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# BIOSYNTHESIS OF AZETIDINE-2-CARBOXYLIC ACID IN *CONVALLARIA MAJALIS*: STUDIES WITH N-15 LABELLED PRECURSORS

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**Key Word Index**—*Convallaria majalis*; Liliaceae; lily-of-the-valley; biosynthesis; azetidine-2-carboxylic acid; [1-$^{14}$C, $^{15}$N]methionine; [1-$^{14}$C, 2- and 4-$^{15}$N]-2,4-diaminobutanoic acid; [1-$^{13}$C, *carboxyl*-$^{14}$C, $^{15}$N]-1-aminocyclopropane-1-carboxylic acid.

**Abstract**—(*R,S*)-[1-$^{14}$C, $^{15}$N]Methionine, (*R,S*)-[1-$^{14}$C, 2-$^{15}$N]- and (*R,S*)-[1-$^{14}$C, 4-$^{15}$N]-2, 4-diaminobutanoic acid, and [1-$^{13}$C, *carboxyl*-$^{14}$C, $^{15}$N]-1-aminocyclopropane-1-carboxylic acid were synthesized and fed to *Convallaria majalis* plants. The labelled methionine was the most efficient precursor of azetidine-2-carboxylic acid (A-2-C), the specific incorporation of the $^{15}$N (0.47%) being essentially the same as that of the $^{14}$C (0.45%). The A-2-C derived from the labelled 2,4-diaminobutanoic acid was radioactive (specific incorporation of $^{14}$C: 0.05, 0.06%); however, a much higher level of activity was found in aspartic and glutamic acid, and 2,4-diaminobutanoic acid is not considered to be a direct precursor of A-2-C in this species. Negligible $^{14}$C activity was found in A-2-C after feeding the labelled 1-aminocyclopropane 1-carboxylic acid.

## INTRODUCTION

We have previously established that L-azetidine-2-carboxylic acid (A-2-C, **6**) is formed in the plant *Convallaria majalis* L. (lily-of-the-valley) from methionine (**1**) and several closely related compounds [1]. Methionine labelled with $^{14}$C at C-1 and with tritium at C-4 is incorporated into A-2-C with complete retention of the tritium relative to $^{14}$C. This result eliminates aspartic acid and aspartic $\beta$-semialdehyde (**4**) as intermediates between methionine and A-2-C. We thus considered that A-2-C is formed via *S*-adenosyl-L-methionine (**3**). An intramolecular $S_N2$ reaction would yield A-2-C with complete retention of the hydrogens at C-4. The adenosyl derivative **3** was indeed a precursor of A-2-C, although the level of incorporation was not significantly higher than that of methionine. One piece of evidence which is apparently inconsistent with the direct incorporation of methionine into A-2-C is the almost complete loss (95%) of tritium from the C-2 position when [1-$^{14}$C, 2-$^{3}$H]methionine serves as a precursor of A-2-C [1]. Sung and Fowden [2] reported that 2,4-diaminobutanoic acid (**7**) is a good precursor of A-2-C in the legume *Delonix regia*. If one accepts that 2,4-diaminobutanoic acid is an intermediate between methionine and A-2-C, a possible mechanism for the loss of tritium from C-2 of methionine is that illustrated in Fig. 1. Reaction of *S*-adenosyl-L-methionine with ammonia, or its biological equivalent, could yield 2,4-diaminobutanoic acid by displacement of the methylthioadenosine group. Transamination at C-2 would afford 4-amino-2-oxobutanoic acid (**11**), in which the tritium originally at C-2 in methionine would be lost. Cyclization of **11** might then yield 1-azetine-2-carboxylic acic (**10**), A-2-C then being formed on reduction. However, chemical and spectroscopic studies on **11** indicate that it shows no tendency to cyclize to the azetine **10** [3]. Another hypothetical route to A-2-C which would result in the loss of tritium from C-2 of labelled methionine is via 1-aminocyclopropane-1-carboxylic acid (**5**). This amino acid is formed from **3** [4] and is an intermediate in the formation of the plant hormone ethylene from methionine [5–7]. Ring enlargement of **5** is proposed via a derivative such as **8**, where X is a leaving group such as $-OPO_3H$. The resultant azetidinium ion **9** could be reduced directly to A-2-C with a hydride anion (from NADH) or could eliminate a proton to yield the azetine **10**, and then to A-2-C on reduction. Analogous ring enlargements of cyclopropylamines have been observed in chemical oxidations [8].

