MICROBIAL CONVERSION OF STEFFIMYCIN AND STEFFIMYCIN B TO 10-DIHYDROSTEFFIMYCIN AND 10-DIHYDROSTEFFIMYCIN B

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It has been shown that steffimycin (**1**) and steffimycin B (**2**) are reduced at the C-10 carbonyl by *Actinoplanes utahensis*, UC®-5885 and *Chaetomium* sp., UC®-4634, respectively. Using cell-free extracts of the latter organism, the optimum conversion time, pH, and enzyme concentration have been determined for the conversion of **2** to **4**. The biochemical conversion of **2** has been found to be TPNH linked.

The reduction of carbonyl groups by microorganisms is a well-known phenomenon[1]. A specific application of this reaction, the reduction of carbonyl groups in anthracycline antibiotics by microbial systems has been reported[2] as has such reductions by mammalian cells[3]. In a research program carried on in these laboratories for several years involving microbial conversions of anthracycline antibiotics as a means of producing new antitumor agents[4~8], carbonyl reduction by anthracycline-producing and other microorganisms has been observed[7]. The present report involves similar studies to bring about carbonyl reduction in steffimycin (**1**) and steffimycin B (**2**) using *A. utahensis*, UC®-5885 and *Chaetomium* sp., UC®-4634.

Methods

1. Microbiological

A. utahensis, UC®-5885 and *Chaetomium* sp., UC®-4634 were stored and maintained on sterile soils in the culture collection of The Upjohn Company. Either source of inoculum was introduced into a seed medium termed GS-7 which contained cerelose and Pharmamedia each added at 25 g/liter of tap H_2O. The presterilization pH of this medium was adjusted to 7.2 with NH_4OH. Following the inoculation of each seed into 100-ml volumes of GS-7 contained in 500-ml fermentation flasks, the cultures were incubated for 72 hours at 28°C and shaken at 250 rpm.

The basal production medium used with both organisms is termed TYG. This medium contains tryptone, 5 g; yeast extract, 3 g; and glucose, 20 g added per liter of deionized H_2O. In the case of *A. utahensis*, $CaCO_3$ was added to TYG at 0.5 g/liter. Both the standard and modified TYG media were used at their natural pH. The growth conditions employed for the production stage of both organisms were identical to those used in the seed stage. In both cases a 5% seeding rate was employed in the inoculation of the production media. Following 48 hours of growth in the production stage, 25 mg of either **1** or **2** was added per liter in 1 ml of dimethylformamide. The fermentations were continued for an additional 48 hours. All media were sterilized by autoclaving at 121°C for 30 minutes.

2. Biochemical

Cell-free extracts (crude enzyme preparations) of TYG-grown *Chaetomium* sp. were prepared as follows: Twenty-five grams of frozen *Chaetomium* sp. mycelia were allowed to thaw at room temperature. The wet mycelia were drained through gauze to remove excess fermentation beer yielding *ca.* 15 g of drained mycelia. The mycelia were suspended in a buffered solution composed of 100 μmoles of potassium phosphate, pH 7.4, and 10 μmoles of β-mercaptoethanol added per ml of deionized H_2O.

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One-half ml of the buffered solution was added per g of *Chaetomium* mycelia. The preparation was sonicated for 10 minutes at 0°C using a Raytheon 10 KC sonic oscillator. Following sonication, the preparation was centrifuged at 10,000×*g* for 10 minutes at 5°C. The supernatant fluid was retained as the cell-free extract. Reaction mixtures used for cell-free conversion of **2** to **4** contained the following components per ml: cell-free extract, 0.80~1.60 mg of protein; TPNH, 1 mg; **2**, 0.1 mg; potassium phosphate, pH 6.5, 100 μmoles; and β-mercaptoethanol, 10 μmoles. The 1-ml reaction was initiated by addition of cell-free extract and was incubated at 25°C in a static condition using a 25-ml Erlenmeyer flask. Reactions were terminated by quick-freezing using $CHCl_3$-dry ice mixtures.

3. Analytical

One ml biochemical reaction mixtures were extracted by shaking for 20 minutes at 350 rpm with 4 ml of $CHCl_3$. The anthracycline components of the extracts were quantitated by analytical HPLC using a chromatic 2200 unit equipped with a Hewlett Packard-Mosely Model 7128A recorder. The absorbent was Partisil, 10 μPAC (Whatman), and the solvent system used was $CHCl_3$ - CH_3OH - H_2O (94.9: 5: 0.1). **4**, monitored at 254 nm, was quantitated by peak height measurement on the basis of standard curves using authentic **4** in the range of 0~0.1 μg.

