-[1-^2H]putrescine (**26**) was fed to *S. pleistocephalus*, only two signals were observed in the ^2H {^1H} n.m.r. spectrum [Figure 3(b)]. The signals at δ 2.58 and 3.05 correspond to rosmarinine (**28**) labelled at C-5β and C-3α, respectively, with enrichment factors of 1.2 and 1.0 ± 0.1% ^2H (Scheme 5). The specific incorporation of ^2H per C_4 unit is thus 1/2(1.2 + 1.0) × 2/90 × 100 = 2.4% [where 90/2 atom % is the average enrichment of ^2H at the terminal carbons of putrescine (**26**)]. The labelling patterns for rosmarinine samples (**25**) and (**28**) are analogous to those observed for retrorsine after feeding the same precursors, and can be explained in the same way. Oxidation of putrescine (**3**) to 4-aminobutanal (**4**) takes place stereospecifically with retention of the *pro-R* and loss of the *pro-S* hydrogens. (Diamine oxidases are known to remove the *pro-S* hydrogen from the methylene group adjacent to the amino group of other primary amines.[27]) Coupling of 4-aminobutanal (**4**) with another molecule of putrescine leads to an imine (**5**) which forms homospermidine labelled as shown [(**24**) and (**27**)], if attack of the hydride donor takes place on the C-*si* face of the imine. These composite labelling patterns [(**24**)

* No molecules of putrescine precursors (**23**), (**26**), (**31**), and (**33**) can contain more than one ^2H atom. The labelling patterns for homospermidine and rosmarinine depicted in Schemes 4, 5, 7 and 8 are therefore composite representations of all the ^2H-labelled species that are present.

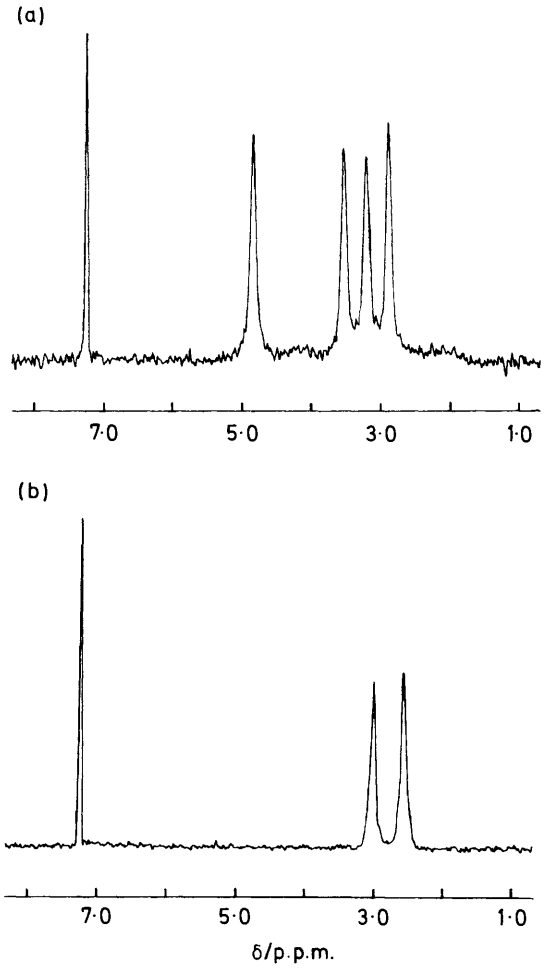
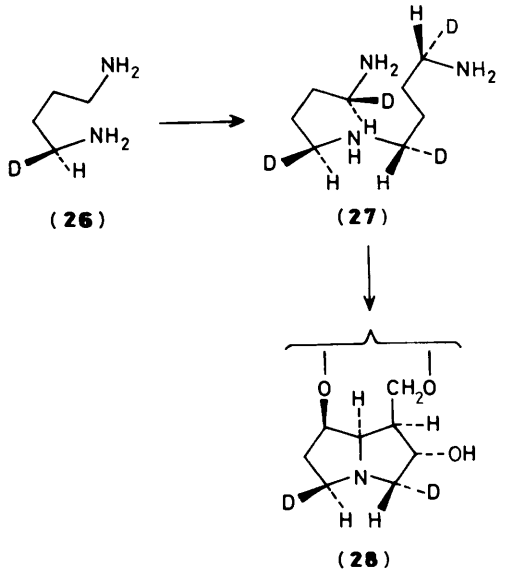


