Biotin Biosynthesis: Synthesis and Biological Evaluation of the Putative Intermediate Thiols

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Abstract: Previous results led to the conclusion that the last step of biotin biosynthesis, the sulfur insertion into dethiobiotin, should involve a thiol as intermediate. The three possible thiols have been synthesized and their biological activity was evaluated: the primary thiol **10** labeled with [^{35}S] or [^{34}S] or with deuterium was transformed into biotin by resting cells of *Bacillus sphaericus* without loss of the label. Hence, it is a possible intermediate. Interestingly, growing cells transformed [^{35}S]- or 5-[$^{2}H_2$]-labeled **10** into biotin with loss of the labels. **10** and one of the secondary thiols **5B** also promote the growth of *Escherichia coli C124*, a biotin auxotroph, but again they lose their sulfur during their conversion to biotin. The growing cells experiments reveal a complex pathway which remains to be elucidated.

Introduction

The last step of the biosynthesis of biotin, i.e., the insertion of sulfur into dethiobiotin (Scheme I) raises a fascinating mechanistic problem. The intermediates and the ultimate sulfur donor have not been identified yet. The type of enzyme involved and the mechanism of C–S bond formation are of course also unknown.[1]

Previous reports allowed the exclusion of a process involving unsaturated precursors.[2,3] We have also shown that an intermediate hydroxylation was very unlikely;[4] the most usual hypothesis is now a direct sulfur insertion into a C–H bond.[3,5] This general assumption leaves many mechanistic possibilities open. The simplest one involves the formation of a thiol, on carbon 2 or on carbon 5 of dethiobiotin, which would then cyclize (Scheme I). The cyclization pathway could be analogous to the one which has now been elucidated in the biosynthesis of penicillin.[6] But no experimental evidence in support of a pathway of this type was yet available.

We decided to prepare the postulated intermediate thiols and to check if they were converted into biotin by *E. coli C124*, the biotin auxotroph strain[7] that we have used in our previous experiments,[2b-4] or *B. sphaericus* which produces biotin from dethiobiotin very efficiently.[15]

Results and Discussion

Synthesis of the Thiols. The synthesis was first achieved starting from the corresponding racemic alcohols.[8] As the configuration at C-2 of the two secondary alcohols **1A** and **1B** had not been established, we prepared the three thiols (±)-**5A**, (±)-**5B** and (±)-**10**, according to the sequence R–OH → R–OMes → R–SCN → RSH outlined in Scheme II.

The primary thiol was also prepared by the same sequence of reactions, starting from the alcohol (−)-**12**, derived from (+)-biotin (Scheme III): oxidation of the biotin methyl ester with H_2O_2 30% in acetic acid gave a mixture of sulfoxides, highly enriched in the (d) isomer. The Pummerer reaction with trifluoroacetic anhydride afforded the thiolactol **11**.[9] Desulfuration with Raney nickel led to the alcohol **12**. The corresponding thiol was isolated as the disulfide (+)-**15**.

Since this work has been carried out, two other syntheses of the same thiol have been published, one by our group,[10] starting from an intermediate in the Hoffmann-La Roche synthesis of biotin, and the second one by R. L. Baxter et al.,[11] using the strategy that we have already reported.[4]

The sulfur-labeled compounds have been obtained with K^{35}SCN or K^{34}SCN, using an excess of the mesylate **2A** or **13**.

5-[$^{2}H_2$]-**15** was obtained from 5-[$^{2}H_2$]-**12**.[12]

Experiments with *Escherichia coli*. The three thiols were tested for their growth promoting activity with the biotin requiring strain *E. coli C124* (Bio A$^-$). These growth experiments were performed with the racemic thiols **5A**, **5B**, and **10** (Scheme II).

At this stage, the configuration of the two secondary thiols was still unknown, and they will be referred to as I and II. The results are given in Table I: one of the secondary thiols II was inactive, while the other one I was almost as efficient as biotin. The primary

Table I. Growth Promoting Activity of Thiols I, II, and **10** for *E. coli C124*

compd	min. concn required for growth[a]	max. growth (O.D. 570 nm) at the given concn	genern time[b] (min)
(±)-I = **5B**	0.5	2	60
(±)-II = **5A**	inactive		
(±)-**10**	10	1.4	110
(+)-biotin	0.1	2	55

[a] Calculated for the active enantiomer in ng/mL. [b] When *E. coli C124* is grown on a synthetic medium with (10 ng/mL) of (±)-compound (I, II, or **10**) or 5 ng/mL of (+)-biotin.

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‖ Laboratoire de Chimie Organique Structurale, Université Pierre et Marie Curie.

(1) (a) Izumi, Y.; Tani, Y.; Yamada, H. *Bulletin of the Institute for Chemical Research*; Kyoto University, 1980; Vol. 58, p 434. (b) Eisenberg, M. In *E. coli and S. typhimurium*; Neidhardt, F. C., Ed.; American Society for Microbiology: 1987. (c) MeMoll, E.; White, R. H.; Shive, W. *Biochemistry* **1984**, *23*, 558.
(2) (a) Parry, R. J.; Kunitani, M. J. *J. Am. Chem. Soc.* **1976**, *38*, 317. (b) Guillerm, G.; Frappier, F.; Gaudry, M.; Marquet, A. *Biochimie* **1977**, *59*, 119.
(3) Frappier, F.; Jouany, M.; Marquet, A.; Olesker, A.; Tabet, J. C. *J. Org. Chem.* **1982**, *47*, 2257.
(4) Frappier, F.; Guillerm, G.; Salib, A. G.; Marquet, A. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 521.
(5) White, R. H. *J. Am. Chem. Soc.* **1980**, *102*, 6607.
(6) Baldwin, J. E.; Bradley, M. *Chem. Rev.* **1990**, *90*, 1079.
(7) *E. coli C124* (His$^-$Bio A$^-$) and C162 (His$^-$Bio B$^-$) were a generous gift of Dr. P. Cleary.
(8) The alcohols **1A** and **1B** were obtained by reduction with $NaBH_4$ of the corresponding ketone which was N,N′ diacetylated (4). One of the acetyl group was lost during the reduction. The position of the remaining one has not been determined.
(9) Lett, R.; Kuroki, Y. *Tetrahedron Lett.* **1982**, *23*, 5541.
(10) Even, L.; Florentin, D.; Marquet, A. *Bull. Soc. Chim. Fr.* **1990**, *127*, 758.
(11) Baxter, R. L.; Camp, J. D.; Coutts, A.; Shaw, N. J. *Chem. Soc., Perkin Trans. 1* **1992**, 255.
(12) Lett, R.; Kuroki, Y., personal communication.

