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BIOSYNTHESIS OF THE AGLYCONES OF PLANT THIOGLUCOSIDES—I.

PRECURSOR STUDIES OF THE AGLYCONE OF PROGOITRIN*

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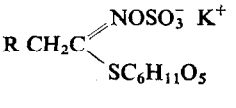
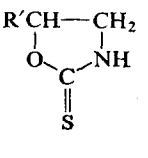
Abstract—Excised leaves from rutabaga plants have been demonstrated to synthesize the thioglucoside progoitrin. Labeled precursor studies have revealed that the most effective precursors of the progoitrin aglycone are methionine and acetate. Carbon label from 2-^{14}C-methionine and 2-^{14}C-acetate is incorporated into the aglycone structure whereas ^{14}C-label from methyl-labeled methionine and carboxyl-labeled methionine or acetate is not. Partial degradation studies with progoitrin have shown that approximately 38 per cent of the incorporated acetate label is present in the oxime carbon of the aglycone. No activity is found in this position, however, when 2-^{14}C-methionine is utilized as a precursor. These data are consistent with a biogenetic scheme for progoitrin involving extension of the methionine chain through successive additions of a chain-building unit derived from acetate.

INTRODUCTION

THIOGLUCOSIDES are a structurally intriguing class of plant products whose mode of biosynthesis is just beginning to be investigated and whose function in plant cell metabolism is now only guessed at. One of the more interesting of these compounds is the thioglucoside progoitrin (Fig. 1) which, upon action of the plant enzyme myrosinase, is converted to the oxazolidine-thione, goitrin (Fig. 1). This derivative has been demonstrated by Greer and his associates to be a potent antithyroid substance.[1]





R	*R'*
CH_2=CH— Sinigrin	CH_2=CH— Goitrin
CH_2=CH CH— Progoitrin (OH)	CH_2=$CHCH_2$— Napoleiferin
CH_3 SCH_2 CH_2— Glucoibervirin	

FIG. 1. STRUCTURES OF THIOGLUCOSIDES AND OXAZOLIDINETHIONES.

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[1] M. A. GREER, *J. Am. Chem. Soc.* **78**, 1260 (1956); *Arch. Biochem. Biophys.* **99**, 369 (1962).

With a number of thioglucosides, structural similarities have linked their aglycone moieties to specific amino acids as precursors.[2] Subsequent labeled precursor studies have verified several of these relationships[4, 5] and have led to the tentative conclusion that amino acids are the primary sources of thioglucoside aglycones. The aglycone of progoitrin, however, bears no direct structural relationship to a known amino acid. It is of interest, therefore, to investigate the mode of biogenesis of the aglycone of progoitrin and determine its prime amino acid precursor. This report represents the results of such a study.

RESULTS

Initial precursor studies were attempted with simple acids labeled with ^{14}C. Table 1 presents the results of these studies obtained by using formate, acetate, propionate, pyruvate, malonate, and lactate. It is apparent from these data that, within this group, the methyl carbon of acetate is the most efficient precursor of progoitrin. Since considerable variation in the specific activities of progoitrin might occur as a function of variables related to plant

TABLE 1. UTILIZATION OF ^{14}C-LABELED ACIDS IN PROGOITRIN BIOGENESIS

Precursor†	Specific activity† (product)	Specific activity relative to acetate
	d/m/mμmole	
^{14}C-Formate	0·4	0·2
1-^{14}C-Acetate	NI*	—
2-^{14}C-Acetate	8·2	1·0
1-^{14}C-Propionate	0·3	<0·1
3-^{14}C-Propionate	NI	—
1-^{14}C-Malonate	NI	—
2-^{14}C-Malonate	1·2	0·4
2-^{14}C-Pyruvate	0·7	0·2
2-^{14}C-DL-Lactate	0·3	0·2

* Not incorporated, i.e. less than 0·3 d/m/mμmole.
† Uptake of precursor was established to be better than 99% in all cases.
All were 0·5 mμc/mμmole.

age, leaf selection and cutting, etc., it was decided that in this and all subsequent precursor studies a simultaneous 2-^{14}C-acetate control would be carried with each precursor compound evaluated. This permitted some assessment of the capability of each group of leaves to incorporate a standard precursor and hence normalized deviations. The relative specific activities expressed in Table 1 are the result of this approach.