4. Chemical

A. 10-Dihydrosteffimycin (**3**)

A 12-liter fermentation of *A. utahensis*, UC®-5885 utilizing 300 mg of **1** as the substrate was mixed with 500 g of filter aid and filtered. The filter cake was washed with 3 liters of water adding the wash to the filtrate. The filter cake was extracted with four 4-liter portions of CH_2Cl_2, and the filtrate was extracted with three 4-liter portions of CH_2Cl_2. The combined extracts were evaporated *in vacuo* to a black oil, wt. 1.247 g. The oil was chromatographed on 125 g of silica gel eluting with $CHCl_3$ - CH_3OH (93: 7) until 326 five-ml fractions had been collected. Fractions 208~318 were combined on the basis of a tlc analysis (SiO_2; $CHCl_3$-CH_3OH, 9: 1; Rf 0.26). Evaporation *in vacuo* gave 211 mg (74%) of **3** homogeneous by tlc in the above solvent system and in $CHCl_3$ - CH_3OH - H_2O (78: 20: 2), Rf 0.49. This material was recrystallized from acetone to give 118 mg; mp 253~255°C; UV (C_2H_5OH) 227 nm (ε 26,120), 268 (17,510), 285 sh (15,120), 434 (12,670); IR (Nujol) 3360, 1670, 1615, 1595, 1565, 1460, 1405, 1385, 1370, 1310, 1290, 1245, 1205, 1160, 1135, 1105, 1085, 1055, 1040, 955, 780, 755 cm^{-1}; ^1H NMR (d_6-DMSO-D_2O)δ 1.19 (s, 3 H, CH_3C), 1.33 (d, 3 H, CH_3CH), 3.41~3.70 (m, 10 H, CH_3O and CHO), 3.86 (s, 3 H, CH_3O), 4.44~5.02 (m, 3 H, CHO), 5.44 (broad singlet, 1 H, anomeric), 6.77 (d, 1 H, aromatic), 7.18 (d, 1 H, aromatic), 8.04 (s, 1 H, aromatic); ^{13}C NMR (d_6-DMSO) δ 191.3 (C-5), 181.2 (C-12), 166.8 (C-2), 164.8 (C-4), 161.4 (C-6), 149.8 (C-10a), 135.0 (C-11a), 132.1 (C-12a), 129.2 (C-6a), 117.7 (C-5a), 113.7 (C-11), 109.8 (C-4a), 107.9 (C-1), 106.5 (C-3), 101.1 (C-1′), 85.7 (C-8), 80.7 (C-2′), 73.6 (C-9), 71.9 (C-4′), 70.5 (C-10), 70.3 (C-3′), 70.0 (C-7), 69.8 (C-5′), 59.2, 58.3, 56.2 (3 CH_3O), 18.5 (CH_3 at C-9), 17.8 (CH_3 at C-5′); mass spectrum (*m/e*) 400 (aglycone+H); (*m/e*) 993.3965 (6 TMS-CH_3), Calcd. for $C_{45}H_{77}O_{13}Si_6$, 993.3980.

Anal. Calcd for $C_{28}H_{32}O_{13}$: C, 58.79; H, 5.55.
Found: C, 58.91; H, 5.61.

B. 10-Dihydrosteffimycin B (**4**)

1) From *Chaetomium* sp., UC®-4634: an 11.5-liter fermentation of *Chaetomium* sp., UC®-4634 utilizing 300 mg of **2** as a substrate was filtered. The filter cake was washed with 1 liter of water, and the washings were added to the filtrate. Both the filtrate and the filter cake were extracted with four 3-liter portions of CH_2Cl_2. The combined extracts were evaporated *in vacuo* to give 1.50 g of residue. The residue was chromatographed on silica gel using $CHCl_3$ - CH_3OH (98: 2) for 239 ten-ml fractions then continuing with a solvent ratio of 95: 5. On the basis of tlc (SiO_2; $CHCl_3$-CH_3OH, 9: 1; Rf 0.46), fractions 146~265 were combined and evaporated *in vacuo*, wt. 238 mg. The residue was crystallized from acetone to give 91 mg. The ML was concentrated to dryness, and the residue was chromatographed on 15 g of silica gel eluting with $CHCl_3$ - CH_3OH (97: 3) and collecting 59 five-ml fractions. A tlc analysis using the above system resulted in combination of fractions 14~26. The pool was evaporated *in vacuo* to give 61 mg, total yield 152 mg (51%). Recrystallization from acetone gave mp 245~248°C; UV (C_2H_5OH) 227 nm (ε 29,795), 267 (18,760), 285 (15,930), 430 (12,500); IR (Nujol) 3360, 1665, 1615, 1595, 1555, 1450, 1405, 1370, 1290, 1235, 1200, 1155, 1100, 1085, 1025, 950, 750 cm^{-1}; ^1H NMR (d_7-

