

Biotin Synthase Mechanism: Evidence for Hydrogen Transfer from the Substrate into Deoxyadenosine

Franck Escalettes,[†] Dominique Florentin,[†] Bernadette Tse Sum Bui,[†] Denis Lesage,[‡] and Andrée Marquet^{*,†}

Contribution from the Laboratoire de Chimie Organique Biologique and Laboratoire de Chimie Structurale Organique et Biologique, Université Paris VI, CNRS UMR 7613, 4, place Jussieu, 75252 Paris Cedex 05, France

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Abstract: Biotin synthase, the enzyme which catalyses the conversion of dethiobiotin (DTB) to biotin has an absolute requirement for S-adenosylmethionine (AdoMet) which is cleaved into methionine (Met) and 5'-deoxyadenosine (DOA) in equimolar amounts (Guianvarc'h, D.; Florentin, D.; Tse Sum Bui, B.; Nunzi, F.; Marquet, A. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 402–406). To look at an eventual [²H] transfer from DTB into DOA, 6,9-[²H₅]DTB (**3a**), 9-[²H₃]DTB (**3b**), 6(*S*)-[²H₁],9-[²H₁]DTB and 6(*R*)-[²H₁],9-[²H₁]DTB (**3c**), (**3d**) (Escalettes, F.; Florentin, D.; Marquet, A.; Canlet, C.; Courtieu, J. *Tetrahedron Lett.* **1998**, *39*, 7499–7502) have been synthesized and incubated with biotin synthase in the presence of AdoMet. Mass spectrometry analysis revealed that deuterium was indeed transferred from the substrate into deoxyadenosine, bringing the first experimental evidence for the involvement of a deoxyadenosyl radical in the activation of the functionalized positions. The results also allow us to conclude that 2 mol of AdoMet are necessary for breaking the C–H bonds at positions 6 and 9.

Biotin synthase, the enzyme which catalyses the conversion of dethiobiotin to biotin (Scheme 1) has an absolute requirement for S-adenosylmethionine.¹

We demonstrated that AdoMet was not the sulfur donor, and this led us to postulate² that it could belong to the very interesting family of enzymes, namely, pyruvate formate-lyase (PFL),³ anaerobic ribonucleotide reductase (ARNR),⁴ and lysine 2,3-aminomutase (LAM),⁵ which use AdoMet as a source of deoxyadenosyl radical (DOA*) produced by the reductive cleavage of the sulfonium moiety (the radical probably being stabilized by some group of the protein).

This hypothesis which is illustrated in Scheme 2 is supported by the following data: Biotin synthase contains an iron–sulfur cluster^{6–8} as do one of the subunits of ARNR⁹ and the activase of PFL.¹⁰ The associated reducing system characterized in *Escherichia coli*, which consists of NADPH, flavodoxin¹¹ and flavodoxin, reductase¹² is identical to that participating in PFL

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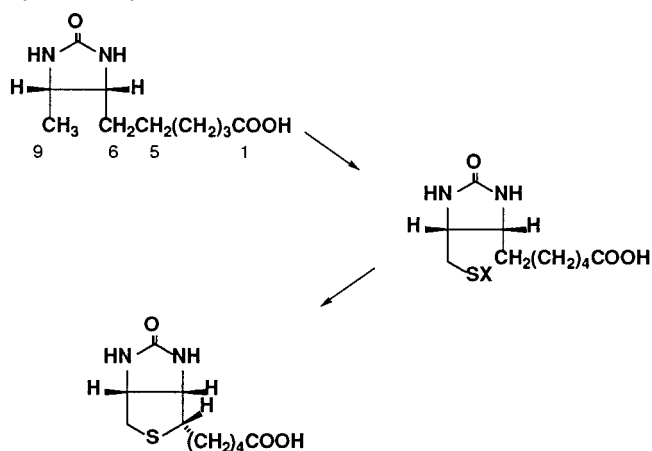
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Scheme 1. Conversion of Dethiobiotin to Biotin Catalyzed by Biotin Synthase



and ARNR. We have also recently established that, during the production of biotin, deoxyadenosine and methionine were generated, as expected, in equimolar amounts.¹³ The observed stoichiometry was about 2.8 mol per mol of biotin produced.

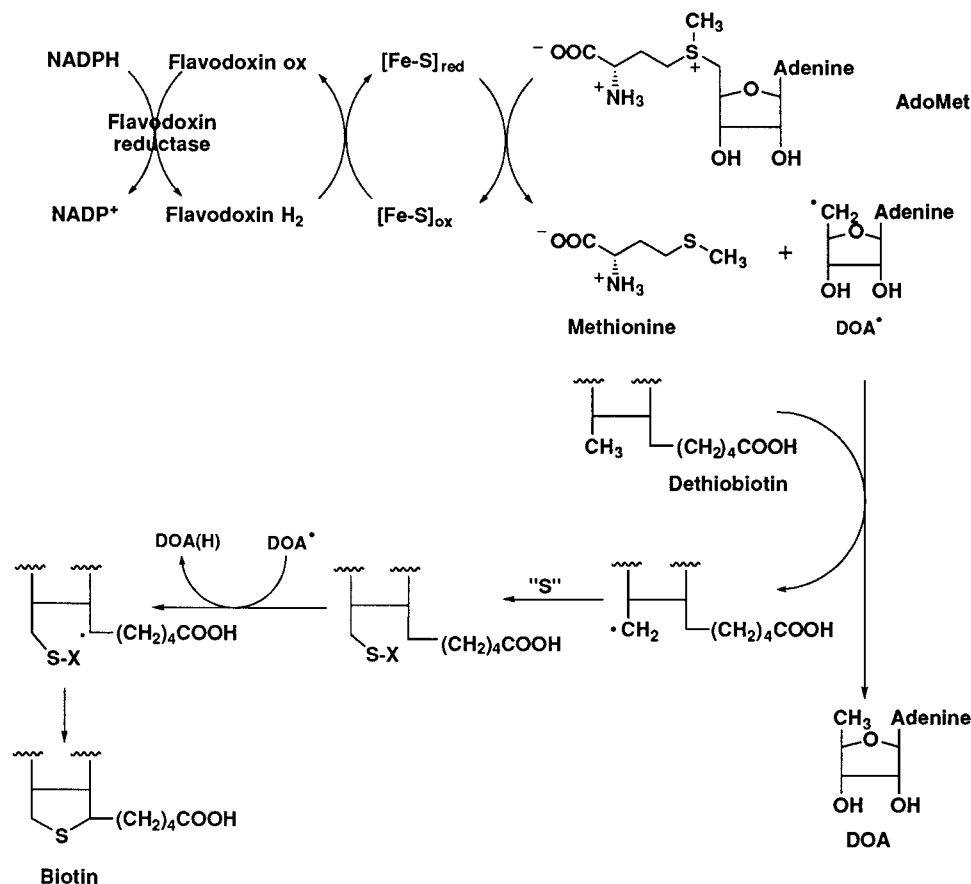
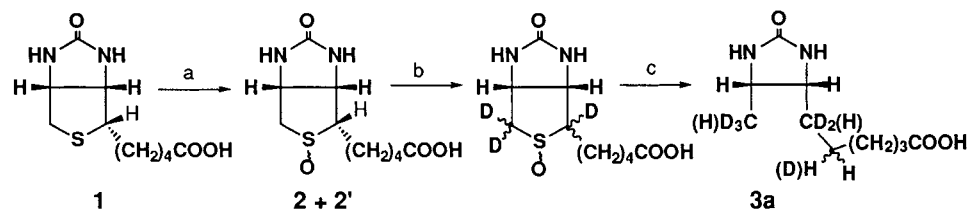
Despite common features, the three AdoMet dependent enzymes described so far follow different pathways. Whereas, in PFL¹⁴ and ARNR,¹⁵ a catalytically competent protein glyceryl radical has been identified, LAM works according to a B₁₂-

