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BIOSYNTHESIS OF SOME SULFUR-CONTAINING NATURAL PRODUCTS. INVESTIGATIONS OF THE MECHANISM OF CARBON-SULFUR BOND FORMATION

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INTRODUCTION

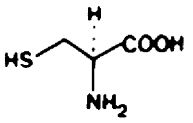
Sulfur-containing natural products occur in virtually all living organisms One group consists of the primary metabolites that are essential to life Examples of compounds falling into this category include the amino acid cysteine (**1**) and the enzyme cofactors biotin (**2**), lipoic acid (**3**), and thiamine (**4**) (Fig 1) The second group of natural sulfur compounds contains the so-called secondary metabolites, which are believed to be non-essential to the producing organisms Representative examples of compounds of this type include the antibiotic isopenicillin N (**5**), the mustard oil glucoside sinigrin (**6**), and α-terthienyl (**7**), which is isolated from marigolds Many additional illustrations can be found in recent reviews [1,2]

It is apparent that naturally occurring sulfur compounds exhibit enormous structural variety Investigations of the biosynthesis of this rich panoply of substances can therefore be expected to reveal a corresponding richness in the underlying biochemical pathways leading to these compounds Nevertheless, one might expect that a limited number of mechanisms would be utilized by Nature to introduce sulfur into these diverse carbon skeletons This review is a somewhat personalized account of the current state of knowledge regarding the mechanism of C-S bond formation in the biosynthesis of biotin, lipoic acid, and asparagusic acid In addition, the final section of the review contains a brief discussion of the mechanism of C-S bond formation accompanying the biosynthesis of the penicillins

BIOTIN

The vitamin biotin (**2**) was first isolated from egg yoke by Kogl and Tönnis in 1936 [3] Elucidation of the structure of the compound by du Vigneaud *et al* required an additional seven years,[4,5] and over a decade elapsed before the role played by biotin as a carbon dioxide carrier in carboxylation reactions became clear [6-12] The mechanistic and structural aspects of biotin containing enzymes are still the object of extensive scrutiny [13]

Clues to the biosynthesis of biotin were first obtained from early observations by Mueller[14-16] and du Vigneaud [17] Mueller found that pimelic acid (**8**) served as a growth factor for *Corynebacterium diphtheriae*, while du Vigneaud showed that this requirement could be satisfied by very low concentrations of biotin du Vigneaud also discovered[18] that dethiobiotin (**13**) was as effective as biotin in supporting the growth of *Saccaromyces cerevisiae* These initial clues were eventually followed up by a number of investigators who utilized radiotracer methods and mutants to elucidate the major steps in the biosynthesis of biotin [19] The studies with *E coli* mutants culminated in the pathway for biotin biosynthesis outlined in Scheme 1 [20] This route proceeds by condensation



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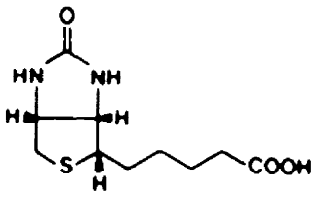

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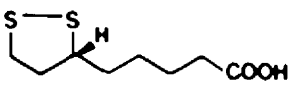

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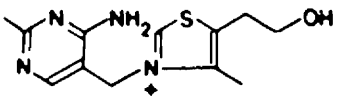

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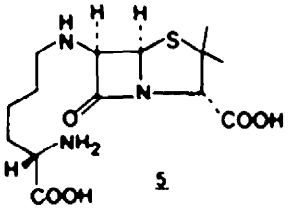

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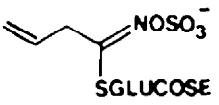

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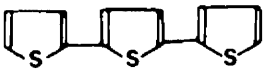

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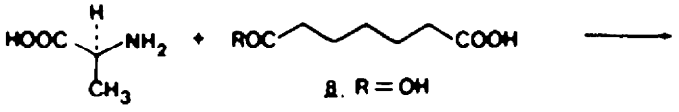
Fig 1



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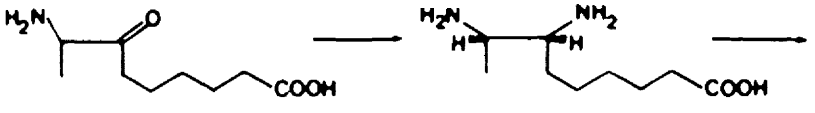
8. R = OH

10. R = SCoA



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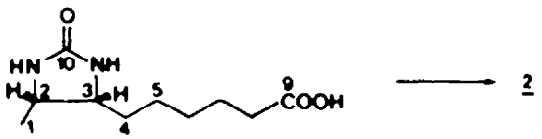


13 2

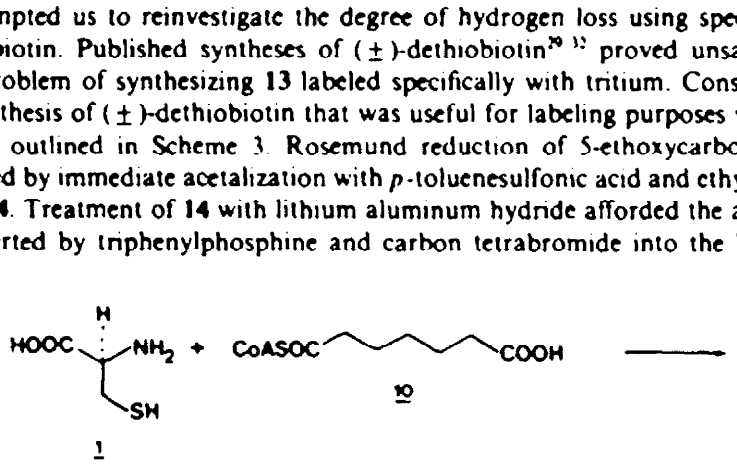
Scheme 1

of *L*-alanine (**9**) with pimeloyl CoA (**10**) to give 7-oxo-8-aminopelargonic acid (**11**). Transamination then leads from **11** to 7,8-diaminopelargonic acid (**12**). The latter substance subsequently reacts with carbon dioxide in the presence of ATP to generate dethiobiotin (**13**). Finally, sulfur is introduced into dethiobiotin to yield biotin. On the other hand, tracer studies with *Achromobacter* led to the hypothesis[21] that biotin is biosynthesized from *L*-cysteine (**1**) and pimeloyl CoA according to Scheme 2. This apparent dichotomy in the pathways to biotin has recently been resolved by Marquet who reexamined[22] the biosynthesis of biotin in *Achromobacter*. Precursor incorporation experiments with [3-^{14}C,^{35}S]-*L*-cysteine failed to yield radioactive biotin. However, the same organism converted [2,3-^{3}H, 9-^{14}C]-(±)-dethiobiotin into biotin without change in the tritium to carbon-14 ratio. It therefore appears that the biosynthesis of biotin in *Achromobacter* follows the same pathway as observed in other microorganisms (*vide infra*).

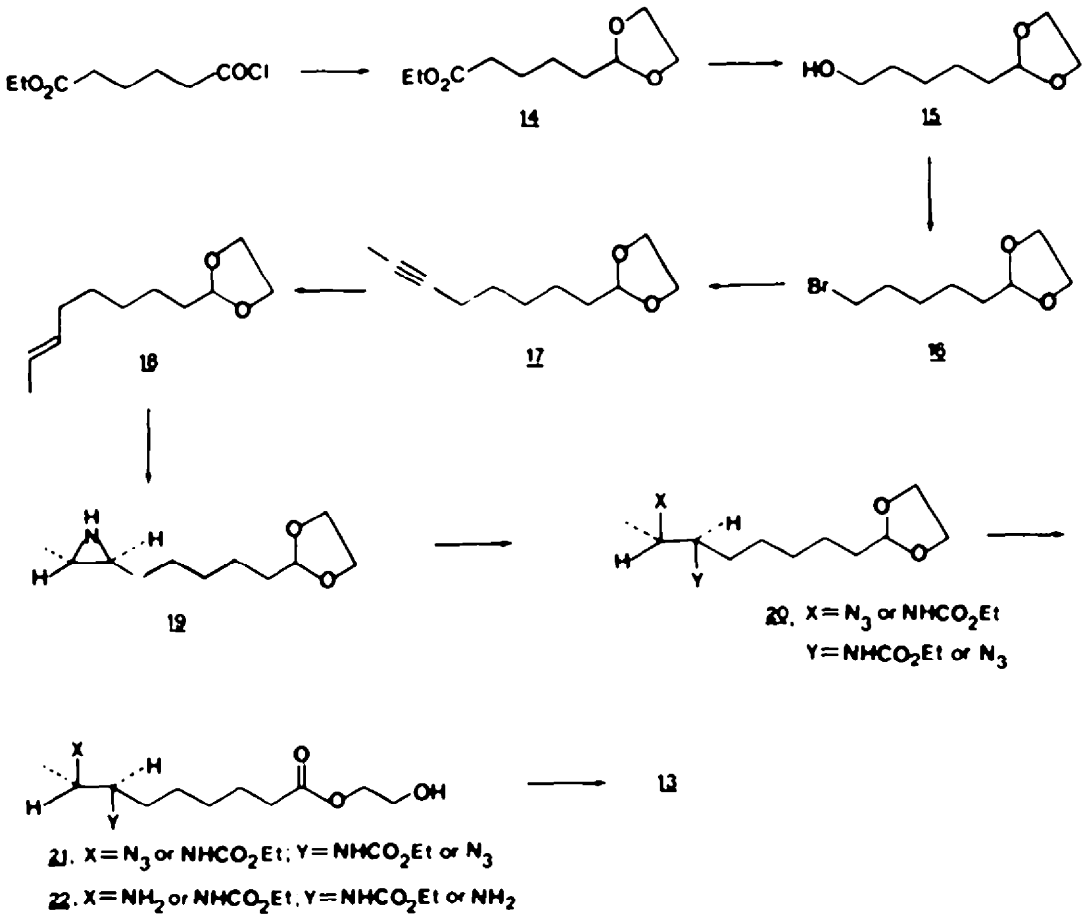
The most unusual step in the biosynthesis of biotin is undoubtedly the conversion of dethiobiotin into biotin. At the present time, there are two major unsolved problems connected with this transformation. One problem is the nature of the sulfur donor. Three groups have investigated the ability of various compounds to act as sources of sulfur in biotin biosynthesis. Niimura,[23] utilizing sulfur-starved cells of *S. cerevisiae*, found the following decreasing order of effectiveness: methionine sulfoxide, methionine, Na_2S, $NaHSO_3$, Na_2SO_4, homocysteine, S-adenosylmethionine, and methyl mercaptan. Cystine, cysteine, methionine sulfone, S-methylthioadenosine, choline sulfate, and glutathione were inactive as sulfur donors. Addition of ^{35}S-methionine to the cells gave radiolabeled biotin.[24,25] A similar experiment with radiolabeled methionine was carried out by Shimada[26] using *Aspergillus niger* and gave the same result. Wright[27] found that lipoic acid and two lipoic acid derivative stimulated biotin production in *A. niger*, but administration of ^{35}S-lipoic acid gave relatively low levels of incorporation into biotin. This result suggests that the stimulating effect of lipoate is due to other factors than the donation of sulfur.

The second problem associated with the conversion of dethiobiotin into biotin concerns the mechanism of the sulfur introduction process. Pioneering experiments to solve this problem were carried out by Li *et al.*[28] These investigators administered a mixture of [9- or 10-^{14}C]-dethiobiotin and randomly tritiated dethiobiotin to cultures of *A. niger* and isolated radioactive biotin sulfone. From the decrease in the ^{3}H/^{14}C ratio, it was suggested that approximately four hydrogen atoms might be lost during the sulfur introduction process. This loss could be accommodated by the involvement of at least two unsaturation steps.

