BACTERIAL METABOLISM OF ANTHRACYCLINE ANTIBIOTICS

STEFFIMYCINONE AND STEFFIMYCINOL CONVERSIONS

PAUL F. WILEY, JAMES M. KOERT, DAVID W. ELROD,
ELIZABETH A. REISENDER and VINCENT P. MARSHALL

Research Laboratories, The Upjohn Company
Kalamazoo, Michigan 49001, U. S. A.

(Received for publication May 16, 1977)

Streptomyces nogalater, UC®–2783, and *Streptomyces peucetius* var. *caesius*, IMRU–3920/UC®–5633, catalyze ketonic carbonyl reduction of steffimycinone (**1**, Scheme 1). Using cell-free preparations of *S. nogalater*, the process of ketonic carbonyl reduction has been shown to be TPNH linked. The product, steffimycinol (**2**), is reduced further by *Aeromonas hydrophila*, 2C/UC®–6303, by the process of microaerophilic conversion of anthracyclinones previously reported[1,2] with the result being the formation of 7-deoxysteffimycinol (**3**). The products (**2** and **3**) were isolated by extraction from the fermentations followed by chromatographic purification. Identification was by comparison of various physical properties and spectral data with those of authentic materials obtained by chemical means. Catalytic activity of the crude enzyme preparations of *S. nogalater* was lost by dialysis but restored by addition of TPNH although not by addition of DPNH demonstrating TPNH dependence. The reaction rate increased linearly with added crude enzyme protein up to 4 mg/ml and was highest between pH 6.5 and 7.0.

An extensive program involving modification of antibiotics as a means for discovering new antitumor agents has been carried on in these laboratories for some time[1~3]. One of the approaches selected was the use of anthracycline antibiotics producing organisms as agents for modification of anthracyclines other than those normally produced by the organisms. Initial efforts involved the use of steffimycin and steffimycin B[4] as substrates for *S. nogalater* which forms nogalamycin[5] as a secondary metabolite. The work was subsequently extended to steffimycinone (**1**), the anthracyclinone of the steffimycins, and to the adriamycin producing organism *S. peucetius* var. *caesius*. These organisms and a cell-free extract of the former were found to catalyze the reduction of the C–10 carbonyl with TPNH as a cofactor in the case of the cell-free system.

A very similar TPNH keto reduction of adriamycin and daunomycin by mammalian cells has been reported[6,7]. It has been suggested[8] that these and other cytoplasmic keto reductases are involved in detoxification of xenobiotic substances in mammals. These ubiquitous enzymes metabolize, in addition to some of the anthracyclines, benzaldehyde[9], acetophenone[10], oxisuran[11] and warfarin[12] with TPNH dependence being the rule. Such reductions by bacteria have also been reported using anthracyclines or anthracyclinones as substrates[3,13~18], but the functions of such enzymes in bacteria are completely unknown.

The reduction of steffimycinone (**1**) to steffimycinol (**2**) made available a new anthracyclinone whose anaerobic reduction by microorganisms was of interest in view of previous work[1,2] in this area. The reduction was found to proceed as did those cases previously reported[1,2].

Methods

1. Microbiological

S. nogalater, UC®–2783, and *S. peucetius* var. *caesius* were maintained on agar slants and cultured in seed media as described by ARCAMONE *et al.*[18]. Following 48 hours of growth in the seed stage, the cultures were used as the inoculum (10%) for a medium (TYG) composed of tryptone, yeast extract, and glucose (5: 3: 20). After 48 hours of culture at 28°C in the latter medium, **1** was introduced to a concentration of 50 mg/liter. Microaerophilic conversions using *A. hydrophila* were done as reported previously[2].

2. Biochemical

Cell-free extracts of noninduced, TYG grown *S. nogalater* were prepared and dialyzed by the methods used previously[2].