DMF-D_2O) δ 1.27 (s, 3 H, CH_3C), 1.30 (d, 3 H, CH_3CH), 3.60 (s, 6 H, CH_3O), 3.67 (s, 3 H, CH_3O), 3.97 (s, 3 H, CH_3O), 4.87 (d, 2 H, CHO), 5.45 (broad s, 1 H, anomeric), 6.62 (d, 1 H, aromatic), 7.02 (d, 1 H, aromatic), 7.97 (s, 1 H, aromatic); ^{13}C NMR (d_6-DMSO) δ 191.5 (C-5), 182.2 (C-12), 167.8 (C-2), 166.0 (C-4), 162.8 (C-6), 151.0 (C-10a), 136.2 (C-11a), 133.3 (C-12a), 130.3 (C-6a), 118.2 (C-5a), 115.0 (C-11), 111.3 (C-4a), 109.8 (C-1), 108.6 (C-3), 102.9 (C-1′), 87.3 (C-8), 84.1 (C-4′), 82.7 (C-2′), 75.2 (C-9), 73.5 (C-3′), 72.1 (C-10), 71.9 (C-7), 70.0 (C-5′), 61.5, 60.7, 60.0, 57.8 (4 CH_3O), 20.2 (CH_3 at C-9), 19.4 (CH_3 at C-5′); mass spectrum (*m/e*) 400.1179, Calcd for $C_{21}H_{20}O_8$, 400.1158 (aglycone+H); *m/e* 863.3336 (4 TMS-CH_3), Calcd for $C_{40}H_{63}O_{13}Si_4$, 863.3345.

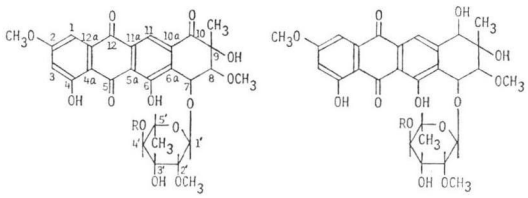
Anal. Calcd for $C_{29}H_{34}O_{13}$: C, 58.97; H, 5.80.
Found: C, 58.91; H, 5.96.

2) From *A. utahensis*, UC®-5885: A similar fermentation and isolation procedure using *A. utahensis*, UC®-5885 gave **4** identified by comparison with the above isolated material by tlc using $CHCl_3$-CH_3OH (95: 5), $C_2H_5COCH_3$ - CH_3COCH_3 - H_2O (70: 20: 11), and $CH_3COOC_2H_5$ - CH_3OH (95: 5) as the solvent systems.

Results and Discussion

Fermentations of *A. utahensis*, UC-5885 converted **1** to **3** in a yield of 74% with some recovery of **1** (Scheme 1). The structure of the product was established by the usual spectral criteria. The disappearance of a carbonyl bond at 1710 cm^{-1} in the infrared spectrum of **1** upon going to **3** indicates reaction at C-10 and probably reduction. The ^{13}C NMR spectrum of **3** lacked the chemical shift at δ 198.5 found in the spectrum of **1**[10] and assigned to the carbonyl carbon at C-10 while a new peak appeared in the C-O region at δ 70.5. These changes were consistent with the conversion indicated in Scheme 1. A similar fermentation using *Chaetomium* sp., UC®-4634 and **2** as a substrate resulted in conversion of **2** to **4** by reduction of the carbonyl group at C-10. Spectral methods were also used to establish the nature of the conversion with arguments similar to those used for the structure of **3** being employed. The stereochemistry of **3** and **4** at C-10 was not ascertained, but no evidence was found for a mixture of isomers so it was presumed that reduction was stereospecific. The conversion of **2** to **4** also occured with *A. utahensis*, UC®-5885 although in this case conversion was established using only tlc.

Scheme 1.



1, R=H
2, R=CH_3

3, R=H
4, R=CH_3

Cell-free extracts of *Chaetomium* sp. cultures also converted **2** to **4** and were used to study various parameters of the reduction. Those extracts derived from cells grown for 2 days showed enormously greater rates of conversion than did extracts from cells grown for 4 or 7 days (Fig. 1). The specific activity of the 2-day cell-free extract was 8.5 nmoles of **4** formed per hour per mg of protein as compared to 0.65 and 0.33 nmoles of **4** per hour per mg of protein attained using 4- and 7-day cell-free extracts, respectively.

As demonstrated in Fig. 2, there is direct proportionality between reaction rate and added crude enzyme protein. The relationship between reaction rate and added crude enzyme protein is linear between 0 and 1.6 mg of crude enzyme protein per ml of reaction mixture. Based on this information, a protein concentration of 0.8~1.6 mg per ml of reaction volume was used in the standard assay.

The reactions were buffered with 0.3 M potassium phosphate over a range of pH 5.0~8.5. Fig. 3 presents the reaction rate studied as a function of buffer pH. This experiment shows the buffer pH optimum to be pH 6.0.

