Phytochemistry, 1974, Vol. 13, pp. 427 to 433. Pergamon Press. Printed in England.

BIOSYNTHESIS OF AZETIDINE-2-CARBOXYLIC ACID IN *CONVALLARIA MAJALIS*

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(*Received* 11 *July* 1973. *Accepted* 17 *August* 1973)

Key Word Index—*Convallaria majalis*; Liliaceae; lily-of-the-valley; azetidine-2-carboxylic acid; biosynthesis from methionine, *S*-adenosyl-methionine, homoserine.

Abstract—*Convallaria majalis* plants were fed DL-methionine-[1-^{14}C], [1-^{14}C, 4-^{3}H], and [1-^{14}C, 2-^{3}H], *S*-adenosyl-L-methionine-[1-^{14}C], and DL-homoserine-[1-^{14}C], resulting in the formation of labeled azetidine-2-carboxylic acid (A-2-C). The complete retention of tritium relative to carbon-14 in the feeding experiment involving methionine-[1-^{14}C, 4-^{3}H] indicates that aspartic acid or aspartic-β-semialdehyde are not intermediates between methionine and A-2-C. However, since the A-2-C derived from methionine-[1-^{14}C, 2-^{3}H] had lost 95% of the tritium relative to the C-14, it is not considered that methionine or its *S*-adenosyl derivative are the immediate precursors of A-2-C. Our data and that of others is consistent with the intermediate formation of γ-amino-α-ketobutyric acid which on cyclization yields 1-azetine-2-carboxylic acid, A-2-C then being formed on reduction.

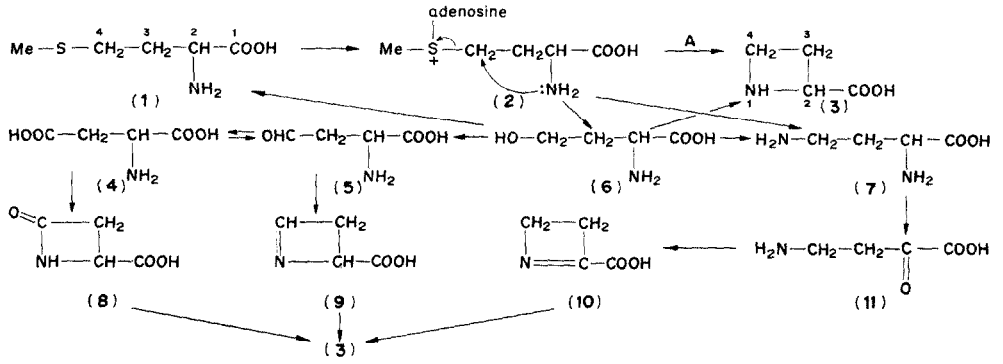
AZETIDINE-2-CARBOXYLIC ACID (**3**, abbreviated A-2-C) was discovered in 1955 in several members of the Liliaceae,[1–4] and in a few species of Agavaceae and Amaryllidaceae.[5] More recently it has been found in the legume *Delonix regia*[6] and in minute amounts in sugar beets.[7] An appreciable amount of work has been published on attempts to determine its biosynthesis and the present article describes our efforts in this area in the last decade.

Scheme 1 outlines various hypothetical routes which have been suggested for the biosynthesis of this imino acid. Linko[8] considered that it would be formed from aspartic acid (**4**). One route would be via aspartic-β-semialdehyde (**5**), which on cyclization yields 1-azetine-4-carboxylic acid (**9**), reduction then affording A-2-C. Another pathway would be via 2-azetidinone-4-carboxylic acid (**8**).[9] To test these hypotheses Linko administered aspartic-[U-^{14}C] acid to the leaves of young Lily-of-the-valley (*Convallaria majalis*) plants. No activity was found in A-2-C, the major metabolites of the aspartic acid being alanine and β-alanine, presumably formed by decarboxylation of the β- and α-carboxyl groups respectively. Radioactive A-2-C was obtained from the leaves of *C. majalis* which had been exposed to $^{14}CO_2$;[10] however, activity was incorporated into alanine and serine

* Contribution No. 126 from this Laboratory.