Among the commonly occurring amino acids, lysine is the only potential precursor of progoitrin with a carbon chain length sufficient to completely supply the aglycone carbon unit. Decarboxylation together with loss of ammonia from the ε-carbon of the amino acid could yield a 5-carbon chain with an ethylenic bond in the appropriate position.[3] Because of

[2] A. KJAER, *Acta Chem. Scand.* **8**, 1110 (1954); M. G. ETTLINGER and A. J. LUNDEEN, *J. Am. Chem. Soc.* **78**, 4172 (1956).
[3] M. E. DAXENBICHLER, C. H. VANETTEN and I. A. WOLFF, *Biochemistry* **4**, 318 (1965).
[4] E. W. UNDERHILL, M. D. CHISHOLM and L. R. WETTER, *Can. J. Biochem. Physiol.* **40**, 1505 (1962).
[5] E. W. UNDERHILL, *Can. J. Biochem. Physiol.* **43**, 189 (1965).

these possible relationships (UL)-^{14}C lysine was the first amino acid evaluated as a precursor of progoitrin. No effective incorporation of lysine radioactivity was observed. As a consequence the study was extended to amino acids with shorter carbon chains. The results of this study are presented in Table 2. It is apparent from these data that (UL)-^{14}C methionine is a relatively specific amino acid precursor of progoitrin. In order to gain some insight into the extent of incorporation of the total methionine molecule, variously labeled methionine preparations were examined with respect to their efficacy as precursors (Table 3). The fact

TABLE 2. INCORPORATION OF UL-^{14}C-AMINO ACIDS INTO PROGOITRIN

UL-^{14}C-amino acid†	Specific activity (product)	Specific activity relative to acetate
	d/m/mμmole	
L-Lysine	NI*	—
L-Threonine	0·5	0·1
L-Tyrosine	NI	—
L-Alanine	1·4	0·2
L-Serine	1·6	0·2
L-Glutamate	NI	—
L-Aspartate*	NI	—
L-Glycine	3·0	0·3
L-Ornithine	0·3	<0·1
L-Methionine	21·8	3·4
L-Cystine	NI	—

* No incorporation, i.e. specific activities less than 0·3.
† Uptake of precursor was established to be better than 99% in all cases.
All were 0·5 mμc/mμmole.

TABLE 3. INCORPORATION OF VARIOUSLY LABELED METHIONINES INTO THE PROGOITRIN MOLECULE

Methionine label	Specific activity of progoitrin relative to acetate
(UL)-^{14}C-L-Methionine	3·4
Carboxyl-^{14}C-DL-Methionine	NI*
2-^{14}C-DL-Methionine	6·6
Methyl-^{14}C-DL-Methionine	NI

* No incorporation.

that neither carboxyl-, nor methyl-labeled methionine is incorporated into progoitrin is suggestive of a biogenetic scheme involving decarboxylation and loss of a thiomethyl group from the amino acid progenitor.

Undoubtedly 2-^{14}C acetate and possibly 2-^{14}C-methionine, as well, contribute to the glucose pool. As a consequence the glucose moiety of progoitrin is probably labeled to some degree when these effective progoitrin precursors are used. In order to determine the relative extent of the incorporation into glucose and the aglycone, samples of progoitrin were isolated from plants fed 2-^{14}C-methionine and 2-^{14}C-acetate and treated in the following way. Specific activity values of the progoitrin samples were determined using the anthrone procedure described in the Methods section. Subsequently the progoitrin samples were degraded, through the action of myrosinase to goitrin and glucose. Specific activity values were

then obtained for both these products as described in the Methods section and compared to the original progoitrin specific activities (Table 4). Greater than 95 per cent of the label from methionine is seen to be incorporated into the aglycone. As expected, a greater percentage of the acetate label is incorporated into the glucose moiety although nearly 80 per cent of the activity in progoitrin derived from this precursor is in the aglycone.

TABLE 4. SPECIFIC ACTIVITIES OF AGLYCONE AND GLUCOSE MOIETIES OF PROGOITRIN

	Specific activity (dpm/mμmole)		
Labeled precursor	Progoitrin	Glucose	Goitrin
2-^{14}C-Acetate	8·7 (100%)*	1·5 (17%)	6·9 (79%)
2-^{14}C-Methionine	75·2 (100%)	2·8 (4%)	75·1 (99%)

* Per cent of original progoitrin specific activity.

