Note

On the Biosynthesis of Biotin: Bioconversion of 1',3'-Dimethyldethiobiotin by *Bacillus sphaericus*

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Even though it is now well established that dethiobiotin (DTB) **1** is the precursor of biotin **2** in many microorganisms,^{1,2)} the mechanism of this transformation has not been elucidated. However, it has been demonstrated that the two C-S bonds of biotin are formed with removal of only one hydrogen, without involvement of the adjacent positions.^{3,4)} Furthermore, an intermediate hydroxylation either at C₅ or C₂ is very unlikely.⁵⁾ This leads to the postulation of a direct sulfur introduction, which raises a puzzling mechanistic problem.

We considered the possible participation of the ureido ring, for instance, through the formation of an intermediate N-derivative responsible for an intramolecular functionalisation at C₅ or C₂. To test this hypothesis, we examined whether 1',3'-dimethyl dethiobiotin (diMe DTB) **3**, in which both ureido nitrogens are alkylated, could be converted into biotin without demethylation.

MATERIALS AND METHODS

Materials. ω ,4-Dibromoacetophenone (DBAP) was obtained from Merck and dibenzo-18-crown-6 from Borregaard (Norway). d-Biotin was a gift from Hoffmann-La Roche (France). DTB was obtained from biotin by desulfuration with Raney nickel,^{6,7)} [3,4,-³H₂]DTB (200 mCi/mmol) was previously prepared in our laboratory.⁸⁾ AG1X2 was purchased from Bio-Rad. [³H]diMe DTB (20 mCi/mmol) was prepared from a tritiated sample

diluted with unlabelled DTB (1/9), by methylation with formol-formic acid according to Eschweiler-Clarke.⁹⁾ After evaporation of the reagents under reduced pressure, the crude extract was purified by chromatography on anion exchange resin (AG1X2 formate form). The labelled material was eluted with a linear gradient from 500 ml of water to 500 ml of formic acid. Yield: 85%; mp 102~104°C. NMR (CF₃COOH, Me₄Si as internal standard) δ 1.2 (d, $J=6$ Hz, 3H, CH₃); 1.5 (m, 8H, CH₂)₄; 2.45 (m, 2H-CH₂COOH); 2.8 (s, 3H) and 2.9 (s, 3H) (-NCH₃, N'CH₃); 3.8 (m, 2H, CH-N). 1',3'-dimethyl biotin **4** (diMe biotin)¹⁰⁾ was obtained by the same procedure. F = 170~172°C (lit. = 172~174°C).

Analytical techniques. TLC was performed on silica gel thin-layer chromatoplates; Merck, 60 F254. Detection was achieved with a 254 UV lamp or by autoradiography with a Berthold beta camera BF 290 HR. Column chromatography was performed on silica gel 60 Merck (70~230 mesh ASTM). Scintillation counting was carried out with an Inter technique SL₃₀ spectrometer in Bray's liquor.¹¹⁾

HPLC studies were performed with a Waters apparatus equipped with two 6000 A pumps, a model 660 solvent programmer and a model U6K injector. Detection was accomplished with an Altex UV detector ($\lambda=254$ nm). Derivatized mixtures were separated on a 330×4 mm column packed with C₁₈ bonded phase (μ -Bondapak, 10 μ m particle size) with methanol-H₂O (60:40) as solvent.

Mass spectrometry. The mass spectra were obtained with a VG 70~70 double focusing mass spectrometer equipped with a dual electron impact (EI)/chemical ionization (CI) source and a PDP8 computer. The source temperature was maintained at about 180°C.

Typical experimental conditions were:

EI: trap current, 100 μ A; ionizing energy, 70 eV; ion repeller, potential zero; direct insertion probe at 50°C.

IC: emission current, 200 μ A; reagent gas, NH₃ at about 1 Torr (NH₃⁺/NH₄⁺ = 0.1).

Bioconversion experiments. The reaction was carried out using resting cells of *Bacillus sphaericus* as described by Izumi *et al.*¹²⁾ The standard reaction mixture contained 100 μ mol of tris-HCl buffer (pH 8.0), 2 μ g of diMe DTB and 30 mg (as dry weight) of intact cells in a total volume of 1 ml. The reaction was performed at 37°C for 5 hr with reciprocal shaking (150 rpm). All the samples (75×1 ml) were collected and centrifuged (10⁴ rpm for 10 min), and the supernatants evaporated to dryness and treated with 99% ethanol.

Each ethanolic solution was concentrated and the residue poured on a column of anion exchange resin AG1X2 formate (100×200 mesh, 25×250 mm). After washing with 200 ml of distilled water, the resin was eluted with a linear gradient of from 500 ml of distilled water to 500 ml

of 0.1N formic acid. The eluted radioactive fractions (10 ml each) were collected, evaporated to dryness and converted to the BAP esters as previously described.¹³⁾ The crude reaction mixture was chromatographed on a silica gel column previously standardized with diMe biotin.

RESULTS AND DISCUSSION

The conversion was performed with *Bacillus sphaericus*,

a bacterium in which all the enzymes involved in biotin biosynthesis have been detected and which shows a high biotin synthetase activity.¹²⁾

The reaction was carried out with resting cells as described by Izumi *et al.*¹²⁾ After a first purification on AG1X2,¹⁴⁾ the radioactive fractions were collected and converted to the BAP esters,¹³⁾ which were purified by a preliminary chromatography on silica gel. Then, the fractions corresponding to the diMe biotin BAP ester were

FIG. 1. Liquid Chromatograms of (a) Biological Extract; (b) Biological Extract + BAP Esters of Biotin (B_1) and DiMe Biotin (B_2).

Conditions as in MATERIALS AND METHODS.

FIG. 2. CI Mass Spectra of a) Fraction B_2 (Fig. 1); b) BAP Ester of DiMe Biotin.

Conditions as in MATERIALS AND METHODS.

analysed by HPLC with UV detection: two peaks corresponding, respectively, to the BAP esters of biotin (B_1) and diMe biotin (B_2) were observed (Fig. 1a). Their intensities increased on addition of authentic materials (Fig. 1b).

To confirm this result, peak B_2 (Fig. 1a) was collected and analyzed by mass spectrometry: the diMe biotin BAP ester was identified in the CI spectrum from its protonated molecular ion ($m/z=469\sim471$) and the fragment ions at $m/z=273$, 241 and 127 observed in the reference spectrum (Fig. 2). Under the EI conditions, the molecular peak was not observed, but the main characteristic ions of the diMe biotin BAP ester, $m/z=112$ (14%), 113 (18%), 125 (100%), 183 (15%), 185, 255 (7%) and 271 (33%), appeared.^{15,16}

From the radioactivity of the diMe biotin fraction, we estimated that the resting cells of *Bacillus sphaericus* convert diMe biotin DTB into diMe biotin in a 1% yield (under the same conditions, DTB was transformed into biotin in a 25% yield). The reaction led also to 0.2% of biotin (based on diMe DTB), identified from its analytical properties (peak B_2 in Fig. 1) and by a microbiological assay with *L. plantarum*.¹⁷ Only traces of DTB were found in the medium and so the isolated biotin probably resulted from demethylation of the biosynthesized diMe biotin.

Since the introduction of the sulfur atom at C_2 and C_5 occurred in the case of the N,N'-dimethyl analog of DTB, it is clear that the hypothesis of the participation of the ureido ring in this transformation has to be rejected.

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