The uncertainties resulting from the use of randomly tritiated dethiobiotin in these double label experiments prompted us to reinvestigate the degree of hydrogen loss using specifically tritiated forms of dethiobiotin. Published syntheses of (±)-dethiobiotin[29-32] proved unsatisfactory when applied to the problem of synthesizing **13** labeled specifically with tritium. Consequently, a new stereospecific synthesis of (±)-dethiobiotin that was useful for labeling purposes was developed.[33] This synthesis is outlined in Scheme 3. Rosemund reduction of 5-ethoxycarbonyl-n-pentanoyl chloride[34] followed by immediate acetalization with *p*-toluenesulfonic acid and ethylene glycol gave the acetal ester **14**. Treatment of **14** with lithium aluminum hydride afforded the acetal alcohol **15** which was converted by triphenylphosphine and carbon tetrabromide into the bromoacetal **16**.



Scheme 2



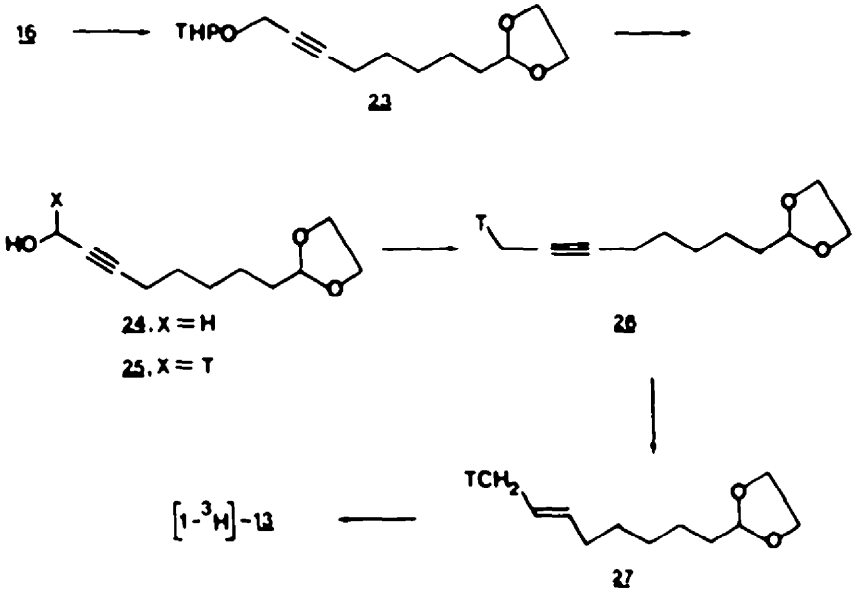
Scheme 3

Alkylation of propyne with **16** in liquid ammonia produced the acetylenic acetal **17**. Sodium-ammonia reduction of **17** then led to the *trans*-acetal olefin **18**. The *trans* olefin **18** was next subjected to the conditions of a stereospecific aziridine synthesis devised by Hassner.[15] Sequential treatment of **18** with iodine azide, triethylphosphite, and lithium aluminum hydride afforted the *trans*-substituted aziridine **19**. Ring-opening of the aziridine with sodium azide in acidic, aqueous alcohol[16] followed by acylation with ethyl chloroformate yielded a mixture of two isomeric azido-urethanes **20**. Ozonolysis[17] of the mixture **20** at $-78°$ in methanol produced a mixture of two isomeric ethylene glycol esters (**21**). Catalytic reduction of the ester mixture gave a mixture of two isomeric amino esters (**22**) which was treated with sodium ethoxide in ethanol to produce the ethyl ester of (±)-dethiobiotin. Acidic hydrolysis of this ester then yielded crystalline (±)-dethiobiotin (**13**).

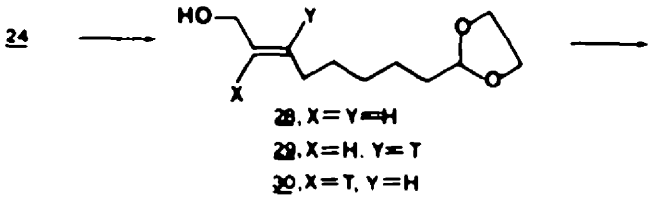
Since the introduction of sulfur into dethiobiotin might involve the removal of hydrogen from any of five positions of the molecule (C-1 to C-5), the synthesis just described was modified to allow the introduction of tritium at each of these sites.[18,19] These modifications are outlined in Scheme 4. Alkylation of the tetrahydropyranyl ether of propargyl alcohol[40] with the bromoacetal **16** yielded the acetylenic acetal **23** (Scheme 4). Deprotection of **23** with aqueous acetic acid containing ethylene glycol yielded the propargyl alcohol **24**. Collins' oxidation of **24** followed by reduction of the resulting propargyl aldehyde with [^{3}H]-potassium borohydride gave the [1-^{3}H]-propargylic alcohol **25**. Treatment of **25** with mesyl chloride and triethylamine in THF generated the primary mesylate which was not isolated but immediately reduced at $-78°$ with lithium aluminum hydride to the [1-^{3}H]-acetylenic acetal **26**. Reduction of **26** with sodium in liquid ammonia proceeded without loss of label to yield the *trans* olefin **27** bearing a tritium label at C-1. **27** was then converted into [1-^{3}H]-(±)-dethiobiotin by the steps outlined in Scheme 3.

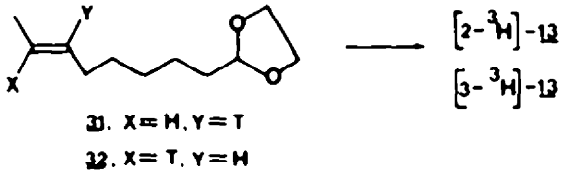
Reduction of the propargyl alcohol **24** with lithium aluminum hydride in THF (Scheme 4b) gave

the anticipated[41,42] *trans* allylic alcohol **28**. When the reduction of **24** was carried out with unlabeled lithium aluminum hydride and the reaction quenched with tritiated water, the [3-^{3}H]-allylic alcohol **29** was obtained, as expected.[43] It was also expected that reduction of **24** with [^{3}H]-lithium aluminum hydride followed by workup with unlabeled water would give the [2-^{3}H]-allylic alcohol **30**, but the labeling pattern in **30** proved to be much less specific than in alcohol **29**. The labeled

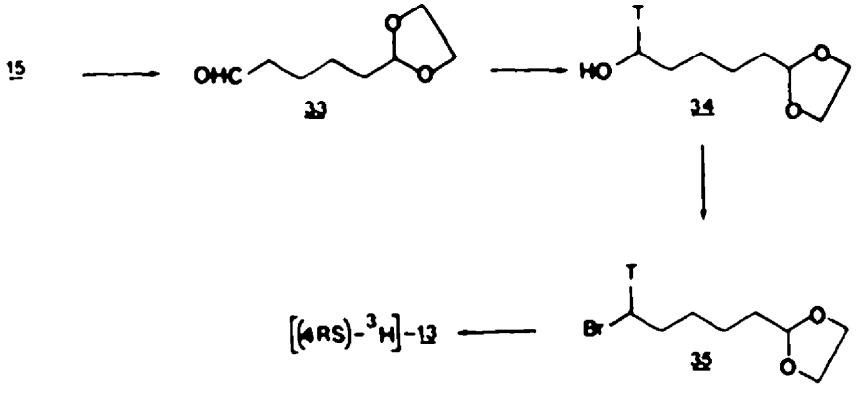


Scheme 4(a)

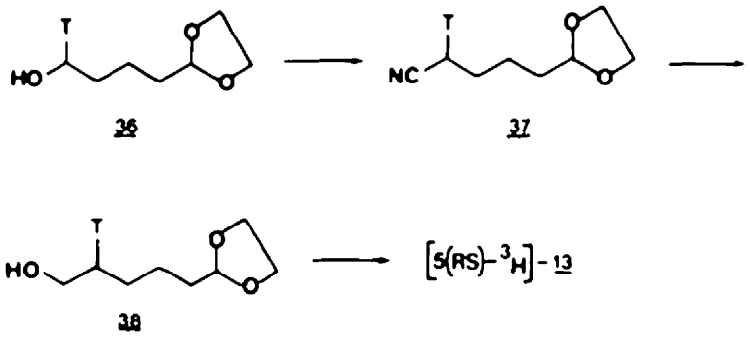




Scheme 4(b)



Scheme 4(c)



Scheme 4(d)

Scheme 4

alcohols **29** and **30** were converted to the corresponding labeled acetal olefins **31** and **32** by generation of the mesylates and reduction at low temperature with lithium aluminum hydride. The precise distribution of the tritium label between C-2 and C-3 in **31** and **32** was determined by ozonolysis of the labeled olefins to give acetaldehyde and the ethylene glycol ester of 6-formyl-n-hexanoic acid. The two labeled aldehydes obtained from each olefin were derivatized as thiosemicarbazones and recrystallized to constant specific activity. In this way, it was established that the labeled alkene **31** carried 17% of the tritium label at C-2 and 83% at C-3, while the alkene **32** carried 58% of its tritium at C-2 and 42% at C-3. The labeling pattern in **31** and **32** having been defined, each of the labeled olefins was converted into the corresponding labeled form of (±)-dethiobiotin by the steps of Scheme 3.

The synthesis of [4(RS)-^{3}H]-(±)-dethiobiotin began with oxidation of the acetal alcohol **15** to the aldehyde **33** using Collins' reagent (Scheme 4c). Reduction of **33** with [^{3}H]-borohydride then yielded the [1-^{3}H]-acetal alcohol **34**. This alcohol was converted into the [1-^{3}H]-bromoacetal **35** which was ultimately transformed into [4(RS)-^{3}H]-(±)- dethiobiotin using known chemistry.

Finally, [5(RS)-^{3}H]-(±)-dethiobiotin was prepared from the [1(RS)-^{3}H]-alcohol **36** (Scheme 4d) which was available from 4-ethoxycarbonyl-n-butanoyl chloride by the methods used to prepare alcohol **34**. Alcohol **36** was converted[39] to the corresponding mesylate and the mesylate treated with aqueous potassium cyanide to produce the nitrile **37**. Basic hydrolysis of **37** followed by lithium aluminum hydride reduction of the resulting acid generated the [2(RS)-^{3}H]-alcohol **38**. This labeled alcohol was then transformed into [5(RS)-^{3}H]-(±)-dethiobiotin in the usual manner.

The samples of tritiated (±)-dethiobiotin prepared as described were each mixed with [10-^{14}C]-(±)-dethiobiotin[45] and the doubly labeled precursors were then administered to cultures of *A. niger* (ATCC 1004) using the methods of Li *et al.*[28] The precursors were not resolved since only (+)-dethiobiotin appears to serve as a biotin precursor.[46] After an incubation period of 5–6 days, the biotin produced from each doubly labeled precursor was isolated as (+)-biotin sulfone,[28] and converted to biotin sulfone methyl ester. The methyl esters were purified by chromatography and by recrystallization to constant activity and constant tritium to carbon-14 ratio. The results of these experiments are summarized in Table 1, expts. no. 1-5.