[1] VIRTANEN, A. I. and LINKO, P. (1955) *Acta Chem. Scand.* **9**, 551.
[2] VIRTANEN, A. I. (1955) *Nature* **176**, 989.
[3] FOWDEN, L. (1955) *Nature* **176**, 347.
[4] FOWDEN, L. (1956) *Biochem. J.* **64**, 323.
[5] FOWDEN, L. and STEWARD, F. C. (1957) *Ann. Botany (London)* **21**, 53.
[6] SUNG, M.-L. and FOWDEN, L. (1969) *Phytochemistry* **8**, 2095.
[7] FOWDEN, L. (1972) *Phytochemistry* **11**, 2271.
[8] LINKO, P. (1958) *Acta Chem. Scand.* **12**, 101.
[9] This compound is formed *in vitro* by heating asparagine in an aqueous phosphate buffer at 100°: TALLEY, E. A., FITZPATRICK, T. J. and PORTER, W. L. (1956) *J. Am. Chem. Soc.* **78**, 5836.
[10] FOWDEN, L. and BRYANT, M. (1959) *Biochem. J.* **71**, 210.

at a much faster rate. Slight labeling of A-2-C occurred when aspartic-[U-^{14}C] or glutamic-[U-^{14}C] acid were fed. α,γ-Diaminobutyric-[U-^{14}C] acid (**7**) was metabolized in the plant to an unknown compound X, which was close to A-2-C on a 2-D PC. This compound X was apparently converted to A-2-C by hydrogenation in the presence of Adams catalyst. It was suggested that compound X is 1-azetine-2-carboxylic acid (**10**). No significant activity was detected in A-2-C when γ-aminobutyric-[^{14}C] acid or glucose-[U-^{14}C] were supplied to the plant.



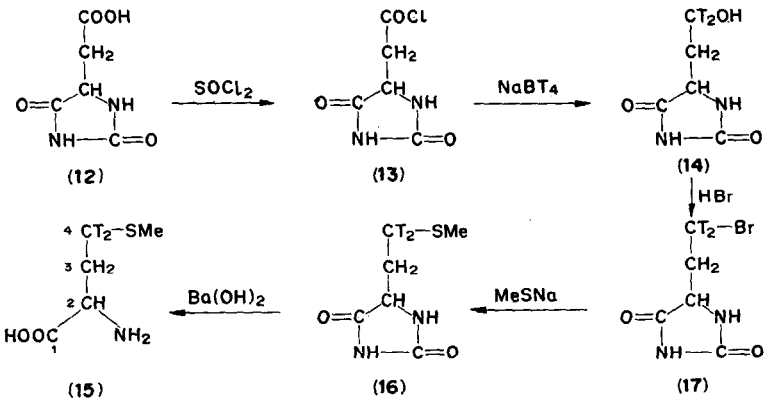
SCHEME 1. HYPOTHETICAL BIOSYNTHETIC PATHWAYS TO AZETIDINE-2-CARBOXYLIC ACID.

Since spermidine has been shown to arise by the nucleophilic attack of putrescine on *S*-adenosyl-L-methionine,[11] we considered in 1964 that A-2-C could be formed by an intramolecular displacement of the thiomethyladenosine by the α-amino group of *S*-adenosylmethionine (**2**) (reaction A, Scheme 1). A similar hypothesis had been suggested by Schlenk and Dainko.[12] However, at the time we commenced our work we were fortunately unaware of their results. They incubated homogenates of various parts of the *C. majalis* plant with DL-methionine-[2-^{14}C], *S*-methyl-DL-methionine-[2-^{14}C], and *S*-adenosyl-L-methionine-[2-^{14}C]. In none of these experiments did they observe significant incorporation of activity into A-2-C. Negative results were also obtained when the stems of young plants were placed in solutions of the above labeled compounds. In our first experiment[13] (expt. 1, Table 1) DL-methionine-[1-^{14}C] was fed to *C. majalis* plants growing out of doors by means of cotton wicks inserted through the leaves near to ground level. At the time of feeding (May) most of the plants were just beginning to produce flowers. After 7 days the leaves and the shoots were harvested, and A-2-C isolated from the amino acid fraction as previously described.[4] There was a reasonable incorporation (1·67%) of radioactivity into A-2-C and a degradation (decarboxylation with ninhydrin[14]) indicated that essentially all the radioactivity was located on the carboxyl group. Later, Su and Levenberg[15] confirmed and extended our findings by feeding DL-methionine-[1-^{14}C], DL-methionine-[2-^{14}C], and L-methionine-[U-^{14}C] to excised shoots of *C. majalis*, and obtained labeled A-2-C with incorporations of 0·88, 0·54 and 0·24% respectively. The distribution of activity in