These hypotheses for the loss of tritium from C-2 of labelled methionine have now been investigated by feeding experiments with [$^{15}$N]methionine, and 2,4-diaminobutanoic acid and 1-aminocyclopropane-1-carboxylic acid labelled with $^{13}$C, $^{14}$C and $^{15}$N.

## RESULTS AND DISCUSSION

The syntheses of the labelled putative precursors of A-2-C are illustrated in Fig. 2. (*R,S*)-[$^{15}$N]Methionine was made by the Strecker reaction on 3-methylthiopropanal (**12**) using [$^{15}$N]ammonium chloride and sodium cyanide [9]. The intermediate aminonitrile (**13**) was hydrolysed with hydrochloric acid and the resultant methionine mixed with commercially available [1-$^{14}$C]methionine prior to feeding. The labelled 2,4-diaminobutanoic acids (**7**) were made by modifications of a previously described synthesis of this amino acid [10]. Reaction of phthalimide with acrolein in the presence of Triton B yielded 3-phthalimidopropanal (**14**). A Strecker reaction on this aldehyde afforded the aminonitrile **15**, which yielded **7** on hydrolysis. By the use of [$^{15}$N]phthalimide,

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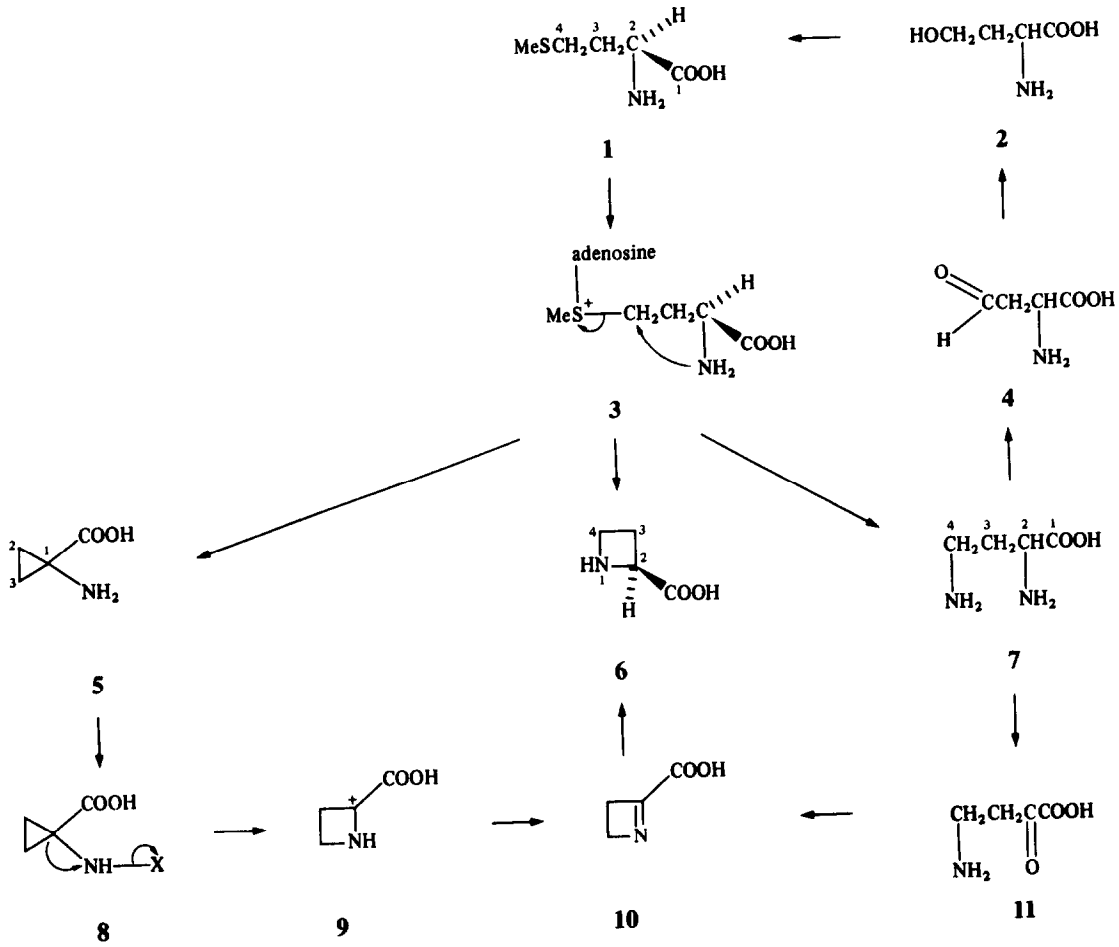


