# Precursor Role of [$^{14}$C,$^{15}$N]-2-Amino-6-(methylthio)caproic Acid in Progoitrin Biosynthesis*

Chiu-Jong Lee and George S. Serif†

ABSTRACT: A new methionine homolog, 2-amino-6(methylthio)caproic acid, has been synthesized, and labeled with $^{14}$C in the C-2 position and $^{15}$N at the amino group. Precursor feeding experiments with rutabaga (*Brassica napobrassica*) leaves demonstrate that the C-2 carbon of the amino acid is incorporated selectively into the C-1 position of product progoitrin while $^{15}$N is effectively incorporated into progoitrin nitrogen. These data support the theory that 2-amino-6-(methylthio)caproic acid is a direct and natural precursor of progoitrin.

The aglycone moieties of plant thioglucosides (glucosinolates) are derived from amino acid precursors (Kjaer, 1954; Ettlinger and Lundeen, 1956). With some thioglucosides common amino acids contribute the total carbon skeleton of the aglycone residue. In other circumstances the amino acid is modified by chain extension through single or multiple condensations with acetate prior to its conversion into thioglucoside. The latter biosynthetic scheme has been shown to obtain with glucobarbarin (Figure 1) derived from phenylalanine (Underhill, 1965) and sinigrin (Figure 1) derived from methionine (Chisholm and Wetter, 1966; Matsuo and Yamazaki, 1966). In the biosynthesis of progoitrin (Figure 2), a homolog of sinigrin, previous work suggests that methionine is converted into the methionine homologs 2-amino-5-(methylthio)valeric acid and 2-amino-6-(methylthio)caproic acid[1] prior to its conversion into progoitrin (Chisholm and Wetter, 1967; Serif and Schmotzer, 1967). The authors, in a preliminary report, have recently demonstrated that [$^{14}$C]$NH_2$MeSCap is an effective precursor of progoitrin (Lee and Serif, 1968). This report extends these preliminary observations to include the chemical synthesis of the new amino acid $NH_2$MeSCap, a verification of the position of the $NH_2$MeSCap carbon chain in the aglycone moiety, and a study of the integrity of the carbon–nitrogen component of $NH_2$MeSCap as a direct precursor of the carbon–nitrogen component of progoitrin.

## Experimental Procedure

*Synthesis of* DL-*2-Amino-6-(methylthio)caproic Acid.* A. DIMETHYL *N*-FORMYL-2-AMINOMALONATE. A solution of sodium nitrite (1.9 g, 27.5 mmoles) in water (2.5 ml) was added dropwise to a stirred solution of dimethyl malonate (1.32 g, 10 mmoles) in glacial acetic acid (1.7 ml) at ice-bath temperatures. After 2 hr the mixture was extracted several times with ether and the ether extracts were washed successively with 1% sodium bicarbonate and with water. Ether was removed from the extracts by a stream of dry nitrogen and the crude product was used directly in the hydrogenation step. Crude dimethyl $\alpha$-nitrosomalonate was dissolved in 10 ml of ethyl alcohol and hydrogenated at atmospheric pressure in the presence of 300 mg of 10% palladium–charcoal catalyst (Hartung *et al.*, 1960) until the theoretical uptake of hydrogen was achieved. The catalyst was filtered from the reaction mixture and solvent was removed under reduced pressure. The residue was dissolved in 25 ml of 90.5% formic acid, 8 ml of acetic anhydride was added to the solution, and the reaction mixture was left overnight at room temperature (Sheehan and Yang, 1958). The reaction was terminated by the addition of 8 ml of ice water. Solvent was removed at reduced pressure. The crude crystalline mass of dimethyl *N*-formyl-2-aminomalonate (1.45 g) was recrystallized first from water and subsequently from toluene. The product recrystallized from toluene melted at 83–84° uncor (lit. (Hellmann and Lingens, 1954) mp 85.5°), and weighed 0.96 g (53% yield based on dimethyl malonate).

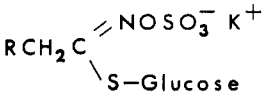
B. 1-CHLORO-4-(METHYLTHIO)BUTANE. Methylmercaptan (5.2 g, 0.108 mole) was added to solution of sodium (2.5 g, 108 mole) in 50 ml of anhydrous ethanol cooled in an ice bath. 1-Bromo-4-chlorobutane (17.1 g, 0.1 mole) was added rapidly with stirring. After formation of NaBr ceased, the reaction mixture was filtered and solvent was removed from the filtrate at reduced pressure. The liquid residue was fractionated by vacuum distillation. 1-Chloro-4-(methylthio)butane (9.6 g 70% yield) was collected over a boiling point range of 68–70° uncor at 11.5 mm. Upon standing the clear distillate became turbid and a white, crystalline, water-soluble deposit formed. Nuclear magnetic resonance spectroscopy was used to identify both the liquid 1-chloro-4-(methylthio)butane and the white crystalline secondary product, *S*-methyltetrahydrothiophenesulfonium chloride, formed by internal cyclization.