[11] TABOR, H., ROSENTHAL, S. M. and TABOR, C. W. (1958) *J. Biol. Chem.* **233**, 907.
[12] SCHLENK, F. and DAINKO, J. L. (1960) U.S. Atomic Energy Commission. Reports of the Argonne National Laboratory, ANL-6200, 94.
[13] This preliminary experiment was reported as a communication: LEETE, E. (1964) *J. Am. Chem. Soc.* **86**, 3162.
[14] COLOWICK, S. P. and KAPLAN, N. O. (1957) in *Methods in Enzymology*, Vol. IV, p. 711. Academic Press, New York.
[15] SU, E. F.-W. and LEVENBERG, B. (1967) *Acta Chem. Scand.* **21**, 493.

the A-2-C was consistent with the four-carbon-chain of methionine serving as a direct precursor of the imino acid.

However, these results and ours do not eliminate the possibility that methionine is metabolized to a sulfur-free compound prior to the utilization of its carbons for the biosynthesis of A-2-C. Homoserine **(6)** can be formed by the enzymatic hydrolysis of *S*-adenosylmethionine.[16,17] Aspartic-β-semialdehyde and aspartic acid formed by the oxidation of homoserine could yield A-2-C as previously outlined. Another possibility would involve the formation of an *O*-ester of homoserine, such as its *O*-phosphate. A-2-C would then be formed by an intramolecular displacement of the ester group by the α-amino group of the homoserine. Methionine was labeled with tritium at C-4 in an effort to decide between these various pathways. DL-Methionine-[4-^{3}H] was prepared by the method illustrated in Scheme 2. This sequence of reactions is based, in part, on the synthesis of Livak *et al.*[18]



SCHEME 2. SYNTHESIS OF METHIONINE-[4-^{3}H].

Treatment of hydantoin-5-acetic acid **(12)** with thionyl chloride yielded the acid chloride **(13)** which was reduced in dioxane with sodium borohydride-[^{3}H] affording 5-(β-hydroxyethyl)hydantoin **(14)**. This alcohol was converted to 5-(β-bromoethyl)hydantoin **(17)** by heating with 48% hydrobromic acid. This bromo compound with sodium methylmercaptide yielded **16**, which on hydrolysis with barium hydroxide afforded methionine-[4-^{3}H] **(15)**. This tritium labeled methionine was mixed with DL-methionine-[1-^{14}C] and fed as before to *C. majalis* plants (expt. 2). The resultant A-2-C was labeled with both tritium and C-14, the ratio of activity of the two isotopes being the same as in the administered methionine-[1-^{14}C, 4-^{3}H].[19] All the C-14 was located on the carboxyl group. To determine the location of the tritium, the A-2-C was heated in a sealed tube with HCl yielding homoserine,[8] which was oxidized with $KMnO_4$ affording aspartic acid which was almost devoid of tritium. This result indicates that all the tritium was located at C-4 of the A-2-C. The complete retention of tritium relative to the C-14 eliminates the formation of A-2-C from methionine via aspartic-β-semialdehyde or aspartic acid. Homoserine could still be an intermediate between methionine and A-2-C, therefore DL-methionine-[1-^{14}C] was fed to

[16] PARKS, L. W. and SCHLENK, F. (1958) *Arch. Biochem. Biophys.* **75**, 291.
[17] SHAPIRO, S. K. and MATHER, A. N. (1958) *J. Biol. Chem.* **233**, 631.
[18] LIVAK, J. E., BRITTON, E. C., VANDERWEELE, J. C. and MURRAY, M. F. (1948) *J. Am. Chem. Soc.* **67**, 2218.
[19] A preliminary account of this work was presented at the 155th National Meeting of the American Chemical Society, San Francisco, 31 March–5 April, 1968, Abstracts P-178.

