Bacterial Degradation of Biotin and Dethiobiotin: Isolation and Identification of β -Hydroxy and Keto Metabolites

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The degradation of biotin and dethiobiotin by resting cells of a biotin-degrading bacterium, *Mycoplana* sp. No. 166, was studied. The metabolites were isolated from reaction mixtures supplemented with biotin or dethiobiotin. By mass and NMR spectral analyses, the biotin metabolites were identified to be bisnorbiotin and β -hydroxybisnorbiotin, and the dethiobiotin metabolites to be methyl tetranordethiobiotinyl ketone, bisnordethiobiotin, β -hydroxybisnordethiobiotin, and tetranordethiobiotin. The two β -hydroxy metabolites and the keto metabolite were new compounds.

Metabolites of biotin and dethiobiotin have been reviewed,¹⁾ but the metabolic pathways of these vitamers in microorganisms are not completely known. Little is known about the relationship between the metabolites and the enzymes that catalyze their formation.

We isolated a *Mycoplana* sp. from a soil sample that strongly degrades biotin, and found that its cell-free extract has biotinyl-CoA synthetase activity.²⁾ Here, we investigated the metabolites of biotin and dethiobiotin using this microorganism; the metabolites of these vitamers were isolated from the resting cell reaction mixture and were identified.

MATERIALS AND METHODS

Chemicals. Authentic bisnordethiobiotin was prepared microbiologically in our laboratory.³⁾ Other chemicals were purchased from commercial sources.

Microorganism and culture medium. *Mycoplana* sp. No. 166, a biotin-degrading bacterium,²⁾ was used throughout this experiment. The composition of the medium was described previously as basal medium BM-1²⁾ containing

0.2% biotin instead of 0.1% biotin.

Culture conditions. The bacterium was cultivated in 200 ml of the medium in 2-liter shaking flasks at 28°C for 24 hr under reciprocal shaking (120 strokes/min).

Resting cell reaction for biotin and dethiobiotin degradation. The cells in the culture broth were harvested by centrifugation at 0°C at 10,000 $\times$ g for 15 min, washed twice with saline, and resuspended in 100 mM potassium phosphate buffer (pH 7.0). This cell suspension was used for the resting cell reaction. Fifty milliliters of the cell suspension (OD_{610} , 55) containing 1.0% biotin or dethiobiotin in a 500-ml Erlenmeyer flask was vigorously shaken (180 strokes/min) at 30°C on a reciprocal shaker for 5 hr for biotin or 22 hr for dethiobiotin.

Assays. To detect the metabolites in the purification procedure, TLC plates (Merck silica gel 60 F₂₅₄, aluminium sheet) were used. Free acid metabolites were developed with solvent I (acetic acid–acetone–methanol–benzene, 1:1:4:14, by vol.) or solvent II (butanol–water–methanol–benzene, 2:1:2:1, by vol.). For methyl esters, solvent I or solvent III (ethyl acetate–methanol, 4:1, by vol.) was used. Solvents I to III were also used for silica gel column chromatography.

The metabolites with an ureido ring were detected on TLC plates as pink spots after being sprayed with *p*-dimethylaminocinnamaldehyde (DACA).⁴⁾

Ammonia in the reaction mixture was absorbed to sulfuric acid using the Conway micro-diffusion method.⁵⁾ The amount of ammonia absorbed was estimated colorimetrically by the phenol-hypochlorite method.⁶⁾

Purification of metabolites of biotin. Fifty milliliters of the reaction mixture was evaporated to dryness under reduced pressure at below 45°C and the metabolites in the residue were repeatedly extracted with 99% ethanol. The extract was evaporated to dryness and the residue was dissolved in a small amount of distilled water. The concentrated solution was applied to a Dowex 1-X2 column (formate, 100 to 200 mesh), 2×24 cm, and eluted with a linear gradient from 1 liter of water to 1 liter of 0.03 M formic acid. Fractions of 12 ml were collected. Biotin and two metabolites were eluted with about 0.025 M formic acid. These fractions positive for DACA were concentrated and put on a Dowex 50W-X8 (H⁺) column, 0.8×25 cm. Two-milliliter fractions eluted with distilled water were collected. Metabolites B and A and biotin appeared in that order on the chromatogram. They were collected separately and metabolites A and B were further purified as described below.

Metabolite A: The Dowex 50W-X8 (H⁺) fractions containing metabolite A were concentrated and further purified by Dowex 50W-X8 (H⁺) column chromatography. The fractions containing metabolite A were collected and evaporated to dryness to give 6 mg of a white powder.