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Scheme 2. Hypothetical Mechanism for *Escherichia coli* Biotin Synthase**Scheme 3.** Preparation of **3a**^a

^a (a) H₂O₂, CH₃COOH; (b) 1 N NaOD, D₂O, 80 °C, 30 h; (c) NiCl₂, NaBD₄, CH₃OD, THF.

like mechanism,¹⁶ where the homolytic cleavage of a C–H bond on the substrate is mediated by the deoxyadenosyl radical itself, as shown by the reversible transfer of ³H from the substrate to deoxyadenosine.¹⁷ It remains to be established to which subfamily biotin synthase belongs. To look at an eventual ²H transfer from the substrate into deoxyadenosine, we prepared different samples of dethiobiotin deuterated at the positions to be functionalized, carbons 6 and 9 (numbering as shown in Scheme 1).

Results

1. Synthesis of Deuterated dethiobiotins. 1.a. 6,9-[²H₅]DTB (3a). Biotin was oxidized into a 75:25 mixture of sulfoxides **2** + **2'** (Scheme 3). After complete exchange of the α hydrogens,¹⁸ as checked by NMR, the trideuterated sulfoxides were reduced into deuterated dethiobiotin **3a** by the very straightforward

method described by T. G. Back et al.¹⁹ using NiCl₂ and NaBD₄ in CH₃OD. Mass spectrometry analysis of the corresponding methyl ester allows the location of deuterium according to the fragmentation pattern (Scheme 4a), previously established.²⁰

It revealed an incomplete deuteration with the ratio of the different species (including the species containing also deuterium at C5) as shown in Scheme 4b.

1.b. 9-[²H₃]DTB (3b). **3b** was synthesized as described in Scheme 5.^{21,22} In that case, the secondary hydrogens were selectively exchanged using milder basic conditions. Then, the sulfoxides were reduced and the bideuterated biotin methyl ester **4** was transformed into the isopropyl sulfonium salt **5**. Reduction with LiAl[²H₄] and then with Li/EtNH₂ gave [²H₅]dethiobiotinol **7** which was oxidized with O₂ and Pt to trideuterated dethio-

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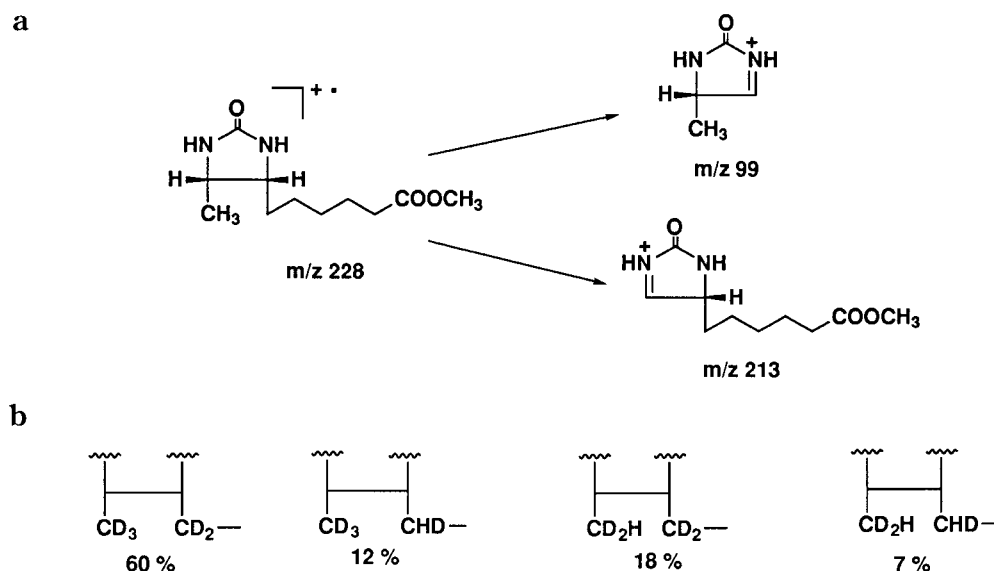
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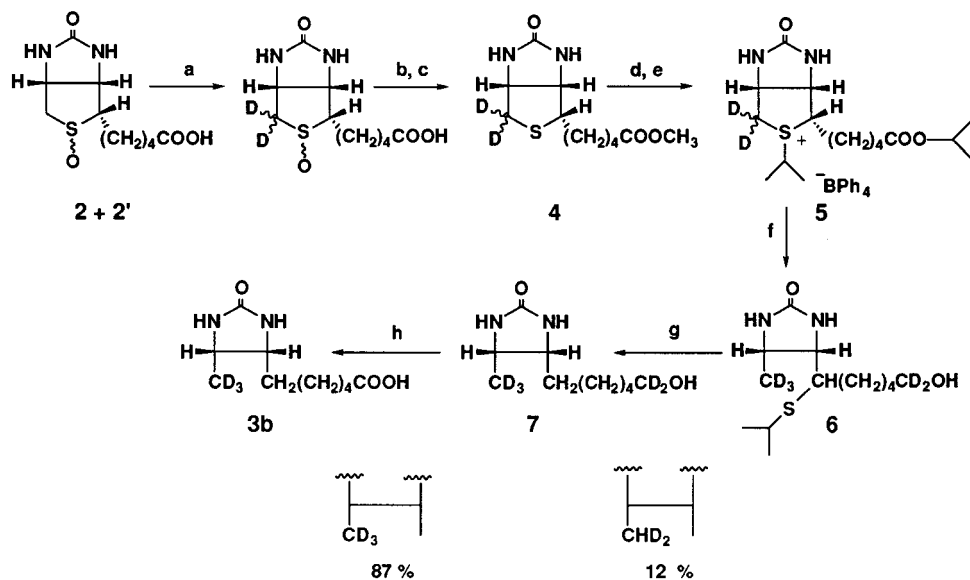
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Scheme 4. (a) Main Fragment Ions Obtained by EIMS from DTB Methyl Ester. (b) Percentages of the Different Deuterated Species of **3a**



Scheme 5. Preparation of **3b** and Percentages of the Different Deuterated Species^a



^a (a) 1 N NaOD, rt, 11 days; (b) TiCl_3 , reflux, 8 h, rt, 12 h; (c) CH_3OH , Amberlite, rt, 4 days; (d) $i\text{PrOH}$, $\text{CH}_3\text{SO}_3\text{H}$, 50 °C, 20 h; (e) NaBPh_4 ; (f) LiAlD_4 , THF, reflux, 1 h; (g) Li/EtNH_2 , 6 h; (h) O_2 , Pt, H_2O , 7 days.

biotin **3b**, with the proportion of labeled species as shown in Scheme 5.