Figure 3. 55.28 MHz ^2H $\{^1H\}$ N.m.r. spectra of rosmarinine in $CHCl_3$ at 60 °C: (a) sample of rosmarinine (**25**) derived from (*R*)-[1-^2H]-putrescine (**23**); (b) sample of rosmarinine (**28**) derived from (*S*)-[1-^2H]-putrescine (**26**). The signal at δ 7.25 is natural abundance ^2H in $CHCl_3$



Scheme 5.

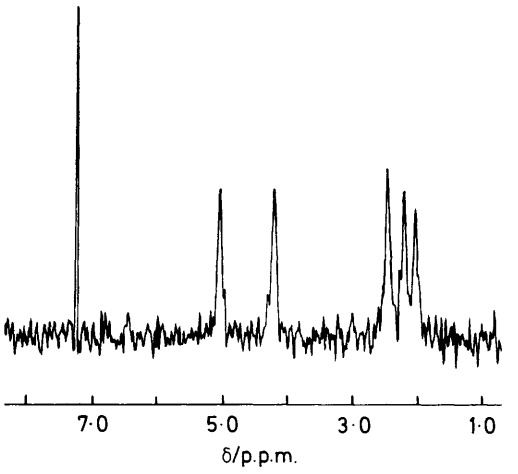
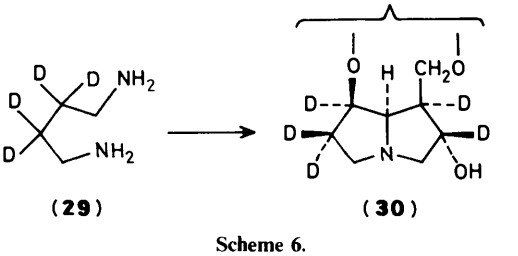


Figure 4. 55.28 MHz ^2H $\{^1H\}$ N.m.r. spectrum of rosmarinine (**30**) in $CHCl_3$ at 60 °C derived from [2,2,3,3-$^2H_4$]putrescine (**29**). The signal at δ 7.25 is natural abundance ^2H in $CHCl_3$

and (**27**)] are a consequence of the C_{2v} symmetry of homospermidine. Two further oxidations on the terminal carbons of homospermidine both take place with stereospecific retention of the *pro-R* and loss of the *pro-S* hydrogens. The 8α-pyrrolizidine aldehyde (**12**) is then formed by attack on the C-*re* face of the presumed iminium ion intermediate. To complete the biosynthesis of isoretronecanol (**13**), reduction of the aldehyde (**12**) takes place by attack of the hydride equivalent on the C-*re* face of the carbonyl group. These processes will lead to rosmarinine (**25**) and (**28**) labelled as shown in Schemes 4 and 5, after feeding the (*R*)-[1-^2H]-and (*S*)-[1-^2H]-putrescines, respectively.

The operation of an intramolecular ^2H isotope effect in the initial enzymic oxidation of the (*S*)-[1-^2H]putrescine (**26**) favours the formation of (*S*)-[4-^2H]-4-aminobutanal over unlabelled material produced by the removal of ^2H at the other end of another molecule of the precursor (**26**). This leads to a sample of rosmarinine (**28**) with retention of ^2H to ^{14}C of $2.4/3.7 \times 100 = 65 \pm 5\%$. This is in good agreement with figures of 68 and 69%, respectively, obtained for incorporation of (*S*)-[1-^2H]putrescine (**26**) into retrorsine[21] and a mixture of pyrrolizidine alkaloids.[17]

In order to investigate the fate of hydrogen atoms on the two central carbon atoms of putrescine, [2,2,3,3-$^2H_4$]putrescine (**29**) dihydrochloride was synthesized and fed to *S. pleistocephalus*. The ^2H $\{^1H\}$ n.m.r. spectrum of the rosmarinine produced showed five signals present at δ 2.02 (6α-H), 2.20 (6β-H), 2.46 (1α-H), 4.20 (2β-H), and 5.00 (7α-H) (Figure 4). The enrichment factors estimated for rosmarinine (**30**) are approximately equal at 7.0 ± 0.5% ^2H, leading to a specific incorporation of ^2H per C_4 unit of $7.0 \times 1/99 \times 100 = 7.1\%$ [because the putrescine (**29**) sample contained 99% $^2H_4$ species]. From consideration of the ^2H labelling pattern for rosmarinine (**30**), it is clear that three ^2H atoms are removed from two molecules of putrescine (**29**) as they are converted into rosmarinine (**30**). These removals



Scheme 6.