TABLE 1. INCORPORATION EXPERIMENTS WITH LABELED AMINO ACIDS IN *Convallaria majalis*

Expt. No.*	Compound fed to plant (wt. activity)	Duration of feeding (days)	Fr. wt of harvested plants (g)	Azetidine-2-carboxylic acid wt isolated (g) Sp. act. dpm/mM	(Absolute inc.) %	Aspartic acid† Sp. act. dpm/mM	Glutamic acid† Sp. act. dpm/mM
1	DL-Methionine-[1-^{14}C] (9·32 mg, 0·2 mCi)	7	2400	1·45 $5·0 \times 10^5$ (1·67) RSA‡ 100		$2·0 \times 10^4$ 4	$1·27 \times 10^4$ 2·5
2	DL-Methionine-[1-^{14}C, 4-^{3}H] (19·34 mg, 0·093 mCi-^{14}C) [^{3}H/^{14}C=0·63]	7	570	0·120 ^{14}C: $1·89 \times 10^6$ (1·1) [^{3}H/^{14}C = 0·66]		Not isolated	Not isolated
3a	DL-Methionine-[1-^{14}C] (7·0 mg, 0·185 mCi)	14	305	0·134 $4·51 \times 10^6$ (1·45) RSA 100		$3·46 \times 10^4$ 0·8	$5·78 \times 10^4$ 1·3
3b	DL-Methionine-[1-^{14}C] (7·0 mg, 0·185 mCi) + L-homoserine (100 mg)	14	256	0·132 $9·33 \times 10^5$ (0·30) RSA 100		$1·08 \times 10^5$ 11·5	$9·79 \times 10^4$ 10·5
4a	DL-Methionine-[1-^{14}C] (10·6 mg, 0·327 mCi)	5	459	1·12 $9·6 \times 10^5$ (1·4) RSA 100		$2·16 \times 10^5$ 22·5	$2·56 \times 10^5$ 26·5
4b	DL-Homoserine-[1-^{14}C] (8·14 mg, 0·312 mCi)	5	437	1·00 $4·6 \times 10^5$ (0·67) RSA 100		$2·56 \times 10^5$ 55·5	$1·0 \times 10^5$ 22
5a	*S*-Adenosyl-L-methionine-[3-^{14}C] (0·546 mg, 0·023 mCi)	7	167	0·080 $2·15 \times 10^4$ (0·034) RSA 100		$3·45 \times 10^4$ 160	$3·66 \times 10^4$ 170
5b	DL-Methionine-[1-^{14}C] (2·34 mg, 0·16 mCi§	7	170	0·095 $3·89 \times 10^5$ (0·115) RSA 100		$2·91 \times 10^5$ 75	$2·68 \times 10^5$ 69
6a	DL-Methionine-[1-^{14}C, 2-^{3}H] (37·25 mg, 0·0185 mCi-^{14}C) [^{3}H/^{14}C=6·44]	5	740	0·95 [^{3}H/^{14}C = 0·32] ^{14}C: $7·1 \times 10^4$ (1·79) RSA 100		[^{3}H/^{14}C = 4·3] $4·5 \times 10^3$ 6·3	$2·2 \times 10^3$ 3·1
6b	*S*-Adenosyl-L-methionine-[1-^{14}C] (50 mg, 0·052 mCi)	5	802	1·20 $3·7 \times 10^5$ (3·84) RSA 100		$2·7 \times 10^3$ 0·7	$4·1 \times 10^3$ 1·1

* Experiments having the same number were carried out at the same time, the plants being subjected to the same environmental conditions. All the experiments except 5 involved the use of intact plants growing in soil out of doors. Most of the feedings were done on the farm of Christine and George Piper, Medina, Minnesota, and we are greatly indebted to them for permitting us to use their plants in this investigation. The feedings were all carried out in May or early June at a time when the plants were flowering. In experiments 5a and 5b excised leaves were used, the cut ends being placed in beakers containing solutions of the labeled compounds.

† In experiment No. 1 the yields of aspartic and glutamic acid were 303 and 491 mg respectively.

‡ RSA = Relative Specific Activity.

§ At the time of harvesting the residual activity not absorbed by the excised leaves fed *S*-adenosyl-L-methionine-[1-^{14}C] and DL-methionine-[1-^{14}C], was 5·0 and 7·8% respectively.

the plant along with a relatively large amount of non-radioactive homoserine (expt. 3b). The incorporation of activity (0·30%) into A-2-C was indeed significantly lower than the incorporation of methionine-[1-^{14}C] (1·45%) fed at the same time to another group of plants (expt. 3a). Another significant difference between these two experiments was the relative amount of activity incorporated into aspartic and glutamic acid. In all the previous feeding experiments the absolute and specific incorporation of activity into aspartic and glutamic acid was quite small compared with the incorporation into A-2-C (see Table 1). However, the co-administration of homoserine with the methionine-[1-^{14}C] apparently suppressed its conversion into A-2-C, and a larger proportion was metabolized to aspartic and glutamic acids.