A number of conclusions can be drawn from the data in Table 1. Experiments 1-3 clearly demonstrate that the introduction of sulfur at C-1 and C-4 of (+)-dethiobiotin takes place without the loss of hydrogen from C-2, C-3 or C-5. It therefore seems unlikely that unsaturation is introduced at any of these sites during the biosynthesis of (+)-biotin from (+)-dethiobiotin; however, the possibility of enzymatic removal of hydrogen from C-2, C-3 or C-5 followed by replacement of the hydrogen without exchange cannot be excluded. Experiment 4 shows that the incorporation of [1-^{3}H]-(±)-dethiobiotin into biotin proceeds with about 30% tritium loss. The nature of the reaction associated with the oxidation of the methyl group of (+)-dethiobiotin is unknown, but the tritium loss observed in experiment 4 is consistent with the removal of one hydrogen atom from the methyl group by a process that exhibits little or no isotope effect. Experiment 5 reveals that [4(RS)-^{3}H]-(±)-dethiobiotin is converted into (+)-biotin with about 47% tritium loss. This figure is within experimental error of that expected (50%) for the stereospecific removal of one hydrogen atom from C-4 of dethiobiotin during the formation of biotin. Thus, it appears that two hydrogen atoms are removed from (+)-dethiobiotin as the result of its conversion to (+)-biotin.

Table 1 Incorporation of specifically tritiated dethiobiotin into biotin by *A. Niger*

Expt. No.	Precursor	$^{3}H/^{14}C$ for Precursor	$^{3}H/^{14}C$ for Biotin Sulfone Methyl Ester	% ^{3}H Retention
1	[2,3-^{3}H,10-^{14}C]-(±)-13[a]	6.05	5.74	95.0
2	[3-^{3}H,10-^{14}C]-(±)-13[b]	2.89	3.04	105.0
3	[5-^{3}H,10-^{14}C]-(+)-13	5.72	5.33[c]	93.2
4	[1-^{3}H,10-^{14}C]-(±)-13	6.88	4.81	69.9
5	[4(RS)-^{3}H,10-^{14}C]-(±)-13	5.88	3.10	52.7
6	[4(R)-^{3}H,10-^{14}C]-(±)-13	3.37	3.07	91.1
7	[4(S)-^{3}H,10-^{14}C]-(±)-13	6.00	0.42	7.0

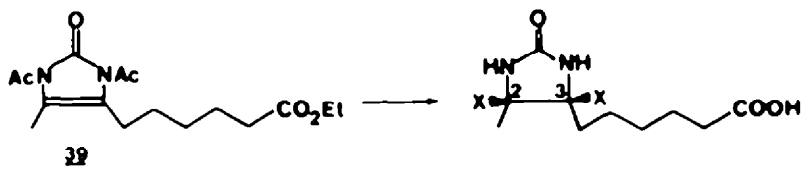
[a] Precursor had 58% ^{3}H at C-2, 42% at C-3.

[b] Precursor had 17% ^{3}H at C-2, 83% at C-3.

[c] $^{3}H/^{14}C$ ratio measured on biotin sulfone.

The experiments just described utilize the eucaryotic organism *Aspergillus niger*. Biotin is also known to be synthesized from dethiobiotin by a number of procaryotes.[47] Marquet has reported the results of experiments[48a,b] with *E. coli* that suggest the dethiobiotin to biotin conversion follows the same route in procaryotic organisms. The N,N'-diacetylimidazolone **39** (Scheme 5a) was reduced with [^{2}H]-triethylsilane in trifluoroacetic acid and the product saponified to yield [2,3-^{2}H]-(±)-dethiobiotin (85% of label at C-2, 15% at C-3). Similarly, [2,3-^{3}H] (±)-dethiobiotin was obtained by reduction with [^{3}H]-triethylsilane and TFA. [5-$^{2}H_2$]-(±)-Dethiobiotin was synthesized according to Scheme 5b. The ester alcohol **40** was converted to its trityl ether and then reduced with lithium aluminum deuteride to the dideutero alcohol **41**. The chloride **42** obtained from **41** by the action of triphenylphosphine and carbon tetrachloride was coupled to the allylic bromide **43** in the presence of cuprous bromide to give the dethiobiotin derivative **44**. Reduction of **44** in the usual way followed by deprotection gave labeled dethiobiotinol (**45**) from which [5-$^{2}H_2$]-(±)-dethiobiotin was prepared by oxidation with Heyn's catalyst. [1-$^{2}H_2$]-(±)-Dethiobiotin was also synthesized. Each of the labeled forms of dethiobiotin was administered to an auxotrophic mutant of *E. coli* (C124) whose biosynthetic pathway to biotin is blocked before dethiobiotin. The results of the experiments are summarized in Table 2.

Administration of [2,3-^{3}H]-(±)-dethiobiotin in conjunction with [9-^{14}C]-(±)-dethiobiotin gave radioactive biotin that had retained all of the tritium label (expt. 1). This result parallels that observed earlier in *A. niger*. The possibility of hydrogen migration from C-2 and/or C-3 was ruled out by administration of [2,3-^{2}H]-(±)-dethiobiotin to the *E. coli* auxotroph followed by mass spectral analysis of the resulting biotin (expt. 2). In the same way, an incorporation experiment



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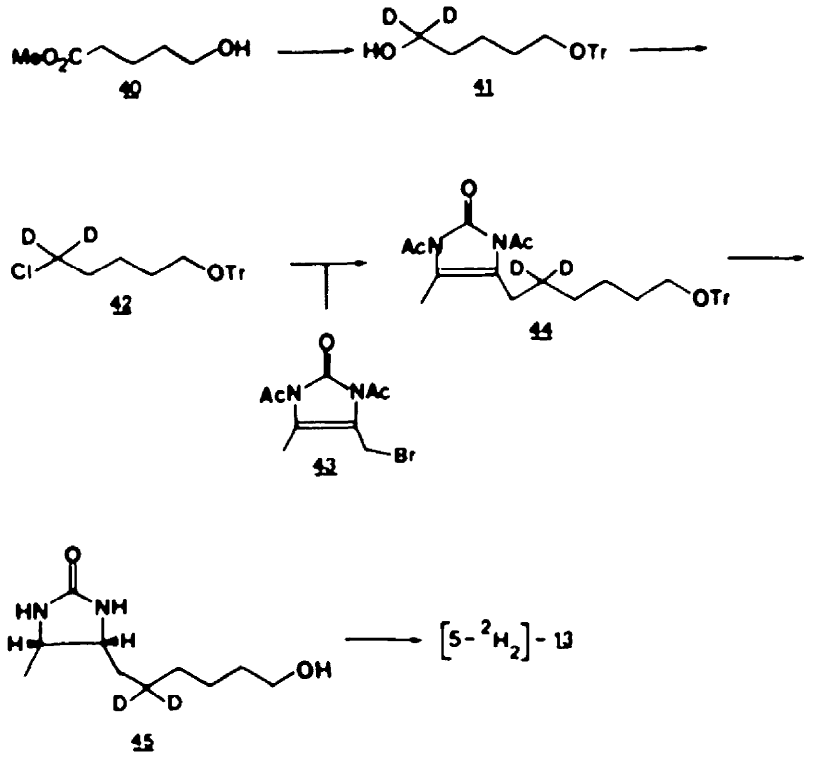
X = D
X = T

Scheme 5(a)



Scheme 5(b)

Table 2 Incorporation of labeled dethiobiotin into biotin by *E. coli*

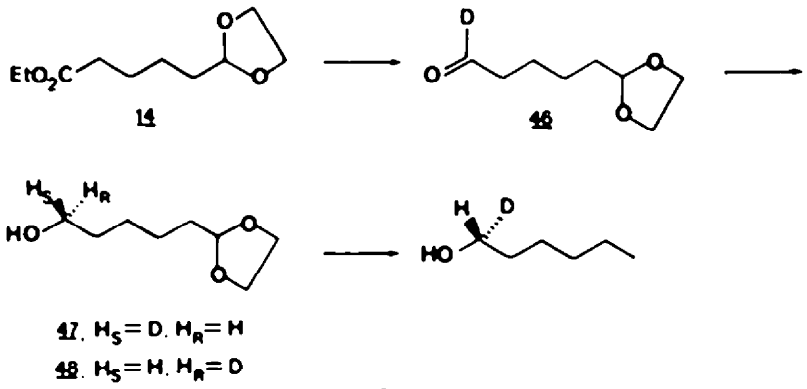
Expt. No.	Precursor	Labeling Pattern of Precursor	Labeling Pattern of Biotin
1	[9-^{14}C,2,3-^{3}H]-(±)-13	85% ^{3}H at C-2	^{3}H/^{14}C = 8.3,
	^{3}H/^{14}C = 8.2	15% ^{3}H at C-3	101% ^{3}H retention
2	[2,3-^{2}H]-(±)-13	85% [2-^{2}H$_1$]	89% [2-^{2}H$_1$]
		15% [3-^{2}H$_1$]	11% [3-^{2}H$_1$]
3	[5-^{2}H$_2$]-(±)-13	88% [5-^{2}H$_2$]	84% [5-^{2}H$_2$]
		12% unlabeled	14% unlabeled
4	[1-^{2}H$_3$]-(+)-13	65% [1-^{2}H$_3$]	71% [1-^{2}H$_2$][a]
		25% [1-^{2}H$_2$]	16% [1-^{2}H$_1$]
		10% unlabeled	14% unlabeled

[a] Calculated values are 73.5% [1-^{2}H$_2$], 16.5% [1-^{2}H$_1$], 10% unlabeled.

with [5-^{2}H$_2$]-(±)-dethiobiotin showed that both deuterium atoms in the precursor are retained at C-5 in resulting biotin (expt. 3). This finding is also consistent with a related experiment carried out using *A. niger*. Finally, administration of [1-^{2}H$_3$]-(±)-dethiobiotin to *E. coli* C124 gave biotin labeled with two deuterium atoms at C-1 (expt. 4). This observation agrees with our interpretation of the change in tritium to carbon-14 ratio accompanying the incorporation [1-^{3}H, 10-^{14}C]-(±)-dethiobiotin into biotin by *A. niger* (*vide supra*).

In order to gain some additional insight into the mechanism of sulfur introduction during biotin biosynthesis, we decided to elucidate the stereochemistry of the introduction of sulfur at C-4 of

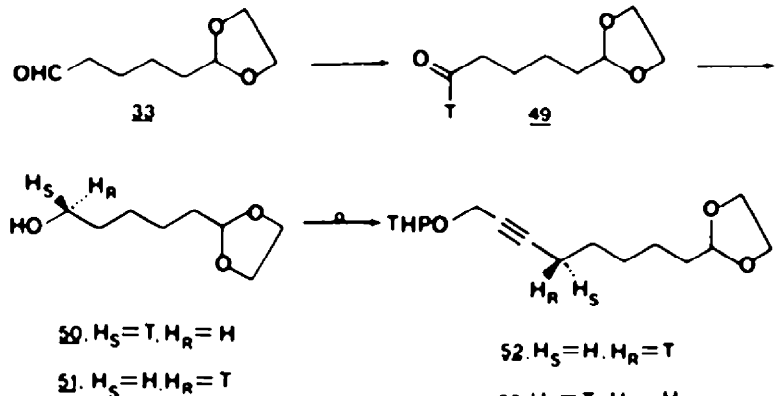
dethiobiotin. This was accomplished by means of precursor incorporation experiments with [4(R)-^{3}H]-dethiobiotin (**54**) and [4(S)-^{3}H]-dethiobiotin (**55**).[49] The synthesis of these two chirally labeled forms of dethiobiotin is summarized in Schemes 6 and 7. The acetal ester **14** was reduced with lithium aluminum deuteride and the resulting 1,1-dideuterio alcohol oxidized to the deuterated aldehyde **46** with pyridinium chlorochromate (Scheme 6). The deuterated aldehyde was then reduced with the adduct of (+)-α-pinene (*ca* 81% optical purity) and 9-borabicyclononane.[50] The alcohol **47** produced in this reduction was expected to have the S configuration.[50] This was confirmed by derivatization of **47** with (−)-camphanoyl chloride and examination of the NMR spectrum of the camphanate ester in the presence of Eu(dpm)$_3$, the diastereotopic hydrogen atom at C-1 of the camphanate ester of **47** resonated at higher field as predicted.[51] Similarly, reduction of aldehyde **46** with the adduct of (−)-α-pinene (75% optical purity) and 9-BBN yielded the R alcohol **48**. As an additional check of the chirality of **48**, it was degraded[49] to [(1R)-^{2}H$_1$]-hexanol whose chirality was verified by the camphanate method. The chirally tritiated alcohols **50** and **51** were then prepared using the same technique (Scheme 7). Reduction of the aldehyde **33** with [^{3}H]-potassium borohydride and oxidation of the resulting labeled alcohol with pyridinium