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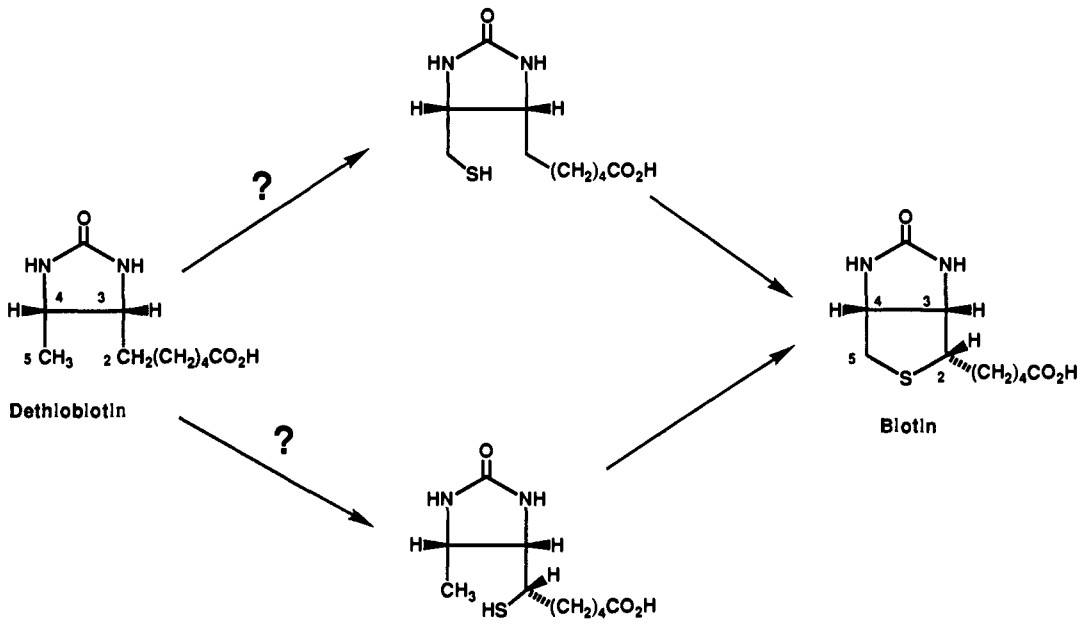
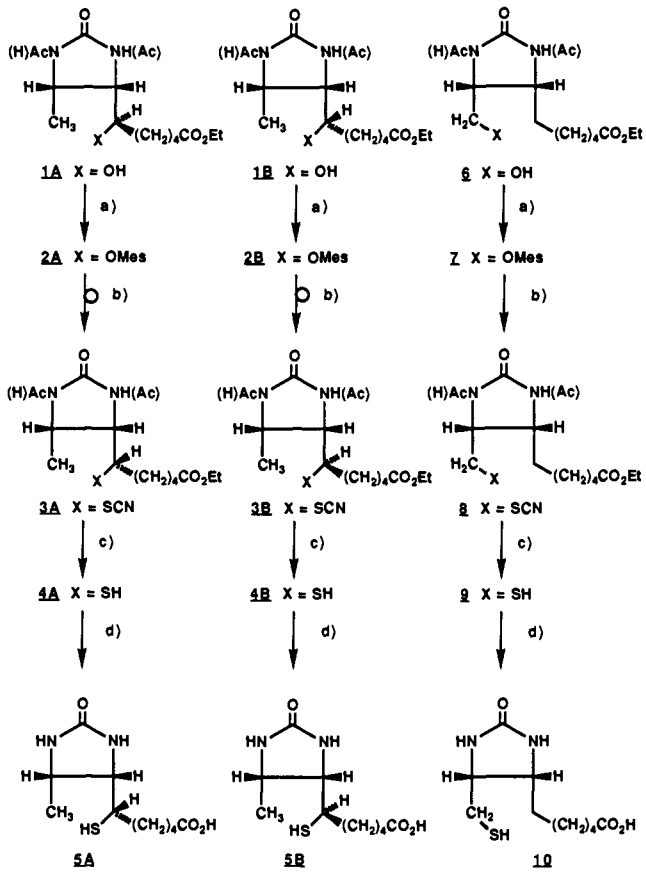
Scheme I



Scheme II[a]



[a] Reagents: (a) CH_3SO_2Cl, $(C_2H_5)_3N$, CH_2Cl_2; (b) KSCN, DMF; (c) $NaBH_4$, C_2H_5OH; (d) NaOH, CH_3OH.

thiol **10** also promoted the growth but at significantly higher concentrations. Thus, structure I appeared to be the best candidate for the first intermediate and the [^{35}S] labeled compound I was synthesized using K^{35}SCN.

In a preparative experiment, *E. coli C124* was allowed to grow in the presence of the labeled thiol, and the biotin was isolated as previously described.[2b] Unexpectedly, the fractions corresponding to biotin (identified and quantified with *Lactobacillus plantarum* and the isotopic dilution technique) were completely devoid of radioactivity.

At this point, it became essential to establish the configuration at C-2 of compound I. This was achieved through a correlation with biotin by R. Lett and Y. Kuroki,[12] according to Scheme IV. The 2-(*S*) thiol was obtained by opening of the thiophane ring of biotin and the 2-(*R*) isomer by the same reaction applied to epibiotin **16**.

This proved that the active thiol corresponded to structure **5B**, with a configuration at C-2 opposite to that of biotin. This result was consistent with the [^{35}S] loss just mentioned and an attractive hypothesis involved an intermediate dithiol which would cyclize with elimination of the sulfur at C-2 (Scheme V).

This hypothesis also allowed the rationalization of the stereochemistry of sulfur insertion which was shown to occur with retention at C-2 by Parry,[13] whereas inversion was found in the case of lipoic acid,[14] a closely related transformation. Our finding was consistent with a sulfur insertion with inversion, whatever the mechanism is, followed by a SN_2 type substitution (Scheme VI).

The postulated disulfide **18** was then also synthesized, starting from epibiotin **16** (Scheme VII). The reaction sequence was first experimented on biotin giving access to the epimer **17**,[9] which was also tested. Both compounds were inactive in the growth assays of *E. coli C124*. However, this negative result has to be interpreted with caution since the radioactive material was not prepared and the permeability of the cells to both compounds could not be checked. The study was run with the disulfide form. An in situ reduction by dithiothreitol would give the dithiol form, but we checked that this treatment inactivated the biotin synthetase (starting from dethiobiotin) of that strain.

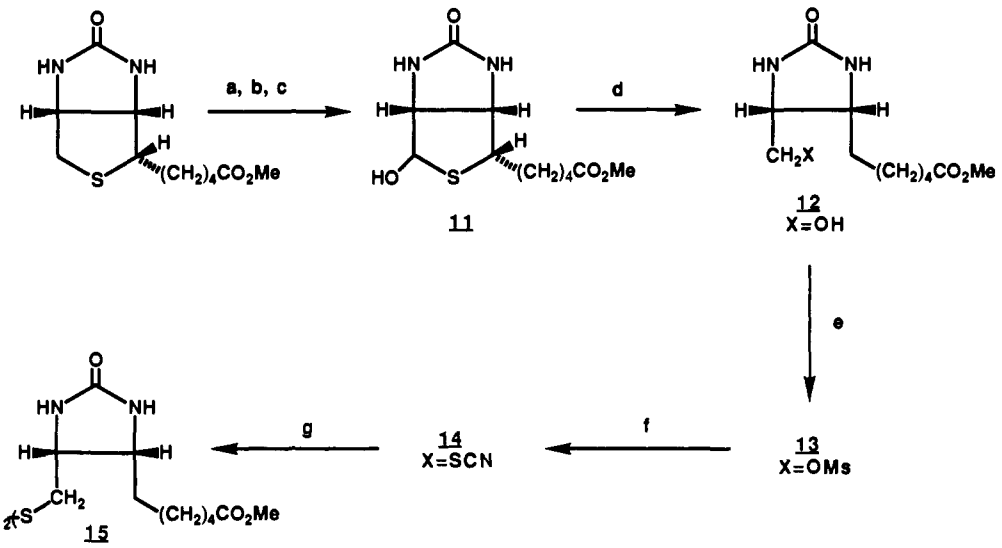
Whatever the role played by the disulfide **18** might be, it was tempting to conclude that if the sulfur atom of **5B** was not incorporated, that of the primary thiol **10** should be. This was studied with (+)-[^{35}S]-**15** as precursor (We checked that with *E. coli*, the methyl esters were converted as efficiently as the free acids. The disulfide form was used without reduction.). Once again, the biotin produced was almost devoid of radioactivity, only 1% was retained.

