Pyrrolizidine alkaloid biosynthesis. Incorporation of 2-aminobutanoic acid labelled with ^{13}C or ^{2}H into the senecic acid portion of rosmarinine and senecionine

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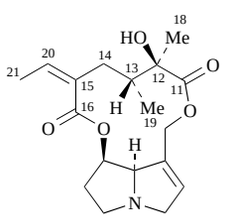
(±)-[3,4-$^{13}C_2$]-2-Aminobutanoic acid 10 and (±)-[3,4-$^{2}H_5$]-2-aminobutanoic acid 11 are synthesized by alkylating diethyl acetamidomalonate with labelled ethyl iodide followed by acid hydrolysis. These compounds are used to obtain complete labelling patterns for the first time in a necic acid by studying the pyrrolizidine alkaloids rosmarinine 3 and senecionine 1 using NMR spectroscopy. The senecic acid portion 12 of both alkaloids shows equal incorporation of [3,4-$^{13}C_2$]-2-aminobutanoic acid 10 into the two C_5 halves of the C_{10} acid consistent with formation of senecic acid *via* two molecules of isoleucine. After feeding [3,4-$^{2}H_5$]-2-aminobutanoic acid 11, retention of ^{2}H at C-13 and C-20 of both alkaloids 13 confirms that the biosynthesis does not involve keto intermediates at these carbon atoms.

Introduction

Pyrrolizidine alkaloids have a wide distribution in a number of unrelated plant families.[1] Many of these alkaloids such as senecionine **1** contain retronecine **2** as the base portion of a dilactone and they are hepatotoxic.[2] Alkaloids such as rosmarinine **3**



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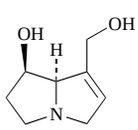

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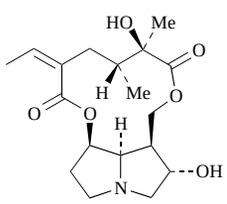

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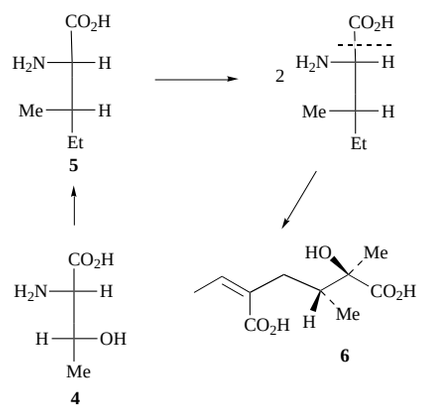
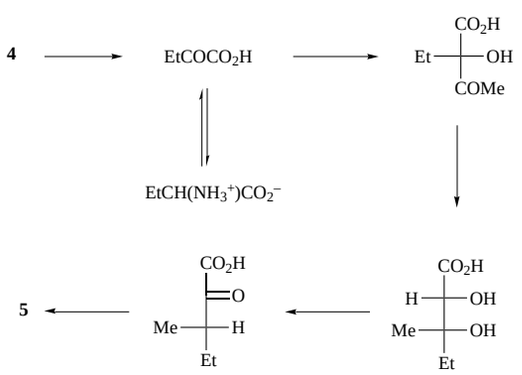
without the double bond in the base portion are not hepatotoxic. Rosmarinine has been isolated from *Senecio pleistocephalus* plants,[3] whereas senecionine is conveniently obtained from transformed root cultures of *S. vulgaris*.[4] Biosynthetic studies[5] have shown that the base portion retronecine **2** is formed from two molecules of 1,4-diaminobutane (putrescine) *via* homospermidine. The biosynthesis of the diacid portions of pyrrolizidine alkaloids has been less intensively studied but experiments with precursors labelled with radioisotopes (^{14}C, ^{3}H) have indicated that these necic acids are derived from several of the common α-amino acids such as L-threonine **4**, L-isoleucine **5**, L-valine and L-leucine, although labelling patterns have been far from complete.[5] The most extensively studied necic acid is senecic acid **6**. Crout *et al*. fed ^{14}C-labelled precursors to *Senecio magnificus* and obtained partial labelling patterns for senecic acid by degradation. These results were consistent with formation of senecic acid from two molecules of isoleucine with loss of both carboxy carbons (Scheme 1).[6]