C. DL-2-AMINO-6-(METHYLTHIO)CAPROIC ACID. Dimethyl *N*-formyl-2-aminomalonate (17.5 g, 0.1 mole) was added slowly to a solution of 2.3 g (0.1 mole) of sodium metal in 45 ml of absolute ethanol. When solution was achieved, 15 g (0.108 mole) of 1-chloro-4-(methylthio)butane was added

* From the Academic Faculty of Biochemistry and Molecular Biology, College of Biological Sciences, Ohio State University, Columbus, Ohio 43210. *Received January 26, 1970.* These studies were supported by a National Science Foundation (Grant GM-15661), by a U. S. Public Health Service grant (AM-11430), and by the Herman Frasch Foundation.

† Person to whom requests for reprints should be addressed.

[1] Abbreviation used is: $NH_2$MeSCap, 2-amino-6-(methylthio)-caproic acid.



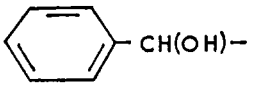
| GLUCOSIDE | R |
|---|---|
| Glucobarbarin |  |
| Sinigrin | $CH_2=CH-$ |
| Progoitrin | $CH_2=CHCH(OH)-$ |

FIGURE 1: Natural thioglucosides with aglycones derived from amino acids.

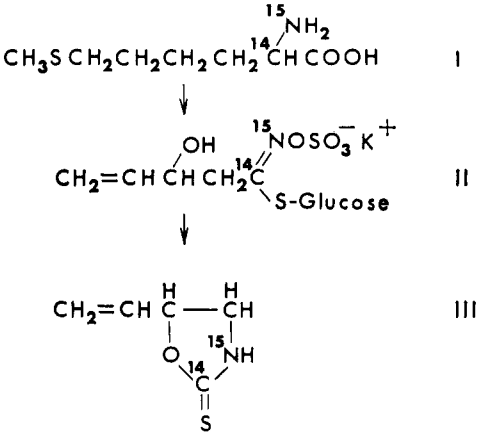


FIGURE 2: Structural interrelationships between $NH_2$MeSCap (I), progoitrin (II), and goitrin (III).

and the reaction mixture was refluxed for 4 hr. Sodium chloride was removed by filtration and the solvent was taken off at reduced pressure. The oily residue was refluxed, first for 1 hr with 10 ml of 2.4 N HCl and then for 3 hr after addition of a further 10 ml of 2.4 N HCl. The hydrolysate was evaporated to dryness at reduced pressure to remove the large excess of HCl. The residue was dissolved in water and passed through an Amberlite IR4B column (free amine form) to remove residual chloride. The effluent solution was decolorized with charcoal and solvent water was removed at reduced pressure. The crude crystalline mass (6.12 g, 34% yield based on *N*-formyl-2-aminomalonate) was recrystallized twice from absolute ethanol. *Anal.* Calcd: C, 47.46; H, 8.47; N, 7.91; S, 18.08. Found: C, 47.44; H, 8.66; N, 8.10; S, 18.02.

*Synthesis of* DL-[$^{15}N$]$NH_2$*MeSCap.* DL-[$^{15}$N]$NH_2$MeSCap was prepared by the above procedure with the exception that [$^{15}$N]sodium nitrite (99%) was used in the nitrosylation step.

*Synthesis of* DL-[2-$^{14}$*C*]$NH_2$*MeSCap.* The synthesis of DL-2-$^{14}$C was begun with commercially available dimethyl [2-$^{14}$C]-*N*-formyl-2-aminomalonate. The above procedures were generally followed with several exceptions. The quantities of starting materials were reduced, *i.e.*, the initial quantity of dimethyl [2-$^{14}$C]-*N*-formyl-2-aminomalonate used was 79.2 mg (0.9 mCi). In addition the free [$^{14}$C]$NH_2$MeSCap product was separated by paper chromatographic procedures. The overall yield of DL-[2-$^{14}$C]$NH_2$MeSCap based on dimethyl [2-$^{14}$C]-*N*-formyl-2-aminomalonate was 35%.