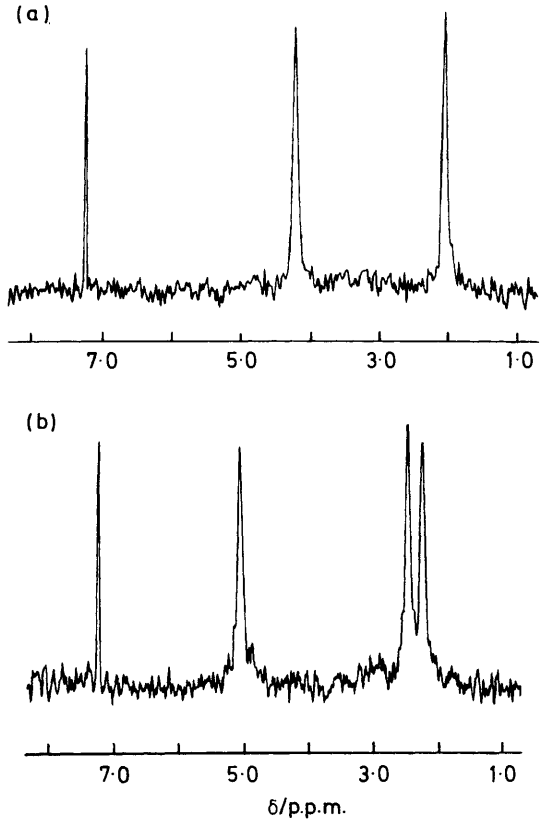
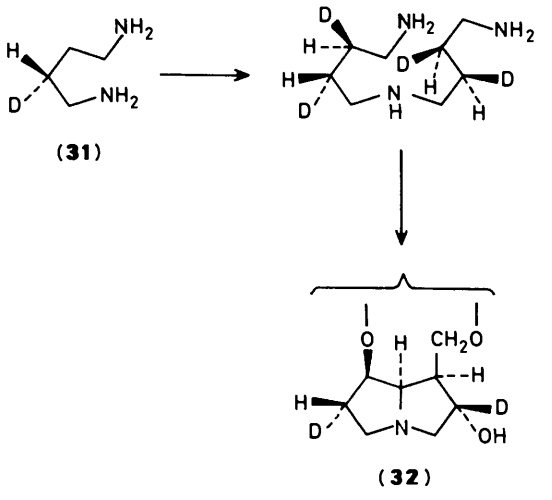
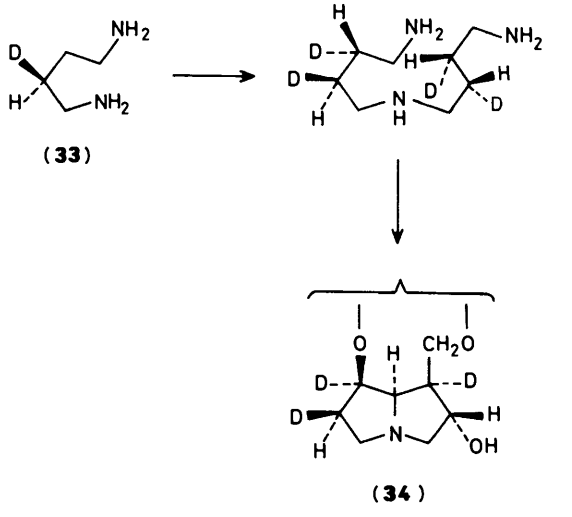


Figure 5. 55.28 MHz ^2H $\{^1H\}$ N.m.r. spectra of rosmarinine in $CHCl_3$ at 60 °C: (a) sample of rosmarinine (**32**) derived from (*R*)-[2-^{2}H]-putrescine (**31**); (b) sample of rosmarinine (**34**) derived from (*S*)-[2-^{2}H]putrescine (**33**). The signal at δ 7.25 is natural abundance ^2H in $CHCl_3$



Scheme 7.