Fig. 1. Hypothetical biosynthetic routes to azetidine-2-carboxylic acid.

[$^{15}$N]ammonium chloride or sodium [$^{14}$C]cyanide, 2,4-diaminobutanoic acid labelled at the 4-amino, 2-amino or carboxyl group, respectively, was obtained. Several syntheses of labelled 1-aminocyclopropane-1-carboxylic acid (**5**) have been described [11–14]. The method used in the present work is based on a procedure of Ghosez *et al.* [15] for alkylating glycine derivatives. Ethyl [2-$^{13}$C, 1-$^{14}$C, $^{15}$N]glycinate hydrochloride (**17**) was condensed with *p*-bromobenzaldehyde in the presence of triethylamine to yield the Schiff base **16**. Reaction of this compound with 1,2-dibromoethane in acetonitrile in the presence of potassium carbonate yielded the cyclopropane derivative **18**, which on hydrolysis with hydrochloric acid yielded labelled **5**. The stable isotopes $^{13}$C and $^{15}$N were introduced with the hope that the direct incorporation of **5** into A-2-C could be established by $^{13}$C NMR. The signal arising from C-2 in A-2-C would be expected to exhibit satellites due to spin-spin coupling with the adjacent $^{15}$N atom. In the labelled **5**, the $^{13}$C at C-1 appeared as a doublet, with a coupling constant of 9.0 Hz, due to the adjacent $^{15}$N, in its $^{13}$C NMR spectrum.

The $^{15}$N content of these putative precursors and the resultant A-2-C was determined by conversion of these compounds to nitrogen gas, which was then analysed by mass spectrometry [16]. Details of the amounts of compounds fed, by the wick method, to *C. majalis* plants are recorded in Table 1. The only compound which showed a significant incorporation of both $^{14}$C and $^{15}$N into A-2-C was [1-$^{14}$C, $^{15}$N]methionine. Furthermore, the specific incorporation of these two isotopes was almost the same, indicative of the direct incorporation of this amino acid into A-2-C. This result also eliminates from consideration 4-amino-2-oxobutanoic acid as an intermediate between methionine and A-2-C. The incorporation of $^{14}$C from the labelled 1-aminocyclopropane-1-carboxylic acid into A-2-C was negligible. Indeed, most of the $^{14}$C activity in the free amino acids isolated from the plant which had been fed this compound was due to unchanged 1-aminocyclopropane-1-carboxylic acid. The specific incorporation ($^{14}$C) of the labelled 2, 4-diaminobutanoic acids into A-2-C was quite low compared with methionine. On the other hand, much higher incorporations were observed in aspartic and glutamic acids. It is thus proposed that the incorporation of 2,4-diaminobutanoic acid into A-2-C proceeds via aspartic $\beta$-semi aldehyde (**4**), by transamination at the 4-amino group. Reduction would then yield homoserine (**2**), which is a precursor of methionine, and has been shown to be an efficient precursor of A-2-C in *C. majalis* [1]. Although the incorporation of the [1-$^{14}$C]-2,4-diaminobutanoic acid