Metabolite B: The Dowex 50W-X8 (H⁺) fractions containing metabolite B were concentrated. Slight contamination by the other compounds positive for DACA was detected, so metabolite B was further purified after conversion to the corresponding methyl ester with diazomethane. The methylated derivative was isolated by repeated silica-gel column chromatography on a 1×80 cm column, the first time with solvent III and the second time with solvent I. The eluate was concentrated, developed on a TLC plate with solvent III, and then extracted from the plate. In the final step, silica gel column chromatography of the extract with an eluent of a mixture of ethyl acetate-methanol (5:1) gave 0.6 mg of a white powder.

Purification of metabolites of dethiobiotin. The metabolites of dethiobiotin were also purified by Dowex 1-X2 (formate) column chromatography by the same procedure as for the metabolites of biotin. Metabolite C passed through the column. After the column was washed with distilled water, metabolite D, dethiobiotin, metabolites E, and F appeared in that order on the chromatogram by elution with formic acid. Metabolites C, D, E, and F were collected separately and further purified as follows.

Metabolite C: Collected fractions of metabolite C were concentrated and further purified by Dowex 50W-X8 (H⁺) column chromatography as for metabolite A. Removal of the solvent gave 2.5 mg of a white powder. The hydrazone derivative of metabolite C, produced by the

treatment with 2,4-dinitrophenylhydrazine,⁷⁾ was purified by TLC, developed with solvent I, and extracted with ethyl acetate.

Metabolite D: The Dowex 1-X2 fractions of metabolite D were concentrated and purified by Dowex 50W-X8 (H⁺) column chromatography, silica gel column chromatography repeated twice (solvent II), Dowex 50W-X8 (H⁺) column chromatography, and finally Dowex 1-X2 (formate) column chromatography. Less than 1 mg of metabolite D was obtained.

Metabolite E: The Dowex 1-X2 fractions of metabolite E were concentrated and purified by Dowex 50W-X8 (H⁺) column chromatography repeated three times. The metabolite was methylated with diazomethane and the methyl ester was further purified by silica-gel column chromatography (solvent III). Less than 1 mg of methylated metabolite E was obtained.

Metabolite F: The Dowex 1-X2 fractions of metabolite F were concentrated and purified by Dowex 50W-X8 (H⁺) column chromatography done twice. Methylated metabolite F (3.7 mg) was obtained as a white powder by the same method as for methylated metabolite D.

Instrumental analyses. Low-resolution mass spectral data were obtained by the In-Beam EI (20 eV) method on a Hitachi M-80 instrument. High-resolution mass spectral data were obtained on the same spectrometer equipped with an M-003 data system. ¹H NMR spectra (400 MHz) were recorded on a JEOL-JNM GX-400 spectrometer in D₂O for the free acid metabolites and in CD₃OD or CDCl₃ for methyl esters. Chemical shifts were given in ppm with sodium 2,2-dimethyl silapentane-5-sulfonate or tetramethylsilane as the internal standard.

RESULTS

Identification of biotin and dethiobiotin metabolites

To isolate and identify the metabolites produced from biotin and dethiobiotin by *Mycoplana* sp. No. 166, reaction mixtures containing the biotin vitamers and the resting cells were incubated as described in MATERIALS AND METHODS. The course of the formation of DACA-positive metabolites was examined qualitatively with TLC. Dethiobiotin was degraded much more slowly than biotin and degraded to more kinds of metabolites than biotin. The optimum reaction time was 5 hr for biotin and 22 hr for dethiobiotin. The metabolites were purified and identified from the following analyses.

Metabolite A. Metabolite A had an *R_f* value

FIG. 1. Structures of Biotin and Dethiobiotin Metabolites.

FIG. 2. 400 MHz ^1H NMR Spectrum of Methylated Metabolite B in CD_3OD .
The ^1H NMR spectrum of metabolite A (bisorbiotin) is given for comparison.

of 0.40 on TLC with solvent I. The mass spectrum showed a molecular ion peak (m/z 216) and fragment ion peaks (m/z 198, $M-18$; 156, $M-\text{CH}_2\text{COOH}$; 97, 2-keto-5-methenyl imidazolium cation).

The NMR spectrum indicated the presence of a propionic acid side chain, δ : 1.84 and 2.01 (1H each, m, H-8a and H-8b) and 2.47 (2H, m, H-9a and H-9b) and a bicyclic ring, δ : 2.79 (1H, d, $J_{6a,6b}=13.2$, H-6a), 3.01 (1H, double d, $J_{6a,6b}=12.7$ and $J_{5,6b}=4.9$, H-6b), 3.33 (1H, m, H-7), 4.45 (1H, double d, $J_{4,5}=8.3$ and $J_{4,7}=4.4$, H-4), 4.61 (1H, m, H-5). These results indicate that metabolite A is bisnorbiotin, which is produced from biotin through the loss of two carbon atoms (1, Fig. 1).

Metabolite B. Metabolite B had an R_f value of 0.22 on TLC with solvent I. This low R_f value suggested that this metabolite had a more hydrophilic group than metabolite A.