47. $H_S = D$, $H_R = H$

48. $H_S = H$, $H_R = D$

Scheme 6

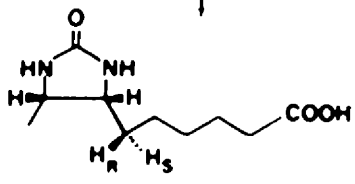


50. $H_S = T$, $H_R = H$

51. $H_S = H$, $H_R = T$

52. $H_S = H$, $H_R = T$

53. $H_S = T$, $H_R = H$



54. $H_S = H$, $H_R = T$

55. $H_S = T$, $H_R = H$

Scheme 7

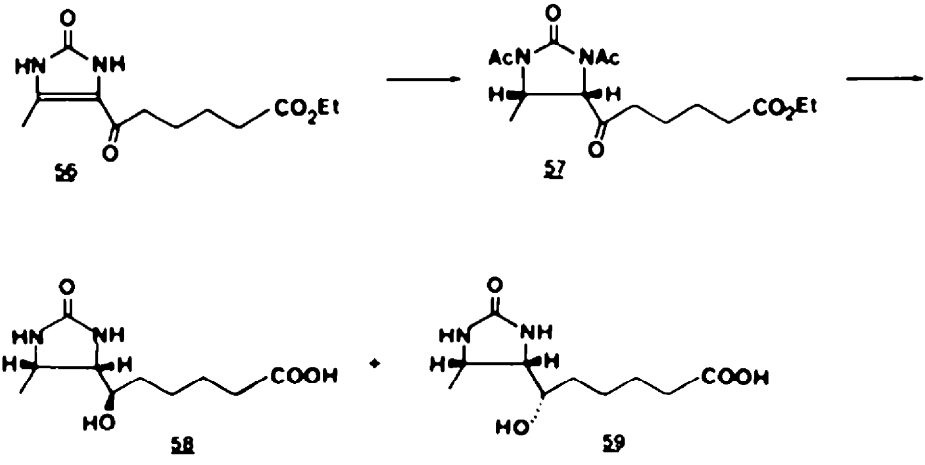
chlorochromate gave the tritiated aldehyde **49**. Reduction of **49** with the adduct of 9-borobicyclononane and (+) or (−)-α-pinene yielded the *S* and *R* alcohols **50** and **51**, respectively. Each of these chirally tritiated alcohols was converted to its tosylate and the tosylates were treated with the lithio derivative of the THP ether of propargyl alcohol according to the method of Corey.[52] On the basis of the assumption that this reaction proceeds with inversion of configuration, the products of the alkylation are the [1(R)-^{3}H] and [1(S)-^{3}H]-acetylenic acetals **52** and **53**, respectively. These chirally tritiated acetylenes were transformed into [4(R)-^{3}H] and (4(S)-^{3}H]-(±)-dethiobiotin (**54**, **55**) using the methods of Schemes 3 and 4.

The samples of chirally tritiated (±)-dethiobiotin were each mixed with [10-^{14}C]-(±)-dethiobiotin and the doubly labeled precursors administered to *A. niger*. The (+)-biotin sulfone isolated in these experiments was purified as its methyl ester to constant specific activity and constant ratio. The results of the incorporation experiments are shown in Table 1 (expts. no. 6, 7). The tritium to carbon-14 ratios of the biotin sulfone methyl ester isolated in these experiments clearly demonstrate that sulfur is introduced at C-4 of dethiobiotin with loss of the 4 *pro*-S hydrogen atom. Since the absolute configuration of (+)-biotin at C-4 is known[53] to be S, it follows that sulfur is introduced at C-4 of dethiobiotin with *retention* of configuration.

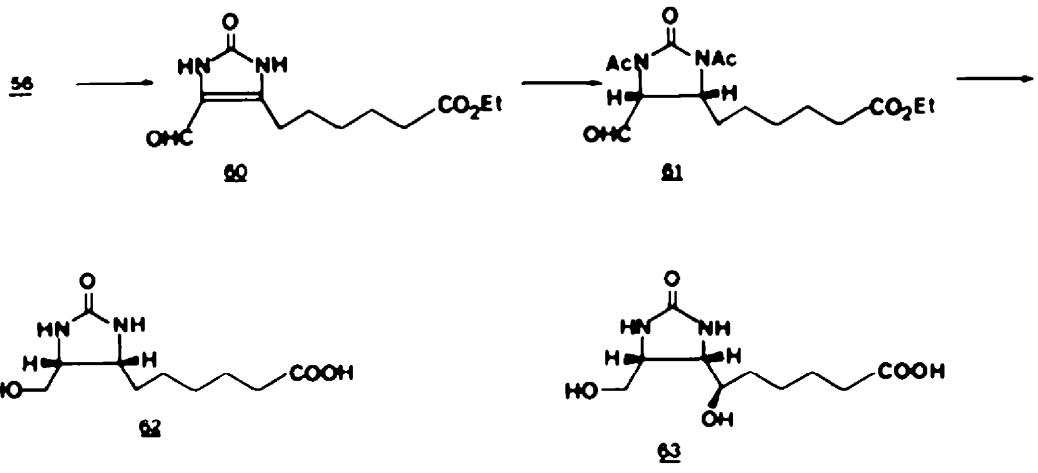
The stereochemistry observed in the introduction of sulfur at C-4 of dethiobiotin suggests that the functionalization mechanism may be a single step process. An alternative possibility, such as hydroxylation, activation of the hydroxyl group, and displacement by a sulfur nucleophile would presumably result in overall *inversion* of configuration since biological hydroxylations of saturated carbon atoms usually proceed with retention of configuration.[54] Additional evidence against the intermediacy of hydroxylated forms of dethiobiotin has been provided by Marquet.[55] The known[31] imidazolone **56** (Scheme 8a) was acetylated and then reduced to give the N,N'-diacetyl derivative **57**. Borohydride reduction of **57** was followed by saponification to produce the two epimers of 4-hydroxydethiobiotin **58** and **59**. Imidazolone **56** was next converted to the aldehyde **60** by the method of Zav'yalnov.[56] The aldehyde was transformed into the N,N'-diacetyl derivative and the latter compound reduced catalytically to the acetylated aldehyde **61**. Treatment of **61** with borohydride and saponification of the reduction product then yielded 1-hydroxydethiobiotin **62** (Scheme 8b).

The three forms of hydroxydethiobiotin were evaluated as precursors of biotin in *E. coli* mutant C124. None of the compounds tested supported the growth of this organism. Transport studies showed that all three compounds can enter the cells and so the absence of growth could not be attributed to permeability problems. The dihydroxydethiobiotin derivative **63**[57] was also evaluated as a precursor and it did not induce growth. It therefore seems unlikely that hydroxylation takes place at C-1 or C-4 of dethiobiotin during the formation of biotin. However, one cannot rule out involvement of hydroxylated intermediates that are tightly bound to the enzyme(s) catalyzing the introduction of sulfur.

In addition to these studies, Marquet has reported the isolation of an intermediate ("X") that apparently lies on the pathway between dethiobiotin and biotin.[58] Intermediate X was isolated



Scheme 8(a)



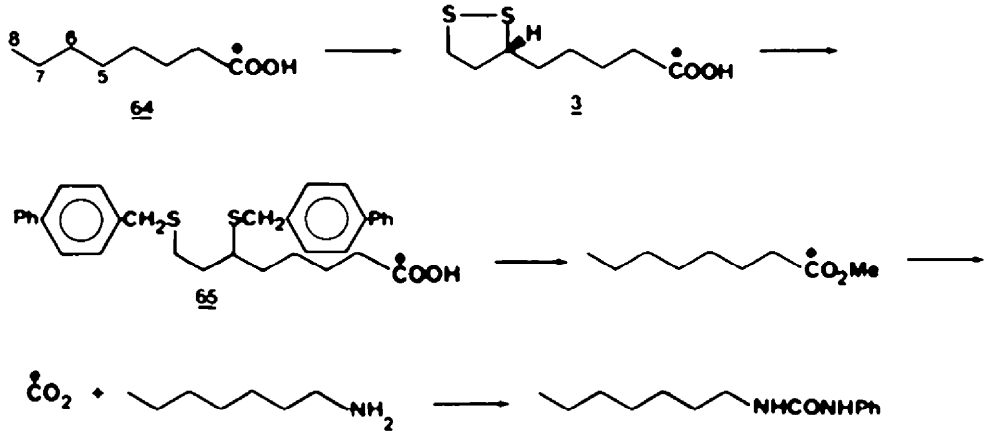
Scheme 8(b)

from the incubation medium of resting cells of *E. coli* mutant C124. The compound appears to contain sulfur and it promotes the growth of *E. coli* C124 and of several *E. coli* mutants blocked between dethiobiotin and biotin. The conversion of X into biotin by growing cells of *E. coli* C124 was also established. Unfortunately, the structure of intermediate X is unknown at present, and since it is unstable and only available in minute quantities, elucidation of the structure may prove to be a formidable problem.

LIPOIC ACID

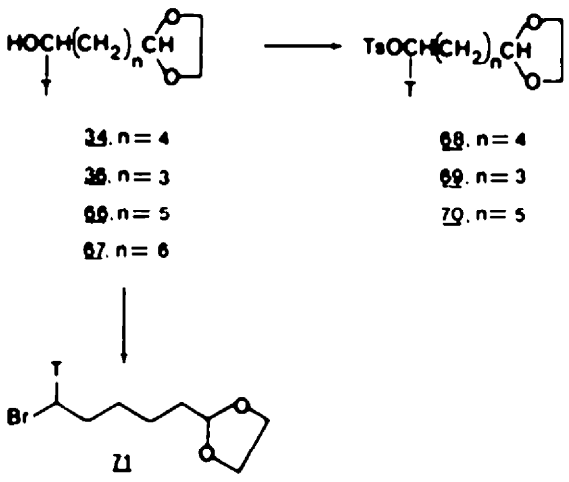
α-(+)-Lipoic acid (6,8-thioctic acid) (3) was independently discovered by several groups in the late 1940s as a growth factor and a requirement for pyruvate oxidation in various microorganisms.[59-61] The substance was first obtained in crystalline form by Reed *et al.*, who processed enormous quantities of the water insoluble residues of beef liver.[64,65] The isolation was rapidly followed by structural [65,66] and synthetic investigations[67,68] culminating in the formulation of lipoic acid as the 1,2-dithiolane derivative 3. The absolute configuration of lipoic acid was determined[69] by Mislow in 1956.

