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NEW ASPECTS OF THE CHEMISTRY OF BIOTIN AND OF SOME ANALOGS

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Abstract - The strategies of the recent total syntheses of biotin are compared and one of them is discussed more extensively. The latter, whose principle relies on the stereoselective α alkylation of sulfoxides, can be used efficiently for the preparation of analogs, some of which are described. The first total synthesis of selenobiotin is reported also. Its *in vitro* biological activity with E. Coli acetyl CoA carboxylase and its *in vivo* activity are investigated. Along with selenobiotin, biotin sulfoxides and sulfone are tested with the biotin carboxylase subunit of the above enzyme, in order to obtain some insight on the role of the sulfur atom in biotin catalyzed reactions.

I. STRUCTURE AND BIOCHEMICAL FUNCTION OF BIOTIN

Biotin was isolated in 1934, by Kögl (1) from egg yolks and its structure established in 1942 by Du Vigneaud (2) and then confirmed by X-ray (3) (Fig. 1). It is also a cristallographic study of a biotin derivative which gave the absolute configuration (4).

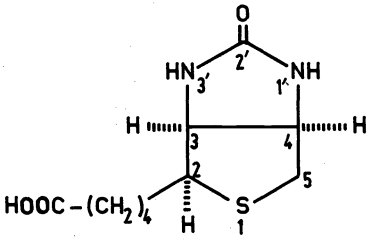


Fig. 1

Very recently, in 1976, two groups redetermined the crystal structure of biotin and obtained results in agreement with the previous ones, but more accurate (5). According to these data, the ureido ring is planar and the thiophane ring has an envelope conformation (Fig. 2).

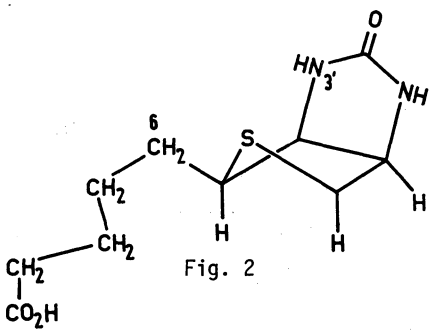


Fig. 2

The valeric side chain is not fully extended but twisted and there is a strong interaction between C_6 and N'_3, a feature of importance in the reactivity of biotin. This envelope conformation of the thiophane ring is also found in solution, as shown by NMR studies by Glasel (6) and by our group (7).

Before its specific biological role (8) was recognized, biotin was studied as a growth factor. The product isolated by Kögl was a yeast nutrient. Its identity with other growth factors, in particular with vitamin H was soon established. Vitamin H was known as an anti egg-white injury factor : rats fed with a diet containing raw egg-white as the sole source of amino-acids developed severe demartitis together with hair loss. These symptoms were relieved by an unknown factor from yeast named vitamin H, (from "Haut" : skin). The toxic properties of raw egg-white were later shown to be due to avidin, a glycoprotein with an extraordinary affinity for biotin. Inhibition of a reaction by avidin is the method which is commonly used when one wants to prove that biotin plays a role in this reaction.

It is now well known that biotin is involved in carboxylation reactions (8). It is the coenzyme of carboxylases, catalyzing fundamental metabolic processes. A very important one is, for instance, *acetyl CoA carboxylase* which carries out the transformation of acetyl CoA into malonyl CoA.

$$CH_3COSCoA \longrightarrow {}^{-}OOC\text{-}CH_2\text{-}COSCoA$$

This is the first step of the biosynthesis of fatty acids. It is also, at least in animal tissues, the rate-determining, and thereby regulating step of the synthetic process.

Other biotin-enzymes catalyze chemically analogous reactions, namely fixation of carbon dioxide at an enolisable position.

α to a thioester

$$CH_3\text{-}CH_2\text{-}COSCoA \xrightarrow[\textit{Carboxylase}]{\textit{Propionyl CoA}} CH_3\text{-}\underset{\displaystyle COO^-}{\underset{|}{CH}}\text{-}COSCoA$$

or at a vinylogous position

$$CH_3\text{-}\underset{\displaystyle CH_3}{\underset{|}{C}} = CH\text{-}COSCoA \xrightarrow[\textit{Carboxylase}]{\beta\ \textit{methylcrotonyl CoA}} {}^{-}OOC\text{-}CH_2\text{-}\underset{\displaystyle CH_3}{\underset{|}{C}} = CH\text{-}COSCoA$$

or α to a ketone

$$CH_3\text{-}CO\ COO^- \xrightarrow[\textit{Carboxylase}]{\textit{Pyruvate}} {}^{-}OOC\text{-}CH_2\text{-}CO\ COO^-$$

The mechanism of biotin catalysis is known in its main features. The overall process involves two successive half-reactions (Fig. 3).

1. N carboxylation of biotin

This step was first elucidated in 1959 by Lynen and his group. They identified the 1' nitrogen as the site of carbon dioxide fixation by isolation of the unstable N-carboxybiotin as a methyl ester. The necessary cofactors for this reaction are Adenosine triphosphate, bicarbonate and a divalent cation Mg^{++} or Mn^{++}.

