Utilization of the Intact Carbon Chain of Methionine for Biosynthesis of Azetidine-2-carboxylic Acid in *Convallaria majalis*

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The biosynthesis of azetidine-2-carboxylic acid in *Convallaria majalis* (Lily-of-the Valley) has been examined using ^{14}C-labeled methionines as precursors. [1-^{14}C] Methionine as well as [2-^{14}C] methionine and uniformly-[^{14}C-labeled] methionine were transformed by excised shoots into the imino acid. With the first two radioisomers, ^{14}C was found exclusively in the carboxyl and azetidine ring carbon atoms, respectively, while with the third the ring contained three times as much label as the carboxyl group. These results provide evidence that the linear four-carbon chain of methionine can serve, intact, as a precursor of azetidine-2-carboxylic acid in this plant.

A zetidine-2-carboxylic acid (A-2-C),** a recently discovered cyclic imino acid,[1a—d] has been found to occur in very high concentrations in many members of the family Liliaceae. Its biosynthesis and accumulation appear to be features peculiar to this family as well as to a few species in the allied families Agavaceae and Amaryllidaceae.[2] Possible pathways for the biosynthesis of A-2-C in *Convallaria majalis* (Lily-of-the Valley) and *Polygonatum multiflorum* (Solomon's Seal) have subsequently been investigated.[3,4] Labeled, putative precursors such as L-aspartic acid, L-glutamic acid, D-glucose, γ-aminobutyric acid, and L-α,γ-diaminobutyric acid, on administration to various portions of growing plants did not result in metabolically significant labeling of A-2-C. More recently, however, Leete [5] noted that [1-^{14}C] methionine could serve as a relatively effective precursor of A-2-C (but not of aspartic or glutamic acids) in intact, growing *C. majalis* plants, the label being confined almost exclusively in such experiments to the carboxyl group of the imino acid. As a result of this observation, Leete re-stated a proposal (advanced earlier by

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** The abbreviation used in this paper is: azetidine-2-carboxylic acid, A-2-C.

Schlenk and Dainko [6] without supporting evidence) that A-2-C may be formed *via* intramolecular displacement of the thiomethyladenosine moiety of S-adenosylmethionine, a mechanism analogous in part to that demonstrated for the biosynthesis of spermidine from S-adenosyl(5')-3-methylmercaptopropylamine and putrescine.[7] Such a route must naturally invoke utilization of an intact carbon chain, C-1 through C-4, of methionine (or a close metabolic derivative of methionine), a stipulation which the above experiment left unchallenged. In the present work, evidence is presented which confirms and extends the experimental finding of Leete, and provides support for the conclusion that the linear four-carbon chain of methionine can serve, without prior scission, as a precursor of A-2-C in excised shoots of *C. majalis*.

MATERIALS AND METHODS

Lily-of-the-valley *(Convallaria majalis)* "pips", which had been given a preliminary cold-shock specifically for the purpose of allowing subsequent rapid indoor growth, were obtained at selected intervals between December and May from Wayside Gardens, Mentor, Ohio. They were usually kept in closed cellophane bags at 3°C for several weeks before use.

Solvent systems for paper chromatography were composed of the following solutions:

A. 1-Butanol:glacial acetic acid:water (18:2:5, v/v).
B. 1-Butanol:2-butanone:conc. NH_4OH:water (5:3:1:1, v/v).
C. Phenol saturated with water (100 g of reagent grade phenol in 25 ml of water), adjusted to pH 6.0 with 5 N NaOH.

The following substances were obtained from the commercial sources indicated: DL-[1-^{14}C] methionine, DL-[2-^{14}C] methionine, and uniformly-L-[^{14}C] labeled methionine from New England Nuclear Corp., Boston, Mass.; L-methionine and L-azetidine-2-carboxylic acid from Calbiochem, Los Angeles, Calif.; DL-methionine sulfone and DL-methionine sulfoxide from Nutritional Biochemicals Corp., Cleveland, Ohio.