Next, a direct comparison of the incorporation of methionine-[1-^{14}C] and homoserine-[1-^{14}C] was made (expts. 4a, 4b). The homoserine-[1-^{14}C] was prepared by refluxing *S*-methylmethionine-[1-^{14}C] hydroxide in water.[20] The A-2-C derived from homoserine-[1-

[20] The decomposition of *S*-alkylated methionines in alkaline solution yielding homoserine and its lactone has been reported: (a) GUNDLACH, H. G., MOORE, S. and STEIN, W. H. (1959) *J. Biol. Chem.* **234**, 1761; (b) LAVINE, T. F., FLOYD, N. F. and CAMMAROTI, M. S. (1954) *J. Biol. Chem.* **207**, 107.

^{14}C] had all its activity located on the carboxyl group; however, the homoserine was a poorer precursor of A-2-C than methionine which was fed at the same time (0·67% vs. 1·4% incorporation). For some inexplicable reason[21] considerably more of both precursors were metabolized to aspartic and glutamic acids. The radioactive aspartic acid derived from homoserine-[1-^{14}C] was found to have 85% of its activity located on its α-carboxyl group, indicative of a direct conversion.

Recently, Sung and Fowden[22] have examined the biosynthesis of A-2-C in *D. regia* seedlings, and found that DL-homoserine-[4-^{14}C] was a better precursor (0·28% incorporation) than L-methionine-[1-^{14}C] (0·11% incorporation).

We also made a comparison of the ability of methionine and *S*-adenosyl-L-methionine to serve as precursors of A-2-C. In our first set of experiments (5a, 5b) the labeled compounds were fed to excised leaves of *C. majalis*. The resultant incorporations into A-2-C were quite low compared with the previous feeding experiments which involved the use of intact plants. These results are in accord with the earlier observations of Fowden and Bryant[10] who obtained a higher incorporation of aspartic acid into A-2-C when it was fed to the roots of *C. majalis*. In a later study (expts. 6a, 6b) the incorporation of DL-methionine-[1-^{14}C, 2-^{3}H] and *S*-adenosyl-L-methionine-[1-^{14}C] were compared, feeding to intact plants. The *S*-adenosyl-L-methionine was apparently a superior precursor (3·84% incorporation). However, if it is assumed that only the L-isomer of methionine is utilized for the production of A-2-C, the incorporation of the DL-methionine (1·79%) was not significantly different. In this last study the methionine was also labeled with tritium at C-2. This methionine-[2-^{3}H] was prepared by exchange of the azlactone of *N*-acetylmethionine with triturated water.[23] The A-2-C derived from the methionine-[1-^{14}C, 2-^{3}H] was almost devoid of tritium (>95% loss of ^{3}H relative to the ^{14}C). This result clearly forces us to abandon the hypothesis that *S*-adenosyl-L-methionine is the direct precursor of A-2-C. In their recent work, Sung and Fowden[22] found that α,γ-diaminobutyric acid was the best precursor of A-2-C in *D. regia*. We therefore propose that this diamino acid is incorporated via γ-amino-α-ketobutyric acid **(11)**, which on cyclization yields 1-azetine-2-carboxylic acid. Reduction then affords A-2-C. It is suggested that the α,γ-diaminobutyric acid can be formed by the amination of *S*-adenosylmethionine, or via homoserine.[24] The tritium at C-2 of methionine will then be lost when the intermediate α,γ-diaminobutyric acid is converted to γ-amino-α-ketobutyric acid. This hypothesis is being investigated using ^{15}N-labeled precursors.

EXPERIMENTAL

General methods. M.ps are corrected. A Nuclear Chicago Model 724 or Mark II liquid scintillation counter was used for assay of the radioactive compounds, using either toluene or dioxane–EtOH with the usual scintillators.[25] Assays were carried out in duplicate and were reproducible to 5%. Elementary analyses were carried out by Clark microanalytical laboratory, Urbana, Illinois.

Compounds fed to Convallaria majalis. The following compounds were obtained commercially from the indicated sources: DL-methionine-[1-^{14}C] (Calbiochem, Los Angeles; Amersham-Searle, Chicago), *S*-adenosyl-L-methionine-[1-^{14}C] (Amersham-Searle).

[21] The plants were a little more advanced than in the previous feeding experiments.

[22] SUNG, M.-L. and FOWDEN, L. (1972) *Phytochemistry* **10**, 1523.