2. Transcarboxylation

Carbon dioxide, activated as N-carboxybiotin is then transfered to the substrate during this second step.

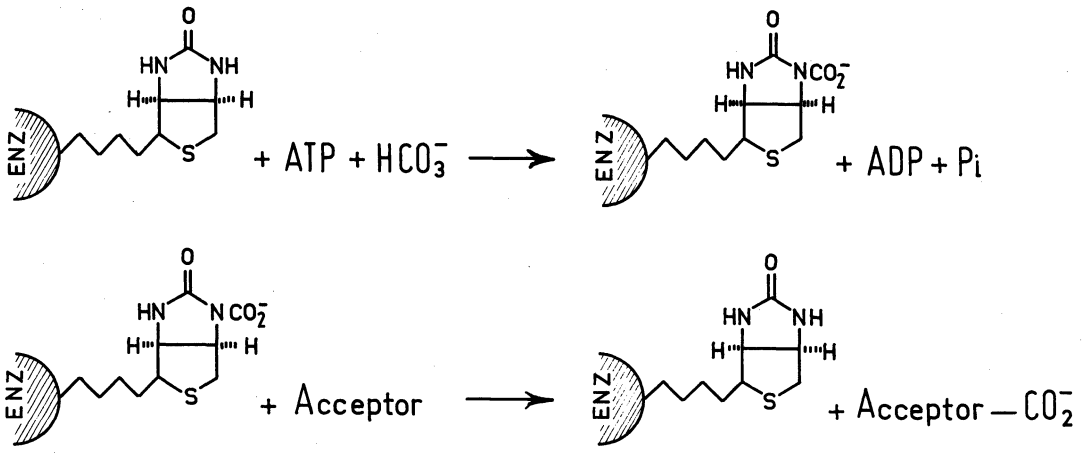


Fig. 3

These two half-reactions occur at different sites and even on different subunits of the enzyme. Biotin is covalently linked to the enzymes through the ε amino group of a lysine. The length of the linkage (valeric chain of biotin and lysine side chain) allows biotin to act as a carboxyl carrier between the two active sites of the enzyme.

In spite of these important findings, many aspects of the mechanism are still obscure ; the amount of information concerning carboxylases is scarce, compared to what is known for other enzymes. Very little data on the structure of the active sites are available and the primary sequences have not been determined. Little is known about the nature of the catalytic processes, the amino-acids involved have not been identified. At this state of knowledge, exploration of the active site by analogs is a very valuable method. Such a work has been undertaken, but it is still uncomplete. The number and the variety of the derivatives which have been tested is rather limited and very often, existing data deal only with growth-promoting properties. Lynen and Lane (8) have studied the carboxylation of some analogs with purified enzymes but have given only relative activities with respect to biotin. Determination of the kinetic parameters, K_M and V_{max} would be most interesting. Analogs bearing functional groups allowing affinity-labeling would also be very useful.

Hence biotin chemistry and biochemistry represent a very wide field of investigation in which our group became interested a few years ago. It is on synthesis problems that I would like to emphasize here. As I just mentioned, it would be interesting to prepare other analogs

or to have an easier access to some of those which have already been described and therefore to dispose of an appropriate synthesis. On the other hand, the industrial synthesis of biotin is also of great interest. Owing to its basic metabolic function biotin is present in practically all the cells, but in very small amounts. All attempts to prepare biotin industrially with microorganisms have not been, up to now, efficient enough to compete with chemical synthesis.

II. BIOTIN TOTAL SYNTHESES

The first syntheses achieved shortly after the structure elucidation, long and non stereoselective, have only an historical interest (9). An industrial synthesis was published in 1949 by the Hofmann La Roche group (10). It remains long (13 steps), but it is very stereoselective and produces only the natural isomer. Except for some improvement to this process (11) nobody has been further interested in biotin synthesis for a long period.

Lately, a renewed interest has suddenly appeared and four new syntheses have been proposed in 1975, all of them very different from one another in their strategy. The first one is due to our group (12), the others have been published shortly after respectively by Ohrui and Emoto (13), Uskokovic' and coworkers (14) and Zav'yalov and coworkers (15). Before discussing with more details our own work, I think it would be interesting to compare very briefly the general scheme of these syntheses. The two first ones yield (d) biotin with an optically active natural product as a starting material : D mannose in the work of Ohrui and Emoto, L cysteine in that of Uskokovic. But these syntheses, of a great academic interest are neither short nor versatile for the preparation of analogs.

The synthesis of Zav'yalov's group (Fig. 4) starts with 4 methyl imidazolone which is transformed in five steps in the thiophene analog of biotin, a compound which has also been obtained in Mukayama's group (16). But, as expected, hydrogenation raises some problems. It was performed by Zav'yalov under ionic conditions with triethylsilane and trifluoroacetic acid. (dl) biotin is thus obtained, but with a 10 % yield only. This synthesis is by far the shortest, but its practical use is linked to the improvement of the last step.