Another experiment was performed with the deuterated thiol (+)-5-[$^2H_2$]-**15**.

The biotin produced was purified as methyl ester, which was analyzed by mass spectrometry. The CI spectrum shows three molecular peaks at m/z 259, 260, and 261, corresponding to $^2H_0$, $^2H_1$, and $^2H_2$ molecules with the respective intensities 70%, 7%, and 23%.[16]

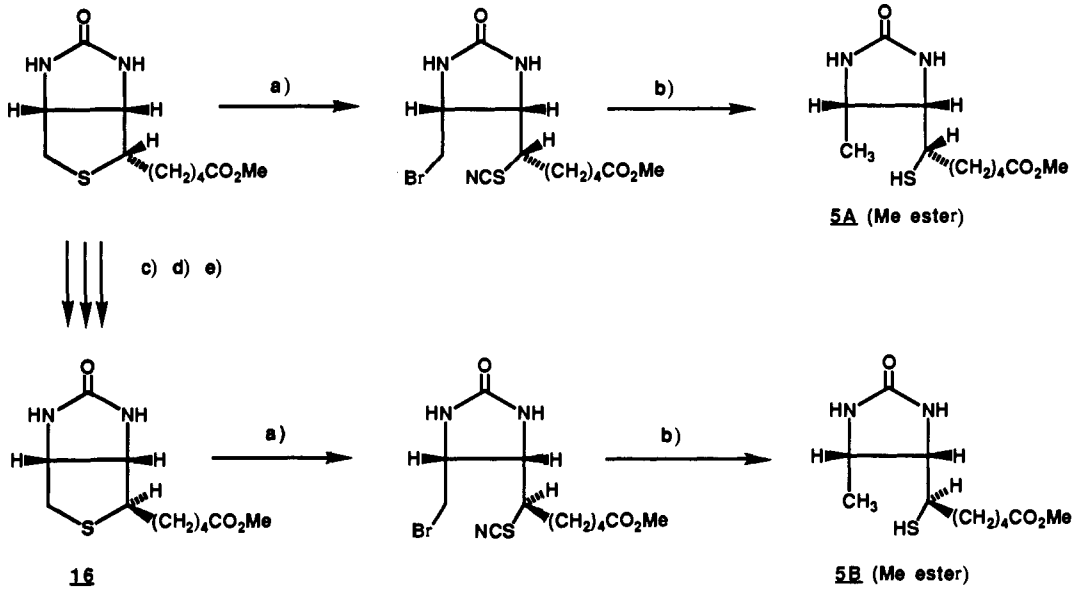
(13) Trainor, D. A.; Parry, R. J.; Gitterman, A. *J. Am. Chem. Soc.* **1980**, *102*, 1467.
(14) Parry, R. J.; Trainor, D. A. *J. Am. Chem. Soc.* **1978**, *100*, 5243.

Scheme III[a]



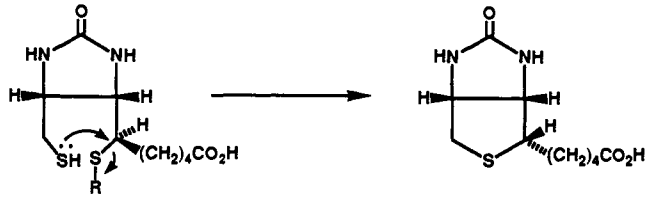
[a] Reagents: (a) 1 equiv of H_2O_2 30%, AcOH; (b) TFAA, $CHCl_3$; (c) CH_3OH, CH_3ONa; (d) Raney Ni, C_2H_5OH; (e) CH_3SO_2Cl, $(C_2H_5)_3N$, CH_2Cl_2; (f) KSCN, DMF; (g) $NaBH_4$, CH_3OH.

Scheme IV[a]



[a] Reagents: (a) BrCN; (b) $NaBH_4$, DMSO; (c) H_2O_2, CH_3CO_2H; (d) NaOH, $C_2H_5OCH_2CH_2OH$, HCl aqueous; (e) $(CH_3)_3SiI$, $CHCl_3$, CH_3OH.

Scheme V



These results reveal that the loss of sulfur is not a simple sulfur exchange but reflects a complex pathway, and we can conclude that compound **15** is used to make biotin (since *E. coli C124* is unable to synthesize DTB) but that the conversion is by no way a direct one. Thiol **10** could even not be on the main biosynthetic route but just be converted into an active intermediate.

(15) Izumi, Y.; Kano, Y.; Inagaki, K.; Kawase, N.; Tani, Y.; Yamada, H. *Agric. Biol. Chem.* **1981**, *45*, 1983.

(16) These three species were always present when the experiment was repeated several times, but their relative proportions varied, probably due to different physiological states of the bacteria.

Table II. Conversion of Biotin Precursors by Resting Cells of *B. sphaericus*

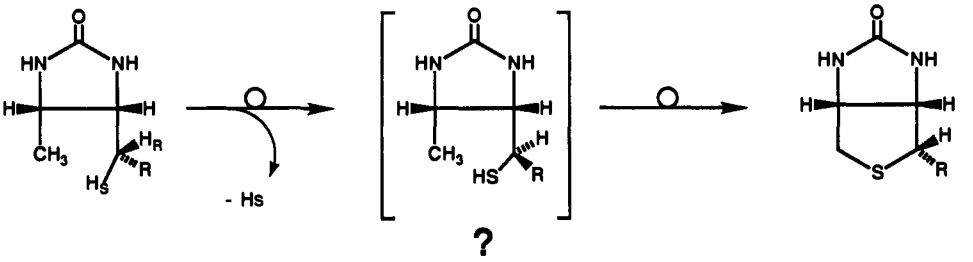
	precursor[a]	concn of precursor[b]	"biotin produced[c]"	yield of convrsn (%)
1	DTB	1	150–175	15–17.5
2	DTB	2	300	15
3	**5A**	2	d	
4	**5B**	2	2	
6	**15** +DTT 0.1 M	2	30	1.5

[a] In this series of experiments, all the precursors were obtained as methyl esters from (+)-biotin and saponified just before use. [b] In μg/mL. [c] Determined microbiologically with *L. plantarum* in ng/mL. [d] The O.D., equivalent to that produced by 80 ng of biotin, is probably due to intrinsic growth-promoting activity of **5A**.

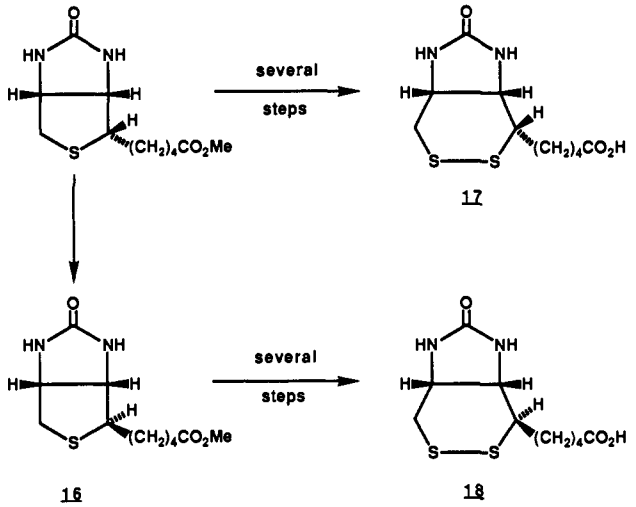
Experiments with *B. sphaericus*. *B. sphaericus* is another bacteria which efficiently converts dethiobiotin into biotin, under resting cells conditions.[15]

Resting Cells Experiments. The three thiols were also tested with this strain. The experiments were run with the methyl esters of **5A** and **5B** derived from biotin and epibiotin (Scheme IV) and

Scheme VI



Scheme VII



with the primary disulfide **15** (Scheme III). The saponification of the methyl esters (which is necessary with this strain) and the following reduction by DTT in the case of **15** were achieved just before use. The results are given in Table II.