Lipoic acid is widely distributed in living systems where it functions as the coenzyme in those multienzyme complexes that catalyze the oxidative decarboxylation of α-keto acids.[70-72] The first information concerning the biosynthesis of lipoic acid was obtained by Reed[70,73] in 1964. In unpublished experiments, he showed that octanoic acid (64) serves as a specific precursor of lipoic acid in *E. coli* (Scheme 9). This is an intriguing observation which suggested to us that the dithiolane ring of lipoic acid might be generated by a mechanism related to the formation of the thiophane ring of biotin. Before addressing ourselves to that possibility, it was necessary to verify that

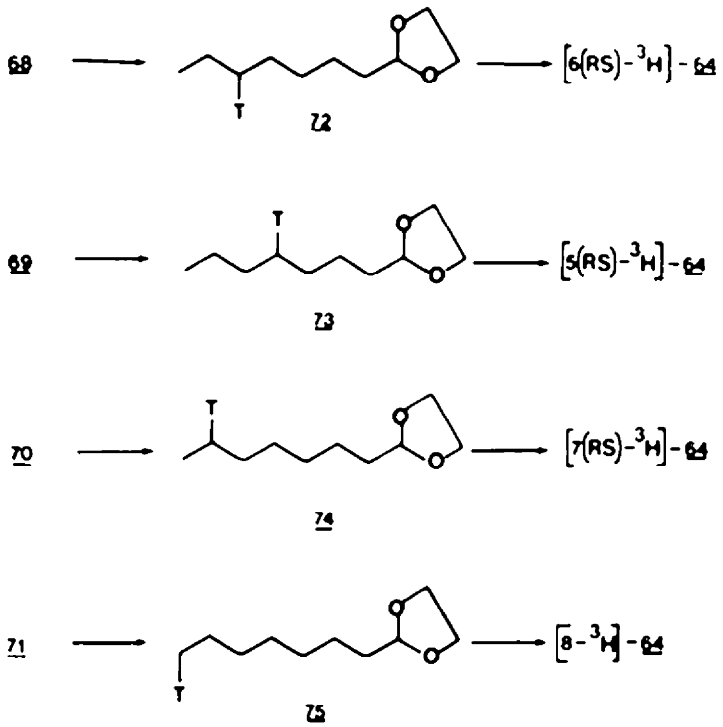


Scheme 9

octanoic acid is specifically incorporated into lipoic acid by *E. coli*. Accordingly, sodium[1-^{14}C]octanoate was administered to shake cultures of *E. coli* (Crookes strain, ATCC 8739) and the cells harvested by centrifiguration after 16 hr at 32–34°. The cells were sonicated, radioinactive lipoic acid was added as carrier, and the mixture was then autoclaved in 6N sulfuric acid at 120° for 2 hr to liberate protein-bound lipoic acid. The crude lipoic acid recovered by benzene extraction of the autoclaved mixture was derivatized by reduction with sodium in liquid ammonia followed by treatment with p-phenylbenzyl chloride to give the bis(p-phenylbenzyl) derivative **65** (Scheme 9). This derivative was recrystallized to constant radioactivity to give an incorporation figure of 0.17%. The purified derivative **65** was converted to the corresponding methyl ester which was recrystallized and then desulfurized with Raney nickel to yield methyl octanoate (Scheme 9). Hydrolysis of the methyl octanoate and Schmidt degradation of the resulting octanoic acid then gave carbon dioxide, trapped as barium carbonate, and n-heptylamine, which



Scheme 10(a)



Scheme 10(b)

Table 3 Incorporation of labeled octanoic acid into lipoic acid by *E. coli*

Expt No.	Precursor	$^{3}H/^{14}C$ for Precursor	$^{3}H/^{14}C$ for Lipoic Acid	Labeling Pattern	% ^{3}H Retention
1	[1-^{14}C]-64	-	-	>90% label at C-1	-
2	[5(RS)-^{3}H,1-^{14}C]-64	4.05	4.11	-	102.0
3	[7(RS)-^{3}H,1-^{14}C]-64	3.95	3.81	-	96.5
4	[8-^{3}H,1-^{14}C]-64	5.02	4.81	-	95.8
5	[6(RS)-^{3}H,1-^{14}C]-64	5.08	2.53	-	49.8
6	[6(S)-^{3}H,1-^{14}C]-64	4.13	3.47	-	84.0
7	[6(R)-^{3}H,1-^{14}C]-64	4.40	0.48	-	10.9

was derivatized with phenyl isocyanate. The results of this degradation (Table 3, expt. 1) prove that octanoic acid is a specific precursor of lipoic acid in *E. coli*.[44]

The role of octanoic acid as a lipoate precursor having been established, samples of octanoic acid specifically tritiated at C-5, C-6, C-7 and C-8 were synthesized from the homologous series of tritiated alcohols **34**, **36**, **66** and **67** (Scheme 10a,b).[44] The labeled alcohols **34**, **36**, and **66** were converted to the corresponding tosylates (**68–70**) and each of the tosylates was treated with the appropriate lithium dialkylcuprate to yield[74] the tritiated octanal derivatives **72–74**. The tritiated octanal derivative **75** was prepared from the labeled alcohol **67** by conversion of **67** to the bromide **71** (Ph_3P, CBr_4) followed by reduction of **71** with lithium triethylborohydride. The tritiated acetals **72–75** were then transformed into specifically tritiated forms of sodium octanoate by ozonolysis[77] followed by alkaline hydrolysis of the resulting ethylene glycol esters. Each of the samples of tritiated sodium octanoate was mixed with sodium [1-^{14}C]-octanoate and the tritium to ^{14}C ratios measured directly and by derivatization of a portion of each mixture with p-bromophenacyl bromide. The four samples of doubly labeled octanoate were then administered to *E. coli* and lipoic acid isolated in the manner already described. The lipoic acid obtained from each experiment was derivatized with p-phenylbenzyl chloride and the derivatives recrystallized to constant radioactivity and constant tritium to ^{14}C ratio. The results of these experiments are shown in Table 3 (expts 2–5).

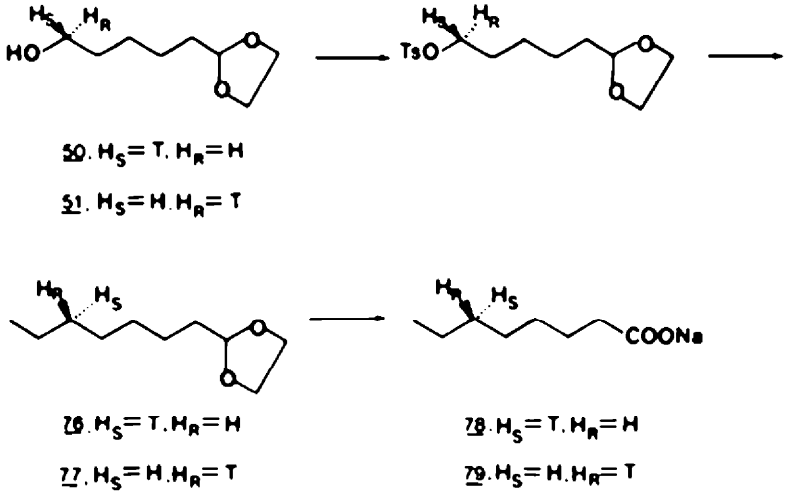
The following conclusions can be derived from the data in Table 3. Experiments 2 and 3 show that the introduction of sulfur at C-6 and C-8 of octanoic acid occurs without hydrogen loss from C-5 or C-7. This result is similar to that obtained when dethiobiotin labeled at C-2, C-3 or C-5 is transformed into biotin and it presumably rules out the introduction of unsaturation at C-5 or C-7 of octanoate during the course of sulfur introduction. Experiment 4 shows that the incorporation of sodium [8-^{3}H]-octanoate into lipoate proceeds without tritium loss, within experimental error. This result is probably the consequence of a substantial tritium isotope effect associated with the removal of a hydrogen atom from C-8. It will be recalled that the incorporation of [1-^{3}H]-dethiobiotin into biotin appears to proceed with little or no isotope effect. Since the nature of the reaction(s) associated with the oxidation of the methyl groups in octanoic acid and dethiobiotin is unknown, the reasons for this difference are presently unclear. Experiment 5 reveals that sodium [6(RS)-^{3}H]- octanoate is incorporated into lipoic acid with about 50% tritium loss. This figure is precisely that expected for the stereospecific removal of one hydrogen atom from C-6 of octanoic acid as a consequence of sulfur introduction, and it parallels the results obtained when [4(RS)-^{3}H]-dethiobiotin is transformed into biotin.

The experiments just outlined suggest that the mechanism(s) of sulfur introduction associated with biotin biosynthesis and lipoic acid biosynthesis may be closely related. This possibility was

further investigated by examining the stereochemistry of sulfur introduction at C-6 of octanoic acid.[75] The synthesis of [(6R)-^{3}H]- and [(6S)-^{3}H]-sodium octanoate was accomplished by the route summarized in Scheme 11. The chirally tritiated alcohols **50** and **51** (Scheme 7) were converted to the corresponding tosylates and the latter compounds reacted with lithium diethylcopper. Since the reaction of dialkylcuprates with tosylates proceeds with inversion of configuration at the sulfonate bearing carbon atom,[76] this reaction sequence transforms the *S*-alcohol **50** into the *S*-acetal **76** and the *R* alcohol **51** into the *R* acetal **77**.[75] Ozonolysis of the chirally tritiated acetals and base-catalyzed hydrolysis of the resulting ethylene glycol esters then generated sodium [(6S)-^{3}H]- and [(6R)-^{3}H]-octanoate (**78**, **79**, respectively). The two chirally labeled compounds were administered to *E. coli* after mixing with sodium [1-^{14}C]-octanoate and radioactive lipoic acid isolated as the S,S'-bis(*p*-phenylbenzyl) derivative. After extensive purification, the derivatives from each experiment gave the tritium to carbon-14 ratios shown in Table 3, expts. 6 and 7.

The tritium to ^{14}C ratios shown in Table 3 clearly prove that sulfur is introduced at C-6 of octanoic acid with loss of the 6 *pro*-R hydrogen atom. Since the absolute configuration of lipoic acid at C-6 is known to be R,[69] it follows that sulfur is introduced at C-6 of octanoic acid with *inversion* of configuration. This result is somewhat surprising in view of the fact that sulfur is introduced at C-4 of dethiobiotin with *retention* of configuration (*vide supra*). At the present time, it is not clear whether the introduction of sulfur in biotin biosynthesis proceeds by a mechanism that is fundamentally different from that involved in lipoate biosynthesis, or whether the mechanism is the same in both cases with the stereochemical outcome being dictated by other factors.[78]

The results from our investigations of lipoic acid biosynthesis have been fully corroborated by White using deuterium labeled precursors.[79,80] Initially, he examined the incorporation of deuterium into C_{12}, C_{14} and C_{16} saturated fatty acids by *E. coli* B grown in a medium containing [2-^{2}H$_3$]-acetate. The isotope distribution observed by mass spectral analysis could be accounted for by the presence of three distinct populations of deuterium. The most enriched of these populations consisted of the terminal methyl group in which 80% of each fatty acid continued a methyl group with three deuterium atoms. This deuterium population was derived directly from the acetate methyl group without exchange. The second population, which was 45% deuterated, consisted of one labeled hydrogen for each even numbered carbon atom. This second population was presumably derived from acetate via malonyl-CoA. The third population (19% deuteration), which carried one labeled hydrogen atom on each odd numbered carbon atom, was formed indirectly from acetate via labeled NADPH. Desaturation of the deuterated C_{16} saturated fatty acid using the *Corynebacterium diphtheriae* desaturation system[81] produced palmitoleic acid. Chemical and mass spectral analyses of derivatives of this acid showed that the NADPH-derived hydrogen on C-9 had been lost, while the acetate-derived hydrogen at C-10 had been retained. In addition, White showed that [2,2',10(S)-^{2}H$_3$]-palmitic acid was converted to palmitoleic acid with retention of three deuterium



50. H_S = T, H_R = H

51. H_S = H, H_R = T

76. H_S = T, H_R = H

77. H_S = H, H_R = T

78. H_S = T, H_R = H

79. H_S = H, H_R = T

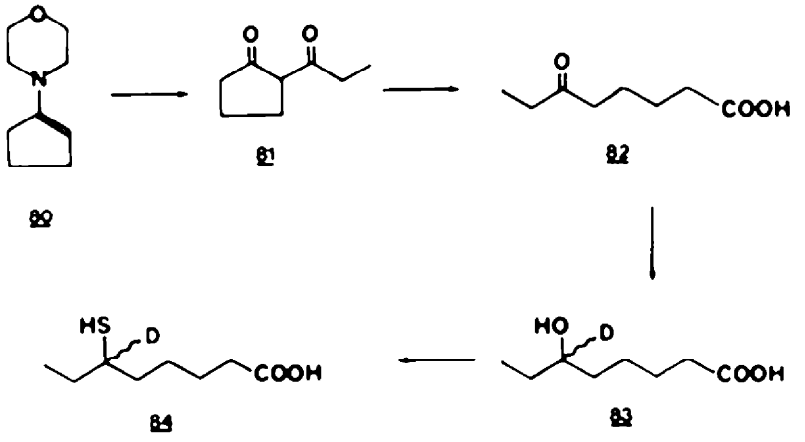
Scheme 11

atoms. He therefore concluded that the hydrogen incorporated from acetate at C-10 occupied the *pro*-S position, while the hydrogen at C-9 derived from NADPH occupied the *pro*-R position.