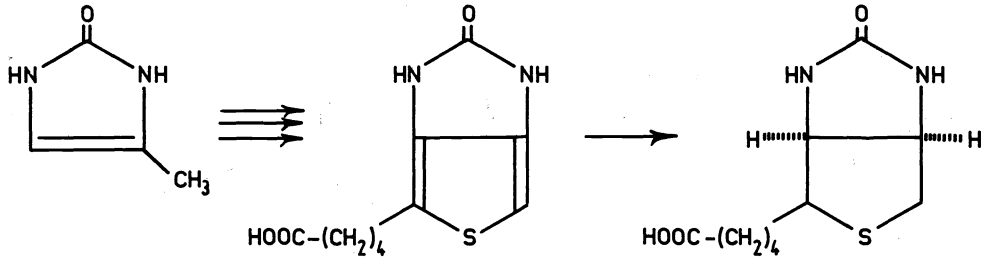


Fig. 4

Our synthesis yields also (dl) biotin. Its principle is very simple and takes advantage of the high selectivity of the alkylation of sulfoxides which was established by several groups in the last few years. We have especially studied the methylation of six membered cyclic sulfoxides and we have shown on many examples that the methylation occurs exclusively axially in the axial sulfoxides and for more than 90 % equatorially in the equatorial sulfoxides, that is to say always trans to the S→O group (17). For instance, for the 4-t.butyl thiacyclo-

hexane oxides (Fig. 5) :

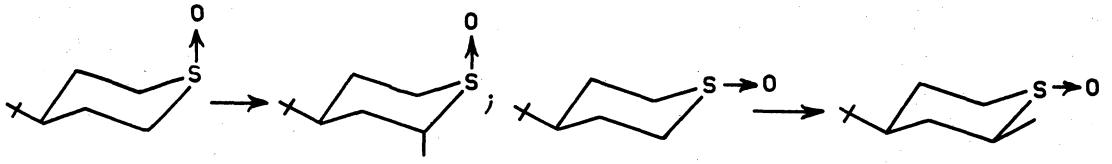


Fig. 5

Supposing that this empirical rule holds for five-membered rings it offers a very simple scheme for a total synthesis of biotin (Fig. 6). One has to prepare the intermediate sulfide, oxidize it and then alkylate the sulfoxide.

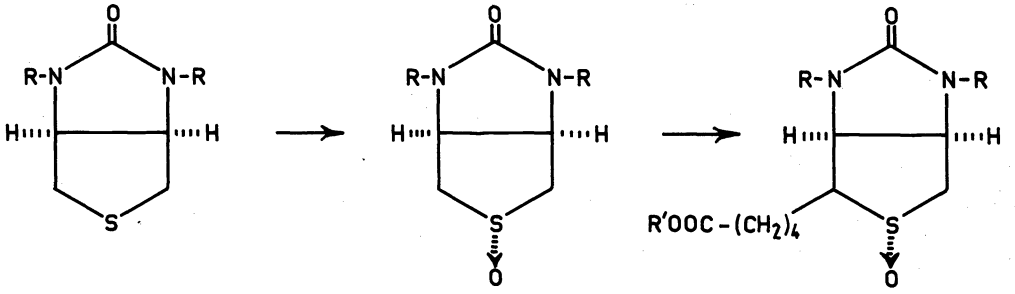


Fig. 6

To introduce the chain with the right stereochemistry, namely trans to the junction hydrogens the alkylation has to be carried out on the sulfoxide which is cis to these hydrogens. The general scheme of the synthesis is represented on Fig. 7. The sulfide is easily prepared with excellent yields by classical reactions starting with mesodibromosuccinic acid, the first two steps being those of the synthesis of Goldberg and Sternbach (10).