The amount of biotin produced was determined microbiologically with *L. plantarum* on the crude supernatant, after centrifugation of the cells, still containing the precursor. It is well-known that *L. plantarum* does not grow on dethiobiotin, but its response to the thiols had to be tested. We found that **5B** and **10** were completely inactive (<0.2%), whereas **5A**, the thiol with the biotin configuration at C-2, had a small activity (3%) compared to biotin introduced at the same concentration. **5A** may possibly bind to the apocarboxylases in place of biotin. The growth of *L. plantarum* observed in entry 4 corresponded roughly to the response due to **5A** at its concentration in the assay, and we conclude that no biotin was produced in experiment 4. Thus, only the primary thiol **10** was converted into biotin by resting cells of *B. sphaericus*. (With the disulfide form **15** without DTT, biotin was not detected in this experiment.) The observed conversion yield was only 10% that of dethiobiotin. But, as the permeability of the cells to that compound has not been checked, we do not know if this quantitative difference is of some mechanistic significance.

When the [^{35}S] labeled disulfide (+)-[^{35}S]-**15** (specific activity: 19.8 mCi/mmol) was incubated with the resting cells in the presence of DTT, the biotin produced in the supernatant, purified by ion exchange chromatography, gave a radioactive spot, revealed by autoradiography, at the expected R_f. One part esterified with CH_2N_2 also had the same R_f as biotin methyl ester. After oxidation with H_2O_2/AcOH it gave a spot with the same R_f as biotin sulfone methyl ester. Finally, the biotin fraction from the ion exchange column was diluted with biotin and recrystallized to constant specific activity (Table III). Most of the radioactivity of the precursor was retained in biotin. This experiment showed clearly that thiol **10** is transformed into biotin without degradation.

An independent argument for its direct bioconversion was given by a similar study carried out with (+)-5-[$^2H_2$]-**15**. The nonradioactive biotin was purified as methyl ester on a silica gel column after addition of a small amount of [^{3}H]biotin methyl ester as tracer. The radioactive fractions were collected and analyzed by

Table III. Conversion of (+)-[^{35}S]-**15** by Resting Cells of *B. sphaericus*

	specific activity
(+)-[^{35}S]-**15**	19.8 mCi/mmol
biotin before dilution[a]	18.2 ± 2.6 mCi/mmol
diluted biotin after 1st crystallization	2.16 ± 0.1 μCi/mmol
diluted biotin after 2nd crystallization	1.84 ± 0.1 μCi/mmol
diluted biotin after 3rd crystallization	1.76 ± 0.1 μCi/mmol

[a] The error on the specific activity of biotin corresponds to the error of the microbiological determination.

Table IV. Conversion of (+)-[^{34}S]-**15** by Resting Cells of *B. sphaericus* TK 502-2-C5 pTG 498

precursor	concn of precursor[a]	"biotin produced[b]"	mass spectrum
(±)-DTB	10[a]	1	
(+)-[^{34}S]-**15**	5[a]	0.2	75 ± 10% ^{34}S
+DTT 2.5 mM			
(+)-[^{34}S]-**15**	5[a]	0.06	80 ± 10% ^{34}S

[a] DTB was introduced at a saturating concentration. **15** was only 80% saturating. [b] In μg/mL. [c] 92 ± 5% of ^{34}S-^{34}S; 8 ± 1% of ^{32}S-^{34}S.

mass spectrometry: the CI spectrum showed molecular peaks MH^+ at m/z 259 and 261 (respective intensities 10/90) which corresponded exactly to the deuterium content of the starting sulfide **15** (analyzed on the corresponding alcohol 5-[$^2H_2$]-**12**). The MIKE spectrum of the molecular ion (m/z = 260) showed peaks at m/z = 199 and 200 in the ratio 0.48 ± 0.03. The same relative intensities were observed in the MIKE spectrum of the molecular ion of an authentic sample of 5-[$^2H_2$]-biotin.[3]

Both experiments thus prove that thiol **10** is converted into biotin without degradation. The absence of dilution by nonlabeled biotin was a priori surprising since *B. sphaericus* is not a biotin auxotroph. But we checked that no biotin is released in the resting cells incubation medium if no dethiobiotin is added.

Resting Cells Experiments with Recombinant Strains. Later on, we had at our disposal recombinant strains of *B. sphaericus*, overproducing biotin synthase, obtained by the research groups at Transgene and Nippon Zeon. We repeated the experiments with TK 502-2-C5 pTG 498 (with Bio B included in a plasmid) using (+)-[^{34}S]-**15** as precursor. Biotin produced under resting cells conditions analogous to those reported in Table III was isolated in the same way, using an additional HPLC purification. The mass spectrum showed that about 80% of [^{34}S] was retained in biotin (Table IV). We also performed the same experiment without adding DTT in the incubation medium, since the culture broth used for growing cells did not contain DTT. Table IV shows that under these conditions, although the conversion yield is smaller, the amount of retained [^{34}S] is about the same.

Growing Cells Experiments. The completely different fate of the thiols with *E. coli* and *B. sphaericus* was very unexpected since, both organisms biosynthesizing dethiobiotin with similar enzymes,[15] it was reasonable to assume that the same held for the last step. There was, however, a difference in the culture conditions, the bioconversion being performed with growing cells of *E. coli* and with resting cells of *B. sphaericus*. Since resting cells of *E. coli C124* do not produce any biotin from dethiobiotin, it was necessary to make a comparison with the bioconversion by growing cells of *B. sphaericus*. This was done with the deuterated thiol (+)-5-[$^2H_2$]-**15**. The CI mass spectrum of the biotin pro-

duced in this experiment showed molecular peaks at 259 (8%), 260 (54%), and 261 (38%), corresponding to $^2H_0$, $^2H_1$, and $^2H_2$ species.[16] Thus, the results are qualitatively similar to those observed with *E. coli*.

Conclusion

We can conclude that the results are qualitatively identical for growing cells of both bacteria. This confirms that, under these conditions, a complex desulfurating pathway[17] competes with the clean cyclization observed with resting cells. Our working hypothesis is that the resting cells results represent the true biosynthetic route.

The first step would be a sulfur insertion in the methyl group, leading to the primary thiol **10**, or some derivatized form. This conclusion, mentioned in our previous paper,[10] was also reached by R. L. Baxter et al.[11] The C–S bond is probably formed through a radical mechanism, according to the stereochemical results obtained by D. Arigoni et al.[19]

The structure of the Bio B gene corresponding to biotin synthase of *E. coli*[20] or *B. sphaericus*[21] indicates that a single protein (M = 38.6 Kd) is able to perform the complete reaction, that is to catalyze the formation of the two C–S bonds very likely by the same kind of mechanism. The low yield of conversion of the disulfide **15** compared with that of dethiobiotin could be accounted for if the thiol normally does not leave the enzyme, when it is formed from dethiobiotin. It could also reflect the fact that a derivatized form of **10**, rather than the free thiol, is the true intermediate.