1.c. 6(S) and 6(R)-[²H₁]₉-[²H₁]DTB (3c** and **3d**).** The synthesis of **3c** and **3d** has been described elsewhere.²³ Basic treatment of the mixture of biotin sulfoxides **2** + **2'** led to the partial epimerization of the side chain, that is to a mixture of starting material and epibiotin sulfoxides **8** + **8'**²³ (Scheme 6). The sulfoxides of each series were treated with NiCl_2 and NaBD_4 in CH_3OD leading to samples of dehydrobiotin deuterated at positions 6 and 9, **3c** and **3d**. As the two diastereotopic hydrogens at C-6 are not differentiated by ¹H NMR, whatever the field or the technique used, the stereochemistry of the reaction was analyzed by ²H NMR in a polypeptide liquid crystal.²⁴ The spectra of **3c** and **3d** showed two deuterium signals corresponding to the two configurations at position 6 with a respective ratio of 80/20 and 20/80.²³ The configuration of the

Table 1. Isotopic Composition of **3a**, **3b**, **3c**, and **3d**

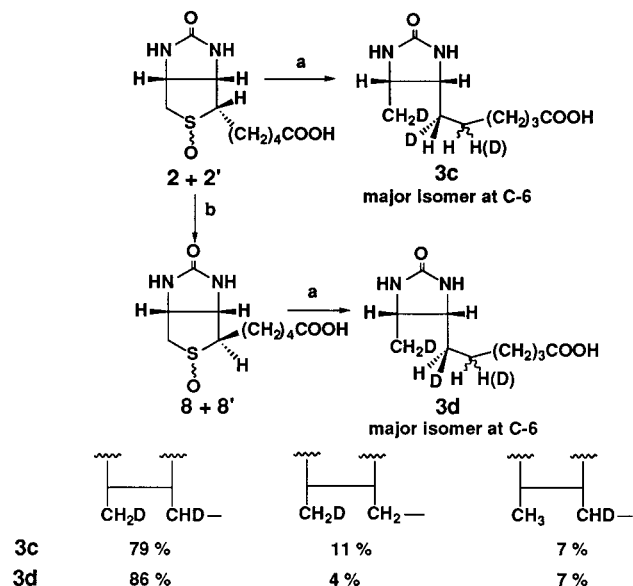
number of D atom	3a	3b	3c	3d
at C-5 ^a	0.3		0.4	0.4
at C-6	1.8		0.9 ^b	0.9 ^c
at C-9	2.7	2.9	0.9	0.9

^a The ²H NMR spectra of **3a**, **3c**, **3d** revealed a partial incorporation of deuterium on the side chain beside position 6. It is very likely located at C5 resulting from the reduction of an intermediate 5–6 double bond.²³ MS gives the total amount of deuterium at C5 + C6. NMR gives the proportions on the two positions. ^b 20% (R) and 80% (S). ^c 80% (S) and 20% (R).

major isomers was attributed, assuming that the hydrogenolysis of the C–S bond occurs stereoselectively with retention of configuration, as described by Back et al.¹⁹ We shall see below that our results confirm this attribution. The labeling content of **3a**, **3b**, **3c** and **3d** are compiled in Table 1.

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Scheme 6. Preparation of **3c** and **3d** and Percentages of the Different Deuteriated Species^a

^a (a) NiCl₂, NaBD₄, CH₃OD, 0 °C, 15 min; (b) 1 N NaOH, 80 °C, 58 h.

Table 2. Comparison of Biotin Production and [²H] Incorporation in DOA

solvent	substrate ^a	biotin (μM) ^{b,c}	[² H]DOA/ ([² H]DOA + DOA)
H ₂ O	DTB	2.9 ± 0.2	
H ₂ O	3a	0.8 ± 0.15	0.32 ± 0.04 ^d
H ₂ O	3b	2.1 ± 0.15	0.18 ± 0.02 ^d
H ₂ O	3c	1.7 ± 0.1	0.17 ± 0.01 ^d
H ₂ O	3d	2.4 ± 0.1	0.05 ± 0.01 ^d
[² H ₂ O]	DTB	2.0 ± 0.2	0.04 ± 0.02 ^e

^a The isotopic composition is shown in Table 1. ^b Determined by microbiological assay. ^c Experimental conditions and error estimation: see Experimental Section. Data represent the mean ± SD of: ^d Duplicate spectra for two independent experiments. ^e Duplicate spectra for five independent experiments. In some of them, the proteins have been lyophilized from [²H₂O] one, two, or three times before running the experiment. The deuterium content of DOA does not depend on lyophilization.

2. Enzymatic Experiments. Enzymatic experiments were carried out with **3a**, **3b**, **3c**, and **3d** which were incubated with 80% pure biotin synthase from *E. coli*, flavodoxin, flavodoxin reductase, NADPH, AdoMet, DTT, cysteine, and Fe²⁺.²⁵ The amount of biotin produced was determined by a microbiological assay, and deoxyadenosine was isolated by HPLC and analyzed by mass spectrometry, as previously described.¹³ It is important to point out that the in vitro system that we are using is not catalytic. In our hands, the amount of biotin produced was less than 1 mol per mol of enzyme (see Experimental Section). The amount of biotin formed and the percentage of deuterated deoxyadenosine molecules in each experiment are reported in Table 2.

The deoxyadenosine recovered from the experiment with **3a** contained 0.32 deuterium. With **3b** deuterated only at position 9, half of this amount (0.18 deuterium) was found. Deoxyadenosine isolated from **3c** (80% 6(*S*)) and **3d** (80% 6(*R*)) contained respectively 0.17 and 0.05 deuterium. To test an eventual exchange with the solvent, several experiments were carried out with nonlabeled dethiobiotin in [²H₂O] and biotin synthase lyophilized 1, 2, or 3 times in the presence of [²H₂O]. The amount of deuterium found in the recovered deoxyadeno-