*Feeding* [$^{14}$*C*,$^{15}$*N*]$NH_2$*MeSCap.* Freshly cut rutabaga (*Brassica napobrassica*) leaves were administered $NH_2$MeSCap by inserting their petioles in small volumes of water containing a mixture of $^{14}$C- and $^{15}$N-labeled amino acid. The leaves were then exposed to light (24 hr) by placing the leaves an average distance of 1 ft from four 40-W fluorescent plant lights (Sylvania Gro-Lux). Additional quantities of water were added as needed.

*Isolation of Goitrin.* At the end of the light exposure period the leaves were dropped into boiling methanol (500 ml) and refluxed for 10 min. This permits destruction of endogenous myrosinase and partial extraction of progoitrin. The leaves were subsequently removed from the extracting fluid and homogenized (Waring Blendor) in a 200-ml volume of 80% methanol. The homogenate was refluxed for 10 min and the extract was removed by filtration. Methanol was removed from the combined extracts by distillation at reduced pressure below 40°. The aqueous residue was filtered through Celite and extracted several times with ether. The Lossen rearrangement of progoitrin to goitrin was effected through use of myrosinase preparation activated with ascorbic acid (Tsuruo and Hatta, 1967). The resulting goitrin was extracted with ether, purified by paper chromatography, and identified by ultraviolet and mass spectroscopy (Serif and Schmotzer, 1967).

*Isolation of* $^{15}$*N-Labeled Amino Acids.* The aqueous residue from the goitrin extraction was passed through a Dowex 50 column in the hydrogen form. After washing the column thoroughly with water the adhering amino acids were eluted with 1 N HCl. The eluate was evaporated under reduced pressure, taken up in aqueous solutions, and passed through an Amberlite IR4B column in the free amine form. The eluate, containing the majority of amino acids, was subjected to two-dimensional paper chromatography. The position of alanine was determined through use of an internal standard consisting of a minute amount of $^{14}$C-labeled alanine. Gluta-

TABLE I: Conversion of the Goitrin Thione Carbon into [$^{14}$C]$CO_2$.

| | Goitrin Added | | Distribution of Radioactivity | | | |
|---|---|---|---|---|---|---|
| Expt | Wt[a] (mg) | Act. (dpm) | [$^{14}$C]-$CO_2$ (dpm) | Residue (dpm) | % $^{14}$C Unde-graded | % $^{14}$C as [$^{14}$C]-$CO_2$ |
| 1 | 10.0 | 4748 | 4244 | 322 | 7 | 89 |
| 2 | 9.9 | 5247 | 4686 | | | 89 |

[a] Isolated labeled goitrin samples were diluted with unlabeled goitrin as carrier.

TABLE II: Incorporation of [$^{14}$C,$^{15}$N]$NH_2$MeSCap into Goitrin.

| Expt | $^{14}$C Content of DL-[$^{14}$C]-$NH_2$MeSCap Administered[a] (m$\mu$Ci/$\mu$mole) | $^{15}$N Content of DL-[$^{15}$N]-$NH_2$MeSCap Administered (atom % excess) | $^{14}$C Content of Isolated Goitrin (m$\mu$Ci/$\mu$mole) | $^{15}$N Content of Isolated Goitrin (atom % excess) | | Ratio of $^{14}$C:$^{15}$N in Isolated Goitrin | |
|---|---|---|---|---|---|---|---|
| | | | | Calcd[b] | Found | Calcd | Found |
| 1 | 104.7 | 93.4 | 1.92 | 1.7 | 1.0 | 1:1 | 1:0.59 |
| 2 | 95.7 | 93.9 | 4.34 | 4.2 | 2.0 | 1:1 | 1:0.48 |
| 3 | 95.7 | 93.9 | 4.04 | 3.9 | 2.6 | 1:1 | 1:0.67 |
| | | | | | | | Av 1:0.58 |

[a] In each experiment the leaves were administered 20 $\mu$moles of precursor [$^{14}$C,$^{15}$N]$NH_2$MeSCap. The quantities of [$^{14}$C,$^{15}$N]-goitrin isolated were 42.3 $\mu$moles, 45.8 $\mu$moles, and 51.2 $\mu$moles in expt 1, 2, and 3, respectively. The quantities of $([^{15}N]NH_4)_2SO_4$ derived from [$^{14}$C,$^{15}$N]goitrin, applied to mass spectroscopic studies are in the range of 7–8 $\mu$moles/study. [b] Based on $^{14}$C dilution.

mate and aspartate adhering to the Amberlite column were eluted with 1 N HCl. This eluate was evaporated under reduced pressure and the glutamate and aspartate were separated by paper chromatography. Alanine, aspartic acid, and glutamic acid were removed from their respective chromatograms with water and the amino acid content of the solutions was determined by the ninhydrin method.