Further experiments are in progress to confirm that thiol **10** or some derivative are on the biosynthetic pathway.[22] If we assume that it is the case, most of the problems remain: what is the final sulfur donor? What is the mechanism of action of biotin synthase? Further studies require an active in vitro system. We are actively trying to obtain it from the strains which overproduce biotin synthase.

The sequences of the Bio B gene of *E. coli* and *B. sphaericus* show 50% homology,[21] whereas there is no homology with isopenicillin *N* synthase (IPNS). On the other hand, the sequence of the *lip* gene of *E. coli* which has been recently established by G. W. Asley et al.[23] shows a region presenting a large similarity with biotin synthase. The authors reasonably assume that the *lip* gene product corresponds to the enzyme which inserts sulfur into octanoic acid. Again no similarity was found with IPNS. If these last results reinforce the hypothesis of a similarity of mechanism for sulfur insertion in the case of biotin and lipoic acid, the analogy with IPNS remains an open question.

Experimental Section

Materials. *Bacterial Strains.* *E. coli C124* [His$^-$, Bio A$^-$], *E. coli C162* [His$^-$, Bio B$^-$], and *B. sphaericus JFO 3525* were generous gifts of Dr. P. Cleary and Dr. Y. Izumi, respectively.

Growth Conditions. *E. coli* was grown on a synthetic medium as previously described[2b] and *B. sphaericus* according to Izumi et al.[15]

The biotin bioassay using *L. plantarum* were monitored using the conventional method.[24]

Chemicals. All chemicals were of the highest purity available. (+)-Biotin was a gift from Hoffmann-La Roche (France). (+)-Biotin methyl ester sulfone was prepared according to Hoffmann et al.[25] (+)-Dethiobiotin was obtained from biotin by desulfuration with Raney nickel.[26] (±)-Dethiobiotin was from Sigma. K^{35}SCN was purchased from the Radiochemical Center Ltd. Amersham. [^{34}S] was from CEA ORIS.

General Methods. Biotin and its precursors were identified using silica gel thin-layer chromatoplates Merck 60F 254. The developing solvents were nBuOH, H_2O, C_6H_6, and CH_3OH (2/1/1/1 + ϵ) for acids and $CH_3COOC_2H_5$ and CH_3OH (4/1 or 9/1) for methyl esters. Detection was achieved by spraying with *p*-(dimethylamino)cinnamaldehyde (PACA)[27] or by autoradiography using a Berthold β camera BF 290 HR. Ellman's reagent, i.e., 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), was used as thiol reagent. Scintillation counting was carried out with an Intertechnique SL 30 spectrometer in Bray's liquor. All the results were corrected for quenching by external standard method.

Column chromatography was performed on silica gel 60 Merck (70–230 Mesh ASTH). Melting points were measured on a Kofler hot stage apparatus and are uncorrected. IR spectra were taken on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained with a Varian HA 100, a Jeol F X-90Q, a Brucker AC200, or a Jeol GSX 400 Q spectrometer. Chemical shifts are expressed in ppm, relative to TMS.

Conventional mass spectra were obtained with a Varian MAT CH5 mass spectrometer under EI (70 eV).

Mass spectra and collision spectra were recorded on reversed mass spectrometer (ZAB 2F, VG micromass Lt) and triple quadripole instrument (R-30-10, Nermag) according to the ion mode preparation (EI and CI).

(a) EI Conditions and High Collision Energy Regime (MIKE and B/E Linked Scan Modes) in ZAB 2F. The enzymatic extracts were introduced via a direct solid probe with a programmed heating (110–250 °C). Typical EI conditions were as follows: 200 μA as trap current (at 70 eV electron energy) under 8 KV as acceleration voltage. MIKE and B/E linked scan spectra were recorded by selection of parent ion under 2000 as mass resolution. For collision experiments (in MIKE and B/E linked scan modes) the height of main beam was reduced by 30% by introducing the collision gas (about 2×10^{-5} Torr of helium). The electric sector was scanned from 100 to 8100 eV (main beam at E_0 = 8065 eV) for full MIKE (and B/E) CID spectra and from 5500 to 6600 eV for recording daughter ions in the m/z 180 to m/z 210 range for fast enzymatic extract analysis. The CAD spectra were recorded using a PDP11 computer.

(b) CI Conditions and Low Collision Energy Regime in Triple Quadrupole Tandem. A DCI probe equipped by a programmed heating (30–300 °C during 1 min) was employed for introducing sample. The CI-NH_3 conditions for producing MH^+ species were as follows: protonating gas NH_4^+/NH_3, pressure in the source 8×10^{-1} Torr, ion repeller 0V, electron current 75 mA, electron energy 100 eV. Argon as collision target was used for inducing collision at E_{lab} = 20 eV under single collision conditions (main beam intensity reduced by 30%, 10^{-2} Torr in primary pomping). The collision spectra were an average of 30 spectra recorded using PDP 11-73 with SIDAR program.

Preparation of Mesylates 2A, 2B, and 7. Triethylamine (300 mg, 3 mmol) was added to a solution of 600 mg (2 mmol) of **1** or **6** in 8 mL of CH_2Cl_2. The solution was chilled to −10 °C and 250 mg (2.2 mmol) of mesyl chloride was added in three times. After 30 min of stirring, the mixture was warmed up to room temperature and extracted with 10 mL of CH_2Cl_2. The organic solution was washed successively with a 5% HCl solution, a saturated $NaHCO_3$ solution, and water. After drying and solvent evaporation, the residue was chromatographed on a silica gel column (ethyl acetate–methylene chloride 50/50). The pure mesylates were obtained.

(±)-**2A** (92%) mp 141–142 °C; mass spectrum m/z 378, 148, 99; [^{1}H]NMR (100 MHz, $CDCl_3$) δ 1.30 (d, 3 H, J = 7, CH_3), 2.30 (t, 2 H, J = 7, CH_2-C(O)), 2.40 (s, 3 H, CH_3-C(O)N), 3.05 (s, 3 H, CH_3SO_2), 3.90 (m, 1 H, C_3HN), 4.50 (m, 1 H, C_4HN), 4.85 (m, 1 H, CH-OSO_2).

(±)-**2B** (91%) mp 113–114 °C; [^{1}H]NMR (100 MHz, $CDCl_3$) δ 1.20 (d, 3 H, J = 7, CH_3), 2.30 (t, 2 H, J = 7, CH_2-C(O)), 2.42 (s, 3 H, CH_3-C(O)-N), 3.10 (s, 3 H, CH_3SO_2), 3.78 (m, 1 H, C_3HN), 4.45 (m, 1 H, C_4HN), 4.88 (m, 1 H, CH-OSO_2).

(17) We do not believe that the loss of [^{35}S] and [^{2}H] can result from an exchange on the initially labeled biotin since it has been shown that the early events in biotin degradation corresponded to the ω-oxidation of the side-chain.[18]

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(±)-**7** (58%) oil; [^{1}H] NMR (100 MHz, $CDCl_3$) δ 2.20 (t, 2 H, J = 7, CH_2-C(O)), 2.40 (s, 3 H, CH_3-C(O)N), 3.00 (s, 3 H, CH_3SO_2), 3.25 (d, 2 H, J = 8, CH_2-S), 4.2–4.5 (m, 2 H, C_3H-N, C_4H-N).