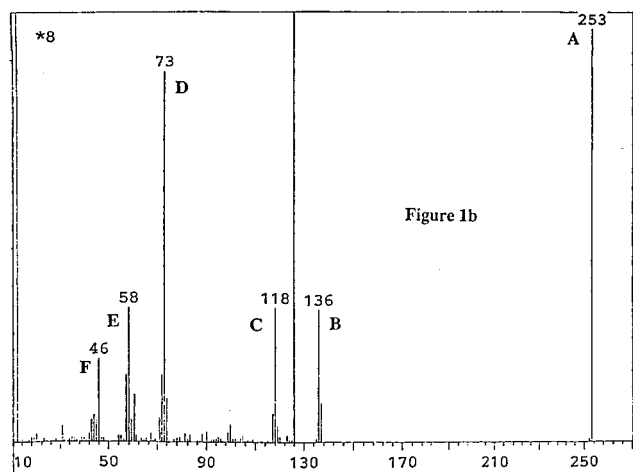
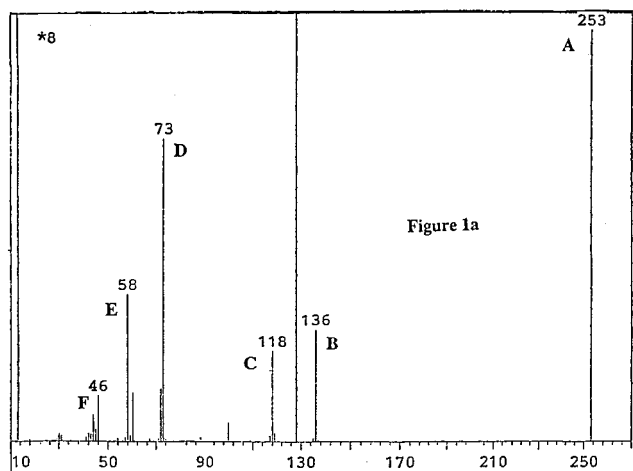


Figure 1. CID spectra of protonated [²H₁]DOA reference sample (a) and [²H₁]DOA isolated from an enzymatic assay with **3c** as substrate (b). Note: The small peaks at *m/z* 57, 74, 117, and 137 correspond to the nonnegligible ¹³C contribution because of the low proportion of [²H₁]DOA biosynthesized (40% of ¹³C in the MS peak at *m/z* 253).

sine in these five independent experiments was 0.04 ± 0.02 which is hardly significant.

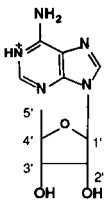
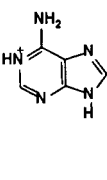
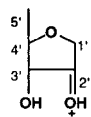
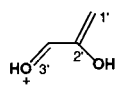
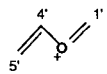

3. Location of Deuterium in Deoxyadenosine. The proposed mechanism implies that deuterium is introduced into deoxyadenosine at position 5'. To confirm this assumption, we tried to locate the deuterium atom by tandem mass spectrometry. A reference sample of 5'-[²H₁]DOA was prepared by reduction of 5'-chloro-5'-deoxyadenosine, according to Wang et al.²⁶ DOA and 5'-[²H₁]DOA were analyzed under CI-NH₃ and CI-ND₃ as reagent gas conditions, and the produced protonated molecules were studied by selective collision experiments. Several product ions could be formally identified according to the *m/z* ratios of the different peaks as shown in Table 3.

As expected, fragment D coming from [²H₁]DOA does not contain deuterium, whereas fragments E and F show a shift of *m/z*, from 57 to 58 and from 45 to 46, respectively. The same shift is observed with [²H₁]DOA isolated from the enzymatic assay (Figure 1). This allows us to conclude that deuterium is located either at position 4' or 5'. Although the two positions cannot be differentiated by mass spectrometry, one can assume on the basis of mechanistic arguments that deuterium is actually located on carbon 5'.

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Table 3. Location of Deuterium Atom in [²H₁]DOA

						
	A	B	C	D	E	F
	Parent ion (m/z)					
[DOA]H ⁺	252	136	117	73	57	45
[² H ₄][DOA]D ⁺	257	140	119	75	57	46
5'-[² H ₁][DOA]H ⁺	253	136	118	73	58	46

Discussion

Previous work by our group has led to the following contributions to the understanding of biotin synthase mechanism: (1) Functionalization of dethiobiotin starts at the methyl group since the primary thiol (Scheme 1, X = H) is transformed into biotin by resting cells of *Bacillus sphaericus*, whereas the two epimeric C-6 thiols are not.²⁷ (2) Looking at the conversion of dethiobiotin into biotin with a mixture of 9-[²H₃]DTB and 9-[²H₂]DTB, we did not observe any isotopic internal selection,²¹ showing thus the absence of isotope effect associated to the first step. (3) The free thiol is probably not the true intermediate since it is converted into biotin less efficiently than dethiobiotin either in vivo²⁷ or in a cell-free system.² It is probably present in the enzyme active site as S-X, either as a free functionalized derivative or bound to the enzyme. An intermediate with the chromatographic properties of the free thiol has been detected in experiments with lavender cell cultures²⁸ showing a possible cleavage of the S-X bond. (4) Experiments with the ³⁵S or ³⁴S-labeled thiol revealed the loss of about 20% of the label during its conversion into biotin, as well in vivo²⁷ as in vitro.² This may indicate some reversibility of the C-SX bond formation. Taking into account the additional data obtained with cell-free systems and purified enzymes mentioned in the Introduction, we are proposing the working hypothesis described in Scheme 2.

In this work we concentrate on the role of the deoxyadenosyl radical issued from AdoMet. We bring evidence that 2 mol of AdoMet are required to functionalize positions 6 and 9 of dethiobiotin, as postulated in Scheme 2. We also discuss the two possible pathways for the homolytic cleavage of the C-H bonds of DTB. Is it mediated by the deoxyadenosyl radical itself like in LAM⁵ or by an intermediate protein radical like in PFL¹⁴ or ARNR.¹⁵ In any case it has to be pointed out that here AdoMet is a cosubstrate and not a catalyst as in the case of the three other AdoMet-dependent enzymes since the intermediate radicals are trapped by the sulfur donor.

The results presented in this paper show clearly a deuterium transfer from the labeled substrates into deoxyadenosine. The availability of the differently labeled substrates **3a-d** allows a

deep insight into the enzyme mechanism. The interpretation of the amount of deuterium found in deoxyadenosine that we observe requires, however, a complex discussion. We will consider successively the two hypotheses, direct transfer or relay through an intermediate protein radical, and see which one fits better with the results.

We shall first discuss the case of a direct deuterium transfer. With [²H₅]DTB (**3a**) as substrate the mass spectrum of the resulting deoxyadenosine shows that only one-third of the molecules have incorporated deuterium. According to the arguments developed above, one DOA[•] has to be involved to generate a radical at C9 where the first functionalization takes place, this radical being trapped by the sulfur donor to give the C-SX intermediate. If the sulfur-donating species does not contain an unpaired electron, this reaction would generate a radical which could be responsible for the homolytic C-H bond cleavage at C6. In that case, only 1 mol of AdoMet should be consumed, i.e., 1 mol of DOA produced per mol of biotin synthesized. The difference with the observed experimental value (2.8 to 3)²⁹ could be attributed to some abortive process. This would account for the amount of deuterated DOA molecules, that is, about 1 per mol of biotin.

However, we have previously observed that AdoMet is required for the transformation of 9-mercaptodethiobiotin into biotin,² which implies that a second DOA[•] is involved in the C₆-H cleavage. To distinguish between the two possibilities, we prepared 9-[²H₃]DTB (**3b**). If only one DOA[•] is involved, the amount of deuterium transfer should be the same when **3a** and **3b** are used as substrates. This is not the case since with **3b** only 18% of the DOA molecules are deuterated, that is, half of the value obtained with **3a**. This strongly confirms our hypothesis postulating that a second DOA[•] is involved in the functionalization at C6.