[23] THOMAS, A. F. (1972) *Deuterium Labelling in Organic Chemistry*, p. 210, Appleton–Century–Crofts, New York.

[24] Homoserine has been shown to be a precursor of α,γ-diamino-butyric acid in the plant *Lathyrus sylvestris*: NIGAM, S. N. and RESSLER, C. (1966) *Biochemistry* **5**, 3426.

[25] FRIEDMAN, A. R. and LEETE, E. (1963) *J. Am. Chem. Soc.* **85**, 2141.

Hydantoin-5-acetyl chloride **(13)**. Hydantoin-5-acetic acid[26] (20 g) was refluxed with $SOCl_2$ (200 ml) for 16 hr. The brown gum (35 g) obtained on removal of the excess $SOCl_2$ *in vacuo* was dissolved in dry EtOAc. On prolonged cooling at −20° the crude acid chloride (17 g) separated. Recrystallization from EtOAc yielded an analytical sample (Found: C, 34·27; H, 3·00; N, 16·13; Cl, 19·89. $C_5H_5N_2O_3Cl$ requires: C, 34·00; H, 2·85; N, 15·86; Cl, 20·08%). M.p. 125–127° dec. IR: (Nujol) 1790 (shoulder acid chloride C=O), 1755, 1740 (hydantoin C=O). MS: no parent peak at 176, but one at 141 (loss of Cl).

5-(β-Bromoethyl-[β-^{3}H])-hydantoin **(17)**. $NaBH_4$ (100 mg) was added to a soln of hydantoin-5-acetyl chloride (1 g) in dioxane (100 ml) and the mixture heated at 100° for 10 min. $NaBH_4$-[^{3}H] (16 mg, nominal activity 50 mCi, International Chemical and Nuclear Co., Calif.) was then added and the mixture heated for 4 hr at 100°. Another portion of non-radioactive $NaBH_4$ (300 mg) was added and the heating continued for an additional hr. The reaction mixture was evaporated to dryness and the residue suspended in H_2O (15 ml). A *n*-BuOH extract (4 × 10 ml) of the mixture yielded on evaporation crude 5-(β-hydroxyethyl)-hydantoin as a colorless oil. This compound was heated with 48% HBr (10 ml) on a steam bath for 3 hr. The residue obtained on evaporation of the solution was diluted with non-radioactive 5-(β-bromoethyl)-hydantoin (1·07 g) and crystallized 2 × from H_2O (8 ml) affording 5-(β-bromoethyl-[β-^{3}H])-hydantoin (1·05 g, 2·6 mCi) as colorless plates, m.p. 139–140° (lit.[18] m.p. 141·5–142°). This compound was converted to DL-methionine-[4-^{3}H] according to the procedure of Livak *et al.*[18]

DL-*Methionine-[2-^{3}H]*. A soln of DL-methionine (152 mg, 1·02 mM) in Ac_2O (120 mg, 1·18 mM) and dioxane (6 ml) was refluxed for 0·5 hr. The residue obtained on evaporation of the solvent was dissolved in dioxane (2 ml) containing 1 drop of pyridine and T_2O (0·5 ml, nominal activity 56 mCi), and the mixture refluxed for 1 hr. The solvent and the excess T_2O was removed and the residue refluxed with 10 ml 18% HCl overnight. The residue obtained on evaporation was dissolved in H_2O (1 ml) and placed on a column of Dowex 50-X8 (1 × 25 cm). The column was washed with distd H_2O until the eluant was both neutral and free of ^{3}H. The methionine was then eluted with 1% aq. NH_3. The fractions containing methionine (detected with ninhydrin) were evaporated to yield the ammonium salt of DL-methionine-[2-^{3}H] (153 mg, $9·28 \times 10^8$ dpm/mM). Dilution of this material with a known weight of non-radioactive DL-methionine and crystallization several times from H_2O yielded methionine having an activity consistent with the undiluted material being radiochemically pure. On subjecting this methionine-[2-^{3}H] to the manipulations used in the isolation of A-2-C from the plants, there was essentially no loss of tritium. When the azlactone formed from methionine and acetic anhydride was exchanged with D_2O instead of T_2O, the only proton signal which decreased in its NMR spectrum was the multiplet centered at $\delta = 4·7$ which is assigned to the hydrogen located on the α-carbon.