Preparation of Thiocyanates 3A, 3B, and 8. **2** or **7** (378 mg, 1 mmol) and 485 mg (5 mmol) of KSCN were dissolved with heating in 8 mL of DMF. The solution was stirred at 100 °C during 48 h and DMF evaporated under reduced pressure. After addition of 15 mL of water, extraction with 3 × 15 mL of $CHCl_3$, drying, and evaporation, the resulting crude product was chromatographed on a silica gel column (ethyl acetate–methylene chloride, 50/50).

(±)-**3A** (64%) mp 97–98 °C; IR (CCl_4) 2160 cm^{-1} (SCN); mass spectrum m/z 341, 296, 282, 141. [^{1}H]NMR (100 MHz, $CDCl_3$) δ 1.22 (d, 3 H, J = 7, CH_3), 2.30 (t, 2 H, J = 7, CH_2-C(O)), 2.4 (s, 3 H, CH_3-C(O)-N), 2.9 (m, 1 H, CH-SCN), 3.85 (m, 1 H, C_3H-N), 4.60 (m, 1 H, C_4H-N).

(±)-**3B** (60%) oil; IR (CCl_4) 2160 cm^{-1}; mass spectrum m/z 341, 296, 282, 141. [^{1}H]NMR (100 MHz, $CDCl_3$) δ 1.22 (d, 3 H, J = 7, CH_3), 2.3 (t, 2 H, J = 7, CH_2-C(O)), 2.4 (s, 3 H, CH_3-C(O)-N), 2.65 (m, 1 H, CH-SCN), 3.45 (m, 1 H, C_3H-N), 4.50 (m, 1 H, C_4H-N).

(±)-**8** (64%) oil; IR (CCl_4), 2160 cm^{-1}. [^{1}H]NMR (90 MHz, $CDCl_3$) δ 2.20 (t, 2 H, J = 7, CH_2-C(O)), 2.4 (s, 3 H, CH_3-C(O)-N), 2.88 (d, 2 H, J = 6, CH_2-SCN), 4.1–4.5 (m, 2 H, C_3H-N, C_4H-N).

Preparation of Thiols 4A, 4B, and 9. $NaBH_4$ (40 mg) was added to a solution of 200 mg (0.58 mmol) of **3** or **8** in 5 mL of C_2H_5OH. The mixture was stirred at room temperature for 8 h. After neutralization of the solution with a few drops of CH_3COOH and evaporation of the solvent under vacuum, the residue was taken with 15 mL of water and extracted with 3 × 10 mL of CH_2Cl_2. After drying and evaporation to dryness, the residue was purified by preparative silica gel TLC (ethyl acetate, DTNB was used as thiol reagent). From **3A** (150 mg): (±)-**4A** (68 mg) along with 18 mg of the desacetylated compound (overall yield: 65%). [^{1}H]NMR (100 MHz, $CDCl_3$) δ 1.22 (d, 3 H, J = 7, CH_3), 2.25 (t, 2 H, J = 7, CH_2-C(O)), 3.5 (m, 1 H, CH-SH), 4.5 (m, 1 H, C_3H-N), 4.8 (m, 1 H, C_4H-N).

From **3B** (200 mg): (±)-**4B** (75 mg) along with 12 mg of the corresponding desacetylated compound (overall yield: 47%). [^{1}H]NMR (100 MHz, $CDCl_3$) δ 1.22 (d, 3 H, J = 7, CH_3), 2.3 (t, 2 H, J = 7, CH_2-C(O)), 3.5 (m, 1 H, CH-SH), 4.5 (m, 1 H, C_3H-N), 4.7 (m, 1 H, C_4H-N).

From **8** (40 mg): (±)-**9** (17 mg) along with 5 mg of the corresponding N desacetylated compound (overall yield: 64%). [^{1}H]NMR (100 MHz, $CDCl_3$) δ 2.2 (t, 2 H, J = 7, CH_2-C(O)), 2.4 (s, 3 H, CH_3-C(O)-N), 2.45 (d, 2 H, J = 8, CH_2-S), 3.7–4.35 (m, 2 H, C_3H-N, C_4H-N).

Preparation of Thiols 5A, 5B, and 10. NaOH (1 mL, 4 N) was added to a solution of 50 mg (0.15 mmol) or **4** or **9** in 0.5 mL of CH_3OH. The solution was stirred under argon at 40 °C for 4 h. After cooling, the pH was adjusted to 7.5 with 1 N HCl, and the mixture was applied to an ion-exchange column. The fractions which gave positive tests with DTNB (SH indicator) and PACA (ureido ring indicator) were collected and gave **5** or **10**, the homogeneity of which was controlled by TLC (benzene–butanol–methanol–water 1/2/1/1).

(±)-**5A** (83%): oil; [^{1}H]NMR (90 MHz, $CDCl_3$) (as methyl ester) δ 0.8–1.8 (m, 6 H, CH_2), 1.1 (d, 3 H, J = 6, CH_3), 1.4 (d, 1 H, J = 9, SH), 2.3 (t, 2 H, J = 7, CH_2-C(O)), 2.8 (m, 1 H, CH-SH), 3.65 (s, 3 H, OCH_3), 3.65–3.70 (m, 1 H, C_3H-N), 3.75 (m, 1 H, C_4H-N), 4.75 (s, 1 H, NH), 5.1 (s, 1 H, NH); [^{13}C]NMR (22.5 MHz, $CDCl_3$) 15.42 (CH_3), 24.49, 26.15, 33.85, 35.17 (CH_2), 41.31 (CH-SH), 50.87 (C-H-N), 51.55 (OCH_3), 62.33 (CH-N), 164.7 (N-C(O)-N), 173.7 (C-(O)-OCH_3).

(±)-**5B** (88%): oil; MS m/z 246, 212, 99; [^{1}H]NMR (as methyl ester) δ 0.8–1.9 (m, 6 H, CH_2), 1.20 (d, 3 H, J = 6.4, CH_3), 1.25 (d, 1 H, J = 8.5, SH), 2.37 (t, 2 H, J = 7, CH_2-C(O)), 2.8 (bq, 1 H, CH-S), 3.66 (s, 3 H, OCH_3), 3.7–3.70 (m, 1 H, C_3H-N), 3.95 (m, 1 H, C_4H-N), 4.83 (s, 1 H, NH), 5.4 (s, 1 H, NH); [^{13}C]NMR (22.5 MHz, $CDCl_3$) (as methyl ester) 15.37 (CH_3), 24.40, 25.56, 33.80, 35.02 (CH_2), 39.7 (CH-SH), 51.3 (OCH_3), 51.50, 62.57 (CH-N), 163.45 (N-C(O)-N), 173.83 (C(O)-O).

(±)-**10** (72%); mp 140 °C (softening) 160 °C, mass spectrum m/z 247 (MH^+), 246 (M^+), 131; [^{1}H]NMR (200 MHz, $CDCl_3$) (as methyl ester) δ 1.20–1.80 (m, 8 H, CH_2), 2.25 (t, 2 H, CH_2-C(O)), 2.50–2.60 (m, 2 H, CH_2-S), 3.60 (s, 3 H, OCH_3), 3.65–3.75 (m, 3 H, C_3HN, C_4HN, SH), 5.86, 5.95 (2 br s, 2 H, NH).