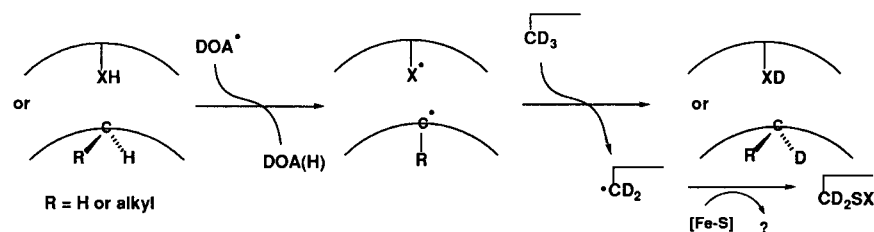
A further proof can be found in the results obtained with two other labeled substrates, **3c** and **3d**. These compounds were obtained by the method used for the preparation of **3a** (see above). The configuration of the major isomer at C6 was tentatively assigned assuming that the hydrogenolysis of the C-S bond occurs stereoselectively with retention of configuration. The results presented in Table 2 confirm this attribution. Indeed, Parry and collaborators³⁰ have already established, using

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(29) This value of 2.8 (found very reproducible) is extracted from our previous work.¹³

Scheme 7



DTB samples stereospecifically tritiated at C6, that the proS hydrogen was removed during the conversion into biotin. We observe, consistently, a higher amount of deuterium transfer into DOA with **3c** where the 6(*S*) configuration was attributed to the major isomer. A quantitative discussion of the deuterium content of DOA issued from these last two experiments is a little hazardous because of the presence of several deuterated species which results in the accumulation of errors in the calculation (the deuterium at C9 should contribute for about 0.05 ^2H and the presence of the “wrong” isomer at C6 lowers or increases the deuterium transfer expected for a 6(*S*) or 6(*R*) configuration, respectively). The qualitative results are, however, consistent with the previous ones and confirm that a DOA^\bullet is involved in both steps.

Nevertheless, an important question remains to be solved: why do we not observe a higher amount of deuterium transfer if a unique pathway is followed? The result obtained with **3b** is particularly clear. The substrate is regioselectively and extensively deuterated and the absence of isotope effect at C9 has been established.²¹ If 1 mol of DOA is issued from the homolytic C–H cleavage at C6 and about 1 mol from an abortive process, the expected $[\text{H}]$ content should be about 0.3–0.35. A value of 0.18 is observed. With **3a** as substrate the $[\text{H}]$ content of DOA is just double, making very unlikely any interpretation based on an (unknown) isotope effect at C6. The hydrogen found in DOA is not issued from the solvent (experiment in $^2\text{H}_2\text{O}$) and may come from the protein. We implicitly assume that the extra amount of DOA produced (0.8 to 1 mol per mol of biotin) arises from an abortive process. Shaw et al. have found about the same ratio (~ 3) between the amount of methionine formed by AdoMet cleavage and the biotin produced.³² They have postulated that it could be correlated with the formation of an intermediate that they have detected. In the conditions of our assay which are different from theirs, we did not observe this intermediate. Anyway if it was the case its formation from **3a** should lead to $\text{DOA}(\text{D})$ and cannot explain the presence of DOA.

A reversible hydrogen transfer between DTB and DOA^\bullet could be considered if the C–S bond formation is the rate-determining step. A detailed analysis of this possibility reveals that it could not account for the large amount of nonlabeled DOA which is produced. Moreover, it would imply the presence of bi(tri)-deuterated DOA molecules. The absence of $[\text{H}_2]$ DOA revealed by the mass spectrum allows to discard this hypothesis.³¹

We have now to consider if the above results fit better with the second hypothesis, that is, the occurrence of an intermediate protein radical. The protein radical in PFL and ARNR is located on a glycylic residue, found respectively in the conserved se-

quence RVSGY¹⁴ and RVCGY.³³ Its formation is catalyzed by an activase (where the DOA^\bullet is produced) which has a number of analogies with biotin synthase. Biotin synthase does not require an activase, and all of the events have to occur on the same protein where the conserved sequence RVS(C)GY is not present.³⁴ We cannot exclude however the relay on another Gly $^\bullet$ or another carbon radical, or an X radical (S^\bullet , O^\bullet ...).

For the discussion of the results in this second hypothesis it becomes very important to consider the turnover of the enzyme. We have seen that in the in vitro system we are using, less than 1 mol of biotin was produced per mol of enzyme. Although biotin synthase is about 80% pure, according to SDS PAGE, it may, however, contain only a small amount of active catalytic protein. According to Duin et al.⁸ the aerobically purified enzyme containing one $[\text{Fe}-2\text{S}]$ cluster per monomer would be converted by anaerobic reduction into another form with a $[\text{Fe}-4\text{S}]$ cluster which could be the active species at the interface of the two monomers. But they did not observe any activity difference between both forms in the in vitro system. Moreover, we have recently found that the inorganic sulfide of $[\text{Fe}-\text{S}]$ reconstituted biotin synthase was efficiently incorporated into biotin.³⁵ We have postulated that the $[\text{Fe}-\text{S}]$ center, besides its supposed role in the electron transfer, was likely the sulfur donor. This implies that the system cannot be catalytic as long as the cluster is not efficiently reconstituted and strengthens the hypothesis of a single turnover in the in vitro experiments.

In that case, the DOA^\bullet would abstract a hydrogen from the protein, and nonlabeled DOA should be produced, deuterium being transferred from the substrate to the protein in the second step (Scheme 7).

In case of multiple turnover $\text{DOA}(\text{D})$ should be produced at each following turn, since the protein is deuterated, provided that the C–D bond is stereospecifically formed or broken (or that the X–D bond does not exchange, which is consistent with the experiment in $^2\text{H}_2\text{O}$).

The observed values of $\text{DOA}(\text{D})$ which is, in each experiment, about half of the expected value would correspond to two turns in each case. We feel that it is a very improbable situation.

Conclusion

All of the data accumulated on the mechanism of action of biotin synthase since the description of an active cell-free system in 1992, strongly supported the hypothesis of an intermediate deoxyadenosyl radical. This work brings the first experimental proof of the involvement of such a radical and also demonstrates that 2 mol of AdoMet are required for the functionalization of positions 6 and 9 of dethiobiotin.

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(31) A 2% $[\text{H}_2]$ content could be deduced from the mass spectrum in the experiment carried out with $[\text{H}_5]$ DTB. We do not know if this value is of significance or within experimental error. $[\text{H}_2]$ species were not detected in the experiment with the other substrates.

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However, the interpretation of the amount of deuterium transfer from the substrate into deoxyadenosine is complex, and our experimental results cannot be completely explained by any of the hypotheses that we have discussed. We feel, however, that the whole set of data is more consistent with a transfer of hydrogen which does not involve an intermediate protein radical. The observed loss of deuterium remains to be explained. Evidently much more work, using other approaches, is necessary to further understand this very complex but fascinating reaction.

