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ON THE MECHANISM OF CONVERSION OF DETHIOBIOTIN TO BIOTIN IN *Escherichia coli*. DISCUSSION OF THE OCCURENCE OF AN INTERMEDIATE HYDROXYLATION

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SUMMARY :

The last step of the biosynthesis of biotin, i.e. the conversion of dethiobiotin to biotin was studied using *E. coli*. The three dethiobiotin derivatives hydroxylated at C-2 or C-5 were synthesized and tested as potential precursors of biotin. It appears that none of these compounds is able to support the growth of *E. coli C124*, a mutant which does not synthesize dethiobiotin, but converts it into biotin. These results strongly disfavour the hypothesis of the activation of the saturated carbons by an hydroxylation process.

The biosynthesis of biotin, 1, has already been intensively studied (1). All the intermediates in the biosynthetic pathway from pimelic acid to dethiobiotin, 2, are presently known and formed by classical biochemical reactions. But the mechanism of the conversion of dethiobiotin into biotin, a very unusual transformation, is still completely unknown.

In our first investigation (2) on biotin biogenesis, we have shown that the formerly proposed hypothesis of an unsatured intermediate of type (i) was highly improbable (scheme 1). These results were in agreement with those obtained separately by Parry and Kunitani with *A. niger* (3).

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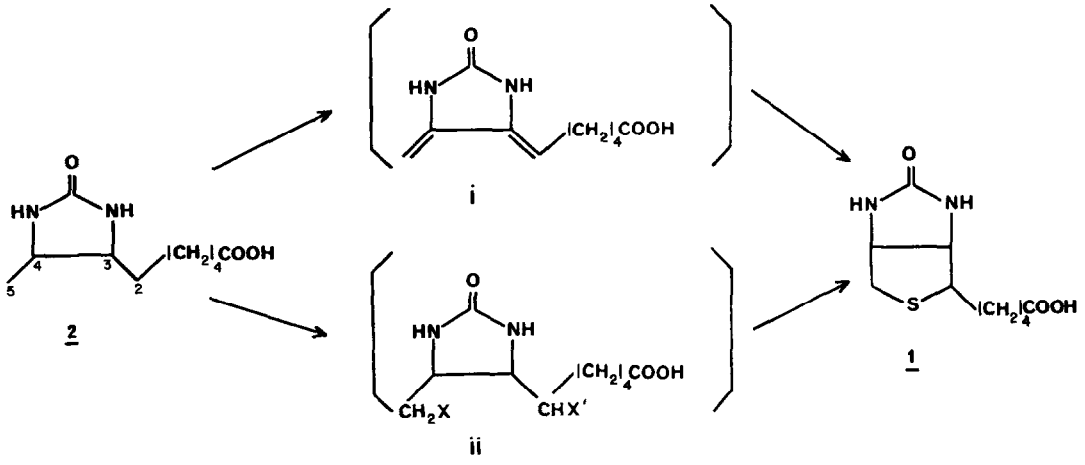
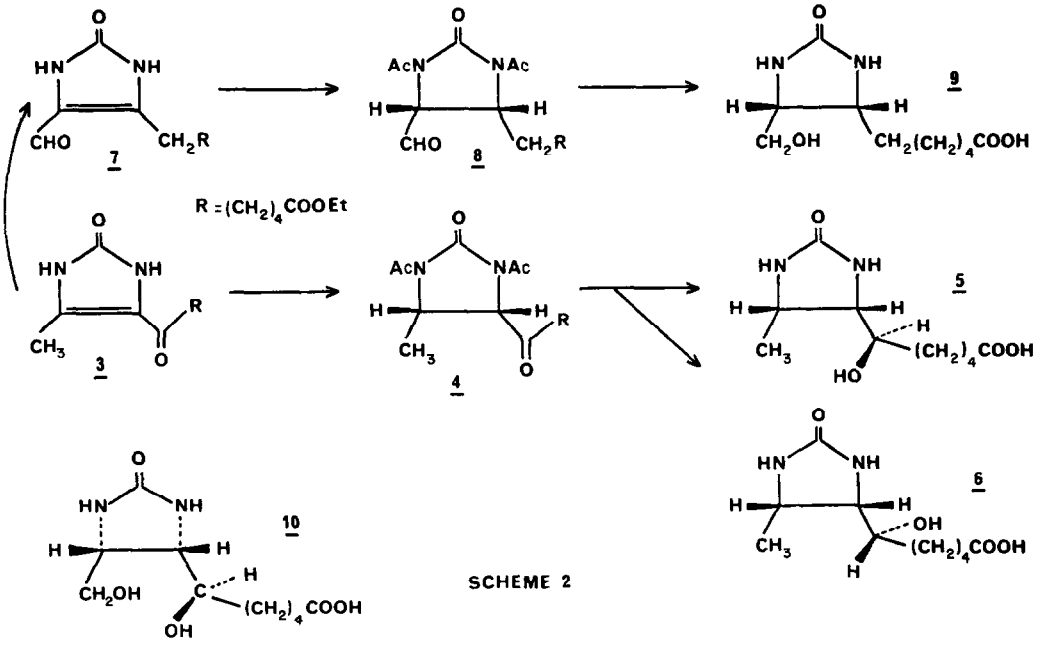