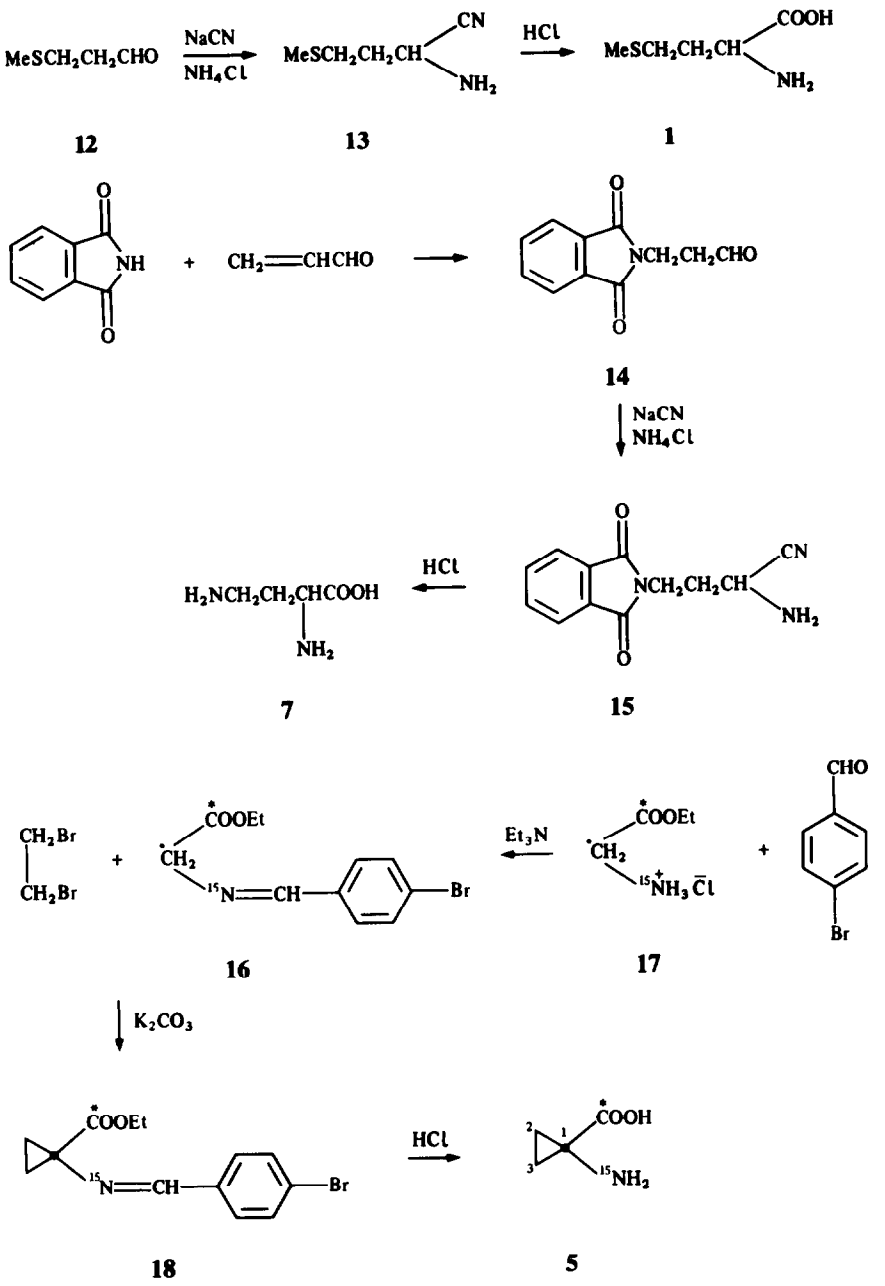


Fig. 2. Syntheses of the putative precursors of azetidine-2-carboxylic acid.

into A-2-C was low, degradation of the labelled A-2-C indicated that essentially all the $^{14}C$ was located on its carboxyl group.