Experimental Section

Chemicals, Materials and Methods. All reactions using nonaqueous reagents were run under a dry argon atmosphere. Flash chromatography was performed on Merck Kieselgel 60 (230–400 mesh) and reversed phase chromatography on Merck Lichroprep RP18 (25–40 μm). The progress of reactions was monitored by analytical TLC on silica gel 60F-254 from Merck. Visualization of TLC was done by UV, phosphomolybdic acid, or paradimethylamino-cinnamaldehyde. **3a**, DOA, and 5'-[$^2\text{H}_1$]DOA were purified on a Nucleosil 5C18 250-4 HPLC column (Merck). ^1H RMN spectra were recorded on a Bruker ARX400 at 400 MHz. ^{13}C RMN spectra were recorded on a Bruker ARX400 at 100 MHz. Tetramethylsilane was used as an internal standard. Elemental analyses were performed by the Service Régional de Microanalyse (SIAR-Jussieu). All chemical and biochemical reagents were purchased either from Aldrich, Acros, or Sigma. DOA and 5'-[$^2\text{H}_1$]DOA were synthesized from 5'-chloro-5'-deoxyadenosine by the procedure of Wang et al.²⁶

4(R),5(S)-4-([1,1- $^2\text{H}_2$]-5-carboxypentyl)-5-[$^2\text{H}_3$]-methyl-imidazolidin-2-one (**[$^2\text{H}_5$]DTB**) **3a**. Sodium deuterioxide (1 N, 10 mL) was added to **2** + **2'** (500 mg, 1.92 mmol). The solution was stirred at 80 °C for 30 h and then acidified with 2 N HCl and purified on a C18 reversed phase column ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$: 100/0 \rightarrow 90/10), yielding a white solid (428 mg, 85%) as a mixture of diastereoisomers. This product (405 mg, 1.52 mmol) was dissolved in a 3/1 mixture of CH_3OD and anhydrous THF (33 mL). NiCl_2 (2.75 g, 21.2 mmol) and then NaBD_4 (2.67 g, 63.8 mmol) were added under argon at 0 °C. The mixture was stirred at room temperature for 15 min. The precipitate was removed by centrifugation and washed with water (10 \times 10 mL). The combined supernatants were evaporated under vacuum. The green residue was dissolved in water (30 mL). The solution was acidified with 1 N HCl and purified by C18 reversed phase chromatography ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$: 100/0 \rightarrow 90/10), yielding a white solid. This solid was treated with CH_3OH (25 mL) and IRN-77 sulfonic Amberlite resin (0.5 g). The solution was stirred under reflux for 11 h and then evaporated under vacuum. The residue was purified by flash chromatography ($\text{CHCl}_3/\text{CH}_3\text{OH}$: 100/0 \rightarrow 90/10) yielding a colorless solid which was saponified with 1 N NaOH and then desalted by C18 reversed phase chromatography ($\text{H}_2\text{O}/\text{CH}_3\text{CN}$: 100/0 \rightarrow 90/10). **3a** (98 mg, 27%) was obtained as a white solid and further purified by HPLC (Nucleosil-C18 Merck column; $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{TFA}$: 85/15/0.1), mp: 157 °C (lit. 159 °C).³⁶ ^1H NMR (D_2O + NaOD): 1.01–1.19 (4H, m, $-\text{CD}_2-(\text{CH}_2)_2-(\text{CH}_2)_2-\text{COOH}$); 1.3–1.39 (2H, m, $-\text{CD}_2-(\text{CH}_2)_2-\text{CH}_2-\text{CH}_2-\text{COOH}$); 1.96 (2H, t, $J = 7.6$ Hz, $-\text{CD}_2-(\text{CH}_2)_3-\text{CH}_2-\text{COOH}$); 3.56 (1H, d, $J = 7.6$ Hz, $-\text{CHN}-$); 3.67 (1H, d, $J = 7.6$ Hz, $-\text{CHN}-$). ^{13}C NMR (D_2O + NaOD): 14.6 ($-\text{CD}_3$); 24.2, 27.66 ($-\text{CD}_2-(\text{CH}_2)_2-(\text{CH}_2)_2-\text{COOH}$); 24.88 ($-\text{CD}_2-(\text{CH}_2)_2-\text{CH}_2-\text{CH}_2-\text{COOH}$); 27.66 ($-\text{CD}_2-(\text{CH}_2)_4-\text{COOH}$); 36.72 ($-\text{CD}_2-(\text{CH}_2)_3-\text{CH}_2-\text{COOH}$); 50.4 ($-\text{CHN}-$); 55 ($-\text{CHN}-$); 165.21 ($\text{C}=\text{O}$); 184 (COOH).

3a(S),4(S),6a(R)-4-(4-methyloxycarbonylbutyl)-[6,6- $^2\text{H}_2$]-2,3,3a,4,6,6a-hexahydro-thieno-[3,4-*d*]imidazol-2-one (**4**). Sodium deuterioxide (1 N, 50 mL) was added to **2** + **2'** (4.75 g, 18.3 mmol). The solution was stirred at room temperature for 11 days and then acidified with 1 N HCl and evaporated under vacuum. The colorless oil obtained was dissolved in a $\text{CHCl}_3/\text{CH}_3\text{OH}$ mixture (2/1, 50 mL). TiCl_3 (10% in H_2O , 2.5 mL, 1.93 mmol) was added at room temperature. The solution was stirred under reflux for 8 h and at room-temperature overnight and then evaporated under vacuum. The black residue obtained was

esterified with CH_3OH (100 mL) and IRN-77 sulfonic Amberlite resin (2 g) at room temperature for 4 days. The mixture was filtered and the solution evaporated under vacuum. The brown residue was dissolved in ethyl acetate and the solution washed with water (3 \times 25 mL). K_2CO_3 was added to avoid emulsion. The organic layer was dried with MgSO_4 and evaporated under vacuum, yielding **4** (3.82 g, 80% from **2** + **2'**) which was crystallized (white solid) from ethyl acetate, mp 162 °C (lit. 163 °C).³⁷