Scheme 1 Proposed biosynthesis of senecic acid **6**

The biosynthesis of L-isoleucine **5** from L-threonine **4** is shown in Scheme 2. Formation of 2-oxobutanoic acid from



Scheme 2 Biosynthesis of isoleucine **5**

L-threonine is catalysed by a dehydratase. Introduction of pyruvate catalysed by acetolactate synthase generates 2-aceto-2-hydroxybutanoic acid. Then ethyl group migration takes place to yield 2,3-dihydroxy-3-methylvaleric acid. The action of a dehydratase and a transaminase complete the formation of L-isoleucine. Experiments with stereospecifically tritiated isoleucine showed that the C-4 *pro-S* hydrogen is lost and the C-4 *pro-R* hydrogen is retained on conversion into senecic

acid.[7] This result helped to demonstrate that the ethyl group migration step in isoleucine biosynthesis (Scheme 2) takes place with retention of configuration.[8]

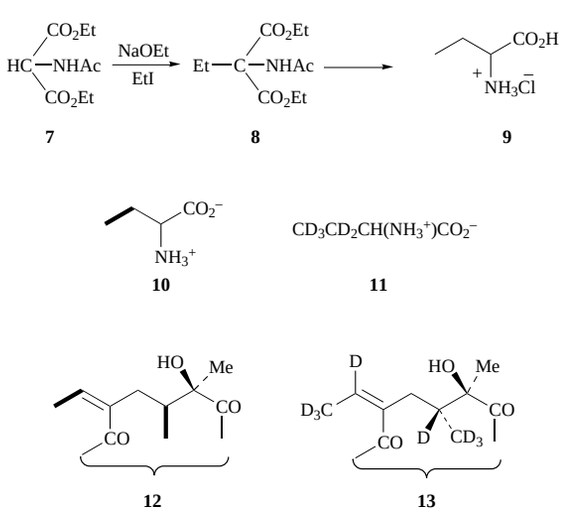
Another known biosynthetic precursor of L-isoleucine is 2-aminobutanoic acid (Scheme 2). Crout *et al.*[7] fed radioactively labelled forms of 2-aminobutanoic acid to *Senecio isatideus* and found that the incorporations were high (up to 3.6%), and that the label was located exclusively in the acid portion of senecionine. It was also observed that both (*R*)- and (*S*)-2-aminobutanoic acid were incorporated into the diacid portion. Only partial labelling patterns could be established. About one half of the ^{14}C radioactivity from (2*R*)- and (2*S*)-[3-^{14}C]-2-aminobutanoic acid was located in the C-20 and C-21 portion of senecionine (numbering as in **1**) obtained by ozonolysis of the acid portion. It was proposed that L-isoleucine was formed from 2-aminobutanoic acid after conversion by the appropriate enzyme (amino acid aminotransferases or oxidases) into 2-oxobutanoic acid (Scheme 2).

Crout *et al.* also showed that in the generation of both of the five carbon units of the C_{10} necic acid the C-3 *pro-S* hydrogen of the 2-aminobutanoic acid is lost and the C-3 *pro-R* hydrogen is retained.[7] There have also been reports of the occurrence of 2-aminobutanoic acid in a number of higher plants.[9] Furthermore, the reported finding of the *R*-isomer in legume seeds[10] and of a 2-oxobutanoate aminotransferase in pea seedlings[11] seems to indicate that the conversion of 2-aminobutanoic acid into 2-oxobutanoic acid may be a normal metabolic process.