Insight into the manner of insertion of acetate and methionine into the progoitrin aglycone may be obtained through an investigation of the accessibility of ^{14}C-label from acetate and methionine to the oxime carbon of this structure. During enzymatic rearrangement of progoitrin the oxime carbon of the thioglucoside becomes the thione carbon of goitrin. Baltzly and Buck[9] have demonstrated that oxazolidones are decomposed to CO_2 and the corresponding hydroxy-amines upon exposure to concentrated mineral acid at room temperatures. Underhill[5] has used this information to obtain the thione carbon from glucobarbarin, an oxazolidinethione. With the sulfur analogue the carbon is released as COS and must be passed over heated CuO in order to generate CO_2. Using the techniques of Underhill, it was observed in the present study that CO_2 was readily obtained from goitrin. Indeed, the release of the thione carbon as CO_2 appears quantitative (Table 5). This quantitative yield permits estimation of the ^{14}C content of the thione (oxime) carbon relative to the total ^{14}C in the molecule by a simple comparison of the ^{14}C present in the CO_2 generated to the initial

TABLE 5. CONVERSION OF THE THIONE CARBON OF GOITRIN TO CO_2

Expt. No.	Goitrin wt.	Barium carbonate		Recovery
		Theoretical	Actual	
	(mg)	(mg)	(mg)	%
1	41·0	62·7	65·9	105
2	40·5	61·8	66·7	108

Goitrin was added to concentrated HCl in a closed system and the solution swept with CO_2-free N/ for a 4 hr period. Evolved COS was passed in the N_2 stream through a quartz tube containing CuO and Ag wire heated to approximately 800°. CO_2 generated was trapped in CO_2-free NaOH and precipitated by the addition of $BaCl_2$. $BaCO_3$ was filtered, washed, dried and weighed.

[6] O.-E. VON SCHULTZ and R. GMELIN, *Z. Naturforsch.* 9b, 27 (1954).
[7] I. TSURUO and T. HATTA, *Agr. Biol. Chem. Tokyo* **31**, 27 (1967).
[8] E. B. ASTWOOD, M. A. GREER and M. G. ETTLINGER, *J. Biol. Chem.* **181**, 121 (1949).
[9] R. BALTZLY and J. S. BUCK, *J. Am. Chem. Soc.* **62**, 164 (1940).

^{14}C content of the goitrin sample. With 2-^{14}C-acetate as the initial precursor, this procedure yielded a ^{14}C content in the oxime carbon of progoitrin of 37–38 per cent (Table 6, Experiments 1 and 2). In order to verify this value, additional experiments were conducted (Table 6,

TABLE 6. ^{14}C INCORPORATION INTO THE THIONE CARBON OF GOITRIN FROM 2-^{14}C-ACETATE AND 2-^{14}C-METHIONE

Experiment no. and precursor	Goitrin wt.	Goitrin activity	Goitrin specific activity	Total activity of $^{14}CO_2$	Specific activity of $^{14}CO_2$	% total ^{14}C in thione carbon
	(mg)	(dpm)	(dpm/μmole)	(dpm)	(dpm/μmole)	(%)
1 (Acetate)	11·9	3125		1197		38*
2 (Acetate)	9·9	3125		1144		37*
3 (Acetate)	10·0		9·9		3·2	33†
4 (Acetate)	41·0		10·8		3·8	35†
5 (Methionine)	6·5	8490		0		0

* Calculation based on 100% conversion of thione carbon to CO_2.

† Calculation based on specific activities of goitrin and CO_2.

Goitrin of known specific activity and weight was treated as described in Table 5 to generate $Ba^{14}CO_3$. For counting purposes the $^{14}CO_2$ was regenerated from this $Ba^{14}CO_3$ in a closed system and trapped in 1 N Hyamine (methanolic). The hyamine solution was then counted by liquid scintillation techniques using internal standards to obtain absolute d.p.m.

Experiments 3 and 4) in which ^{14}C content of the thione (oxime) carbon was determined by a comparison of the specific activities of the initial goitrin sample and generated $^{14}CO_2$. Closely agreeing values were obtained by these two methods. When 2-^{14}C-methionine was the initial precursor, no ^{14}C activity was detected in the thione (oxime) carbon (Table 6, Experiment 5). This absence of incorporation of label from 2-^{14}C-methionine into the thione (oxime) carbon clearly indicates that the carbon–nitrogen unit of the aglycone is not derived from its counterpart in the precursor amino acid.

DISCUSSION

This study implicating methionine in biogenesis of the aglycone of progoitrin is, in the authors' knowledge, the second instance of a precursor role for methionine in thioglucoside formation. Reports by Chisholm and Wetter[10] and Matsuo and Yamazaki[12] have shown that methionine is a prime precursor of the aglycone of sinigrin (Fig. 1). The two thioglucosides bear some resemblance in that they both possess terminal ethylenic bonds. Matsuo and Yamazaki[12] and Chisholm and Wetter[11] have suggested in the case of sinigrin that this double bond arises during the elimination of the thiomethyl group of the original methionine precursor. They base this conclusion on the precursor studies with sinigrin and on the observation that the thioglucoside glucoibervirin (Fig. 1), isolable from the same plant sources as sinigrin, possesses the same structure as sinigrin with the addition of a methyl thiol group across the double bond. Tentatively assuming the validity of this prediction, it is most probable that the ethylenic bond of progoitrin has a similar origin. In order that methionine function as a precursor of sinigrin and progoitrin in the above manner, it is necessary that extension of the carbon chain occur through condensation at the carboxyl end.