SCHEME 1

The hypothesis of an intermediate hydroxylation, (ii), for the functionalisation of the saturated carbons is now considered.

This paper reports the synthesis of hydroxy derivatives of dethiobiotin 5, 6, 9 (scheme 2) and the biochemical experiments undertaken to test them as biotin precursors.



SCHEME 2

MATERIALS AND METHODS

Synthesis of dl-{4-^{3}H} dethiobiotin (200 mCi/mmol) has been described previously (4). d-{2'-^{14}C} biotin (38 mCi/mmol) was obtained from the Radio Chemical Center, Amersham ; {^{3}H}$NaBH_4$ (20 Ci/mmol) from CEA FRANCE.

Synthesis of Alcohols 5, 6, 9 :

These three compounds have been synthesized starting from 4-methyl 5-(ω-carboethoxyvaleryl) imidazolone-2, 3, (5). The aldehyde, 7 was obtained according to Zav'yalov (6).

The same reaction sequence has been used to obtain 5 and 6 from 3 and 9 from 7 : acetylation of imidazolone nitrogens, hydrogenation of the double bond in dioxane over Pd/C, reduction of the cis isomers by $NaBH_4$ in ethanol, saponification and purification on anion exchange resin (AG.1-X_2, formate 100-200 mesh).

The N,N'-diacetylated derivatives of 3 and 7 were hydrogenated over Pd/C (10%) at room temperature and atmospheric pressure. Mixture of cis and trans isomers are obtained. The predominant cis products, 4 (95%) and 8 (60%) are isolated in 63% and 60% yield after separation of the mixture by preparative layer chromatography (Silicagel, AcOEt/$CHCl_3$ 2 : 8). By treatment with 1N NaOH solution in methanol the cis isomers are converted into the more stable trans isomers.

Examination of molecular models, shows that the coupling constant $J_{H_3-H_4}$ should be greater in the cis isomer than in the trans. Such a relation had been previously observed for the trans and cis dethiobiotin, respectively 4.5 and 8 Hz.

The configurations of the ketone 4 and aldehyde 8 were deduced on the basis of $J_{H_3-H_4}$ constant after comparison of their NMR spectra (Varian HA 100) with these of their trans isomers.

The reduction of N,N'-diacetyl derivatives 4 and 8 by $NaBH_4$ gave a mixture of mono and diacetylated alcohols which were saponified to afford the corresponding acids 5 and 6 (78%) and 9 (67%). The separation of 5 and 6 was performed on their AcOEt/MeOH 4 : 1). Because of the easy epimerisation of cis derivatives, we had to check that no isomerisation was occuring during the reduction of 4 and 8. So we prepared the alcohols corresponding to the trans isomers of 4 and 8 and checked by NMR and TLC, that these compounds were different from the cis alcohols 5, 6 and 9.