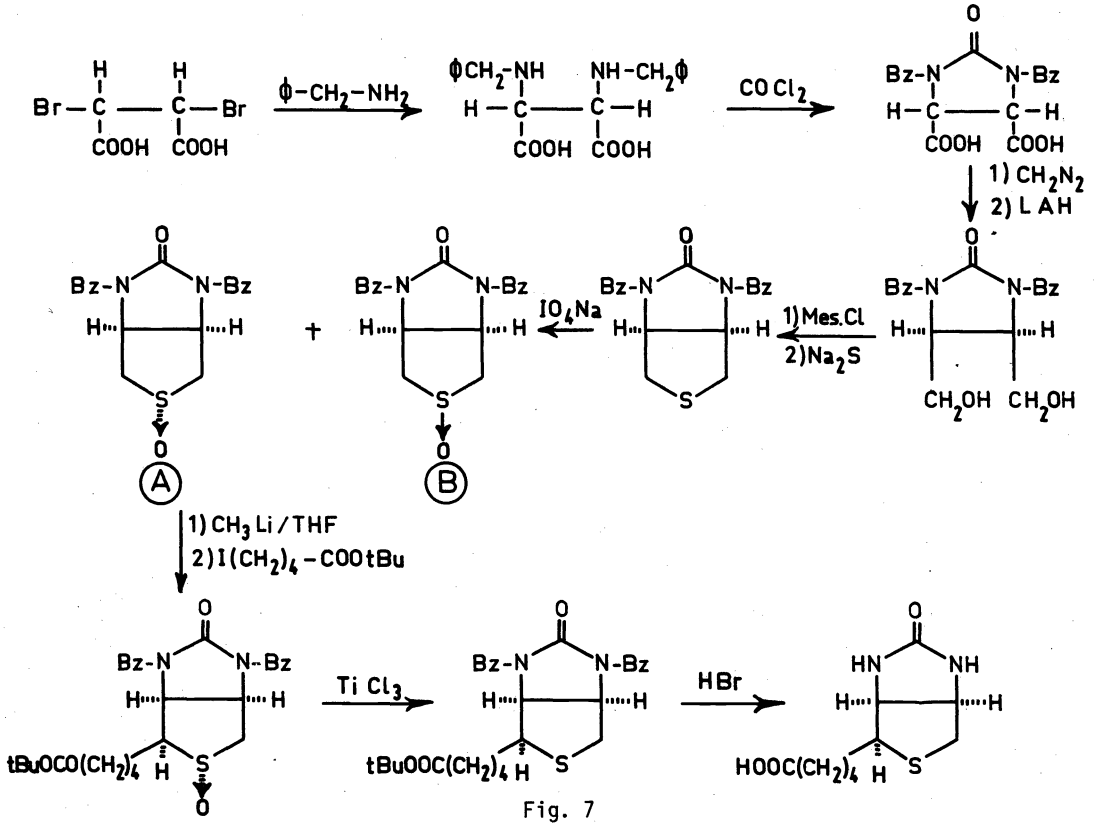


Fig. 7

Oxidation with sodium metaperiodate yields two sulfoxides A and B in a 90-10 ratio. Their configuration was readily determined owing to our previous experience in this field. In a recent work concerning the configuration of biotin sulfoxides (7), we could show that biotin and its sulfoxides have the envelope conformation shown on Fig. 2. Conformational analysis of five-membered rings is generally not straightforward, but in this case we were greatly helped by the fact that a zero or very small coupling constant is observed between H_β and one of the H_α protons. A careful examination of the pseudorotation cycle shows that this envelope conformation, where the dihedral angle between H_β and $H_{\alpha A}$ is nearly 90°, is the only one where this is possible. The NMR spectrum of the minor isomer B shows this pattern and has hence the same conformation (Fig. 8).

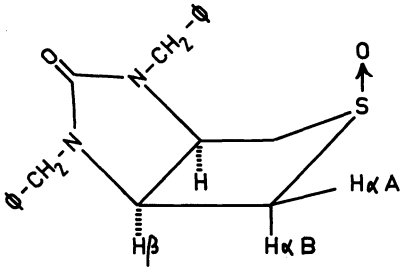


Fig. 8

This is not the case for the major isomer A which exhibits two $H_\beta H_\alpha$ coupling constants of the same order of magnitude. Its conformation is not yet established. As the conformation of B is well defined, the configuration at sulphur can be determined from the NMR data : as shown by our previous studies (18), the J gem of an α methylene group is a very reliable criterium. It is different for an axial and an equatorial sulfoxide, respectively 14.5-15 Hz and 13-13.5 Hz. The observed value of 14.5 Hz proves that the sulfoxide is axial. This is confirmed by benzene-induced shifts. The $H_{\alpha B}$ protons (those which are coupled with H_β) are shielded by ca. 1 ppm whereas $H_{\alpha A}$ by only 0.5 ppm.

Hence in the predominant isomer, the S→O bond is cis to the junction-hydrogens. It is the right isomer for alkylation. It is well known, since the classical work of C.R. Johnson (19) that the stereochemistry of a sulfide oxidation depends on the nature of the oxidizing agent. In the hope of increasing the selectivity, we investigated other oxidation methods. Results in Table 1 show that this is not possible. Sodium metaperiodate is the best reagent, reaction with ozone being more difficult to control.

Table 1. Oxidation stereochemistry

Oxidizing agent	A	B
$NaIO_4$/MeOH H_2O	90	10
O_3/CH_2Cl_2	90	10
H_2O_2/ACOH	80	20
Φ ICl_2/Pyr.	55	45

The alkylation was first classically carried out in tetrahydrofuran with butyllithium as a base. The sulfoxide is treated with butyllithium at -78° and then at -30° by t-Butyl ω-iodovalerate, yielding the expected alkylated product. Only one isomer could be detected. We chose to use a t-Butyl ester to prevent the addition of the lithiated sulfoxide on the ester function. It is actually necessary : the methyl ester is attacked selectively at the ester group to give an ω-iodo side chain (Fig. 9).