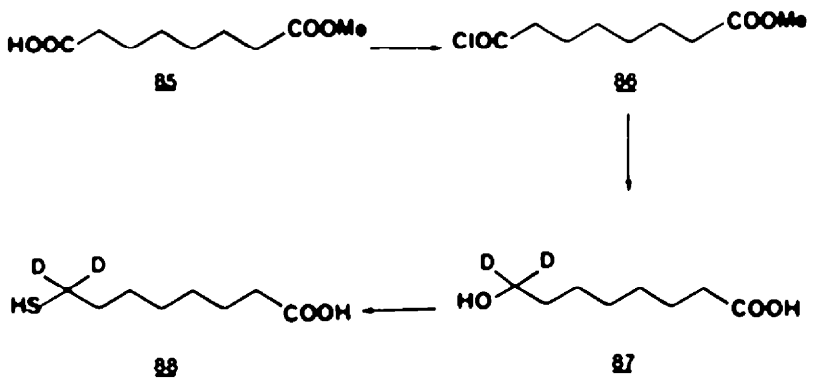
These experiments were extended to an examination of the incorporation of [2-$^2H_3$]-acetate into lipoic acid by *E. coli* B. The lipoic acid produced from this precursor was isolated as 6,8-bis (benzylthio)octanoate and analyzed for deuterium content by GC-MS. The lipoate derivative was found to contain up to eight deuterium atoms, a labeling pattern that is consistent with the biosynthesis of lipoic acid from a saturated fatty acid. Confirmation of this was obtained by administration of [U-$^2H_{15}$]-octanoate to *E. coli*. GC-MS analysis of the isolated lipoic acid derivative showed that 90% of the sample had been biosynthesized from the labeled octanoate and, further, that the labeled lipoic acid contained thirteen deuterium atoms. This result not only demonstrates that octanoic acid is a precursor of lipoic acid in *E. coli*, but it also clearly shows that the sulfur atoms are inserted into octanoic acid with the loss of only two hydrogen atoms. Finally, it was determined from the deuterium incorporation data that the deuterium present at C-6 of octanoic acid biosynthetically derived from labeled acetate is still present after conversion to lipoic acid. Since it had already been shown that the acetate-derived hydrogen on C-10 of palmitic acid is incorporated into the *pro*-S position, it follows that sulfur is introduced at C-6 of octanoic acid with inversion of configuration.

The stereochemistry of sulfur introduction at C-6 of octanoic acid suggests that sulfur might be introduced by hydroxylation with retention of configuration[34] followed by S_N2 displacement of the activated hydroxyl group with a sulfur nucleophile. White[82] has tested this possibility by precursor incorporation experiments with hydroxylated forms of octanoic acid. [6(RS)-$^2H_1$]-6-Hydroxyoctanoic acid (**83**) was synthesized by the methods outlined in Scheme 12a. 1-Morpholino-1-cyclopentene (**80**) was acylated with propionyl chloride and the resulting adduct hydrolyzed to 2-propionyl-1-cyclopentanone (**81**). Treatment of **81** with alkali yielded 6-oxooctanoic acid (**82**) which was reduced with sodium borodeuteride to **83**. The corresponding mercapto acid **84** was also prepared by treatment of **83** with thiourea and hydroiodic acid followed by alkaline hydrolysis of the intermediate thiuronium salt. [8-$^2H_2$]-8-Hydroxyoctanoic acid (**87**, Scheme 12b) was synthesized from suberic acid monomethyl ester (**85**) by conversion to the acid chloride **86** and reduction with sodium borodeuteride in monoglyme. [8-$^2H_2$]-8-Mercaptooctanoic acid (**88**) was in turn obtained from **87** via the thiuronium salt. Finally, [8-$^2H_2$]-(±)-6,8-dihydroxyoctanoic acid (**93**) (Scheme 12c) was prepared from 2-carbethoxymethylcyclohexanone (**89**) by reduction of the protected ester **90** with lithium aluminum deuteride, acetylation of the resulting alcohol, and deprotection to produce the acetoxyketone **91**. Baeyer-Villiger oxidation of **91** with peracetic acid generated the lactone **92** that was hydrolyzed to the requisite octanoic acid derivative **93**.

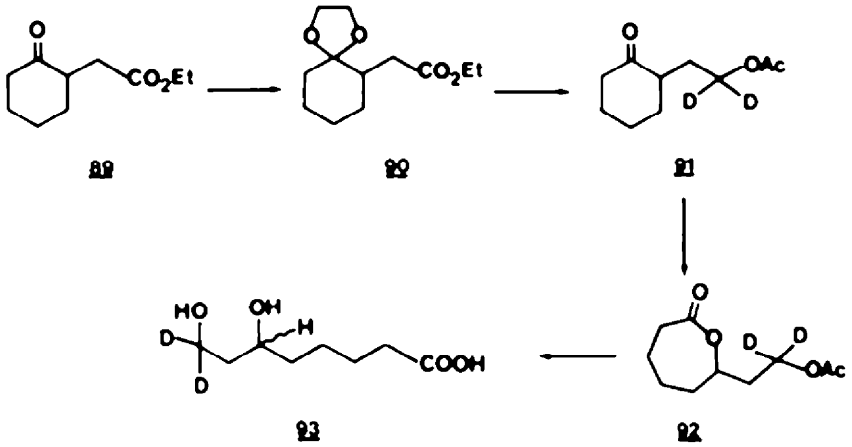
Each of the deuterated acids **83**, **84**, **87**, **88** and **93** was administered to *E. coli* and lipoic acid isolated as 6,8-bis(benzylthio)octanoate. GC-MS analysis of the lipoic acid derivatives gave the data presented in Table 4. From this incorporation data, it is clear that the thiooctanoic acids are much more efficiently incorporated into lipoic acid than are the hydroxyoctanoic acids. Since the



Scheme 12(a)



Scheme 12(b)



Scheme 12(c)

Table 4 Incorporation of deuterated precursors into lipoic acid by *E. coli*

Expt. No.	Precursor	% of Lipoic Acid Derived from Precursor
1	[8-$^2H_2$]-8-Hydroxyoctanoic acid	< 0.5
2	[6(RS)-$^2H_1$]-6-Hydroxyoctanoic acid	< 0.2
3	[8-$^2H_2$]-8-Thiooctanoic acid	19.0[a] 27.9[b]
4	[6(RS)-$^2H_1$]-6-Thiooctanoic acid	2.1
5	[8-$^2H_2$]-(±)-6,8-Dihydroxyoctanoic acid	< 0.5

[a] 15.8 mg of precursor added to culture.

[b] 30.0 mg of precursor added to culture.

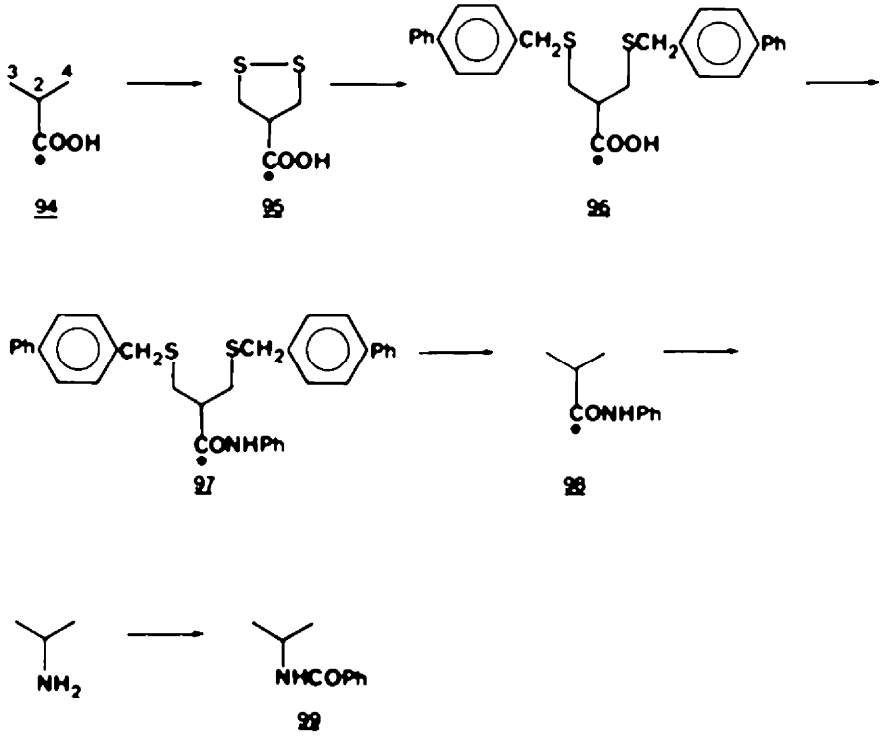
lack of incorporation of the hydroxyoctanoic acids could be due to transport problems, a stable isotope dilution analysis was developed to determine the distribution of [8-$^2H_2$]-8-hydroxyoctanoic acid at the end of incorporation experiment no. 1. The results of the analysis showed that almost all of the deuterated hydroxy acid (35.0 ± 0.1 mg) was present in the medium. The cells, however, did contain 26.5 μg of [8-$^2H_2$]-8-hydroxyoctanoic acid per gram weight of cells, of which *ca* 90% was present in non-esterified form. This represents only a small amount of the compound

administered to the culture, but if only 1% of it had been converted into lipoic acid by the cells, then *ca* 10% of the lipoic acid in the cells would have been derived from the labeled hydroxy acid.