DL-*Homoserine-[1-^{14}C]*. DL-Methionine-[1-^{14}C] (25 mg, 0·77 mCi) was dissolved in a mixture of HOAc (2 ml) and 90% HCO_2H (2 ml) containing MeI (160 mg), and allowed to stand for 3 days at room temp. in the dark. The solid obtained on evaporation of the solvent was subjected to chromatography on thick cellulose sheets, developing with *n*-BuOH–HOAc–H_2O (4:1:1). *S*-Methylmethionine iodide (R_f 0·27) was well separated from unreacted methionine (R_f 0·65). The former was extracted from the paper with 30% EtOH. The extract was evaporated, redissolved in H_2O (7 ml) and stirred with AgOH (from 100 mg of $AgNO_3$) for 30 min. The filtered reaction mixture (pH 8) was then refluxed for 4 hr, Me_2S emanating from the top of the condenser. The solution was evaporated to dryness and the residue (24 mg) subjected to chromatography on a thick sheet of cellulose using the same solvent mixture previously described. The main zone of homoserine (R_f 0·29) was extracted with hot H_2O, which yielded on evaporation DL-homoserine-[1-^{14}C] (8·1 mg, 0·312 mCi). Samples of this product were subjected to PC in several different solvents. A single radioactive spot (detected by exposure of the dried chromatogram to X-ray film) was found in each case, corresponding in position to authentic homoserine.

Isolation of A-2-C and the other amino acids from C. majalis. The fresh plants were macerated with 70% EtOH, and this extract processed as described by Fowden.[4] Final purification of A-2-C was achieved by sublimation (200°, 10^{-4} mm).

Degradation of the A-2-C derived from methionine-[1-^{14}C, 4-^{3}H]. A-2-C (50 mg, ^{14}C: $2·00 \times 10^5$ dpm/mM, $^3H/^{14}C = 0·66$) was dissolved in 6 N HCl (5 ml) and heated in a sealed tube at 100° for 24 hr. The contents of the tube were evaporated to dryness. The residue was dissolved in 3 N H_2SO_4 (10 ml) and $KMnO_4$ (50 mg) added to the stirred soln during 1 hr. After stirring for 6 hr at room temp., the filtered reaction mixture was placed on a column of Dowex 1 (acetate form, 1·8 × 45 cm). The column was washed with H_2O and the amino acids eluted with increasing concentration of AcOH. The fractions containing aspartic acid were pooled affording >99% pure (assayed with an amino-acid analyser) aspartic acid (12 mg, 18% yield from A-2-C). To improve the solubility of aspartic acid in toluene for liquid scintillation counting, it was converted to *N*-(2,4-dichlorophenoxyacetyl)aspartic acid[27] (^{14}C: $1·95 \times 10^5$ dpm/mM, $^3H/^{14}C = <0·01$).

Degradation of aspartic acid to determine activity at C-1. This degradation was carried out on the aspartic acid obtained from plants which had been fed DL-homoserine-[1-^{14}C] (expt. 4b). L-Aspartic acid (1·0 g, $2·30 \times 10^4$ dpm/mM) was suspended in EtOH (100 ml) which was then saturated with HCl gas. After refluxing overnight the soln was evaporated and the residue neutralized with 5% Na_2CO_3. Et_2O extraction of the mixture yielded

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the diethyl ester of aspartic acid which was distilled (100°, 10^{-2} mm). A 5% soln of $LiAlH_4$ in Et_2O (10 ml) was added carefully to a soln of the diester (660 mg) in Et_2O (10 ml). After stirring for 30 min, H_2O (3 ml) was added, the mixture filtered and the residue washed with EtOH. The combined filtrates were evaporated and the residue distilled (170°, 10^{-2} mm) yielding 2-aminobutane-1,4-diol as a colorless viscous oil (120 mg). This compound was dissolved in H_2O (20 ml) and a few drops of AcOH added until the solute was acidic. $NaIO_4$ (300 mg) was added, and after standing for 1 hr the solution was distilled into an aq. soln of dimedone (300 mg). Formaldehyde-dimedone separated (125 mg, $1{\cdot}95 \times 10^4$ dpm/mM). When this sequence of reactions was carried out with aspartic-[4-^{14}C] acid, the ultimate formadehyde-dimedone had less than 2% of the sp. act. of the starting aspartic acid.

Acknowledgements—This investigation was supported by research grants GB-363 and GB-3696 from the National Science Foundation, and by research grant GM-13246 from the National Institutes of Health, U.S. Public Health Service.