Growth of plants. As required, each *C. majalis* pip was planted in sterile, nutrient Vermiculite (Enriched Growth Medium HEA-2, Westinghouse Electric Co., Detroit, Michigan) contained in a small clay pot 9 cm in height and 11 cm in diameter. The pots were placed in a Westinghouse Portable Electric Greenhouse, Model HE-10-A, at 22°C, and the growth medium was watered twice daily. The plants were illuminated continuously by means of four overhead fluorescent lights (20-watt GE Standard Cool White type). During the first day of planting, the pips showed little or no change in size, but from the second to the sixth day a rate of growth of between 1.0 and 1.5 cm per day was recorded. After six days, the height of the shoot (leaves still rolled) was 8—9 cm.

Infiltration of ^{14}C-labeled methionines. Three days after planting (shoot height about 5 cm), the shoot was excised at the base of the stem and placed in a short plastic centrifuge tube (25 × 65 mm) containing 1 ml of the appropriately-labeled methionine in 0.01 N HCl. After about 40 h, nearly all the liquid had been imbibed. Without removing the shoot, 0.5 ml of water was added to the container. When most of the water had been absorbed, another 0.5 ml portion of water was added. Three days after the start of feeding of radioactive methionine the shoot was removed from the tube and used for the isolation of A-2-C.

Extraction and isolation of A-2-C. The excised shoot (or root) was washed carefully with distilled water to remove all radioactive substances superficially attached to the specimen, after which it was sliced very thinly (about 20—25 slices per cm) with a razor blade. (With root tissue a pair of sharpened scissors was used to cut the material into very small pieces.) The tissue was ground in a small mortar with fine glass beads (Superbrite, type 100—5005, Minnesota Mining and Manufacturing Co., St. Paul, Minn.) to disrupt most of the cells and fibrous tissue. The paste was next transferred to a small blendor (Model 1002 Waring blendor, Waring Products Co., Winsted, Conn.) and homogenized for 5 min with 70 % ethanol, approximately 25 ml of liquid being used for each

5 g of tissue. Blending was interrupted at intervals to avoid excessive overheating of the contents. The mixture was then centrifuged at 23 500 g and the supernatant liquid carefully decanted and saved. The residue was again blended with another aliquot of 70 % ethanol. This extraction procedure was performed five times in all. The supernatant liquids were then combined and concentrated *in vacuo* at 40°C to one-tenth of the original volume. The resulting mixture was centrifuged to remove all particles that had precipitated during evaporation, after which the clear supernatant liquid was twice extracted with two volumes of chloroform to remove pigments. The combined aqueous portion was evaporated to about 0.5 ml and streaked on Whatman No. 3 chromatography paper (length of streak, 35 — 38 cm). Descending chromatography was carried out in solvent A for 60 h. Four thin guide-strips from various sections of the paper chromatogram were cut out and dipped in ninhydrin solution (0.2 % ninhydrin in acetone). The area of the paper that contained A-2-C was readily located by observing the development of a characteristic greyish-beige ninhydrin reaction.[1] The imino acid was eluted from the paper with water, and the eluate then concentrated *in vacuo* at 40 — 50°C and rechromatographed in descending fashion either in solvent B for 110 h or in solvent C for 28 h. A-2-C was located as before and the radioactivity on the chromatogram was monitored by means of a Vanguard Model 880 Autoscanner. Perfect coincidence of the ¹⁴C and ninhydrin-reactive A-2-C areas was regularly observed at this stage of purification. After elution of A-2-C from the paper with water, aliquots of the solution were plated on etched-glass planchets one inch in diameter (Autonomos planchets, No. PL-f, B and Z Enterprises, Columbus, Ohio) and counted in the Nuclear-Chicago Model C-110-B low-background counter to precision of better than 5 %.

The commercially-obtained ¹⁴C-labeled methionines were contaminated to the extent of 1 % or less with methionine sulfoxide, methionine sulfone and several other unidentified minor radioactive components. All of the contaminating substances present in the DL-[1-¹⁴C] methionine sample could be completely separated from A-2-C by a paper chromatographic sequence involving the use of solvent A followed by rechromatography of the A-2-C area in solvent C. The contaminants in DL-[2-¹⁴C] methionine were separated from A-2-C by the use of solvent A alone. All the contaminating activities in the uniformly-L-[¹⁴C] methionine preparation were separated from A-2-C by sequential chromatography first in solvent A, followed by rechromatography of the A-2-C area in solvent B. In cutting out A-2-C strips for elution and further chromatography, particular care was taken to use only regions of the paper corresponding to the central location of the A-2-C area as indicated by the ninhydrin reaction. All peripheral areas were sacrificed for the sake of radioactive purity of the final material. As an example of the isotopic homogeneity achieved, the following data give the specific activities (in cpm/mg) on successive recrystallizations from hot, 90 % methanol of the ¹⁴C-labeled A-2-C preparation obtained in an experiment (described below) using uniformly-[¹⁴C] methionine as precursor: 55, 47, 48, 48.*