The results of White's experiments with hydroxylated forms of octanoic acid are similar to those obtained by Marquet in her evaluation of hydroxylated forms of dethiobiotin as biotin precursors (*vide supra*). The evidence amassed by both these investigators strongly suggests that hydroxylated compounds are probably not intermediates in the sulfur introduction processes. On the other hand, White's experiments with deuterated 6-mercaptooctanoic acid (**84**) and 8-mercaptooctanoic acid (**88**) (Table 4, expts 3,4) provide support for the notion that 8-mercaptooctanoic acid lies on the pathway to lipoic acid. If **88** actually is an intermediate in lipoate biosynthesis, however, it is probably tightly enzyme bound. We have repeatedly failed to detect labeled 8-mercaptooctanoic acid by isotope dilution in *E. coli* after administration of [1-^{14}C]-octanoic acid, even when strongly hydrolytic workup conditions were used to free any bound mercapto acid.[81]

ASPARAGUSIC ACID

Asparagusic acid (**95**) (Scheme 13) is a naturally occurring 1,2-dithiolane that has been isolated from both the roots and edible portions of *Asparagus officinalis* L.[84,86] The substance is a plant growth inhibitor exerting activity comparable to abscisic acid,[84,85] and it also possesses potent nematicidal activity.[86] Since the 1,2-dithiolane lipoic acid (**3**, Scheme 9) is derived from octanoic acid, one might expect that asparagusic acid would be derived from isobutyric acid (**94**). Precursor incorporation experiments in our laboratory have shown this to be the case.[87] Administration of commercially available [1-^{14}C]-sodium isobutyrate to young *A. officinalis* plants by the cotton work method gave radioactive asparagusic acid after four days. The asparagusic acid was isolated by dilution with synthetic asparagusate[88] and it was derivatized as the bis (*p*-phenylbenzyl) thioether **96** (Scheme 13). Purification of **96** by chromatography and repeated recrystallization gave the incorporation figure shown in Table 5, expt. 1. Degradation of the thioether **96** was accomplished by conversion to the anilide derivative **97** with DCC and aniline followed by Raney nickel desulfurization to the anilide of isobutyric acid (**98**). The anilide **98** was then hydrolyzed and the resulting isobutyric acid subject to Schmidt degradation to yield isopropylamine. Finally, the isopropylamine was converted to its crystalline benzamide derivative **99** for purposes of final purification. The amide **99** proved to be devoid of radioactivity (Table 4, expt. 1) thereby showing

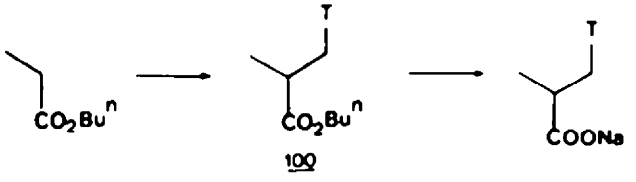


Scheme 13

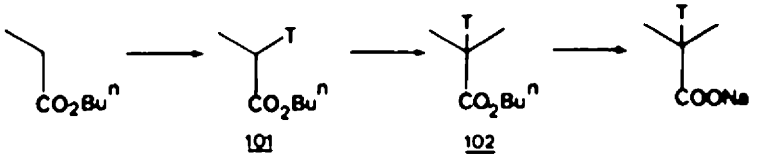
Table 5 Precursor incorporation experiments with *Asparagus officinalis*

Expt No	Precursor	Precursor Isotope Ratio	% Incorpn and/or Isotope Ratio in Product	Labeling Pattern or % Isotope Retention in Product
1	[1-^{14}C]-Sodium isobutyrate	-	1.2% into 95	No label at C-2 to C-4 of 95
2	[3,4-^{3}H,1-^{14}C]-Sodium isobutyrate	$^{3}H/^{14}C$ = 5.72	$^{3}H/^{14}C$ = 5.33 for 95	96.7% ^{3}H retention in 95
3	[2-^{3}H,1-^{14}C]-Sodium isobutyrate	$^{3}H/^{14}C$ = 6.36	$^{3}H/^{14}C$ = 0.16 for 95	2.5% ^{3}H retention in 95
4	[1-^{14}C]-Sodium methacrylate	-	0.38% into 95	No label at C-2 to C-4 of 95
5	[2-^{3}H,1-^{14}C]-(±)-Sodium 3-mercaptoisobutyrate	$^{3}H/^{14}C$ = 6.76	0.35% into 95 $^{3}H/^{14}C$ = 0.154 for 95	2.3% ^{3}H retention in 95 No label at C-2 to C-4 of 95
6	[^{35}S,3(RS)-^{3}H]-(±)-Sodium 3-mercaptoisobutyrate	$^{35}S/^{3}H$ = 0.341	1.1% into 95 $^{35}S/^{3}H$ = 0.322 for 95	94.4% ^{35}S retention in 95
7	[1-^{14}C]-Sodium isobutyrate	-	0.04% into 109	No label at C-2 to C-7 of 109
8	[^{35}S,3(RS)-^{3}H]-2(RS)-S-(ε-carboxy-n-propyl)-L-cysteine	$^{35}S/^{3}H$ = 0.254	1.2% into 95 $^{35}S/^{3}H$ = 0.256 for 95	101% ^{35}S retention in 95

that isobutyric acid had been specifically incorporated into asparagusic acid. Experiments with tritiated forms of isobutyric acid were then carried out to obtain clues to the mechanism of sulfur introduction. [3,4-^{3}H]-Sodium isobutyrate was synthesized as outlined in Scheme 14a. Treatment of n-butyl propionate[89] with LDA in THF–HMPA followed by alkylation with [^{3}H]-methyl iodide yielded [3,4-^{3}H]-n-butyl isobutyrate (**100**). Basic hydrolysis of **100** followed by acidification, steam distillation, and titration with alkali yielded the desired [3,4-^{3}H]-sodium isobutyrate. [2-^{3}H]-Sodium isobutyrate was synthesized in similar fashion (Scheme 14b). The anion formed from n-butyl propionate and LDA was quenched with [^{3}H]-trifluoroacetic acid to generate [2-^{3}H]-n-butyl propionate (**101**). Deprotonation of **101** with LDA was then followed by alkylation with unlabeled methyl iodide to produce [2-^{3}H]-n-butyl isobutyrate (**102**). The labeled ester **102** was finally converted into [2-^{3}H]-sodium isobutyrate by hydrolysis, steam distillation, and titration. Each of the samples of specifically tritiated sodium isobutyrate was mixed with [1-^{14}C]-sodium isobutyrate and the tritium to carbon-14 ratios checked directly and by derivatization of a portion of each mixture as the corresponding anilide. The two samples of doubly labeled sodium isobutyrate were then administered to *Asparagus* and aspargusic acid isolated and derivatized as already described. The results of



Scheme 14(a)

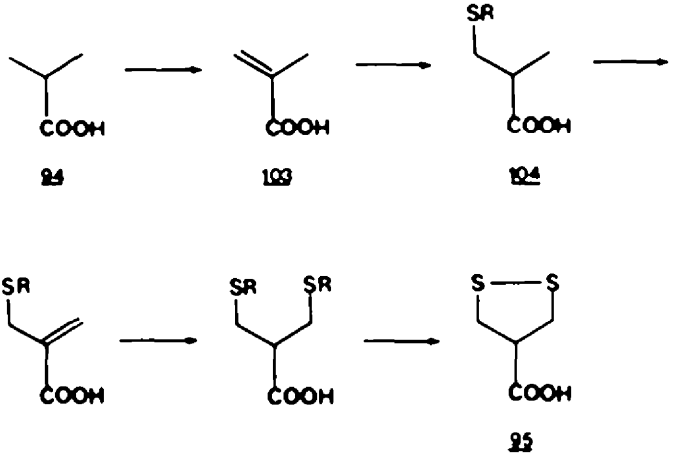


Scheme 14(b)

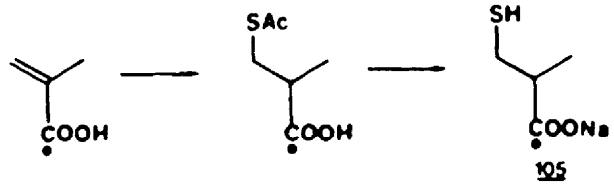
these experiments appear in Table 5 (expts. 2, 3). The incorporation of [3,4-^{3}H]-sodium isobutyrate into asparagusic acid proceeded without tritium loss, within experimental error. The high degree of tritium retention observed in this experiment is presumably the consequence of a substantial tritium isotope effect associated with the removal of a hydrogen atom from each of the methyl groups of isobutyrate. It will be recalled that a similar degree of tritium retention accompanies the conversion of [8-^{3}H]-octanoic acid into lipoic acid (*vide supra*). On the other hand, the incorporation of [2-^{3}H]-sodium isobutyrate into asparagusate resulted in the loss of virtually all of the tritium label. This result stands in complete contrast to the behavior observed during lipoic acid biosynthesis. In the case of the latter 1,2-dithiolane, no tritium is lost from carbon atoms adjacent to the sites of sulfur introduction (*vide supra*). It therefore appears that Nature has at least two ways to create the 1,2-dithiolane ring system. A plausible explanation for the loss of tritium from [2-^{3}H]-sodium isobutyrate would involve dehydrogenation to methacrylic acid (**103**) (Scheme 15), a process that is reported to occur in animals and in microorganisms.[90,91] Consequently, [1-^{14}C]-sodium methacrylate was synthesized by carboxylation of isopropenyl magnesium bromide with [^{14}C]-carbon dioxide and administered to *Asparagus* plants. After four days radioactive asparagusic acid was obtained (Table 4, expt. 4) and degradation in the usual manner (Scheme 13) proved that the incorporation was specific.

The results of the aforementioned experiments suggest the biosynthetic pathway for asparagusic acid outlined in Scheme 15. Dehydrogenation of isobutyric acid (**94**) (or isobutyryl CoA) to methacrylic acid (**103**) could be followed by a Michael-type addition of an unknown sulfur nucleophile. A second dehydrogenation step would then lead to an unsaturated acid that could undergo addition of a second mole of the sulfur nucleophile to ultimately yield asparagusic acid. Additional incorporation experiments[92] will now be outlined that completely support this pathway.

Scheme 15 suggests that 3-mercaptoisobutyric acid (**104**, R = H) may lie on the pathway from isobutyric acid to asparagusic acid. This possibility was evaluated by incorporation experiments with doubly labeled forms of **104** (R = H). [1-^{14}C]-Sodium 3-mercaptoisobutyrate (**105**) was synthesized from [1-^{14}C]-methacrylic acid by treatment with thiolacetic acid followed by alkaline hydrolysis (Scheme 16a). Similarly, [2-^{3}H]-sodium 3-mercaptoisobutyrate (**106**) was prepared by Michael addition of thiolacetic acid to methacrylic acid in tritiated water with subsequent alkaline hydrolysis (Scheme 16b). [^{35}S]-3-Mercaptoisobutyric (**107**) acid was obtained in two steps from methacrylic acid. Reaction of methacrylic acid with hydrogen bromide gave 3-bromoisobutyric acid which was then treated with [^{35}S]-sodium sulfide to yield **107** (Scheme 16c). Finally, [3(RS)-^{3}H]-sodium 3-mercaptoisobutyrate (**108**) was produced from [(Z)-2-^{3}H]-methacrylic acid[93] by thiolacetic acid addition and alkaline hydrolysis (Scheme 16d). The labeled acids **105** and **106** were mixed to give [1-^{14}C,2-^{3}H]-(±)-3-mercaptoisobutyrate and the labeled acids **107** and **108** were mixed to produce [^{35}S,3(RS)-^{3}H]-(±)-3-mercaptoisobutyrate. The ratios of each doubly labeled precursor were measured directly and by derivatization of a portion of each mixture with *p*-bromophenacyl bromide. The two doubly labeled precursors were then administered to *Asparagus* plants to yield the results contained in Table 5 (expts. 5,6).

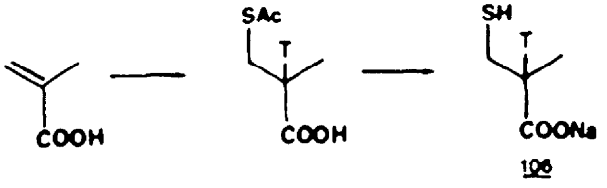


Scheme 15



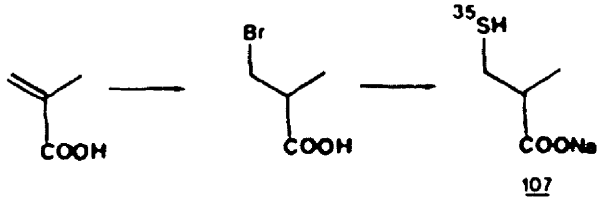
105

Scheme 16(a).