The C-2 configuration of the alcohols 5 and 6 has not been definitely established. Since the biochemical experiments have shown that these alcohols are very probably not intermediates between dethiobiotin and biotin (*vide infra*), we did not complete this configuration attribution.*

* The reduction of 4 yielded an O-acetyl derivative in position -2, as a secondary product, resulting probably of a transacetylation reaction with the neighbouring N-acetyl group. Examination of Dreiding models shows that the transition state for this reaction is highly improbable for isomer 6 but favoured for isomer 5. So, we have tentatively attributed the represented configuration to 5 and 6 on this basis.

The corresponding labelled alcohols {^{3}H} 5 (180 mCi/mmol), {^{3}H} 6 (1 Ci/mmol), {^{3}H} 9 (7.5 Ci/mmol) were easily prepared by reduction of their carbonyl precursors 4, 8 by NaB^3H_4, using the same reaction sequence.

The diol 10 has also been tested as biotin precursor. This compound prepared by Ohrui (7) is a synthetic intermediate which, treated by Na_2S, affords directly biotin with inversion of configuration at C-2 (8).

Bacterial Strain :

E. coli C124 {His^-, bio A^-} is generous gift from Dr. P. Cleary. The strain was grown on synthetic medium as described by Rickenberg et al (9). Before autoclaving the medium was treated with Norit to remove traces of biotin. The growth was monitored at 570 nm.

Growth test :

E. coli C124 was grown on synthetic medium supplemented with minimal quantities of biotin (0.1 ng/cm^3. After 24 h., ($\simeq$ 7.5 x 10^8cells/cm^3), 5 cm^3 of the culture was centrifuged and the cells were washed with 5 cm^3 of 0.9% saline sterile solution. A dilute saline suspension ($\simeq$ 1.5 x 10^6cells/cm^3) was prepared to inoculate three types of flasks containing 10 cm^3 of synthetic medium : flask A : blank ; flask B : standard, supplemented with 1 ng or 5 ng/cm^3 of dethiobiotin ; flask C : supplemented with increasing quantities (5 to 200 ng/cm^3) of the product to be tested.

The inoculated flasks were incubated at 37°C on a rotatory shaker. The growth was monitored at 570 nm.

Transport studies :

The transport studies were performed as described by Eisenberg (10), using *E. coli C124* cells grown on synthetic medium supplemented with 0.1 ng/cm^3 of biotin, either to early stationary phase or to mid-log phase. The washed cells were resuspended (3.5 to 4 x 10^9cells/cm^3) in the uptake medium (10) and 75 ng/cm^{3}of the tested samples were added. After incubation at 0°C or 37°C, portions of 2 cm^3 were withdrawn at various times (2, 5, 10, 20, 30 and 60 min.) and added to 4 cm^3 of cold 0.9% saline. The cells were collected by filtration. The filter, washed with 2 x 4 cm^3 of cold saline, was placed in a counting vial, dried at 80° for 1 hour and the radioactivity on the filter was counted after adding 10 cm^3 of toluene containing per liter 6 g PPO and 0.3 g POPOP. The intracellular concentrations were deduced from the specific radioactivity of the sample and calculated as ng/cm^3. The intracellular free space was estimated to 0.25 µl per 2.5 x 10^8cells (10).

For the passive transport assay, 3 cm^3 of the cells suspension, described above, were supplemented with 50 to 200 ng/cm^3 of the labeled alcohol and maintained at 4°C for 60 h. After this delay, an aliquot of 2 cm^3 was withdrawn, rapidly filtered and washed with 2 x 4 cm^3 of cold saline. The intracellular concentrations were determined according to the same procedure.

RESULTS AND DISCUSSION

The biosynthetic experiments were carried out with *E. coli C124*, a mutant of *E. coli* which is unable to biosynthesize dethiobiotin but carries out the biotransformation of dethiobiotin to biotin (P. Cleary - Personal communication).