Scheme 8.

of ^2H take place at the carbon atoms destined to become C-1, C-2, and C-7 of rosmarinine, but the observed retention of ^2H at C-2 and C-7 leads to the conclusion that the introduction of hydroxy groups at C-2 and C-7 of rosmarinine (**14**) does not involve keto or enol intermediates.

In order to establish the stereochemistry of the remaining enzymic processes involved in rosmarinecine biosynthesis, it was necessary to carry out feeding experiments on *S. pleistocephalus* with the enantiomeric (*R*)-[2-^{2}H]-(**31**) and (*S*)-[2-^{2}H]-putrescine (**33**). The ^2H $\{^1H\}$ n.m.r. spectrum of rosmarinine derived from the (*R*)-presursor (**31**) showed two major peaks at δ 2.05 and 4.20 ([Figure 5(a)], corresponding to rosmarinine (**32**) labelled with ^2H at C-6α and C-2β, respectively (Scheme 7). The enrichment factors are nearly equal at 2.5 ± 0.2% ^2H, resulting in a ^2H specific incorporation per C_4 unit of 2.5 × 2/98 × 100 = 5.1% [where 98/2 atom % is the average enrichment of ^2H at the two central carbons of putrescine (**31**)]. Thus, ^2H is stereospecifically removed from the carbon atoms which become C-1 and C-7 of rosmarinine.

Three main peaks were evident in the ^2H $\{^1H\}$ n.m.r. spectrum of rosmarinine derived from the (*S*)-isomer (**33**) [Figure 5(b)]. The signals at δ 2.23, 2.45, and 5.06 are assigned to rosmarinine (**34**) labelled with ^2H at C-6β, C-1α, and C-7α (Scheme 8). Enrichment factors of *ca.* 2.9 ± 0.3% ^2H were estimated for these three enriched sites, leading to a specific ^2H incorporation per C_4 unit of 2.7 × 2/83 × 100 = 6.5% [where 83/2 atom % ^2H is the average content of $^2H_1$ species at the central carbon atoms of putrescine (**33**)]. One ^2H atom is lost from the carbon atom destined to become C-2 of rosmarinine during the biosynthesis from two molecules of putrescine (**33**). {It should be noted that the sample of putrescine (**33**) dihydrochloride contained *ca.* 6% of an impurity presumed to be [1,1-$^2H_2$]putrescine (**19**) dihydrochloride.[25] It was therefore expected that small signals for C-3α and β, C-5α and β, C-8, and C-9*pro-S* should be discernible in the ^2H $\{^1H\}$ n.m.r. spectrum of rosmarinine [Figure 5(b)]}. Examination of the labelling patterns established for rosmarinine [(**32**) and (**34**)] after feeding the (*R*)- (**31**) and (*S*)-precursors (**33**) allows certain stereochemical details to be deduced. Formation of the pyrrolizidine aldehyde (**12**) involves stereospecific removal of the *pro-R* hydrogen and retention of the *pro-S* hydrogen at the carbon which becomes C-1 of the aldehyde (**12**). Loss of this hydrogen from the iminium ion derived from the dialdehyde (**7**) may generate an enolate anion (or equivalent species) which then attacks the C-*re* face of the iminium ion to give the 8α-pyrrolizidine aldehyde (**12**). Furthermore, both hydroxylation processes at C-2 and C-7 of isoretronecanol (**13**) occur with complete retention of configuration. This is the usual stereospecificity observed for direct hydroxylation at sp^3 carbon atoms.[28]

A common structural feature in pyrrolizidine alkaloids, and one which is important for the hepatotoxic action, is the 1,2-unsaturation as in retrorsine (**11**).[1] The introduction of the 1,2-double bond in retronecine (**10**) may occur by hydroxylation at

C-2 followed by dehydration. (A number of necines occur with hydroxy groups at C-2, whereas none are known with hydroxy groups at C-1 of the necine.) It may be significant that formation of the 1,2-double bond from rosmarinecine (**14**) would require *cis*-elimination of the elements of water. (Formation of rosmarinecine by dehydrogenation, followed by hydration of the double bond is precluded because of the ^2H labelling patterns observed.) By contrast, formation of the unsaturation in retronecine (**10**) may take place by hydroxylation at C-2α of trachelanthamidine (**9**) with normal retention of configuration, followed by *trans*-elimination of the elements of water.