*Conversion of the [$^{14}$C]Thione Carbon of Goitrin into [$^{14}$C]$CO_2$.* An aliquot (0.2 ml) of [$^{14}$C]goitrin solution of known specific activity was added to a reaction vessel containing 0.8 ml of concentrated hydrochloric acid. The reaction mixture was heated at 45° for 15 min and then allowed to stand at room temperature for 4 hr. During the entire reaction period a slow stream of $N_2$ swept the [$^{14}$C]COS generated through a quartz tube packed with cupric oxide and silver wire heated at 800°. [$^{14}$C]$CO_2$ formed was trapped directly in Hyamine hydroxide (0.5 M in methanol) and aliquots were subsequently counted by liquid scintillation techniques using internal standards to obtain absolute disintegrations per minute.

*Preparation of $^{15}$N Samples for Mass Spectrometry.* Samples of goitrin and $NH_2$MeSCap to be assayed for $^{15}$N content were digested by microKjeldahl techniques, the resulting $NH_3$ generated was trapped in 0.1 N $H_2SO_4$. Immediately prior to isotopic analysis the $NH_3$ was converted into $N_2$ by oxidation with sodium hypobromite. Nitrogen isotopic abundance was determined using a Finnegan quadrupole mass spectrometer.

Quantitation of $NH_2$MeSCap and amino acids samples was achieved using ninhydrin. $NH_3$ was quantitated by both ninhydrin and Nesslerization procedures. Goitrin concentrations were determined using the goitrin extinction coefficient of $1.51 \times 10^5$ at 240 m$\mu$.

TABLE III: $^{15}$N Content of Selected Amino Acids Derived from Rutabaga Leaves Fed [$^{14}$C,$^{15}$N]$NH_2$MeSCap.

| Expt[a] | Amino Acid | Sample Size ($\mu$moles) | $^{15}$N Content (atom % excess) |
|---|---|---|---|
| 1 | Glutamic acid | 9 | 0.08 |
| 2 | Alanine | 11 | 0.63 |
| 2 | Aspartic acid | 27 | 0.12 |

[a] Experiment numbers refer to those of Table II.

## Results

The results of degradation studies with [$^{14}$C]goitrin derived from rutabaga leaves fed [2-$^{14}$C]$NH_2$MeSCap are presented in Table I. Small quantities of residual $^{14}$C are seen to remain in the reaction vessel after conversion of the thione carbon into $CO_2$. This $^{14}$C residue may be due to a minor $^{14}$C impurity in the goitrin preparation or to incomplete reaction. Approximately 89% of the $^{14}$C in the goitrin sample, however, is converted into [$^{14}$C]$CO_2$.

Table II presents a summary of the data concerning the $^{14}$C,$^{15}$N content of precursor $NH_2$MeSCap and isolated product goitrin. [$^{15}$N]$NH_2$MeSCap is approximately 55% as effective as [$^{14}$C]$NH_2$MeSCap as a precursor of progoitrin.

Table III demonstrates the exchange of $^{15}$N label from $NH_2$MeSCap with selected amino acid pools. Alanine appears most heavily labeled with $^{15}$N. However the variables of pool sizes and turnover rate limit the evaluation of the significance of this observation.

## Discussion

The data of this study support the theory that the C-2 position of the amino acid $NH_2$MeSCap is a specific and efficient precursor of the thione carbon of goitrin and hence the C-1 carbon of progoitrin (Figure 2). This information, taken in conjunction with earlier studies with $^{14}$C-labeled methionine and acetate (Chisholm and Wetter, 1967; Serif and Schmotzer, 1967), strongly suggest that $NH_2$MeSCap may provide the total carbon skeleton of the progoitrin aglycone.

Although the efficiency of incorporation of [$^{15}$N]-$NH_2$MeSCap into progoitrin is less than that of [$^{14}$C]$NH_2$-

MeSCap ($^{14}C$:$^{15}N$ = 1.0:0.55) this deviation from a 1:1 relationship of $^{14}C$:$^{15}N$ in product progoitrin may reflect transamination or oxidative deamination of DL-$NH_2$MeSCap with subsequent loss of $^{15}N$ label to the amino acid pool (Table III). Matsuo and Yamazaki (1966) observed similar losses of $^{15}N$ when studying DL-[$^{14}C$,$^{15}N$]homomethionine incorporation into sinigrin. They ascribed these losses to utilization of the unnatural D form of the amino acid after its deamination to the corresponding keto acid. Similar circumstances may obtain with D-$NH_2$MeSCap.