3a(S),4(S),6a(R)-4-(4-methylethylloxycarbonylbutyl)-5-methylethyl-[6,6- $^2\text{H}_2$]-2,3,3a,4,6,6a-hexahydro-thieno-[3,4-*d*]imidazol-2-one tetraphenylborate (**5**). To a solution of **4** (2.69 g, 10.35 mmol) in methanesulfonic acid (40 mL) was added freshly distilled 2-propanol (17 mL). The mixture was stirred at 50 °C for 20 h and then neutralized with 5 N NaOH. The solution was washed with CH_2Cl_2 (4 \times 50 mL), and sodium tetraphenyl borate (3.54 g, 10.35 mmol) in H_2O (35 mL) was added to the aqueous layer. The product was extracted by CH_2Cl_2 (4 \times 50 mL). The combined organic layers were treated with brine, dried with MgSO_4 , and evaporated under vacuum, yielding **5** (3.87 g, 58%). ^1H NMR (acetone- d_6): 1.20 (6H, d, $J = 6.1$ Hz, $-\text{O}-\text{CH}(\text{CH}_3)_2$); 1.67, 1.72 (6H, 2d, $J = 6.6$ Hz, $-\text{S}^+-\text{CH}(\text{CH}_3)_2$); 1.59–1.75 (4H, m, $-\text{CH}_2-(\text{CH}_2)_2-\text{CH}_2-\text{COO}-$); 2.12–2.22 (2H, m, $-\text{CH}_2-(\text{CH}_2)_3-\text{COO}-$); 2.35 (2H, t, $J = 6.6$ Hz, $-(\text{CH}_2)_3-\text{CH}_2-\text{COO}-$); 4.16 (1H, m, $J = 6.6$ Hz, $-\text{S}^+-\text{CH}(\text{CH}_3)_2$); 4.39 (1H, ddd, $J = 4.6, 5.6, 9.7$ Hz, $-\text{S}^+-\text{CH}(\text{CH}_2)_4-\text{COO}-$); 4.91–5.02 (3H, m, 2- $\text{CHN}-$, $-\text{O}-\text{CH}(\text{CH}_3)_2$); 6.37, 6.57 (2H, 2s, NH); 6.80 (4H, t, $J = 7.1$ Hz, arom); 6.94 (8H, t, $J = 7.6$ Hz, arom); 7.30–7.38 (8H, m, arom). ^{13}C NMR (acetone- d_6): 18.44, 19.19 ($-\text{S}^+-\text{CH}(\text{CH}_3)_2$); 21.73 ($-\text{O}-\text{CH}(\text{CH}_3)_2$); 24.70, 26.94, 27.36 ($-(\text{CH}_2)_3-\text{CH}_2-\text{COO}-$); 33.19 ($-(\text{CH}_2)_3-\text{CH}_2-\text{COO}-$); 46.18 ($-\text{CD}_2-$); 47.96 ($-\text{S}^+-\text{CH}(\text{CH}_3)_2$); 63.63 ($-\text{S}^+-\text{CH}-$); 56.94, 60.50 (2x- $\text{CHN}-$); 67.05 ($-\text{O}-\text{CH}(\text{CH}_3)_2$); 121.78, 125.53, 136.51 (arom); 164.64 ($\text{C}=\text{O}$); 174.13 ($\text{COO}-$).

4(S),5(S)-4-[[6,6- $^2\text{H}_2$]-6-hydroxyhexyl-1-((1-methylethyl)thio)]-5-[$^2\text{H}_3$]-methyl-imidazolidin-2-one (**6**). To a solution of **5** (1.24 g, 1.69 mmol) in anhydrous THF (35 mL) was added LiAlD_4 (0.546 g, 13 mmol) under argon at 0 °C. The mixture was stirred under reflux for 1 h. NaOH (5 N, 1.5 mL) was added to the solution which was then neutralized with 1 N HCl. The precipitate was removed by filtration on Celite which was washed with THF (5 \times 25 mL). The combined solutions were evaporated under vacuum, and the residue was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CH}_2\text{OH}$: 100/0 \rightarrow 80/20), yielding **6** (279 mg, 59%). ^1H NMR (acetone- d_6): 1.29, 1.31 (6H, 2d, $J = 6.6$ Hz, $-\text{S}-\text{CH}(\text{CH}_3)_2$); 1.33–1.80 (8H, m, $-(\text{CH}_2)_4-\text{CD}_2\text{OH}$); 2.72 (1H, dt, $J = 3.1$ Hz, $J = 10.2$ Hz, $-\text{S}-\text{CH}(\text{CH}_2)_4-\text{CD}_2\text{OH}$); 3.10 (1H, m, $J = 6.6$ Hz, $-\text{S}-\text{CH}(\text{CH}_3)_2$); 3.76 (1H, dd, $J = 7.1, 9.6$ Hz, $-\text{CHN}-$); 3.86 (1H, d, $J = 6.6$ Hz, $-\text{CHN}-$); 5.55, 5.97 (2H, 2s, NH). ^{13}C NMR (acetone- d_6): 24.20, 24.63 ($-\text{S}-\text{CH}(\text{CH}_3)_2$); 26.41, 26.99 ($-\text{CH}_2-(\text{CH}_2)_2-\text{CH}_2-\text{CD}_2\text{OH}$); 33.31, 33.50 ($-\text{CH}_2-(\text{CH}_2)_2-\text{CH}_2-\text{CD}_2\text{OH}$); 36.49 ($-\text{S}-\text{CH}(\text{CH}_3)_2$); 48.57 ($-\text{S}-\text{CH}(\text{CH}_2)_4-\text{CD}_2\text{OH}$); 52.10 ($-\text{CHN}-$); 61.34 ($-\text{CHN}-$); 164.33 ($\text{C}=\text{O}$).

4(R),5(S)-4-[[6,6- $^2\text{H}_2$]-6-hydroxyhexyl)-5-[$^2\text{H}_3$]-methyl-imidazolidin-2-one (**7**). Lithium (46 mg, 6.6 mmol) was added, at -15 °C, to anhydrous liquid ethylamine (20 mL). The solution was stirred at -15 °C for 1 h, and a solution of **6** (205 mg, 0.73 mmol) in anhydrous THF (8 mL) was added. The mixture was stirred for 6 h at -15 °C and then warmed to room temperature, and ethylamine was evaporated. MeOH (10 mL) was added and the solution evaporated under vacuum. CH_2Cl_2 (20 mL) was added to the residue. The precipitate was removed by filtration and washed with CH_2Cl_2 (5 \times 10 mL), and the combined solutions were evaporated under vacuum. The residue was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CH}_2\text{OH}$: 100/0 \rightarrow 80/20), yielding **7** (70 mg, 46%). ^1H NMR (CDCl_3): 1.10, 1.24 (2H, m, $-(\text{CH}_2)_2-\text{CH}_2-(\text{CH}_2)_2-\text{CD}_2\text{OH}$); 1.24–1.35 (6H, m, $-(\text{CH}_2)_3-(\text{CH}_2)_2-\text{CD}_2\text{OH}$, $-\text{CH}_2-\text{CH}_2-(\text{CH}_2)_3-\text{CD}_2\text{OH}$); 1.45–1.54 (2H, m, $-\text{CH}_2-(\text{CH}_2)_4-\text{CD}_2\text{OH}$); 3.65 (1H, dt, $J = 5.1, 8.1$ Hz, $-\text{CHN}-$); 3.78 (1H, d, $J = 7.6$ Hz, $-\text{CHN}-$). ^{13}C NMR (CDCl_3): 23, 25.91, 29.87 ($-\text{CH}_2-\text{CH}_2-\text{CH}_2-(\text{CH}_2)_2-\text{CD}_2\text{OH}$); 30.09 ($-(\text{CH}_2)_2-\text{CH}_2-(\text{CH}_2)_2-\text{CD}_2\text{OH}$); 31.35 ($-\text{CH}_2-(\text{CH}_2)_4-\text{CD}_2\text{OH}$); 51.9 ($-\text{CHN}-$); 56.52 ($-\text{CHN}-$).