Reaction mixtures used for cell-free conversions of **1** to **2** contained the following components per ml: cell-free extract, 1~3 mg of protein; TPNH, 1 mg; **1**, 0.5 mg; potassium phosphate, pH 7.0, 100 μ-moles; and mercaptoethanol, 10 μmoles. The reaction was initiated by addition of cell-free extract and was incubated at 25°C while shaken at 250 reciprocations/minute. Reactions were terminated by quick-freezing in acetone-dry ice mixtures.

3. Analytical

One ml reaction mixtures made up as above were subjected to mixing for 1-minute periods in the presence of 2 ml volumes of $CHCl_3$. The phases were separated by centrifugation for 5 minutes at 5,000 $\times g$. The contents of these extracts were quantitated by high performance liquid chromatography using a chromatic 2200 unit equipped with a Hewlett Packard-Mosely Model 7128A recorder and a Hewlett Packard 3370B integrator. The absorbent was partisil, 10 μ PAC (Whatman), and the solvent system used was $CHCl_3$ - CH_3OH - H_2O (96: 5: 1). The anthracycline aglycones, monitored at 254 nm, were quantitated on the basis of standard curves made using authentic **1** and **2** in a range of 0~4 μg.

4. Chemical

A. Steffimycinol (**2**)

1) From *S. nogalater*: A 4.8-liter fermentation of *S. nogalater* utilizing 1 g of **1** as the substrate was mixed with 240 g of filter aid and filtered. The filter cake was washed with 1.2 liters of water. The filtrate (5.6 liters) was extracted with four 1.4-liter portions of $CHCl_3$. The extracts were combined and evaporated under reduced pressure to give 828 mg of residue which was chromatographed on 83 g of silica gel eluting with $CHCl_3$ - CH_3OH (97: 3) until one hundred and sixty 10-ml fractions had been collected. A tlc analysis (SiO_2; $CHCl_3$ - CH_3OH, 9: 1; Rf 0.45) resulted in combination of fractions 49~65. Evaporation under reduced pressure gave 195 mg of residue identified as **2** by tlc in the above system. Similar evaporation of fractions 66~90 gave 257 mg of slightly impure **2** as judged by tlc. The second fraction was combined with 120 mg of similar material and chromatographed on 38 g of silica gel eluting with $CHCl_3$ - CH_3OH (98: 2). Eighty-six 5-ml fractions were collected. On the basis of a tlc analysis as above fractions 50~70 were combined and evaporated under reduced pressure to give 182 mg of material identified as pure **2** by tlc as above. The two lots were combined and recrystallized from CH_3OH to give a product, mp 238~240°C dec. [lit. 4), 230°C]; mass spectrum *m/e* 416 (M^+); tlc in $CHCl_3$ - CH_3OH (9: 1) indicated homogeneous **2**.

2) From *S. nogalater* Cell-free Extract: A 75-ml fermentation in which 75 mg of **1** had been exposed to the effect of *S. nogalater* cell-free extract was mixed with 4 g of filter aid and filtered. The filter cake was washed with 20 ml of water, and the combined filtrate and washings were extracted with four 50-ml portions of $CHCl_3$. The filter cake was extracted with five 25-ml portions of $CHCl_3$. Combination of the extracts and evaporation under reduced pressure gave 134 mg. This material was placed on a 20 cm × 20 cm × 1 mm preparative thick-layer plate which was developed with $CHCl_3$ - CH_3OH (95: 5). The slowest moving band above the origin was removed and eluted with CH_2Cl_2 - CH_3OH (1: 1). The organic solution was evaporated under reduced pressure, yield 17 mg (22.6%). The material was found to move with **2** on tlc on SiO_2 in $CHCl_3$ - CH_3OH (95: 5); cyclohexane - EtOAC - EtOH (5: 3: 2), Rf 0.29; and butan-2-one - acetone - water (70: 20: 11), Rf 0.66; mass spectrum *m/e* 416 (M^+).