If one of the above hydroxy compounds is an intermediate between dethiobiotin and biotin, it should promote the growth of this mutant.

Growth tests :

The use of the seeding technique employed for microbiological biotin determination (11) ensures the absence of contamination by intracellular biotin as checked by the absence of growth in the blank. After incubation at 37°C for 40 h., neither the dethiobiotin deficient blank, nor anyone of the test flasks supplemented with 5 to 200 ng/cm^3 of alcohols 5, 6 or 9, showed growth of *E. coli* whereas in the standard flask, containing synthetic medium supplemented with 1 ng/cm^3 of dethiobiotin, bacteria had grown to stationary phase ($\geqslant 10^9$cells/cm^3) after 24 h.

The same results were obtained with dethiobiotin dihydroxylated at C-2 and C-5, 10 : the growth test remained negative with external concentrations of 10 up to 500 ng/cm^3. As expected, the corresponding trans isomers of alcohols 5, 6 and 9 were also inactive. However, before claiming that these negative experiments prove that the hydroxy dethiobiotins are not precursors of biotin, it was necessary to check that these molecules enter cells in the experimental conditions used.

Transport experiments :

The transport of {^{14}C} biotin, {^{3}H} dethiobiotin and {^{3}H} alcohols 5, 6, 9 into cells of *E. coli* was then compared.

The biotin uptake, as expected, (10, 12, 13) was temperature dependent and was operating against a concentration gradient. On the other hand, the transport of the alcohols 5 and 6 is temperature independent (Table 1). Moreover, the bacteria do not accumulate the alcohols against a concentration gradient : the intracellular concentration is inferior or equal to the initial extracellular concentration.

Table 1

Intracellular concentration in *E. coli C124* cells after incubation for 1 h. at 0°C or 37°C in the presence of 75 ng of d-{^{14}C} biotin, dl-{4-^{3}H} dethiobiotin or dl-{^{3}H} hydroxydethiobiotin per cm^3 of uptake medium.

	Intracellular concentration (ng/cm^3)				
	Biotin	Dethiobiotin	5	6	9
0°C	250	80	75	25	75
37°C	2600	600	100	25	300

In view of these results, we can conclude that there is no active transport process for the uptake of alcohols 5 and 6 by *E. coli C124*. Concerning dethiobiotin and alcohol 9, the conclusions are not so clear : the transport is temperature dependent, but the intracellular concentrations, higher than the initial extracellular concentration, remain low compared to biotin (Table 1).

Whatever the nature of the transport process of 5,6,9, it is possible to let these hydroxy compounds enter the cells in sufficient amount by a process similar to diffusion. The intracellular concentration in *E. coli C124* vary from 25 to 300 ng/cm^3 at 37°C and from 25 to 75 ng/cm^3 at 0°C (Table 1). By increasing the extracellular concentration up to 200 ng/cm^3, the endogenous concentration at 0°C is increased to 100-140 ng/cm^3. With dethiobiotin, under normal growth conditions, a 1 ng/cm^3 concentration in the incubation medium ensures a 15 to 25 ng/cm^3 content in the intracellular space. Therefore in our growth tests, the endogenous concentration is always superior to the dethiobiotin concentration which support the growth of *E. coli C124*.

The fact that no growth is observed under these conditions enables us to conclude that an intermediate hydroxylation is very unlikely. However, this hypothesis cannot be completely excluded since the conversion of dethiobiotin into biotin, which is certainly a multistep process, could involve a multienzymatic complex carrying out the whole transformation, or part of it, without releasing the intermediates (or incorporating a free intermediate from the medium).

Parry has shown recently (14) that during the conversion of octanoic acid into lipoic acid, the introduction of sulfur on carbon 6 occurs with inversion. He suggests on this basis that the C-S bond could be formed through the sequence : hydroxylation (with retention) nucleophilic substitution of the activated hydroxyl group (with inversion) and points out that the same could be valid for biotin. Our results don't support this hypothesis.

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