The efficiency of utilization of $NH_2$MeSCap in progoitrin formation (Lee and Serif, 1968), the retention of the carbon–nitrogen bond of $NH_2$MeSCap and the specific incorporation of the C-2 of $NH_2$MeSCap into the C-1 position of progoitrin all support the precursor role for $NH_2$MeSCap outlined in Figure 2 and suggest the existence of $NH_2$MeSCap as a normal plant metabolite.

Acknowledgment

The authors are indebted to Dr. William Bulen of the Kettering Foundation at Yellow Springs for his kindness in providing the mass spectrometer and techniques for analysis of $^{15}N$ samples. The authors also acknowledge the excellent technical assistance of Mr. Samuel Lough, Mr. Robert Land, and Mrs. Ann Moreland.

References

Chisholm, M. D., and Wetter, L. R. (1966), *Can. J. Biochem. 44*, 1625.

Chisholm, M. D., and Wetter, L. R. (1967), *Plant Physiol. 42*, 1726.

Ettlinger, M. G., and Lundeen, A. J. (1956), *J. Am. Chem. Soc. 78*, 4172.

Hartung, W. H., Beaujon, J. H. R., and Cocola, G. (1960), *Org. Syn. 40*, 24.

Hellmann, H., and Lingens, F., (1954), *Z. Physiol. Chem. 297*, 283.

Kjaer, A. (1954), *Acta Chem. Scand. 8*, 1110.

Lee, C., and Serif, G. S. (1968), *Biochim. Biophys. Acta 165*, 569.

Matsuo, M., and Yamazaki, M. (1966), *Biochem. Biophys. Res. Commun. 25*, 269.

Serif, G. S., and Schmotzer, L. A. (1967), *Phytochemistry 7*, 1151.

Sheehan, J. C., and Yang, D. H. (1958), *J. Am. Chem. Soc. 80*, 1154.

Tsuruo, I., and Hatta, T. (1967), *Agr. Biol. Chem. Tokyo 31*, 27.

Underhill, E. W. (1965), *Can. J. Biochem. 43*, 189.

# Solid-Phase Synthesis of [5-Glutamine]-α-melanotropin*

James J. Blake, Robert W. Crooks, and Choh Hao Li

ABSTRACT: The tridecapeptide [5-glutamine]-α-melanotropin, a new analog of the hormone, has been synthesized by the solid-phase method.

The fully protected tridecapeptide was cleaved from the resin by methanolysis in dimethylformamide–methanol–triethylamine. Ammonolysis of the methyl ester was effected in dimethylformamide–ethylene glycol–water; the product was then reduced with sodium in liquid ammonia and purified chromatographically to give the highly purified and biologically active tridecapeptide.

Fractionation of the pituitary extracts (Lerner and Lee, 1962; Li, 1959) from several animal species has led to the isolation of a tridecapeptide designated as α-melanotropin (α-MSH).[1] The structure has been shown to be identical in the species investigated, namely *N*-acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalinamide. In addition to its ability to darken the skin of certain amphibians, α-MSH has been shown to possess a variety of other biological activities (Bowers *et al.*, 1964; Krivoy and Guillamin, 1961). Synthesis of the natural hormone by classical techniques has been reported by two groups (Guttmann and Boissonnas, 1959; Schwyzer *et al.*, 1963), and the synthesis of a variety of tridecapeptide and smaller peptide analogs has also been described (Schröder and Lübke, 1966). Recently we reported the synthesis by the solid-phase method of a heptapeptide, methionylglutamylhistidylphenylalanylarginyltryptophylglycine (Blake and Li, 1968), an active core of α-MSH. As an extension of that synthesis, we have synthesized the peptide [5-glutamine]-α-MSH, a new analog of α-MSH, which is reported here.

The synthesis of [5-glutamine]-α-MSH followed the Merrifield procedure (Merrifield, 1964) with the modifications previously reported (Blake and Li, 1968). Deblocking of the Boc group was accomplished with 3.6 N HCl in dioxane, using β-mercaptoethanol to protect tryptophan from acid-

* From the Hormone Research Laboratory, University of California, San Francisco, California 94122. *Received February 2, 1970.* This work was supported in part by grants from the National Institutes of Health of the United States Public Health Service (GM-2907).

[1] Abbreviations used are: MSH, melanocyte-stimulating hormone, melanotropin; Boc, *t*-butyloxycarbonyl. All amino acids occurring in the peptides mentioned in this paper are of the L configuration with the exception of glycine.