These results indicate that none of the previously discussed hypotheses for the loss of tritium from C-2 of labelled methionine, when it is converted to A-2-C, is valid. It is thus proposed that the tritium is lost prior to its conversion to A-2-C, as illustrated in Fig. 3. Reaction of [2-$^{3}H$]methionine with pyridoxal phosphate affords the Schiff base **19**. A tautomeric shift to **20** results in loss of the tritium. A reversal of the tautomerism involving hydrogen yields **21**. Hydrolysis then affords tritium-free methionine; however, $^{15}N$ originally present at C-2 will be retained by this mechanism. This is the accepted mechanism whereby the racemization of α-amino acids occurs [17]. This proposal will be examined by administering [1-$^{14}C$, 2-$^{3}H$]methionine to *C. majalis*, and then reisolating the methionine from the plant after several days to determine whether tritium is lost from the C-2 position.

## EXPERIMENTAL

*General methods.* Mps are corr. Radioactive materials were assayed by liquid scintillation counting using dioxane–EtOH as the solvent with the usual scintillators [18]. $^{13}C$ NMR spectra were determined in a Nicolet 300 spectrometer operating at

Table 1. Incorporation of labelled compounds into azetidine-2-carboxylic acid, aspartic acid and glutamic acid in *C. majalis*

| Compound fed to plant (amount, activity, $^{15}N$ %) | Fresh weight of harvested plants | Specific activities ($^{14}C$) (dpm/mmol) | | |
|---|---|---|---|---|
| | | Azetidine-2-carboxylic acid | Aspartic acid | Glutamic acid |
| (*R,S*)-[1-$^{14}C$, $^{15}N$]Methionine 0.2 mmol, $4.8 \times 10^8$ dpm/mmol, $^{15}N$: 96% excess | 260 | (120)* $^{14}C$: $2.17 \times 10^6$ $^{15}N$: 0.45% excess Spec. inc.‡ $^{14}C$: 0.45% $^{15}N$: 0.47% $BaCO_3$§ $2.06 \times 10^6$ | (24) $^{14}C$: $1.19 \times 10^5$ $^{15}N$: n.d.† 0.025% | (45) $^{14}C$: $1.13 \times 10^5$ $^{15}N$: n.d. 0.024% |
| (*R,S*)-[1-$^{14}C$, 2-$^{15}N$]-2,4-Diaminobutanoic acid · 2HCl, 0.2 mmol, $3.9 \times 10^7$ dpm/mmol, $^{15}N$: 52% excess at C-2 | 284 | (138) $2.10 \times 10^4$ Spec. inc. $^{14}C$: 0.05% $BaCO_3$ $2.03 \times 10^4$ | (32) $3.95 \times 10^5$ 1.01% | (65) $4.35 \times 10^5$ 1.1% |
| (*R,S*)-[1-$^{14}C$, 4-$^{15}N$]-2, 4-Diaminobutanoic acid · 2HCl, 0.2 mmol, $4.4 \times 10^7$ dpm/mmol, $^{15}N$: 62% excess at C-4 | 265 | (129) $2.62 \times 10^4$ Spec. inc. $^{14}C$: 0.06% $BaCO_3$ $2.58 \times 10^4$ | (11) $5.3 \times 10^5$ 1.2% | (54) $3.12 \times 10^5$ 0.71% |
| [1-$^{13}C$, *Carboxyl*-$^{14}C$, $^{15}N$]-1-aminocyclopropane-1-carboxylic acid, 0.5 mmol, $^{13}C$: 92% excess, $7.86 \times 10^7$ dpm/mmol, $^{15}N$: 99% excess | 450 | (221) $> 5.6 \times 10^3$ Spec. inc. $^{14}C$: > 0.01% | n.d. | n.d. |

*Weight in mg.
†Not determined.
‡Specific incorporation = specific activity of the isolated amino acid/specific activity of the administered compound.
§Obtained from the decarboxylation of A-2-C with ninhydrin.