[10] M. D. CHISHOLM and L. R. WETTER, *Can. J. Biochem.* **42**, 1033 (1964).
[11] M. D. CHISHOLM and L. R. WETTER, *Can. J. Biochem.* **44**, 1625 (1966).
[12] M. MATSUO and M. YAMAZAKI, *Biochem. Biophys. Res. Commun.* **24** 786 (1966).

Since aspartate is an effective precursor of methionine in plants, it is puzzling to observe little incorporation of this amino acid into progoitrin (Table 2), particularly since aspartate has been shown to be an effective precursor of sinigrin.[4] One possible explanation of this phenomenon is that the aspartate pool in rutabaga may be of sufficient size to effectively dilute the aspartate label.

Both progoitrin and sinigrin utilize the methyl carbon of acetate as effective precursors of what is probably the chain-building unit. Chisholm and Wetter have proposed a chain-building sequence for sinigrin involving a condensation of acetate with the α-keto acid derived from methionine.[10] A similar reaction sequence is possible for the aglycone of progoitrin with the addition of one further acetate moiety to provide the 5-carbon chain (Fig. 2).

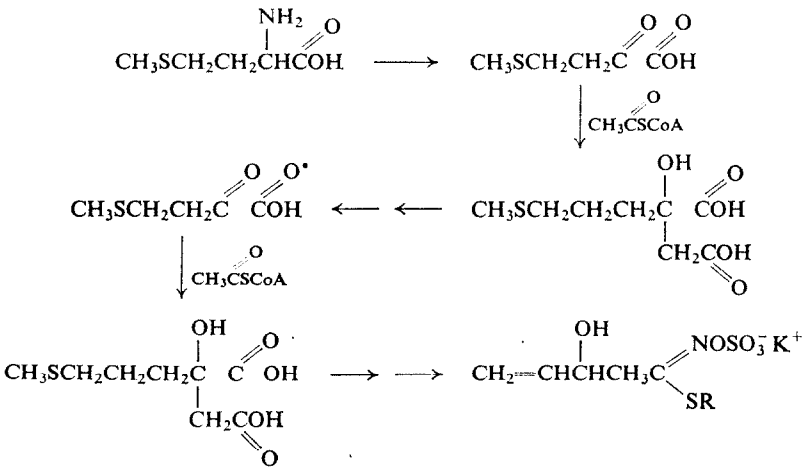

FIG. 2. POTENTIAL BIOGENETIC SCHEME FOR PROGOITRIN.

The degradation data of this report are consistent with such a pathway. Thus label from 2-^{14}C-methionine is not incorporated into the oxime carbon of progoitrin whereas label from 2-^{14}C-acetate is extensively incorporated into this position.

Matsuo and Yamazaki have reported that malonate, as well as acetate, is effective as a chain-building unit for sinigrin.[12] Indeed, they suggest that the incorporation of acetate observed by Chisholm and Wetter in sinigrin[10] is accomplished through prior conversion of acetate to malonate, the primary condensing unit. Evidence from the current study, supporting a similar role for malonate in progoitrin synthesis, is slight. Label from 2-^{14}C-malonate is incorporated into the aglycone of progoitrin but the compound appears to be much less effective as a precursor when compared to acetate. In addition, in experiments in which unlabeled malonate (40 μmoles) was added to the 2-^{14}C-acetate (20 μmoles) fed to rutabaga leaf preparations, no dilution in the specific activity of the derived goitrin was observed. Caution is necessary in interpretation of these data, however, since it is possible that the precursor role of malonate is masked in whole cell plant studies through inherent differences in the membrane transport of doubly and singly charged molecules.[13] More intensive study of the precursor role of malonate is indicated.

Chain extensions in rutabaga beyond the carbon chain length observed in progoitrin is a distinct possibility. Tapper and MacGibbon have recently characterized napoleiferin

[13] S. L. RANSON, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 493. Academic Press, New York (1965).

(C_6H_9ONS) the methylene analogue of goitrin (Fig. 1), from turnip and rape.[14] In addition, samples of rutabaga goitrin, when subjected to high resolution mass spectrometry, occasionally display evidence of an impurity with a prominent parent peak at $M/e=143$ whose calculated empirical formula is C_6H_9ONS. Further studies with rutabaga will be directed to an evaluation of the limit to which chain extension is carried as well as to a detection of the intermediates involved in the overall process.