The good incorporations obtained for 2-aminobutanoic acid suggested that it would be a simple and useful probe for investigations of the necic acid biosynthetic pathway using precursors labelled with stable isotopes. We report the first studies showing the incorporation of a precursor labelled with different stable isotopes into senecic acid **6** in senecionine **1** and rosmarinine **3**. We anticipated that whole plants of *Senecio pleistocephalus* would give higher total incorporations of deuteriated precursors into rosmarinine, whereas transformed root cultures of *S. isatideus* might lead to higher enrichment of ^{13}C-labelled precursors because of less dilution with endogenous unlabelled material.

Results and discussion

A convenient synthesis of (±)-2-aminobutanoic acid was adapted for our purposes.[12] Alkylation of diethyl acetamidomalonate **7** with ethyl iodide using sodium ethoxide as base gave diethyl (±)-2-ethyl-2-acetamidomalonate **8** in 75% yield (Scheme 3). Hydrolysis of **8** in concentrated hydrochloric acid



Scheme 3

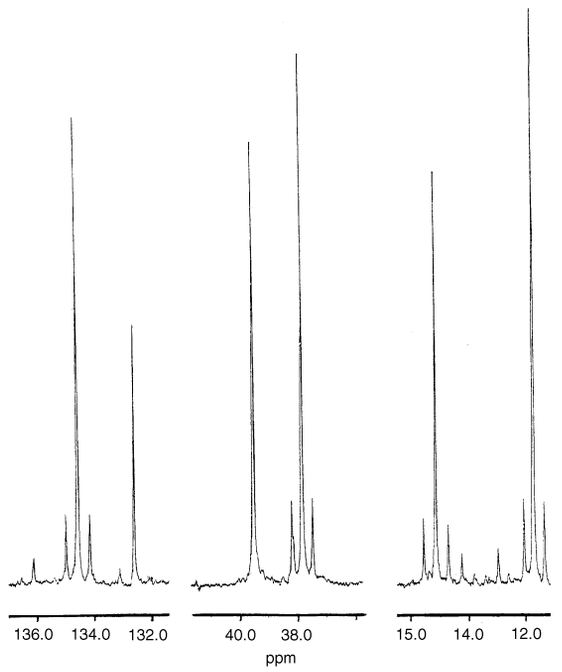


Fig. 1 50 MHz ^{13}C NMR spectrum of rosmarinine **12** in $CDCl_3$ with Me_4Si as reference derived from [3,4-$^{13}C_2$]-2-aminobutanoic acid **10**

gave (±)-2-aminobutanoic acid isolated as the hydrochloride salt **9**.

The ^{13}C and ^{2}H labels were introduced using either [$^{13}C_2$]-iodoethane (98 atom%) to give (±)-[3,4-$^{13}C_2$]-2-aminobutanoic acid **10,** or [$^{2}H_5$]iodoethane (99 atom%) to afford (±)-[3,4-$^{2}H_5$]-2-aminobutanoic acid **11**. The ^{13}C NMR spectrum of **10** contained two large doublets at δ 9.3 and 24.2, with a coupling constant of 34 Hz, along with very small natural abundance signals and an estimated $^{13}C_2$ content of 96%. The ^{2}H NMR spectrum of **11** showed two broad singlets at δ 1.20 and 2.16 of relative intensities 3:2. The $^{2}H_5$ content was greater than 95% estimated from the ^{1}H NMR spectrum.

The ^{13}C-doubly labelled and ^{2}H-labelled 2-aminobutanoic acids **10** and **11** were fed by the wick method to *Senecio pleistocephalus* plants. The $^{13}C_2$-labelled material **10** was fed to young plants to limit the amount of endogenous unlabelled material. As the ^{2}H signals should be easier to observe over natural abundance by ^{2}H NMR spectroscopy, the deuterium-labelled 2-aminobutanoic acid **11** was fed to well established plants.