Quantitative estimation of A-2-C. Since apparently all of the major ninhydrin reactive substances in extracts of *C. majalis* were successfully separated from A-2-C by the above described techniques of descending chromatography in solvent A followed by rechromatography in either solvent B or solvent C, it was both practicable and convenient to determine the imino acid by the ninhydrin method of Moore and Stein.[8] In this procedure 1.0 ml of an aqueous solution of A-2-C (0.02 to 0.20 μmole) derived from the final chromatogram was treated with 1.0 ml of ninhydrin reagent. After mixing, the tube was placed in a boiling water bath for 30 min. On cooling, the absorbance at 570 mμ was recorded in a Beckman DU spectrophotometer against an appropriate blank lacking A-2-C. Standard solutions of A-2-C at three different concentrations were analyzed in each set of determinations.

Decarboxylation of A-2-C. Radioactive A-2-C, isolated from *C. majalis* shoots in the above-described manner, was decarboxylated in Thunberg tubes on heating in the presence of ninhydrin.[9] The contents of each tube consisted of: labeled A-2-C (200 to 400 cpm), crystalline ninhydrin (an amount at least four times greater than the number of micromoles of A-2-C taken), and sodium citrate buffer, pH 2.5 (an amount equal in

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* 100 mg of carrier L-A-2-C were employed, and 3.4 mg of each successive batch of crystals were plated from water and assayed for ¹⁴C.

weight to the ninhydrin used). The total volume in most cases was 3.0 ml.* Each side arm contained an amount of KOH solution (0.40 ml) equivalent to at least twice the amount of A-2-C used, to assure quantitative trapping of CO_2 liberated during the reaction. Using a small amount of silicone grease, the side arm was attached to the tube and held securely in place by rubber bands to be certain of an air-tight system throughout the reaction. The tube was then placed in a boiling water bath for 30 min to effect decarboxylation of A-2-C. After the tube was removed from the bath, 0.3 ml of 12 N sulfuric acid was added quickly through the vent at the side of the tube to provide strongly acidic conditions for the complete liberation of CO_2 from the reaction mixture. The vent was closed and the tube shaken gently for several hours on a rotating platform shaker. An aliquot of the solution in the side arm was then plated into 0.3 ml of a solution of ethanol:0.1 M $BaCl_2$:water (5:3:2 v/v) on an etched-glass planchet, dried under an infrared lamp, and assayed for radioactivity in the Nuclear-Chicago low-background counter.

RESULTS AND DISCUSSION

Net synthesis of A-2-C in growing C. majalis. Before attempting tracer studies, it was of obvious importance to determine whether more A-2-C was accumulated by the aerial or by the root portions of growing *C. majalis* pips. To this end, four pips of identical size and weight were chosen; two were used as controls (*i.e.* the A-2-C content in pooled extracts was determined without allowing the pips to grow), while the other two were grown for six days under conditions described in the section on Methods. At the end of this time, the indicated plant portions were combined, and extracts of each were prepared by the procedures already detailed. After an initial chromatographic separation in solvent A, the appropriate A-2-C-containing areas were eluted and rechromatographed in solvent B. Solutions of A-2-C obtained by elution of the correct areas from the second chromatogram were used for the quantitative estimation of the imino acid with the ninhydrin reagent. The data in Table 1 show that while the amount of A-2-C in the root was not significantly altered, an increase of 30 % in the A-2-C content of the aerial portion of the plants

Table 1. Accumulation of A-2-C in root and aerial portions of *C. majalis*.

Growth period (days)	μmoles A-2-C** in	
	Shoots	Roots
0	35.2	25.2
6	45.6	23.8

occurred during the growth period. Although perhaps less than expected, this extent of A-2-C accumulation seemed to be of sufficient magnitude to justify carrying out subsequent radioactive tracer experiments solely with the operationally-more-convenient aerial portions of the plant.