EXPERIMENTAL

Rutabaga seeds (American Purple Top) were germinated and grown in soil under greenhouse conditions to a minimum age of 2 months. Preliminary studies with the specific precursor ^{35}S-sulfate indicated that excised leaves derived from these and older plants were capable of synthesizing progoitrin when exposed to labeled precursor and light for a 24-hr period. Consequently these preparations were used for subsequent precursor studies. Sixty g lots of leaves were removed and fed labeled materials through insertion of the freshly cut petioles in small volumes of water containing 20 μmoles of ^{14}C-labeled precursors whose specific activities were maintained at 0·5 mc/mmole. Continuous exposure to light (24 hr) was effected by placing the plant leaves at an average distance of 1 ft from four 40 W fluorescent plant lights (Sylvania Gro-Lux). Progoitrin was extracted and purified in the following typical way. At the end of the exposure period, the leaves were dropped into boiling methanol (500 ml) and refluxed for 10 min. This permits destruction of myrosinase and partial extraction of thioglucoside. Leaves were subsequently removed from the extracting fluid and homogenized (Waring blendor) in a 200 ml volume of 80 per cent methanol. The homogenate was refluxed for 10 min and the extract removed by filtration. Methanol was removed from the combined extracts by distillation at reduced pressure below 40°. The aqueous residue was extracted with ether and then passed through an Amberlite IR-4B column (7 × 100 mm) in the Cl form. The column was washed extensively with distilled water until neither color nor radioactivity was detectable in the effluent. The thioglucoside was then eluted with 0·1 N NaCl. The eluate was lyophilized, the residue extracted with ethanol, spotted to Whatman 3 MM and developed by the descending technique in an ethanol:water solvent (85:15). Labeled components were visualized by radio-autography. Considerable streaking is caused by the presence of NaCl in the ethanol extract. Since NaCl remains at the origin in this solvent, the principal thioglucoside spot was eluted and rechromatographed in the same solvent system. The major thioglucoside (R_f 0·7) was eluted from the chromatogram with water. Identity of these small quantities of the labeled thioglucoside with authentic samples of progoitrin* was established by using several criteria. R_f values were determined for both known and unknown samples in several solvents and found to be identical. Degradation of labeled progoitrin to goitrin permitted similar R_f comparisons for this form of the aglycone. An u.v. absorption maximum at 240 nm, typical for oxazolidinethiones, was observed for both the labeled and authentic goitrin samples. Low resolution mass spectrometer patterns for labeled goitrin and authentic samples were similar with a strong parent peak at $M/e+129$. High resolution mass spectrometry established this labeled peak as possessing an empirical formula identical with the theoretical formula of goitrin (C_5H_7ONS).

Specific activity determinations were conducted on isolated labeled progoitrin samples in the following manner. Thioglucoside concentrations were determined by a minor modification of the anthrone procedure of Schultz and Gmelin.[6] The original protocol of these authors suggested incubation of thioglucosides with anthrone reagent in boiling water bath temperatures for 10 min followed by rapid cooling and direct optical density determination at 620 nm. Color intensity values obtained in this manner for thioglucosides were less than one-half those obtained with stoichiometric amounts of glucose. In addition, in our hands, the method provided variable color values with progoitrin. As a consequence, the protocol was altered such that after initial heating at boiling water temperatures for 10 min, the reaction tubes were permitted to stand at room temperature for a minimum period of 4 hr. At the end of this time color values were stable and nearly identical to those obtained with stoichiometric amounts of free glucose. The ^{14}C-content of isolated progoitrin was determined using standard liquid scintillation techniques.

Conversion of progoitrin to goitrin and glucose was effected through use of a myrosinase preparation activated with ascorbic acid.[7] Goitrin formed in this manner was extracted with ether and rechromatographed in the ethanol:water solvent. Free glucose was obtained from the reaction mixture after lyophilization and chromatography in the ethanol:water solvent. Specific activity of goitrin was determined through use of its extinction coefficient at 240 nm[8] and measurement of ^{14}C content by liquid scintillation techniques. Free glucose specific activities were determined through use of the anthrone reagent and liquid scintillation techniques.

Degradation of the thione carbon of goitrin to carbon dioxide was achieved by the procedure of Underhill.[5]

[14] B. A. Tapper and D. B. MacGibbon, *Phytochem.* **6**, 749 (1967).

* Authentic samples of goitrin and progoitrin were kindly supplied by Dr. Monte Green.