After feeding [3,4-$^{13}C_2$]-2-aminobutanoic acid **10** rosmarinine was extracted and examined by ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of the labelled rosmarinine showed four doublets of approximately equal intensity at δ 11.7, 15.1, 37.8 and 134.6 (Fig. 1) with coupling constants of 36.4, 42.0, 36.3 and 42.0 Hz, respectively. The labelling pattern **12** corresponds to intact incorporation of the double label into the C-13 and C-19 positions, plus the C-20 and C-21 positions of rosmarinine. There was equal labelling in both halves of the necic acid. The estimated ^{13}C incorporation of the 2-aminobutanoic acid into rosmarinine was 0.43%.

The ^{2}H NMR spectrum of the rosmarinine sample obtained after feeding [3,4-$^{2}H_5$]-2-aminobutanoic acid to *S. pleistocephalus* showed three signals at δ 0.88, 1.75 and 5.79 with relative intensities of approximately 3:4:0.7, respectively (Fig. 2). The signals correspond to rosmarinine **13** labelled on the methyl group at C-19, the C-21 methyl group superimposed on the C-13 deuterium, and the C-20 vinylic deuterium, respectively. The incorporation of the amino acid, calculated from integration of the ^{2}H signals and compared to the natural abundance deuterium in chloroform was 0.7%. The relative integrals of the signals are consistent with equal incorporation of ^{2}H into both methyl groups and incorporation of *ca.* 1 and

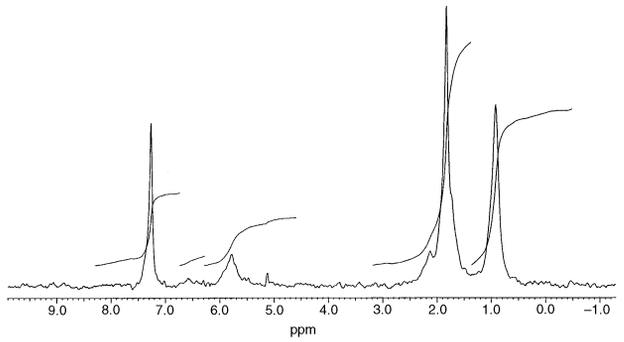


Fig. 2 30.72 MHz $^2H\{[^1H]\}$ NMR spectrum of rosmarinine **13** in $CHCl_3$ derived from [3,4-$^2H_5$]-2-aminobutanoic acid **11.** The signal at δ 7.25 is natural abundance ^2H in $CHCl_3$.

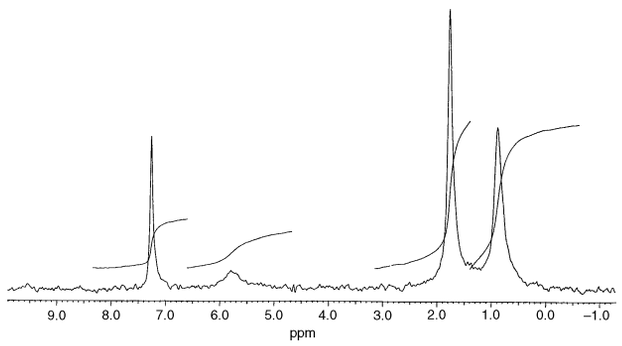


Fig. 3 30.72 MHz $^2H\{[^1H]\}$ NMR spectrum of senecionine **13** in $CHCl_3$ derived from [3,4-$^2H_5$]-2-aminobutanoic acid **11.** The signal at δ 7.25 is natural abundance ^2H in $CHCl_3$.

0.7 deuterium into C-13 and C-20, respectively. This rules out carbonyl groups at C-13 and C-20 as intermediates in the biosynthetic pathway. Loss of 0.5 of the deuterium at C-13 and C-20 would be consistent with previous work using tritium-labelled compounds[7] but ^2H isotope effects and lack of precision of the ^2H integrals could account for this discrepancy.

It is clear from these results for rosmarinine **3** produced by *S. pleistocephalus*, that the C-3 and C-4 positions of 2-aminobutanoic acid are exclusively and equally incorporated intact into the two halves of the senecic acid **6**, labelling solely the C-13 and C-19 alkaloid positions of the right hand portion and the C-20 and C-21 positions of the left hand C_5 unit.