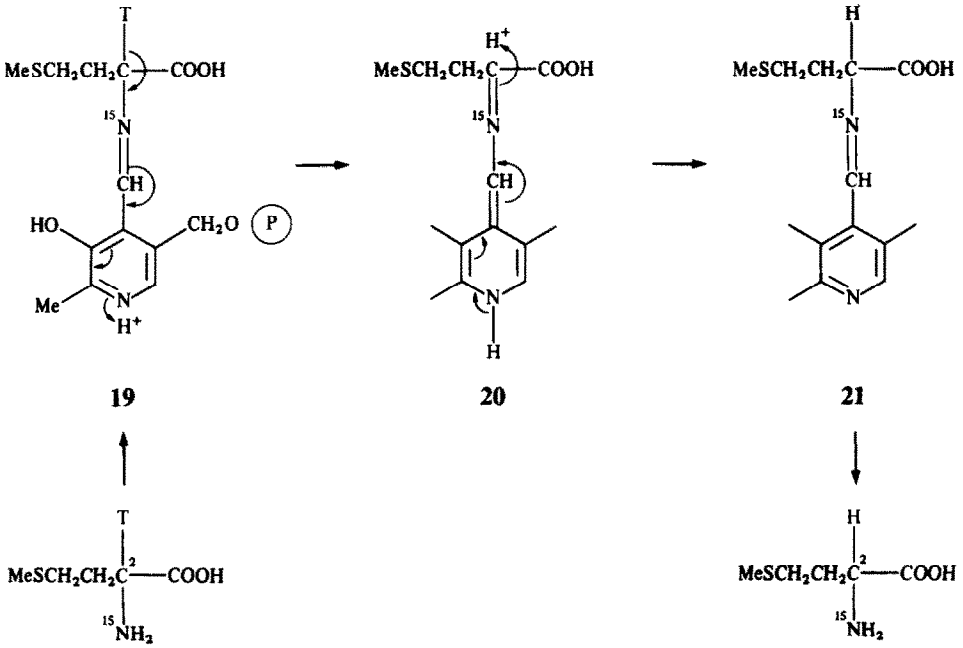


Fig. 3. Hypothesis for the loss of tritium from C-2 of methionine.

75.5 MHz, with the assistance of Dr. S. B. Philson. Mass spectra were determined by Dr. E. Larka and his assistants at the Univ. of Minnesota. Elemental analyses were carried out by M-H-W Laboratories, Phoenix, AZ. The *Convallaria majalis* plants were growing in the garden of the senior author in St. Paul, Minnesota. At the time of feeding (May), some of the plants were just beginning to flower. The average day-time temp. was 70–80°F.

(R,S)-[$^{15}$N]*Methionine* [9]. A cold soln of $^{15}NH_4Cl$ (0.10 g, 1.9 mmol, Malinckrodt) and NaCN (93.1 mg, 1.9 mmol) in $H_2O$ (2 ml) was added to a soln of 3-methylthiopropanal (**12**) (0.21 g, 2.0 mmol) in $H_2O$ (5 ml) at 0°. After stirring at this temp. for 15 min, the reaction mixture was stirred at 20° for 18 hr. The reaction mixture was then acidified with HCl and evaporated to dryness. The residual aminonitrile (**13**) was refluxed with conc HCl (5 ml) for 14 hr. The soln was decolourized with charcoal and evaporated to dryness. The residue was dissolved in $H_2O$ and added to a column of Dowex AG-50W-X8 ($H^+$ form, 15 × 0.5 cm). The column was washed with $H_2O$ until the eluant was neutral. Elution with 1% aq. $NH_3$ afforded after evaporation the ammonium salt of (*R,S*)-[$^{15}$N]methionine (141.5 mg, 42%) which was crystallized from hot $H_2O$. Prior to feeding, this material was mixed with (*R,S*)-[1-$^{14}$C]methionine (Amersham-Searle).