The NMR spectrum of methylated metabolite B is compared to that of metabolite A (bisorbiotin) in Fig. 2 and analyzed as follows: δ : 2.52 and 2.62 (1H, each, AB part of ABX system, $J_{8,9a}=9.3$, $J_{8,9b}=2.9$ and $J_{9a,9b}=15.6$, H-9a and H-9b), 2.78 (1H, d, $J_{6a,6b}=12.7$, H-6a), 3.01 (1H, double d, $J_{6a,6b}=12.7$ and $J_{5,6b}=4.4$, H-6b), 3.70 (3H, s, $-\text{CO}_2\text{CH}_3$), 4.14 (1H, m, H-8), 4.52 (2H, m, H-4 and H-5). The two peaks corresponding to H-8a and H-8b at around 1.80~2.00 ppm that appeared in the case of metabolite A (bisor-

FIG. 3. Mass Spectrum of Methylated Metabolite B.

biotin, Fig. 2) were absent here. A peak at a lower magnetic field (4.14 ppm) was attributed to H-8 and the number of protons corresponding to H-8a or H-8b was only one. This evidence suggests that one of these protons is substituted by a hydroxy group in metabolite B. The NMR spectrum is consistent with the assumption that metabolite B is β -hydroxybis-norbiotin. The mass spectrum of methylated metabolite B is shown in Fig. 3. Although the expected mass ion at m/z 246 was not seen, a strong dehydration ion peak (m/z 228, $M-18$) and other fragment ion peaks (m/z 215, $M-CH_3O$; 144, $M-[CH(OH)CH_2COOCH_3-H]$; 97, 2-keto-5-methenyl imidazolium cation; 84, $CHNHCONHCH$) were present. The molecular formula of methylated metabolite B was found to be $C_9H_{14}N_2O_4S$ from the data of high-resolution mass spectrometry (HR-MS): M^+ , m/z 246.0718 (calcd. for $C_9H_{14}N_2O_4S$, 246.0673). The melting point of this metabolite (methyl ester form) was 145~147°C.

These results indicate that metabolite B is β -hydroxybisnorbiotin, 3-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]-4-imidazolyl)-3-hydroxy propionic acid (**2**, Fig. 1).

Metabolite C. This metabolite had *R_f* values of 0.48 and 0.66 on TLC with solvents I and II, respectively. It was not methylated by treatment with diazomethane, indicating the absence of carboxylic groups.

The NMR spectrum of this compound showed the presence of the methyl protons of an acetyl group δ : 2.24 (3H, s, H-9) and methylene protons δ : 2.88 and 2.94 (1H each, AB part of ABX system, $J_{7a,7b}=18.1$, $J_{4,7b}=4.9$ and $J_{4,7a}=8.3$, H-7a and H-7b) adjacent to an acetyl group. Metabolite C reacted with 2,4-

FIG. 4. Mass Spectrum of Metabolite C.

(a): Mass spectrum of metabolite C.

(b): Mass spectrum of the 2,4-dinitrophenyl hydrazone derivative of metabolite C.

The preparation of the hydrazone derivative is described in MATERIALS AND METHODS.

dinitrophenylhydrazine to give a bright-yellow product. The mass spectrum of metabolite C is shown in Fig. 4(a). Though a molecular ion peak at m/z 156 was not detected, the ion peak at m/z 113 ($M-43$) expected for cleavage of the methyl ketone and the characteristic 2-keto-5-methyl imidazolium fragment at m/z 99 were present. In addition, the 2,4-dinitrophenylhydrazone derivative of metabolite C gave the expected molecular ion peak at m/z 336 (Fig. 4(b)). The molecular formula of metabolite C was decided to be $C_7H_{12}N_2O_2$ from the HR-MS data: M^+ , m/z 156.0903 (calcd. for $C_7H_{12}N_2O_2$, 156.0898). These results indicate that metabolite C is methyl tetranordethiobiotinyl ketone, 1-(5-methyl-2-oxo-4-imidazolidyl)-2-oxo-propane (**3**, Fig. 1). The melting point of this metabolite was 104~107°C.

Metabolite D. The mass spectrum of metabolite D showed a molecular ion peak (m/z 186) and fragment ion peaks (m/z 168, $M - H_2O$; 99, 2-keto-5-methyl imidazolium cation). The NMR spectrum showed peaks corresponding to a methyl ureido ring and a propionic acid side chain δ : 1.48~1.70 (4H, m, H-7a, H-7b, H-8a and H-8b), 2.43 (2H, m, H-9a and H-9b). These results indicated that metabolite D was bisnordethiobiotin (**4**, Fig. 1). In addition, the R_f values of metabolite D with solvents I and II (0.47 and 0.52, respectively) were identical to those of authentic bisnordethiobiotin.