The two precursors were also fed to *Senecio vulgaris* root cultures and senecionine was extracted and purified by preparative TLC. Although the incorporation of the precursor **10** into senecionine was comparable to that obtained for rosmarinine **3**, less alkaloid was obtained and signal to noise ratios in the ^{13}C NMR spectrum were poorer. However, in the ^{13}C NMR spectrum of senecionine, doublets could be observed around the signals at δ 11.1, 15.1, 38.4 and 134.3 with coupling constants of 36, 42, 36 and 42 Hz, respectively. Again, the amounts of label in each half of senecic acid were approximately equal.

After feeding [3,4-$^2H_5$]-2-aminobutanoic acid **11** to *S. vulgaris* the ^2H NMR spectrum showed three peaks at δ 0.91, 1.81 and 5.78 corresponding to labels at H-19, H-13 plus H-21 and H-20, respectively (Fig. 3). The ratios of the relative intensities (approximately 3:3.5:0.5) indicate that there is equal incorporation into the two methyl groups and *ca.* 0.5 deuterium is lost from C-13 and C-20. The specific incoporation of ^2H was 0.5%. These experiments confirmed our findings using rosmarinine.

Stable isotopes have been incorporated into senecic acid in *S. pleistocephalus* and *S. vulgaris* and the first complete labelling patterns for necic acids have been obtained. These labelling patterns show conclusively that in the two *Senecio* species senecic acid **6** is formed from two units of 2-aminobutanoic acid. The C-3 and C-9 positions of senecic acid are generated from the C-3 of 2-aminobutanoic acid, and the C-8 and C-10 diacid positions were originally the C-4 of the amino acid. It has also been shown that there is equal incorporation into both halves of the necic acid. More information on the biosynthesis of necic acids may now be produced by use of other precursors labelled with stable isotopes.

Experimental

General details

Mps were measured on a Kofler hot-stage apparatus and are uncorrected. EI Mass spectra were obtained with AEI MS 12 or 902 spectrometers. NMR Spectra were recorded with a Bruker WP200-SY spectrometer operating at 200 MHz (δ_H), 50.3 MHz (δ_C) or 30.72 MHz (δ_D). *J* Values are given in Hz. The multiplicities of the ^{13}C NMR resonances were determined using DEPT spectra with pulse angles of $\theta = 90°$ and $\theta = 135°$. IR Spectra were obtained on either a Perkin-Elmer 983 spectrophotometer or a Philips PU 9800 FTIR spectrophotometer. Elemental analyses were performed using a Carlo-Erba 1106 elemental analyser. TLC was carried out on Merck Kieselgel G plates of 0.25 mm thickness, eluting with $CHCl_3$–MeOH–conc. NH_3 (85:14:1). Alkaloids were visualised with Dragendorff's reagent.[13]

Diethyl 2-ethyl-2-acetamidomalonate 8

Ethyl iodide (0.38 cm^3, 0.756 g, 4.85 mmol) was added dropwise to a solution of sodium (0.110 g, 4.78 mmol) and diethyl acetamidomalonate (0.944 g, 4.35 mmol) in absolute ethanol (20 cm^3). After addition was complete the solution was heated at reflux for 1 h. The mixture was then cooled to room temperature and the solution was decanted from the sediment. The ethanol was removed *in vacuo*. Recrystallisation of the crude product gave **8** as white crystals (0.878 g, 75%); R_f 0.53 (EtOAc); mp 82 °C (from 95% aqueous acetone) (lit.,[12] 83 °C) (Found: M^+, 245.1267; C, 53.4; H, 7.8; N, 5.8. $C_{11}H_{19}NO_5$ requires *M*, 245.1263; C, 53.88; H, 7.76; N, 5.71).