The stereochemical courses of all the enzymic processes involved in the biosynthesis of rosmarinecine from two molecules of putrescine have been determined after carrying out feeding experiments on *S. pleistocephalus* with samples of the enantiomeric [1-^{2}H]- and [2-^{2}H]-putrescines. Further information about the stereochemical details in pyrrolizidine alkaloid biosynthesis will be obtained using these precursors to establish labelling patterns in other pyrrolizidine alkaloids.

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. N.m.r. spectra were obtained on a Bruker WP200-SY spectrometer operating at 200 MHz for ^1H and 30.72 MHz for ^2H. ^2H N.m.r. spectra were also obtained at 55.28 MHz on a Bruker WH-360 spectrometer. Mass spectra were obtained with A.E.I. MS 12 or 902 spectrometers. 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 were recrystallised to constant specific radioactivity and they were counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for the radioscanning of t.l.c. plates.

[1,1-$^2H_2$]*Butane*-1,4-*diamine* (**19**) (*Putrescine*) *Dihydrochloride.*—4-Phthalimidobutanenitrile (2.36 g, 11 mmol) was added to a suspension of platinum(IV) oxide (0.32 g) in monodeuterioacetic acid. The mixture was stirred under a deuterium atmosphere at atmospheric pressure for 60 h. The mixture was filtered through Celite, and the filtrate was concentrated to give crude 4-phthalimido-[1-$^2H_2$]-1-aminobutane. The crude product was heated at reflux in 4M hydrochloric acid (70 ml) for 6 h. The reaction mixture was cooled to 0 °C, and the precipitated phthalic acid was filtered off. The filtrate was evaporated to dryness, and the residue was recrystallised from aqueous ethanol to yield [1,1-$^2H_2$]butane-1,4-diamine (**19**) dihydrochloride (1.51 g, 84.2%); δ_H (D_2O) 1.77 (4 H, br s) and 3.06 (2 H, br s); δ_D (H_2O) 3.05 (s). The bis(phenylaminothiocarbonyl) derivative had m.p. 177—178 °C (lit.,[29] for unlabelled material, 177—179 °C); δ_H [$(CD_3)_2SO$; 80 °C] 1.58 (s, 4 H), 3.48 (s, 2 H), 7.25 (m, 10 H), 7.75 (s, 2 × NH), and 9.35 (s, 2 × NH); *m/z* 360 (unlabelled material *m/z* 358).

Feeding Methods.— *Senecio pleistocephalus* plants were propagated from stem cuttings and grown in a standard compost in a greenhouse. One plant was used for each experiment. An aliquot of [1,4-^{14}C]putrescine (10 μCi) was added to each ^2H precursor. Each precursor was divided into 10 equal portions, which were dissolved in sterile water and fed by the wick method on each day for a 10-day period to *S. pleistocephalus*. Ten days after administration of each precursor was complete, the plant was harvested and rosmarinine (**15**) was isolated and recrystallised to constant specific radioactivity from dichloromethane–acetone, m.p. 204 °C (decomp.) (lit.,[20] 202—204 °C). The results obtained from feeding the ^2H-labelled precursors are summarised in the Table. Radioscans of silica gel G t.l.c. plates of 0.25 mm thickness developed with chloroform–methanol–conc. ammonia (85:14:1) showed one radioactive band for each labelled sample of rosmarinine, coincident with authentic unlabelled rosmarinine at R_F 0.30. Rosmarinine was visualised by the modified Dragendorff reagent.[30] Rosmarinine: δ_H ($CDCl_3$) 0.94 (3 H, d, *J* 6.5 Hz, 19-H), 1.32 (3 H, s, 18-H), 1.75 (1 H, m, 13-H), 1.82 (3 H, dd, *J* 7.2 and 1.5 Hz, 21-H_3), 1.98 (1 H, m, 6α-H), 2.04 (1 H, m, 6β-H), 2.21 (2 H, m, 14-H_2), 2.48 (1 H, m, 1-H), 2.59 (1 H, m, 5β-H), 2.90 (1 H, dd, *J* 11.1 and 8.0 Hz, 3β-H), 3.03 (1 H, dd, *J* 11.1 and 7.5 Hz, 3α-H), 3.24 (1 H, ddd, *J* 9, 7 and 2 Hz, 5α-H), 3.54 (1 H, dd, *J* 7.7 and 3.2 Hz, 8-H), 4.08 (1 H, dd, *J* 12.5 and 1.0 Hz, 9-H*pro-R*), 4.20 (1 H, ddd, *J* 9.5, 8.0, and 7.3 Hz, 2-H), 4.87 (1 H, dd, *J* 12.6 and 5.2 Hz, 9-H*pro-S*), 5.00 (1 H, ddd, *J* 3.5, 3.3, and 1.2 Hz, 7-H), and 5.76 (1 H, dq, *J* 7.2 and 1 Hz, 20-H).