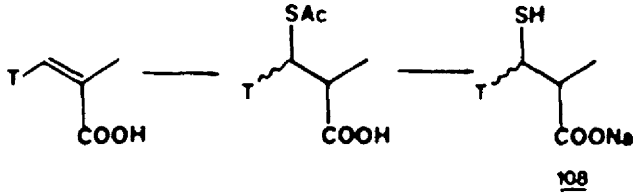
106

Scheme 16(b)



107

Scheme 16(c)

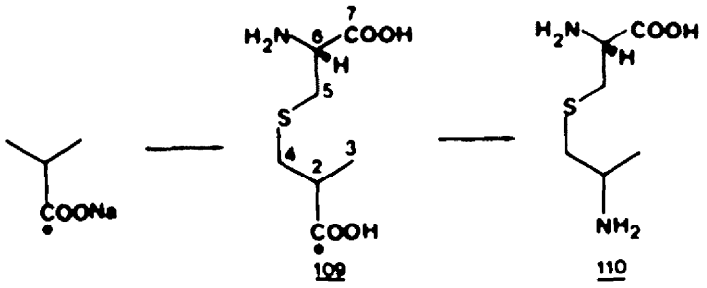


108

Scheme 16(d)

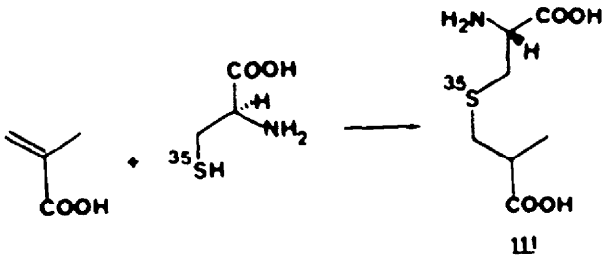
Three conclusions can be drawn from the data obtained in these experiments. First, it is clear that 3-mercaptoisobutyric acid is a specific precursor of asparagusic acid. Second, the incorporation of 3-mercaptoisobutyric acid into asparagusic acid proceeds with the introduction of unsaturation between C-2 and C-4. Finally, asparagusic acid is formed from 3-mercaptoisobutyrate with complete retention of the sulfur atom present in the latter compound. This last conclusion rules out the possibility that 3-mercaptoisobutyrate is incorporated into asparagusic by reversion to methacrylic acid.

The incorporation experiments with 3-mercaptoisobutyrate suggest that sulfide may be the sulfur donor in asparagusate biosynthesis. However, an alternative possibility is that cysteine is the immediate sulfur donor. This possibility arises from the fact that S-(β-carboxy-n-propyl)-*L*-cysteine [**104**, R = $-CH_2CH(NH_2)COOH$, Scheme 15] has been reported to occur in both onion and garlic plants.[94,96] Both of these plants are *Allium* species and they are therefore members of the *Liliaceae*. Since the genus *Asparagus* is also a member of the *Liliaceae*, it seemed probable that S-(β-carboxy-n-propyl)-*L*-cysteine might also occur in *A. officinalis*. This was shown to be the case by administration of [1-^{14}C]-isobutyrate to *Asparagus* plants and isolation of radioactive S-(β-carboxy-n-propyl)-L-cysteine (**109**, Scheme 17a) after four days (Table 5, expt. 7). Degradation of **109** via a Schmidt reaction to the diamino acid **110** proved that the incorporation was specific. Shortly after this experiment had been performed, the isolation of S-(β-carboxy-n-propyl)-L-cysteine from *A. officinalis* was reported by Kasai *et al.*[97] The presence of this amino acid in *Asparagus* having been established, experiments were carried out to evaluate the compound as a precursor of asparagusic acid. For this purpose, [^{35}S]- and [^{3}H]-labeled forms of S-(β-carboxy-n-propyl)-L-cysteine (**111**, **112**) were synthesized as shown in Scheme 17b,c. The two labeled amino acids were then mixed to obtain



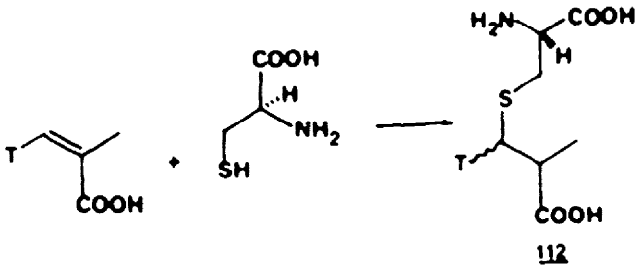
109 110

Scheme 17(a)



111

Scheme 17(b)



112

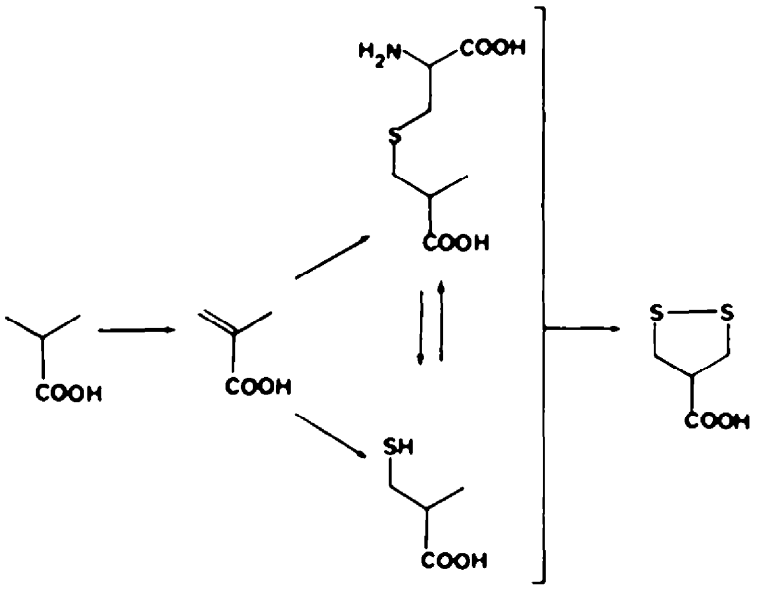
Scheme 17(c)

[^{35}S, 3(RS)-^{3}H]-2(RS)-S-(β-carboxy-n-propyl)-L-cysteine which was administered to *Asparagus* plants. The results of this experiment (Table 5, expt. 8) reveal that the amino acid is specifically incorporated into asparagusic acid with retention of the sulfur atom derived from cysteine.

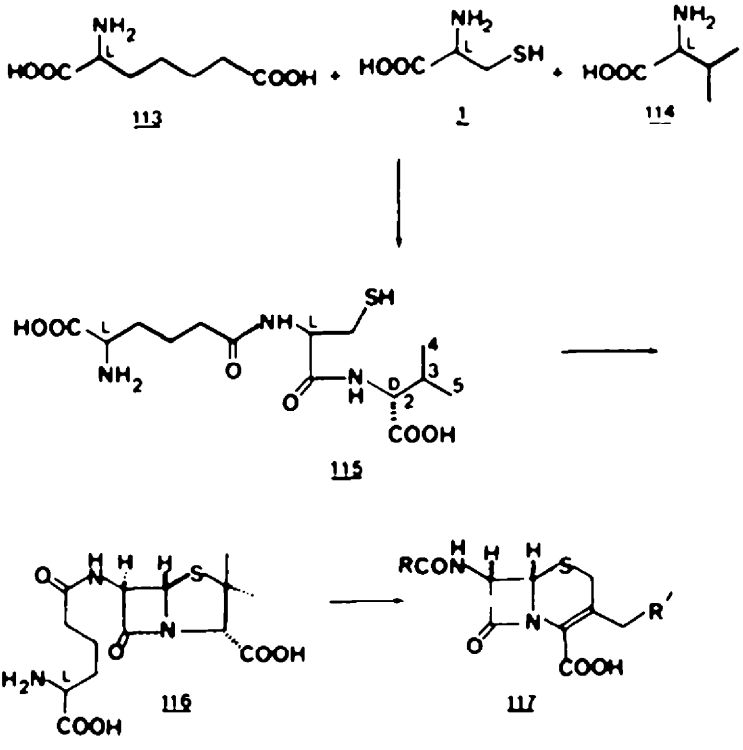
The combined data from experiments 5–8 (Table 5) can be interpreted in two ways. One interpretation is that both S-(β-carboxy-n-propyl)-L-cysteine and 3-mercaptoisobutyrate lie on the pathway to asparagusic acid, with the former compound most likely preceding the latter. An alternative interpretation is that the two thio compounds are interconvertible *in vivo* (Scheme 18). If this is the case, then only one of the two sulfur compounds need lie on the pathway in order to explain the results. The interconversion between the two thio compounds could presumably be mediated by pyridoxal phosphate.

PENICILLINS

The mechanism of carbon–sulfur bond formation in penicillin biosynthesis appears to bear a close relationship to the sulfur introduction steps in biotin and lipoic acid biosynthesis. The biosynthesis of the penicillins has been the subject of intensive scrutiny and all but the most current developments are summarized in recent reviews.[98,99] The biosynthetic picture that has emerged from these numerous investigations is illustrated in Scheme 19. It is now well established that L-α-aminoadipic acid (**114**), L-cysteine (**1**), and L-valine (**113**) are first linked to form δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (**115**), which is also known as ACV or the LLD-tripeptide. This tripeptide next undergoes ring-closure to form isopenicillin N(**116**). The latter compound then serves as the precursor of all other penicillin derivatives and is also rearranged to yield the cephalosporins (**117**). The feature of penicillin biosynthesis that is of especial interest in the context of this review is the ring-closure of the tripeptide **115** to isopenicillin N (**116**). The mechanism of this transformation which leads to the creation of a new carbon–sulfur bond, has been subjected



Scheme 18



Scheme 19

to a number of experimental probes. The key observations resulting from these investigations are as follows.

First, incorporation experiments with methyl deuterated valines have shown that all six deuterons are retained in the bicyclic products. In addition, δ-(L-α-aminoadipyl)-L-cysteinyl-D-[2-^{3}H]-valine is converted to isopenicillin N with retention of at least part of the valine α-proton. These results have recently been confirmed by conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-[2-^{2}H,Me$_2$-^{2}H$_6$]-valine into isopenicillin N by a cell-free system from *Cephalosporium acremonium*.[100]

Second, incorporation experiments with chiral forms of valine in which one or the other of the diastereotopic methyl groups (C-4, C-5) is labeled with deuterium or carbon-13 established that the introduction of sulfur at C-3 of valine occurs with overall *retention* of configuration. Since the

possibility of inversion at C-3 of valine during its incorporation into ACV has been ruled out,[101] it therefore appears that ACV is converted to isopenicillin N with retention of configuration at the site of carbon-sulfur bond formation.

Finally, the possible intermediacy of a form of the tripeptide **115** that is hydroxylated at C-3 of the valine residue can be discounted since incubation of synthetic hydroxy-ACV with a cell-free system from *C. acremonium* failed to produce detectable quantities of isopenicillin N.[102]

It is clear from these observations that the mechanism of C–S bond formation in penicillin biosynthesis exhibits a remarkable similarity to the sulfur introduction processes accompanying the formation of both biotin and lipoic acid. In all three cases, functionalization is accomplished without apparent involvement of adjacent carbon atoms and hydroxylated compounds do not apear to be intermediates. Furthermore, C–S bond formation at C-4 of dethiobiotin and at C-3 of the valine residue of ACV takes place with retention of configuration. It has been suggested that this stereochemistry may result from formation of a carbon radical which is then trapped by a nearby disulfide linkage.[101] Such a mechanism could also account for the formation of the C–S bond at C-6 of octanoic acid with inversion of configuration provided that the stereochemical outcome of radical trapping is dictated by the functionalizing enzyme.

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