(−)-4-(5′-Methoxycarbonylpentyl)-5-(hydroxymethyl)-2-oxoimidazolidine 12. Thiolactol **11** (500 mg, 1.8 mmol) was added to a suspension of 6 g of Raney nickel in 100 mL of absolute ethanol. The mixture was refluxed with stirring for 45 min. After centrifugation, nickel was washed with ethanol (2 × 50 mL), methanol (2 × 50 mL), and methanol–water 1/1 (3 × 50 mL). After solvent evaporation, the residue was purified on silica gel (chloroform–methanol 95/5): 235 mg (53%) of **12** were obtained; mp 131–132 °C; lit.[10] mp 132–133 °C.

(+)-4-(5′-Methoxycarbonylpentyl)-5-(mercaptomethyl)-2-oxoimidazoline Dimer (+)-15. Mesylate **13** (56 mg, 0.162 mmol) obtained from **12** as described for **2A**, **2B**, and **7** and 25 mg (0.26 mmol) of KSCN, in 2 mL of DMF, were heated at 90 °C for 16 h, and DMF evaporated under reduced pressure and worked up, with $CHCl_3$, as usual. The crude product chromatographed on silica gel ($CHCl_3$/CH_3OH 95/5) yielded 23 mg of **14** as an oil (46%): IR 2170 cm^{-1} (−SC≡N). To 8 mg of thiocyanate **14** dissolved in 2 mL of CH_3OH was added 8 mg of $NaBH_4$. After 2 h at room temperature, TLC analysis ($CHCl_3$, CH_3OH, 9/1) revealed two spots giving a pink color with PACA, one of them (R_f = 0.52) gave a yellow color with Ellman's reagent whereas the other one (R_f = 0.42) did not react. After further stirring in air for 3 more h, only that last spot remained, showing a complete conversion of the sulfide into disulfide. After evaporation, the residue was dissolved in $CHCl_3$, washed, and dried on $MgSO_4$. Chromatography on silica gel (chloroform–methanol, 9/1) gave 3.5 mg of **15** (yield: 50%), identical to the previously obtained product.[10]

Synthesis of the Labeled Compounds. (±)-[^{35}S]-**5B.** [^{35}S]-**3B**, **4B**, and **5B** were obtained as described in the general procedures, except that an excess of mesylate **2B** was used in the first step: 0.13 mmol of **2B**, 0.078 mmol of K^{35}SCN (specific activity: 30 mCi/mmol). Purification of [^{35}S]-**3B** was achieved by TLC (yield: 11.25 mg, 42% specific activity: 30 mCi/mmol).

[^{35}S]-**4B** was obtained by reduction of the above sample (yield: 3.07 mg, 37.5%, specific activity: 28 mCi/mmol). [^{35}S]-**5B** recovered after Dowex chromatography corresponded to a total activity of 0.32 mCi.

(±)-[^{35}S]-**15.** The [^{35}S]-labeled compound was prepared as described for the cold compound starting with 26 mg of **13** and 3.9 mg of K^{35}SCN (sp act. 40 mCi/mmol). [^{35}S]-**14** (6.5 mg) was obtained and diluted with the same amount of cold **14**. Reduction gave 4 mg of [^{35}S]-**15** (specific activity 19.8 mCi/mmol).

(+)-[^{34}S]-**15.** K^{34}SCN was prepared from [^{34}S]-sulfur according to the procedure described by J. K. Bartlett et al.:[28] a solution of potassium cyanide (10 mg, 0.154 mmol) in 15 mL of a mixture of toluene and methanol 9/1 was added slowly to a solution of [^{34}S] (4 mg, 0.117 mmol) in 15 mL of methanol. The solution was stirred for 30 min at room temperature, and the solvents were evaporated under vacuum. Colorimetric titration with $FeCl_3$, by comparison with a standard curve established with K^{32}SCN, gave K^{34}SCN: 10 mg (86%). The procedure described for (+)-[^{35}S]-**15** was used, starting with 145 mg of **13** (0.45 mmol) and 25 mg of K^{34}SCN (0.25 mmol). [^{34}S]-**14** (52 mg) was obtained: 73% yield calculated for K^{34}SCN. It was transformed into (+)-[^{34}S]-**15** as described above: F, 205–206 °C. The mass spectrum indicates 92 ± 1% of [^{34}S]-[^{34}S] and 8 ± 1% of [^{34}S]-[^{32}S].

Conversion of Dethiobiotin and of the Thiols by *E. coli C124*. *Growth Assays.* A synthetic medium (KH_2PO_4 13.6 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$ 0.2 g/L, $FeSO_4$ 0.5 mg/L pH = 7.0) was treated with Norit to remove traces of biotin before autoclaving and then supplemented with sterile solutions of glucose (2 g/L) and L-histidine (35 mg/L).

E. coli was grown on this synthetic medium supplemented with 0.1 ng/mL of biotin.

After 24 h, 5 mL of culture (~7.5 × 10^8 cells/mL) were centrifuged, and the cells were washed with 5 mL of 0.9% saline sterile solution. A dilute saline suspension (~1.5 × 10^6 cells/mL) was prepared to inoculate three flasks containing 10 mL of synthetic medium (1:100 v/v): flask A, blank; flask B, standard, supplemented with (+)biotin; flask C, supplemented with increasing quantities of the thiol to be assayed (sterilized by filtration). The inoculated flasks were incubated at 37 °C on a rotatory shaker and the growth was monitored at 570 nm.

Conversion of (±)-[^{35}S]-**5B** *into Biotin by Growing Cells of E. coli C124.* The [^{35}S]-labeled thiol **5B** (specific activity: 28 mCi/mmol) was added aseptically to 8 L of synthetic culture medium inoculated with a small amount of a biotin-deficient preculture of *E. coli C124* (see growth assay). The final concentration of **5B** was 10 ng/mL, and the total amount of radioactivity was 1.8 × 10^7 dpm. The microorganism was grown at 37 °C for 24 h, with reciprocal shaking. The cells were harvested, washed with distilled water, and then autoclaved at 120 °C for 15 min. After centrifugation, the residue was hydrolyzed in 4 N H_2SO_4 for 2 h at 120 °C to extract "bound biotin". The pH of the solution was adjusted to 3–4 with 4 N NaOH, and the solution was stirred with 10 g of Norit overnight. After filtration Norit was eluted with NH_4OH, H_2O, and C_2H_5OH (1/10/10) (the total radioactivity of the eluate was 3.75 × 10^5 dpm). The eluate was concentrated to 20 mL under vacuum, and proteins were precipitated with ethanol and centrifuged. The residual solution was chromatographed on a Dowex AG50 WX_2 (formate form 100–200 mesh) column, eluted with a linear gradient of formic acid (0.05 → 0.1 N). The column had been precalibrated with [^{3}H]-dethiobiotin, [^{3}H]-biotin, and [^{35}S]-**5B**, the last two being eluted together. No significant radioactivity was detected in the fractions corresponding to biotin and **5B**. These fractions were concentrated and analyzed by TLC on

silica gel (benzene–butanol–methanol–water 1/2/1/1). No trace of radioactive biotin could be detected by autoradiography.

The presence of biotin in these fractions was ascertained by their growth promoting activity for *L. plantarum*. It was quantified using the avidin method.[29]

Conversion of (+)-[^{35}S]-**15** *by Growing Cells of E. coli C124.* The experiment was conducted as above in a 8-L culture supplemented with 100 ng/mL of [^{35}S]-**15** (specific activity 14.9 mCi/mmol). The amount of "bound biotin", isolated as described and quantified after the Dowex column with *L. plantarum*, was 7–8 μg (28–32 nmol), and the total radioactivity was 4.7 nCi (specific activity 0.15 ± 1 mCi/mmol).