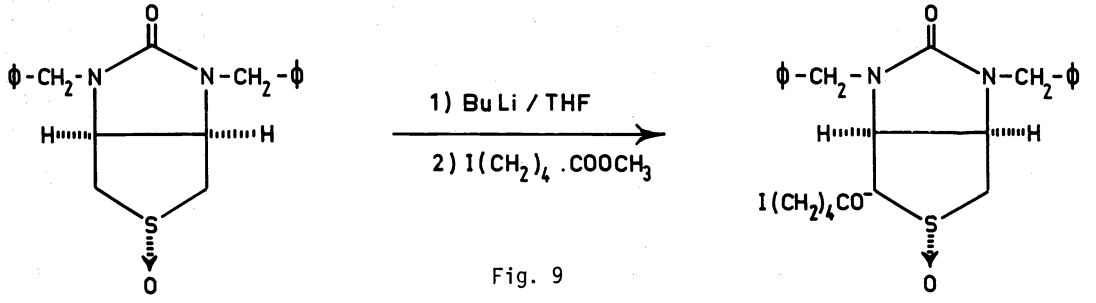


Fig. 9

The sulfoxide is easily reduced either with triphenylphosphine in carbon-tetrachloride (20) or with titanium (III) chloride in methanol-water (21). After hydrolysis of the t-Butyl ester and debenzylation by 48 % aqueous hydrobromic acid, (dl) biotin is obtained. The configuration of the side chain is thus established and this result proves that our empirical rule, alkylation trans to the S→O bond, holds also for five membered rings.

Most of these reactions are very simple. They occur with high yields and require no further comments. However, there is one step which has raised many problems, namely the key step, the alkylation of the sulfoxide.

In the case of sulfoxide A, the intermediate carbanion can undergo a β-elimination which is competitive with the alkylation. There were even doubts as to whether the β-elimination would not be the only reaction. Indeed, in the first experiments carried out with butyllithium in tetrahydrofuran, the alkylation yield did not exceed 30 %. Along with the starting material, always present, we found the monoelimination product and also dibenzyl urea formed by double elimination (Fig. 10).

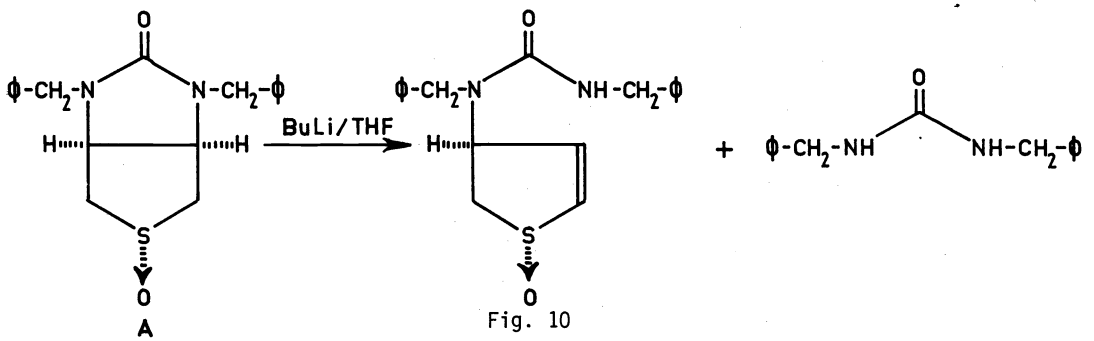


Fig. 10

But we found that by replacing butyllithium by methyllithium to generate the carbanion, the reaction was completely suppressed. In this case, the lithium salt is stable for several hours at -30°C. We checked that this rather surprising result was not due to a salt effect. Butyllithium, prepared with lithium metal and methyl iodide contains lithium iodide and lithium hydroxyde whereas methyllithium obtained by an exchange reaction between butyllithium and methyl iodide in hexane is supposed to be salt-free (22). In the case of butyllithium,

elimination might be accelerated owing to electrophilic assistance due to a coordination of Li^+ with the ureido ring. But no elimination is observed when the reaction is carried out with methyllithium in presence of lithium iodide. The different behaviour of the two bases, is probably due to a change in the transition state of the reaction and requires further investigation.

When this competitive reaction lowering the yield of alkylation was thus eliminated, we found that there was still another one, namely protonation of the intermediate carbanion by the solvent. It is known that butyllithium cleaves tetrahydrofuran at -30°C contrarily to methyllithium(23). An α sulfinyl lithium, a base weaker than methyllithium should not attack the solvent, but it does in fact. This side-reaction occurs also in another etheral solvent such as diglyme, but is a little slower and in this case the alkylation can be achieved with a 60 % yield. The remaining product is only pure starting material.

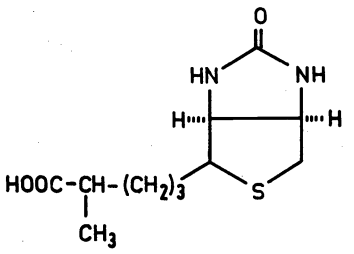
It should be possible to further increase the alkylation yield by using a more reactive alkylating agent, with a better leaving group. This point is now under investigation.

This is, to my opinion a very straightforward synthesis and it could be of industrial interest. But its main advantage over the others is that it is much better adapted to the preparation of analogs since different substituents in particular various side-chains can be introduced on the key intermediate sulfoxide.

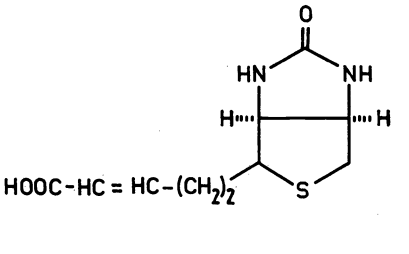
III. PREPARATION OF BIOTIN ANALOGS

1. Side-chain modifications

It is most interesting to synthesize this type of derivatives since two of them, α methylbiotin and α dehydrobiotin (Fig. 11) isolated from microorganisms cultures (24, 25) present interesting antibiotic properties, namely as probable antimetabolites of biotin.