(±)-2-Aminobutanoic acid hydrochloride 9

Diethyl 2-ethyl-2-acetamidomalonate (0.904 g, 3.69 mmol) and concentrated hydrochloric acid (35 cm^3, 37%) were heated at reflux for 4 h. The reaction mixture was then cooled and concentrated *in vacuo*. The residue was washed with acetone, filtered and the filtrate was evaporated to dryness. Recrystallisation gave **9** as white crystals (0.407 g, 79%); mp >230 °C (from 95% ethanol) (Found: C, 34.25; H, 7.0; N, 10.1. $C_4H_{10}NO_2Cl$ requires: C, 34.41; H, 7.17; N, 10.04%).

(±)-[3,4-$^{13}C_2$]-2-Aminobutanoic acid 10

The procedure was repeated as described above but with [$^{13}C_2$]-iodoethane: $\delta_H(D_2O)$ 0.81 (3 H, dm, *J* 130, $^{13}CH_3$), 1.76 (2 H, dm, *J* 130, $^{13}CH_2$), 3.76 (1 H, m, CH); δ_C 9.3 (d, *J* 34, $^{13}CH_3$–$^{13}CH_2$), 24.2 (d, *J* 34, $^{13}CH_2$–$^{13}CH_3$).

(±)-[3,4-$^2H_5$]-2-Aminobutanoic acid 11

The procedure was carried out as previously described but using [$^2H_5$]iodoethane: $\delta_D(H_2O)$ 1.20 (3 D, br s, CD_3), 2.16 (2 D, br s, CD_2).

Feeding experiments with labelled (±)-2-aminobutanoic acid hydrochloride

Senecio pleistocephalus S. Moore plants were obtained from the Royal Botanic Garden, Edinburgh, and were propagated by stem cuttings and grown in five inch pots in a standard compost in a greenhouse. The labelled amino acids were dissolved in distilled water and fed by the wick method.

(a) (±)-[3,4-$^{13}C_2$]-2-Aminobutanoic acid **10** (11 mg) was fed to three six-month-old plants over four days. After a further ten

days rosmarinine was extracted and recrystallised (dichloromethane–acetone; 1:1).[3]

(b) (±)-[3,4-$^{2}H_5$]-2-Aminobutanoic acid **11** (150 mg) was fed to two well established plants over ten days. After a further five days the plants were harvested and rosmarinine was isolated and recrystallised; mp 205–207 °C (lit.,[3] 204 °C); $\delta_H(CDCl_3)$;[14] $\delta_C(CDCl_3)$ 11.6 (C-19), 15.1 (C-21), 25.6 (C-18), 34.4 (C-6), 37.8 (C-13), 39.5 (C-14), 49.1 (C-1), 53.5 (C-5), 61.3 (C-3), 62.2 (C-9), 69.1 (C-2), 69.3 (C-8), 75.3 (C-7), 77.5 (C-12), 132.7 (C-15), 134.4 (C-20), 167.5 (C-16) and 180.6 (C-11).

Establishment and propagation of root cultures

Hairy root cultures of *S. vulgaris* transformed with *Agrobacterium rhizogenes* were established as described for *Nicotiana* sp.[15] These cultures were grown on Gamborg's B5 basal medium with 80 mM of sucrose added, with a 90 rpm shake rate. Subcultures were prepared every three weeks, *ca*. 0.1 g roots were transferred into 50 cm^3 medium in each 250 cm^3 conical flask.

(a) (±)-[3,4-$^{13}C_2$]-2-Aminobutanoic acid **10** (15 mg) was dissolved in sterile water and divided among 10 flasks containing six day old roots. After a further 14 days the cultures were drained and the roots blended in methanol.

(b) (±)-[3,4-$^{2}H_5$]-2-Aminobutanoic acid **11** (150 mg) was dissolved in sterile water (1 cm^3) and divided among 10 flasks each containing seven day old roots. The flasks were shaken at 90 rpm at 25 °C in normal laboratory light for a further 14 days before the cultures were drained and the roots were blended in methanol.

The alkaloids were extracted and separated by preparative TLC. Crystallisation afforded senecionine **1**, mp and mixed mp with authentic sample, 245 °C; $\delta_H(CDCl_3)$.[16]

Acknowledgements

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