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References

1 D. J. Robins, *Fortschr. Chem. Org. Naturst.*, 1982, **41**, 115; *Nat. Prod. Rep.*, 1984, **1**, 235: 1985, **2**, 213; 1986, **3**, 297.
2 E. Nowacki and R. U. Byerrum, *Life Sci.*, 1962, **1**, 157: C. A. Hughes, R. Letcher, and F. L. Warren, *J. Chem. Soc.*, 1964, 4974.
3 W. Bottomley and T. A. Geissman, *Phytochemistry*, 1964, **3**, 357.
4 N. M. Bale and D. H. G. Crout, *Phytochemistry*, 1975, **14**, 2617.
5 D. J. Robins and J. R. Sweeney, *J. Chem. Soc., Perkin Trans. 1*, 1981, 3083.
6 D. J. Robins and J. R. Sweeney, *Phytochemistry*, 1983, **22**, 457.
7 G. Grue-Sorensen and I. D. Spenser, *Can. J. Chem.*, 1982, **60**, 643.
8 H. A. Khan and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 146.
9 H. A. Khan and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1981, 554; *J. Chem. Soc., Perkin Trans. 1*, 1985, 101.
10 H. A. Khan and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1985, 619: J. Rana and D. J. Robins, *J. Chem. Res.*, 1983, (*S*), 146.
11 D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1982, 1289.
12 H. A. Kelly and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1987, 177.
13 E. K. Kunec and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1986, 250.
14 J. Rana and E. Leete, *J. Chem. Soc., Chem. Commun.*, 1985, 1742.
15 J. Rana and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1983, 1222.
16 J. Rana and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1984, 517.
17 G. Grue-Sorensen and I. D. Spenser, *J. Am. Chem. Soc.*, 1983, **105**, 7401.
18 E. K. Kunec and D. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1985, 1450.
19 M. Kinns and J. K. M. Sanders, *J. Magn. Res.*, 1984, **56**, 518.
20 J. N. Roitman, *Aust. J. Chem.*, 1983, **36**, 1203.
21 J. Rana and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1986, 983.
22 H. Stoeckli-Evans, *Acta Crystallogr., Sect. B*, 1979, **35**, 2798.
23 A. A. Freer, H. A. Kelly, and D. J. Robins, *Acta Crystallogr., Sect. C*, 1986, **42**, 1348.
24 D. J. Robins, *Phytochemistry*, 1983, **22**, 1133; J. C. Richards and I. D. Spenser, *Can. J. Chem.*, 1982, **60**, 2810.
25 E. K. Kunec and D. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1089.

26 P. S. Callery, M. S. B. Nayer, E. M. Jakubowski, and M. Stogniew, *Experientia*, 1982, **38**, 431.
27 A. R. Battersby, J. Staunton, and M. C. Summers, *J. Chem. Soc., Perkin Trans. 1*, 1976, 1052.
28 R. Bentley, 'Molecular Asymmetry in Biology,' Academic Press, New York, 1970, Vol. 2, p. 18.
29 T. Tarantelli, *J. Chem. Soc., Dalton Trans. 1*, 1974, 837.
30 R. Munier and M. Macheboef, *Bull. Soc. Chim. Biol.*, 1951, **33**, 846.

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