Fig. 1. Cell-free conversion of steffimycin B to 10-dihydrosteffimycin B studied as a function of reaction time and culture age.

Cell-free extracts were prepared from *Chaetomium* sp. UC®-4634 mycelia cultured 2 days (▲), 4 days (●), and 7 days (□).

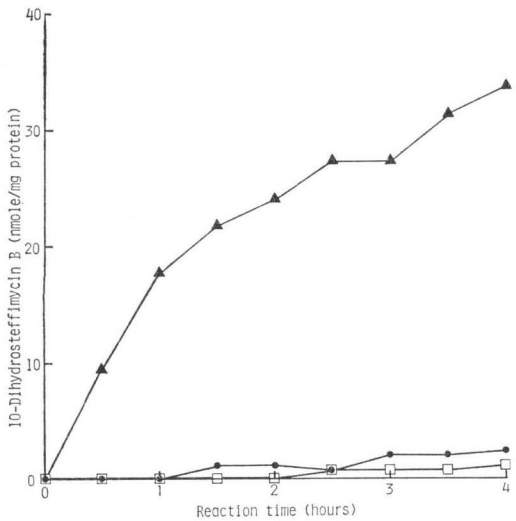


Fig. 2. Cell-free conversion of steffimycin B to 10-dihydrosteffimycin B studied as a function of crude enzyme protein concentration.

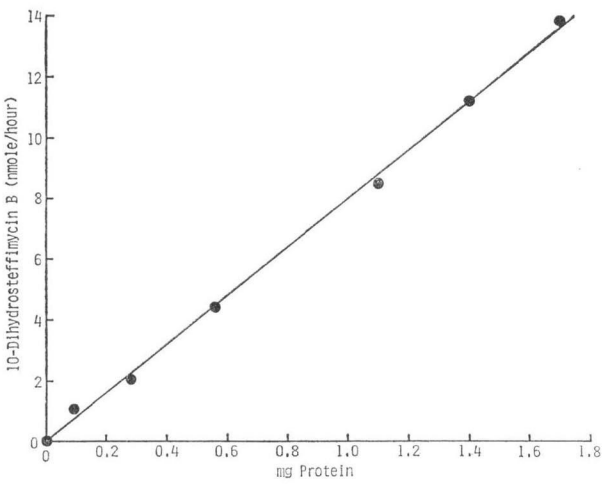


Fig. 3. The buffer pH optimum for cell-free conversion of steffimycin B to 10-dihydrosteffimycin B using a crude enzyme preparation of *Chaetomium* sp. UC®-4634.

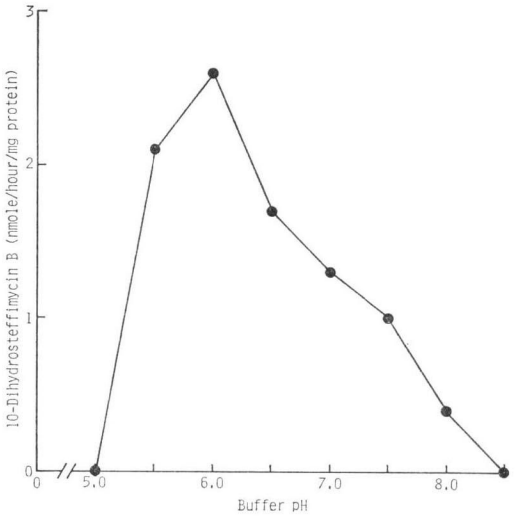
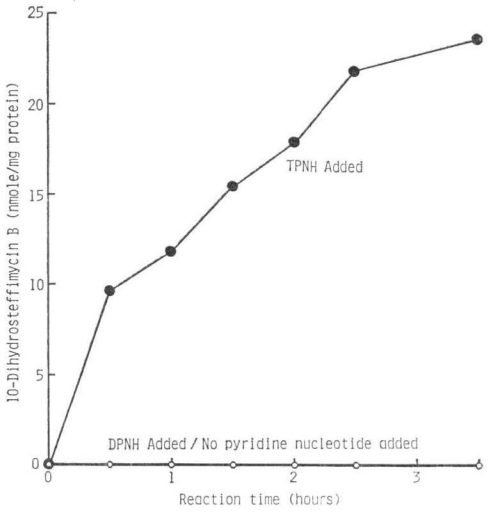


Fig. 4. Cell-free conversion of steffimycin B to 10-dihydrosteffimycin B studied as a function of reduced pyridine nucleotide addition.



The conversion of **2** to **4** was investigated as a function of cofactor addition (Fig. 4). The data from this study demonstrated an absolute requirement for TPNH with no conversion being evident in the presence of DPNH or in the absence of reduced pyridine nucleotide.

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