4(R),5(S)-4-(5-carboxypentyl)-5-[$^2\text{H}_3$]-methyl-imidazolidin-2-one (**3b**). Platinum oxide (72 mg, 0.24 mmol) in H_2O (10 mL) was

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reduced with a stream of H₂ for 30 min. After the solution was purged with argon, O₂ was introduced, and a solution of **7** (39 mg, 0.19 mmol) and Na₂CO₃ (47 mg, 0.44 mmol) in H₂O (2 mL) was added. The mixture was stirred for 20 h at room temperature under a stream of O₂. Platinum was removed by centrifugation, and fresh PtO₂ (85 mg) was added. After treatment by H₂ as before, the mixture was stirred for 6 days at room temperature under O₂. The catalyst was removed by centrifugation and the solution was neutralized and chromatographed on a DOWEX column (1 × 2–200 ion-exchange resin, 0.03N HCOOH), yielding **3b** (26 mg, 63%, white solid). Fifteen milligrams was purified by C18 silica reversed phase chromatography (H₂O/CH₃CN: 100/0 → 80/20), yielding 10 mg of pure product, mp: 156 °C (lit. 159 °C).³⁶ ¹H NMR (D₂O + NaOD): 1.10–1.29 (4H, m, -CH₂-(CH₂)₂-(CH₂)₂-COOH); 1.3–1.46 (4H, m, -CH₂-(CH₂)₂-CH₂-CH₂-COOH); 2.02 (2H, t, *J* = 7.6 Hz, -(CH₂)₄-CH₂-COOH); 3.63 (1H, dt, *J* = 7.6, 6.2 Hz, -CHN-); 3.73 (1H, d *J* = 7.6 Hz, -CHN-). ¹³C NMR (D₂O + NaOD): 14.35 (-CD₃); 25.65 (-(CH₂)₂-CH₂-(CH₂)₂-COOH); 25.97 (-(CH₂)₃-CH₂-CH₂-COOH); 28.86, 29.3 (-(CH₂)₂-(CH₂)₃-COOH); 37.78 (-(CH₂)₄-CH₂-COOH); 51.57 (-CHN-); 56.31 (-CHN-); 165.77 (C=O); 184.31 (COOH).

Biochemicals. Bacterial Strains. The *E. coli* strain TK101 [pL3-BLS2]³⁸ overexpressing biotin synthase was a generous gift from Professor Y. Izumi (Tottori University, Japan). The *E. coli* strains C-1a [pEE1010] and DH01 overexpressing flavodoxin reductase and flavodoxin, respectively, were generous gifts from Professor P. Reichard (Karolinska Institute, Sweden).

Purification of the Enzymes. Biotin synthase, flavodoxin, and flavodoxin reductase were purified, with some modifications, as described by Sanyal et al.,⁶ Osborne et al.,³⁹ and Bianchi et al.,⁴⁰ respectively. Biotin synthase was about 80% pure as judged from SDS-PAGE, and the UV-vis spectra of pure flavodoxin and flavodoxin reductase were essentially identical to those reported by Fujii and Huennekens.⁴¹

Enzymatic Assay. A mixture containing 12 μM biotin synthase, 2 μM flavodoxin, 0.36 μM flavodoxin reductase, 50 μM DTB or [²H]-DTB, 200 μM AdoMet, 5 mM DTT, 2 mM NADPH, and 1 mM cysteine in 40 mM Tris-HCl pH 8 buffer was prepared and equally distributed in several Eppendorfs. Fe(NH₄)₂(SO₄)₂ (1 mM) was then added to each Eppendorf in a final volume of 300 μL. The cofactors and the enzymes were in H₂O for all of the experiments except for the control experiments performed in D₂O where the cofactors were solubilized in D₂O and the enzymes lyophilized 1, 2, or 3 times before resolubilization in D₂O. A reference sample with nonlabeled DTB as substrate and H₂O was tested in parallel for each independent experiment. The Eppendorfs were left under argon for 1 h at room temperature and incubated for 3 h at 30 °C. Two 5 μL-aliquots of each sample were withdrawn to determine the amount of biotin formed by

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means of a microbiological assay with *Lactobacillus plantarum*.⁴² The average concentration of biotin in all of the Eppendorfs (four to six per experiment) was calculated and the standard deviation deduced. The data were normalized to correspond to the formation of 2.9 μM of biotin (mean value of biotin concentration obtained for reference samples (DTB + H₂O) of each independent experiment).

Purification of 5'- or 5'-[²H]-Deoxyadenosine. The proteins of each sample were precipitated with 30 μL of 12% (w/v) trichloroacetic acid. After centrifugation, the supernatants were pooled and analyzed by reversed phase HPLC (Nucleosil 5C18 250-4, Merck) using a 20-min linear gradient of H₂O/acetonitrile (100:0 to 68:32) at a flow rate of 1.2 mL/min. The product which eluted between 13 and 14 min was collected, concentrated under vacuum, dissolved in acetonitrile, and analyzed by mass spectrometry.

Mass Spectrometry Analysis. All EI or CI mass spectra and low energy collision-induced dissociations (CID) spectra of selected ions were performed using a triple quadrupole tandem mass spectrometer (R-30-10 Nermag). The samples were introduced into a high-pressure ion source by using a direct desorption DCI probe with heating of the tungsten filament. Mass and CID spectra corresponded to averages of 10 full spectra recorded on an EZSCAN acquisition system. The source operating conditions were electron energy, 100 eV; emission current, 100 μA; repeller, 0 V; pressure of ammonia as reagent gas, as well as its deuterated analogue (purchased from CEA Eurisotope, France), close to 10⁻⁴ Torr in the source housing (measured above the secondary pumping). To perform Ar-induced collision activation, a pressure, *P*_{ar} = 4 × 10⁻⁵ Torr in the collision cell (essentially single collision conditions) and a laboratory collision energy of 45 eV were applied. The lens potentials are chosen to optimize the product *m/z* 117 ion.

Deuterium Content of the [²H]Dethiobiotins. Mass spectrometry performed under chemical ionization and electron impact ionization conditions with dethiobiotin methyl ester provided respectively the protonated molecule MH⁺ (*m/z* 229) and the fragment ions (*m/z* 213 and *m/z* 99) as major peaks.²⁰ The shift of the *m/z* 99 ion allowed the determination of the labeling at position 9. The labeling at positions 6 and 5 of **3a**, **3c**, and **3d** was evaluated from the shift of peaks at *m/z* 213 and at *m/z* 229 and from ²H NMR data.²³

Content and Localization of Deuterium in [²H]Deoxyadenosine. The proportion of [²H]DOA was determined by chemical ionization using NH₃ as ionization gas. The location of deuterium in [²H]DOA was determined by fragment ion analysis from the CID experiments using DOA or 5'-[²H₁]DOA as reference samples and NH₃ or ND₃ as ionization gas. The results with standard deviations are compiled in Table 2.

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