α methylbiotin



α dehydrobiotin

Fig. 11

The preparation of some other compounds of this type and the knowledge of their biological activity would certainly bring valuable information on the properties of carboxylases. We have already prepared by the present method α-methylbiotin whose total synthesis had previously been achieved by another route (25).
Other syntheses are in progress.

2. Thiophane-ring modifications

Another type of analogs that can readily be obtained by our method are the compounds substituted at the 5 position of the thiophane ring. Indeed we have observed in our model studies on thiane oxides, that alkylation of an α substituted sulfoxide is very regioselective and occurs exclusively at the α' position (17). Taking advantage of this property, we have prepared the two isomeric 5-methylbiotins, according to the scheme depicted in Fig. 12.

Starting with sulfoxide A it is possible to introduce successively the methyl and the valeric chain with the same orientation. Using sulfoxide B, the methyl group can be introduced with the opposite stereochemistry, that is, cis to the junction hydrogens. Inversion of configuration at sulphur with Meerwein's salt allows then the linkage of the side-chain with the natural orientation.

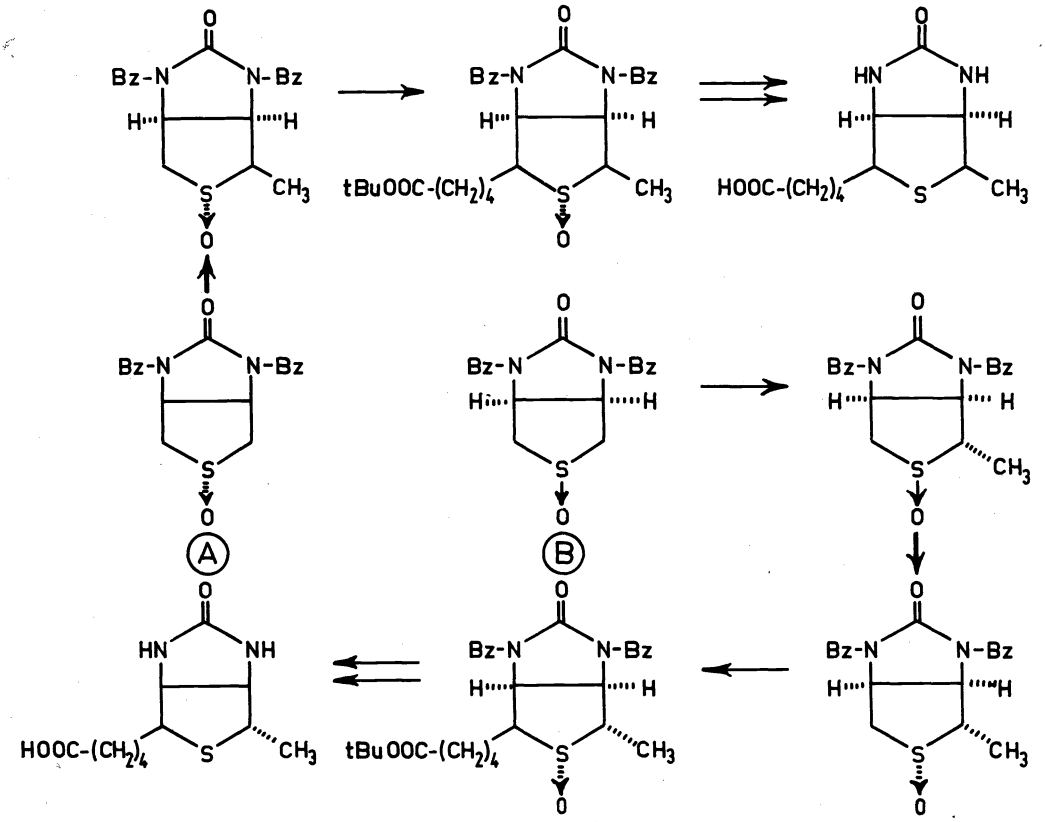


Fig. 12

3. Selenobiotin

Since selenium has been recognized as a very important micronutrient for animals and bacteria, the behaviour of selenium analogs of important sulfur containing natural compounds has received much attention. However, in the field of coenzymes and vitamins, only coenzyme A derivatives were prepared and tested (26). It was interesting to obtain information on the biological activity of selenobiotin.