*Labelled* 2,4-*diaminobutanoic acid* [10]. The following general procedure (with slight changes in the weights when $^{15}$N-labelled intermediates were used) was used for the synthesis of (*R,S*)-[1-$^{14}$C]-, [2-$^{15}$N]- and [4-$^{15}$N]-2,4-diaminobutanoic acid, the labelled starting materials being $Na^{14}CN$ (Mallinckrodt), $^{15}NH_4Cl$ (Mallinckrodt) and [$^{15}$N]phthalimide (Prochem), respectively. A mixture of phthalimide (1.0 g, 6.8 mmol) and acrolein (0.383 g, 6.84 mmol) was stirred in EtOAc (4 ml) at 65°. Two drops of Triton B (40% soln of benzyltrimethylammonium hydroxide in MeOH) were added and the suspended phthalimide dissolved quickly. After stirring for 15 min the solvent was removed and the residue washed with $Et_2O$. Recrystallization of the residue from hot $H_2O$ yielded 3-phthalimidopropanal (**14**) as colourless plates (1.01 g, 73%), mp 124–125°, lit. [10] mp 125–126°. The aldehyde **14** (203 mg, 1 mmol) was dissolved in 50% aq. dioxane (5 ml) and cooled to 0°. An aq. soln (2 ml) containing $NH_4Cl$ (53.5 mg, 1 mmol) and NaCN (49 mg, 1 mmol) was added and the mixture stirred at 20° for 18 hr. The soln was then acidified with HCl and evaporated to dryness. The residue was then refluxed with conc HCl (5 ml) for 18 hr. The cooled reaction mixture was diluted with $H_2O$ (10 ml) and extracted with $CHCl_3$ to remove phthalic acid. The aq. soln was evaporated and the residue redissolved in $H_2O$ and chromatograhed on Whatman No. 17 paper, developing with a mixture of *n*-BuOH–HOAc–$H_2O$ (3:1:1). The paper was dried and the zone ($R_f$ 0.27) corresponding to 2,4-diaminobutanoic acid (detected by spraying a thin strip with ninhydrin) was cut out and extracted with boiling $H_2O$. The soln was acidified with HCl and evaporated to dryness, affording the 2,4-diaminobutanoic acid as its dihydrochloride salt (77 mg, 40%), mp 176°, lit. [19] mp 175–180°, after crystallization from aq. EtOH.

[1-$^{13}$C, Carboxyl-$^{14}$C, $^{15}$N]-1-*aminocyclopropane*-1-*carboxylic acid*. A mixture of [2-$^{13}$C, $^{15}$N]glycine (92.4% $^{13}$C, 99% $^{15}$N) (MDS Isotopes) and [1-$^{14}$C]glycine (nominal activity 250 $\mu$Ci, 56 mCi/mmol) (Amersham-Searle) was added to a mixture of EtOH (10 ml), $C_6H_6$ (30 ml) and conc HCl (2.5 ml) and refluxed for 18 hr, $H_2O$ being separated from the reaction mixture with a Dean–Stark trap. Evaporation of the solvent afforded ethyl [2-$^{13}$C, 1-$^{14}$C, $^{15}$N]glycinate hydrochloride (**17**) (0.92 g, $7.9 \times 10^7$ dpm/mmol), which was washed with $C_6H_6$ and hexane. $^{13}$C NMR ($D_2O$): the enriched C-2 carbon appeared at 44.1 ppm (from TMS) as a doublet, $^1J_{^{13}C,^{15}N} = 7.3$ Hz. This ester (0.90 g, 6.36 mmol), *p*-bromobenzaldehyde (1.18 g, 6.4 mmol), $Et_3N$ (1.7 ml) and anhydrous $MgSO_4$ (0.7 g) were stirred in $CH_2Cl_2$ (15 ml) at 20° for 20 hr. $H_2O$ (20 ml) was then added and the mixture extracted with $Et_2O$ (4 × 75 ml). The dried ($Na_2SO_4$) extract was evaporated and the residue crystallized from hexane at −20°, yielding the Schiff base of *p*-bromobenzaldehyde and ethyl glycinate (**16**) as colourless needles (1.25 g, 73%), mp 27–28°. MS (unlabelled material) *m/z* (rel. int.): 272 (2.5), 270 (2.2) (M ± 1, Br isotopes). 242 (33), 240 (33), 198 (93), 196 (100), 171 (96), 169 (96). Analysis (unlabelled): calc. for $C_{11}H_{12}BrNO_2$: C, 48.91; H, 4.48; Br, 29.58; N, 5.19. Found: C, 48.78; H, 4.45; Br, 29.71; N, 5.06%.