* With the recrystallized sample of A-2-C derived from the experiment with uniformly-L-[^{14}C] methionine, a final volume of 6 ml was employed because of the greater amount of A-2-C oxidized.

** Values shown are the sum of A-2-C in portions of the two plants used for each isolation.

Biosynthesis of A-2-C from different ^{14}C-labeled radioisomers of methionine.
Table 2 summarizes the data obtained in three experiments that utilized
[1-^{14}C] methionine, [2-^{14}C] methionine, and uniformly-[^{14}C] methionine,
respectively, as tracer substrates for the biosynthesis of A-2-C. Confirmation
of the finding of Leete [5] was provided from the result shown in experiment
1, where it is seen that the carboxyl of methionine effectively labeled A-2-C
solely at the carboxyl group of the imino acid. With ^{14}C at C-2 of methionine
(experiment *2*), a similar extent of labeling of A-2-C occurred. From the de-
gradation, however, it was clear that in this case essentially none of the tracer
was located at the carboxyl position. These combined results were taken as
evidence that the fate of the two labeled carbon atoms was such as to ensure
that they remained metabolically distinct from one another throughout the
course of metabolism of methionine by this plant.

Table 2. Incorporation and distribution of radioactivity in A-2-C isolated from excised shoots
of *C. majalis* exposed to ^{14}C-labeled methionines.

	Methionine administered*			A-2-C isolated			
Experiment	labeled carbon atom(s)	spec.act. (cpm/ mμmole)	amount (μmoles)	spec.act. (cpm/ μmole)	cpm	carboxyl group (cpm)	% ^{14}C in carboxyl group
1	1	1 200	1.57	640	16 600	17 270	104
2	2	1 170	2.14	585	13 600	200	1.5
3	1—4 and methyl	76 600	0.051	295	9 500	2 240**	24

* Both the [1-^{14}C] and [2-^{14}C] labeled compounds were DL mixtures; the uniformly-labeled
sample was the L-isomer.
** Decarboxylation was carried out using thrice-recrystallized A-2-C.

To establish unequivocally that C-2 and C-1 of methionine remained
chemically bonded to one another during the biosynthesis of A-2-C, a chemical
degradation would, of necessity, have to be devised whereby C-2 of the imino
acid could be separated from the other two ring carbon atoms. As an indirect,
although considerably more practical alternative to such an endeavor, an
examination was made of the labeling pattern in A-2-C formed from uni-
formly-[^{14}C] methionine (experiment *3*). Obviously, if biosynthesis took place
with linear retention of C-1 through C-4 of methionine in the manner indicated
below one should expect to find a proportion of 1:3 for the relative distribu-

Methionine   *A-2-C*

tion of label between the carboxyl carbon and the sum of ring carbons 2, 3, and 4, respectively, of the imino acid. A relationship other than this would indicate with certainty that the carbon chain of the precursor had been disrupted prior to incorporation of label from C-1 and C-2 into A-2-C. The results, given under experiment *3* in the table, show that the carboxyl group of A-2-C contained very close to one-fourth of the total ^{14}C in the molecule. Thus, it is apparent that the isotope distribution predicted for direct retention of C-1 through C-4 has been obtained. Although a small extent of non-uniformity in the labeling pattern of uniformly-$[^{14}C]$ methionine could possibly have arisen during the course of its commercial production by the biosynthetic procedures employed,* it is inconceivable that this could be of sufficient magnitude to provide the basis of an alternative explanation for the isotope distribution pattern obtained in experiment *3*.

The foregoing results serve to implicate the linear carbon skeleton of methionine as an ultimate biosynthetic source of A-2-C in *C. majalis*. Clearly, however, it is still not possible at the present stage of the problem to invoke a strong argument for or against the mechanism proposed by Schlenk and Dainko [6] and by Leete.[5] If it were possible to establish that methionine did not initially become converted to a simpler, metabolically-related derivative (*e.g.* homoserine) prior to utilization of its carbon atoms for A-2-C synthesis, the suggested role of S-adenosylmethionine as an intermediate would indeed become an attractive hypothesis. Experimental testing of this idea, however, will doubtless have to await the development of *in vitro* systems capable of carrying out the biosynthesis of A-2-C.

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* Obtained from the mixed proteins of *Chlorella* grown photosynthetically in the presence of $^{14}CO_2$ as sole carbon source.