We did not try to prepare this compound according to our synthesis scheme because what is known about the properties of selenoxides is not very encouraging. We used the strategy of Hofmann La Roche's biotin synthesis (10,11) which appeared the best suited for a rapid achievement of our goal. In this process, the previously mentioned diacid is transformed

into a lactone which can be resolved. The optically active lactone is then converted into a thiolactone. We could prepare the corresponding (+) selenolactone with a 50 % yield by successive treatment of the lactone by bis (methoxymagnesium) diselenide and hypophosphorous acid. The consecutive reactions represented on Fig. 13 are similar to those which have been

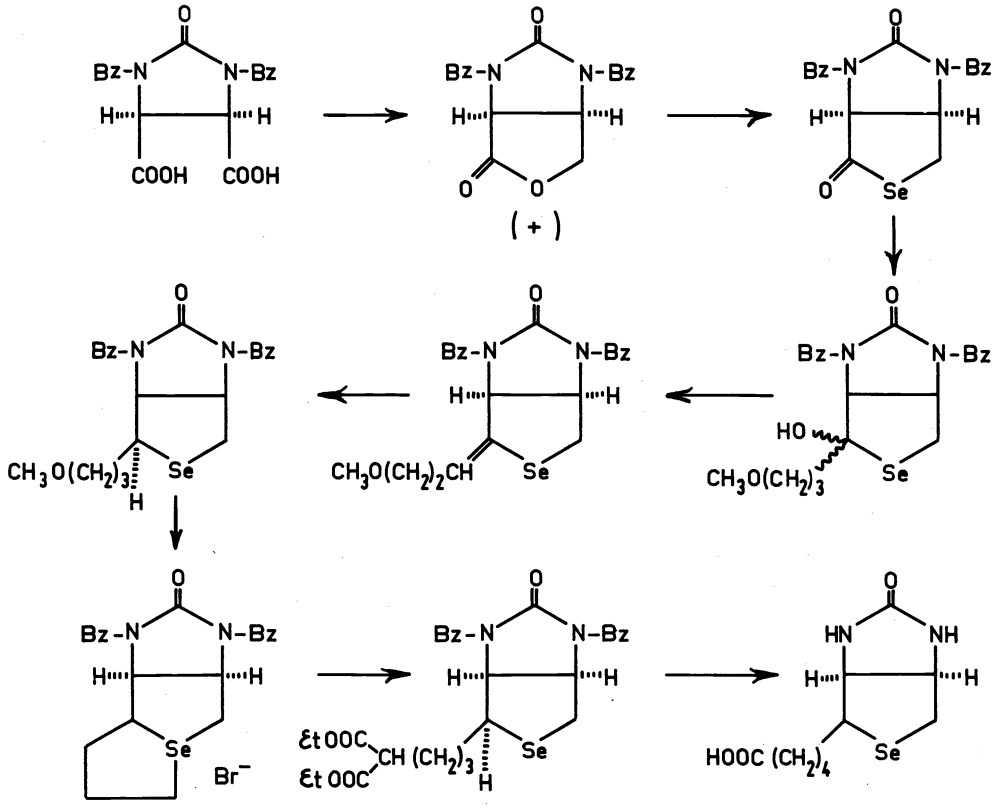


Fig. 13

used for biotin. Replacement of sulphur by selenium could have changed the course of some of them. It is not the case. Hydrogenation of the double bond occurs quantitatively, with a high selectivity. The intermediate selenium salt obtained in the demethylation step is treated with sodio diethylmalonate and the resulting product is classically transformed into (+) selenobiotin. An analysis of its NMR spectrum shows that the coupling constants of the thiophane ring are very close to those found in biotin. Hence selenobiotin and biotin have very similar conformations (27).

IV. BIOLOGICAL ACTIVITY OF BIOTIN ANALOGS

We first tested selenobiotin as a growth factor for two biotin-requiring organisms, Lactobacillus arabinosus and a mutant of E. Coli. L arabinosus is commonly used for the microbial determination of biotin. It is very specific for biotin itself and does not respond to other biotin vitamers. We have observed that under the conditions used for the titration, selenobiotin can fulfil the biotin requirement of that strain *with the same efficiency*. Likewise, E. Coli C 162, a biotin requiring auxotroph (28), grows *exactly at the same rate* on a synthetic medium in the presence of equal amounts of biotin and selenobiotin.

Among the numerous biotin analogs tested so far for growth promoting activity, the only one which has been shown to exhibit activity without being transformed into biotin is oxybiotin (29). We think that selenobiotin is also intrinsically active. Among the available biotin auxotrophs of E. Coli we selected the C 162 strain which is unable to carry out the last step of the biotin biosynthesis, namely the conversion of dethiobiotin into biotin. With such a strain, the transformation of selenobiotin into biotin through dethiobiotin is impossible. Of course, as the intermediates of the dethiobiotin - biotin conversion are still unknown (30), it is impossible to rule out a transformation of selenobiotin into an intermediate located after the blocking step. However, this appears very improbable. Moreover the degradation pathway of selenobiotin must resemble closely that of biotin and must start with the β-oxidation of the valeric chain, yielding norderivatives which are not involved in the biosynthesis (31). But in order to rule out definitely this transformation we are now making attempts to detect selenium in the protein of bacteria grown on selenobiotin.