The radioactive extracts were diluted with 20 mg of cold biotin (specific activity ~57 nCi/mmol) and recrystallized from water. The specific activity was 42 nCi/mmol after the first crystallization and 44 nCi/mmol after the second one. The amount of retained radioactivity was thus about 1%.

Conversion of (+)-**5**-[$^2H_2$]-**15** *by Growing Cells of E. coli. E. coli* was grown as described above in a biotin-free, synthetic medium. The disulfide **15** (100 ng/mL), ester form) was added when the flasks were inoculated with the preculture. The disulfide was not reduced since DTT partially inhibits the growth of *E. coli*.

From a 8-L culture, 3.5 mg of biotin were obtained (*L. plantarum* assay) which gave 0.9 μg of purified methyl ester analyzed by mass spectrometry: mass spectrum CI (NH_4^+) MH^+ *m/z* 261 (22%), 260 (7%), 259 (71%). The MIKE spectrum of the ion at *m/z* 260 showed peaks at *m/z* 199 and 200, in the ratio 0.48 ± 0.02.

Conversion of Dethiobiotin and the Thiols by *B. sphaericus*. The experiments were conducted as described by Izumi et al.[13]

B. sphaericus is grown on a complex medium containing glycerol 20 g/L, proteose peptone 50 g/L, casamino acids 5 g/L, KCl 0.5 g/L, $MgSO_4$ 0.5/L, $MnSO_4$ 10 mg/L, $FeSO_4$ 10 mg/L, K_2HPO_4 1 g/L, thiamine chlorohydrate 10 μg/L, adjusted to pH 7 with KOH before sterilization.

Preparation of the Resting Cells Assay. Five milliliters of the above complex culture medium were inoculated with cells of *B. sphaericus* grown on agar slant and incubated overnight at 28 °C with reciprocal shaking. The culture broth was transferred to a 2-L Fernbach flask containing 500 mL of medium. After 24 h incubation at 28 °C with reciprocal shaking, the cells were harvested by centrifugation at 10000 rpm at 0 °C. They were washed twice with saline and finally suspended in 0.1 M Tris-HCl buffer (pH 8.0) with final O.D. of 48 corresponding to 28.8 mg of dry cells/mL.

Resting Cells Reactions. To 1 mL of this cells suspension in a test tube were added 20 μL (2 μg) of dethiobiotin or thiol. Contrary to *E. coli*, *B. sphaericus* does not use dethiobiotin methyl ester. Thus all the synthetic compounds obtained as methyl esters were saponified just before use (4 h at room temperature in a 1/1 mixture of MeOH–NaOH 0.2 N). After neutralization with HCl 0.2 N the solution was diluted with water and used directly.

The reaction mixture was incubated at 37 °C for 5 h on a reciprocal shaker (160 rpm) and stopped by heating in a boiling water bath for 2 min. In the experiments with the thiols, variable quantities of dithiothreitol (DTT) were added to the reaction. The best conversion rate was obtained with a 10^{-4} M concentration.

After centrifugation of the cells, biotin was quantified in the supernatant with *L. plantarum*.

Preparative Experiments. They were conducted as described, in test tubes containing 1 mL of medium. In these conditions, the biotin yield is higher than in a bulk culture. All the samples were collected and centrifuged (10^4 rpm for 10 min), and the supernatant evaporated to dryness and was treated with 99% ethanol. After centrifugation, the ethanolic solution was evaporated to dryness. The residue was dissolved in water and purified on a AG1 X2 column as described above for the experiments with *E. coli*.

Conversion of (+)-[^{35}S]-**15** *by Resting Cells of B. sphaericus.* The experiment was conducted with 325 mL of cells suspension with 2 μg of **15** (specific activity 19.8 mCi/mmol)/mL of suspension, and the biotin produced was isolated as described. The fractions of the AG1 X2 column corresponding to biotin were evaporated, and the residue dissolved in CH_3OH.

TLC analysis (benzene–butanol–methanol–water, 1/2/1/1) showed that the main radioactive spot had the same R_f as biotin.

One-third of this product was esterified with ethereal CH_2N_2 (2 × 30 min) and purified on a silica gel column (ethyl acetate–methanol, 9/1). The fractions corresponding to biotin methyl ester were collected and checked by TLC (ethyl acetate–methanol, 9/1).

The specific activity of the pure methyl ester was 17.6 mCi/mmol. An aliquot of this ester corresponding to 70 ng dissolved in 100 mL of CH_3OH and 100 mL of CH_3COOH was oxidized with 20 mL of H_2O_2 30% for 20 h. After solvent evaporation the product was analyzed by TLC (ethyl acetate–methanol, 8/2). The main radioactive spot corresponded to biotin sulfone methyl ester. The remaining two-thirds of the crude biotin fraction of the Dowex column were diluted with 24 mg of cold biotin and recrystallized 3 times in water (Table III). The specific activity has been measured by counting samples of respectively 2.41, 2.93, and 2.80 mg.

Conversion of 5-[$^2H_2$]-**15** *by Resting Cells of B. sphaericus.* The experiment was conducted with 150 mL of cells suspension and a concentration of **15** of 2 μg/mL. The biotin produced in the supernatant estimated with *L. plantarum* was about 5 μg, corresponding to a conversion yield of ~2%. After esterification and chromatography on silica gel, ~3 mg of biotin methyl ester was obtained (*L. plantarum* assay on the acid). A last silica gel chromatography was carried out after addition of 25 ng of [^{3}H]-biotin methyl ester (specific activity: 500 mCi/mmol).

The radioactive fractions were collected and studied by mass spectrometry: CI (NH_4^+) (MH^+) *m/z* 261 (90%), 259 (10%); no peak at 260. The MIKE spectrum of the molecular ion $M^{\cdot+}$ (*m/z* 260) showed peaks at *m/z* 199 and 200 in the ratio 0.48 ± 0.03.

Conversion of 5-[$^2H_2$]-**15** *by Growing Cells of B. sphaericus. B. sphaericus* was grown in 1 L of complex medium at 28 °C, as described above for 24 h. Then **5**-[$^2H_2$]-**15** (1 mg in 1 mL CH_3OH) was added, and stirring was continued for 3 days.

Biotin was isolated as described. The methyl ester was chromatographed twice on silica gel, after addition of [^{3}H]-biotin methyl ester as tracer, and about 800 ng were obtained (*L. plantarum* assay on the acid).

It was analyzed by mass spectrometry: CI (NH_4^+) (MH^+) *m/z* 261 (35%), 260 (57%), 259 (8%).

Resting Cells Reactions with B. sphaericus TK 502-2-C5 pTG 498. The culture conditions were the same as for the wild strain, except the presence of kanamycin (10 mg/mL). The reactions were carried out with 14 × 5 mL of cells suspension (OD 50) in 50-mL flasks. Biotin was titrated at each step of the purification by the disc diffusion technique. An HPLC step was introduced after the two silica gel columns (RP8 column 10 × 0.32; eluent CH_3OH/H_2O 4/6, 0.8 mL/min.

Mass Spectrometry Analysis. (a) For the experiment with DDT, the amount of [^{34}S] and [^{32}S] in biotin methyl ester was measured in the conventional CI spectrum after verification of the purity of the molecular peaks (261 and 259) by MS/MS (Table I). (b) For the experiment without DTT, the sample was not pure enough for the direct determination of the [^{32}S]/[^{34}S] ratio. It was calculated on the parents peaks obtained from the daughter common ion: *m/z* 55.

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