The Schiff base **16** (1.25 g) was dissolved in MeCN (30 ml) containing 1,2-dibromoethane (5 ml) and anhydrous $K_2CO_3$ (7 g) and the mixture refluxed with stirring for 3 days. The cooled reaction mixture was diluted with $H_2O$ (15 ml) and extracted with $Et_2O$ (3 × 75 ml). Evaporation of the dried ($Na_2SO_4$) extract yielded an oil which was subjected to a high vacuum at 25° to remove excess dibromoethane. The residual oil (**18**) was refluxed with 2 M HCl (30 ml) for 24 hr. The cooled soln was extracted with $Et_2O$ (to remove *p*-bromobenzaldehyde) and the aq. soln then applied to a column of Dowex AG-50W-8X ($H^+$ form) (15 g). The column was washed with $H_2O$ and then with M $NH_4OH$. The residue obtained on evaporation of the fractions which contained 1-aminocyclopropane-1-carboxylic acid was dissolved in a little $H_2O$, filtered and $Me_2CO$ added when the amino acid crystallized (219 mg, 46%). Sublimation (200°, $10^{-4}$ mm Hg) afforded material with a specific activity of 7.86 × $10^7$ dpm/mmol. $^{13}$C NMR ($D_2O$, pH 7): $\delta$37.9 (*d*, C-1, $^1J_{^{13}C,^{15}N} = 9.0$ Hz), 14.4 (*d*, C-2, C-3, $^1J_{1,2} = 13.2$ Hz). (This small one-bond $^{13}$C–$^{13}$C coupling constant is typical for cyclopropane rings [20].) TLC on silica gel eluting with *n*-BuOH–HOAc–$H_2O$ (80:20:1) indicated that > 99% of the radioactivity was located at a position coincident with authentic 1-aminocyclopropane-1-carboxylic acid ($R_f$ 0.28). No activity was found where glycine ($R_f$ 0.12) runs in this solvent system.

*Feeding the putative precursors to* C. majalis *and isolation of A-2-C and other amino acids*. The plants were fed by the wick method, the cotton wick passing through the stems of several closely spaced plants before returning to the beakers (10 ml) which contained aq. soln of the labelled compounds. All feeding expts were carried out for 7 days. The fresh plants were then pulled up, washed free of dirt, and then chopped up in a Waring blendor with 70% EtOH. This extract was then processed as described by Fowden [21]. Final purification of the A-2-C was carried out by sublimation (200°, $10^{-4}$ mm Hg).

*Degradation of A-2-C to determine the* $^{14}$C *activity on its carboxyl group*. A-2-C was decarboxylated with ninhydrin [22], the evolved $CO_2$ being collected as $BaCO_3$, by absorption in aq. $Ba(OH)_2$. The $Ba^{14}CO_3$ was assayed by direct soln in Aquasol II (New England Nuclear) according to a previously described method [23]. The activities of these samples of $BaCO_3$ are recorded in Table 1.

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