Metabolite E. Metabolite E also had a low R_f value of 0.26 on TLC with solvent I.

Figure 5 shows the NMR spectrum of metabolite E. Like metabolite B, the peak corresponding to H-8 appears in the lower mag-

FIG. 5. 400 MHz 1H NMR Spectra of Metabolite E in D_2O .

netic field (4.05 ppm). However, the peak of H-8 was very close to another peak and the assignment of three protons at 3.94~4.05 ppm was not certain. They could be analyzed by spin decoupling experiments; the peaks at 3.94, 4.00, and 4.05 ppm were assigned to H-5, H-4, and H-8, respectively, and the low field chemical shift of H-8 suggested the presence of a hydroxy group at the β -carbon.

An NMR spectrum was also recorded for methylated metabolite E in $CDCl_3$. The peaks corresponding to H-5, H-4, and H-8 were more separated and two imido protons of the ureido ring and a hydroxy proton were also observed at 4.27, 4.77, and 3.26 ppm, respectively. The mass spectrum of metabolite E is shown in Fig. 6. There was an M^+ peak with low intensity at m/z 202 and also a stronger one at $M - 18 = 184$. The characteristic m/z 99 fragment was also present. The molecular formula of methylated metabolite E was found to be $C_9H_{16}N_2O_4$ from the HR-MS data; M^+ , m/z 216.1125 (calcd. for $C_9H_{16}N_2O_4$, 216.1109). The results from NMR and mass analysis were consistent with metabolite E being β -hydroxybisanordethiobiotin, 4-(5-methyl-2-oxo-4-imidazolidyl)-3-hydroxy butanoic acid (**5**, Fig. 1).

Metabolite F. Metabolite F had an R_f value of 0.37 on TLC with solvent I. The methylated metabolite gave a molecular ion peak at m/z 172. In addition, the characteristic m/z 99 fragment was also clearly seen in the spectrum. The NMR spectrum of methylated metabolite F indicated the presence of only one methylene group in the side chain δ : 2.52 and 2.58 (1H each, AB part of ABX system, $J_{4,7a} = 9.3$, $J_{7a,7b} = 16.6$ and $J_{4,7b} = 4.4$, H-7a and H-7b).

From the above evidence, metabolite F was identified as being tetranordethiobiotin (**6**, Fig.

FIG. 6. Mass Spectrum of Metabolite E.

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DISCUSSION

A few microbial β -oxidation products from biotin and dethiobiotin are known: bisnorbiotin,^{8~10)} β -dehydrobisnorbiotin,¹⁰⁾ tetranorbiotin,¹⁰⁾ bisnordethiobiotin,^{3,11)} and tetranordethiobiotin.¹¹⁾ Furthermore, there have been some reports of the isolation of β -hydroxybiotin derivatives and methyl ketone derivatives from the culture filtrate of pseudomonads grown on biotin: β -hydroxybiotin,¹²⁾ β -hydroxybiotin *l*-sulfoxide,¹³⁾ β -hydroxybiotin sulfone,¹²⁾ methyl bisnorbiotinyl ketone,¹²⁾ and methyl tetranorbiotinyl ketone.¹²⁾

In this work, we found a new biotin metabolite, β -hydroxybisnorbiotin, and new dethiobiotin metabolites, β -hydroxybisnordethiobiotin and methyl tetranordethiobiotinyl ketone. These have not been reported before either as biological compounds or as chemically synthesized compounds. As Kazarinoff *et al.*¹²⁾ proposed for the methyl ketone derivatives of biotin, it seems likely that methyl tetranordethiobiotinyl ketone was produced from β -ketobisnordethiobiotin, which is probably one of the metabolites in the β -oxidation of dethiobiotin. Because of its unstability, the β -keto derivative might be decarboxylated to give more stable methyl tetranordethiobiotinyl ketone. The possibility of the chemical decarboxylation of β -ketobisnorbiotin during the purification procedure is excluded by the results of TLC when we analyzed the reaction mixture before its purification. From these facts, the biotin-degrading bacterium *Mycoplana* sp. No. 166 was also confirmed to degrade biotin and dethiobiotin through β -oxidation of their side chains.

As mentioned in the results section, β -oxidation products were formed much faster from biotin than from dethiobiotin. Though the data were not shown, this bacterium released ammonia from biotin, but not from dethio-

biotin in a 7-hr reaction. This result strongly suggested that the bacterium can degrade biotin to ammonia rapidly through ureido ring cleavage, while it can degrade dethiobiotin to ammonia slowly if at all. Such slow β -oxidation and poor ureido ring cleavage of dethiobiotin by this organism may bring about its release of more kinds of DACA-positive degradative intermediates than is the case with biotin. The difference in the degradation rate between biotin and dethiobiotin may be due to the substrate specificities of some enzymes concerned in β -oxidation and ureido ring cleavage.

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