It was also interesting to study the properties of selenobiotin at the molecular level for *in vitro* carboxylation reactions. The carboxylases are essential for growth but the fact that E. Coli grows at the same rate in the presence of biotin or selenobiotin does not bring any information about the relative activities of seleno and normal carboxylase beyond the fact that both enzymes are active. We have compared the specific activities of acetyl CoA carboxylases present in crude cell-free extracts of E. Coli C 162 grown on biotin or on selenobiotin. The test used to measure the acetyl CoA carboxylase activity involves the carboxylation of acetyl CoA with radioactive bicarbonate and the measurement of radioactivity incorporated into malonyl CoA (32). With cultures grown under identical conditions (except for growth factor) the enzymic assay shows that the specific activity of the selenocarboxylase is about 60 % of that of the natural carboxylase. This represents the first example described so far of an active acyl CoA carboxylase with a modified prosthetic group. The only known example of such a replacement of biotin by an analog is due to Lane who demonstrated *in vitro* the incorporation of oxybiotin in the Propionibacterium Shemanii transcarboxylase. However in this case the transformed carboxylase obtained is inactive (33).

We selected acetyl CoA carboxylase of E. Coli for this study because it is a rather simple and convenient material. Contrarily to most acetyl CoA carboxylases which are active only under polymeric forms, with a molecular weight 4 to 8 millions, acetyl CoA carboxylase of E. Coli is active in the monomeric form. It is constituted by three subunits (8) as schematically represented on Fig. 14.

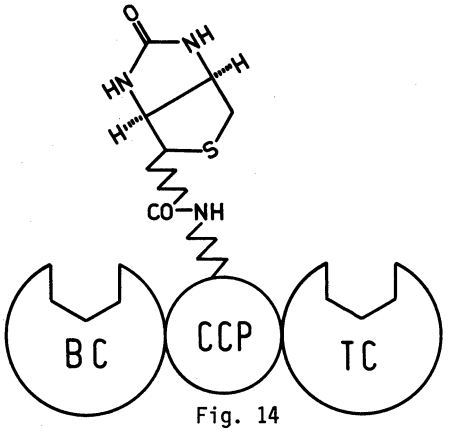


Fig. 14

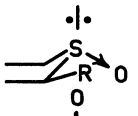
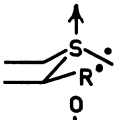
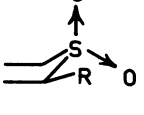
Biotin is covalently attached, through a lysine, to a small protein, named "Carboxyl Carrier Protein" (CCP). A second subunit, free of biotin, "Biotin carboxylase" (B.C.) catalyses the carboxylation of biotin linked to the C.C.P. The third subunit, "Carboxyl Transferase" (C.T.) catalyses the transfer of CO_2 between N-carboxybiotin and acetyl CoA. The utility of the long arm as a link between biotin and the protein appears clearly.

An attractive property of this enzyme is its dissociation into active subunits which can be separated and thoroughly purified. The biotin carboxylase, which under normal conditions carboxylates the prosthetic group of the enzyme, is able to carboxylate free biotin. This constitutes a very appealing but elaborate model for the first half reaction and makes possible the study of the active site with the help of biotin analogs. The carboxylation of biotin or analogs can be followed either by use of radioactive bicarbonate or spectrophotometrically through a serie of coupled enzymatic reactions (34).

We observed that selenobiotin is a very good substrate for biotin carboxylase, as it has almost the same affinity (K_M Sebiotin/K_M biotin = 1) along with a good reactivity (V_{max} Sebiot/V_{max} biot = 0.84). Lane has already tested oxybiotin among other analogs with the Biotin Carboxylase of E. Coli (34), but he has not been able to reach the K_M and V_{max} values, which are of interest for this kind of study. Under the conditions he used, the activity of selenobiotin is 84 % of that of biotin, compared with 20 % for oxybiotin. Thus the reactivity decrease is less important when sulfur is replaced by selenium than by oxygen.

This argument has to be taken in account in the discussion of the role of the sulfur atom in the biotin function. Among the numerous hypotheses which have been discussed, the most favored is that sulfur may facilitate binding of the coenzyme to the active site through hydrogen bonding. To get further unsight in this problem, we also investigated biotin analogs modified at sulfur, namely the two isomeric sulfoxides and the sulfone. The results obtained are listed in table 2.

TABLE 2. Activity of biotin sulfoxides and sulfone with E. Coli Biotin Carboxylase

		K_M analog/K_M biotin	V_{max} %
d sulfoxide		8	20
l sulfoxide		3	3
sulfone		3	3

The two sulfoxides exhibit a very different behaviour. The K_M values, which give a good picture of the affinity, are not very different. However, it appears that the (d) sulfoxide has the lowest affinity. This cannot be interpreted on the basis of unfavorable steric

interactions due to the equatorial oxygen in this isomer since the affinity of the sulfone, which has also an equatorial oxygen is higher. This may be explained by a destabilizing polar interaction, for it is well known that the polarity of the $S^{\delta+}-O^{\delta-}$ bond is larger for a sulfoxide than for a sulfone.

The reactivity differences, expressed by the V_{max} values are more important. The (d) sulfoxide has a relatively high V_{max}. The (l) sulfoxide and the sulfone exhibit a very low reactivity. It is tempting to suggest that an axial free electro-pair is essential for reactivity.

Of course, this is a prelimary discussion and this study must be completed with other analogs to establish the topology of the active site.

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