3) From *S. peucetius* var. *caesius*: A 3.44-liter *S. peucetius* var. *caesius* fermentation to which 200 mg of **1** had been added was filtered with the aid of filter aid. The filtrate was extracted with three 1.72-liter portions of CH_2Cl_2 which was washed with two 50-ml portions of H_2O. The combined water washes were extracted with 50-ml of CH_2Cl_2 which was added to the original extract. The combined CH_2Cl_2 solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure, yield 388 mg. The residue was dissolved in 3 ml of $CHCl_3$ - CH_3OH (97: 3). Standing gave 91 mg of ppt. The material in the filtrate was chromatographed by HPLC using a 60-g porosil A column with the above solvent system. Analysis of fractions was done by tlc using $CHCl_3$ - CH_3OH (95: 5). Combination and evaporation of the appropriate fractions gave 73 mg which was chromatographed similarly on a 15.5-g column, yield 20 mg. This material was combined with the 91 mg fraction and recrystallized from CH_3OH to give 68 mg (34%) of compound which was homogeneous and identical with **2** as judged by tlc in $CHCl_3$ - CH_3OH (95: 5). Its ^{13}C NMR spectrum was the same as that of **2**[4].

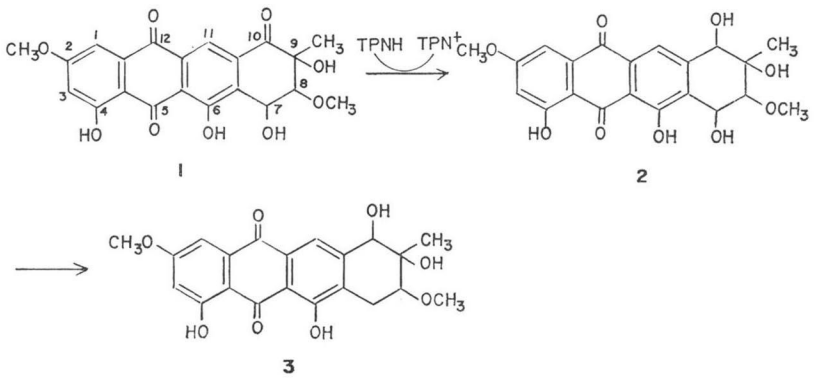
Anal. Calcd. for $C_{21}H_{20}O_9$: C, 60.57; H, 4.84.
Found: C, 60.55; H, 4.97.

B. 7-Deoxysteffimycinol (**3**)

A 2-liter anaerobic fermentation to which 100 mg of **2** had been added was filtered. Both the filtrate and the filter paper were extracted with four 500-ml portions of CH_2Cl_2. The combined extracts were evaporated to dryness under reduced pressure to give 195 mg of residue. This material was chromatographed on 20 g of silica gel eluting with $CHCl_3$ - CH_3OH (98: 2). Forty 5-ml fractions were collected followed by elution with $CHCl_3$ - CH_3OH (95: 5). Another forty-seven 5-ml fractions were collected. Analysis by tlc (SiO_2; $CHCl_3$ - CH_3OH, 9: 1) resulted in combination into pools 1 (9~11), 2 (12~18), 3 (19~23), 4 (24~45), and 5 (46~80). Evaporation of the pools *in vacuo* gave respectively 1.4 mg, 20.1 mg, 49.3 mg, 30.7 mg, and 11.4 mg. The material from pool 3 was judged by tlc to be pure **3** (fraction A). Recrystallization of material from pool 4 from $CHCl_3$ - CH_3OH (97: 3) gave 14.6 mg (fraction B) of pure **3** as judged by tlc. Materials from pools 2 and 5 and the material from the mother liquor of fraction B was combined and chromatographed on 9.6 g of silica gel eluting with $CHCl_3$-CH_3OH (98: 2) and collecting fifteen 5-ml fractions. On the basis of tlc analysis, these fractions were combined as pools 1 (5~7), 2 (8~9), and 3 (10~15). Evaporation of pool 2 *in vacuo* gave 8.1 mg of pure **3** (fraction C) as judged by tlc. Pool 1 on evaporation gave 12.3 mg of material which, on the basis of tlc, contained 40~60% of **3** and two faster moving components. Pool 3 on evaporation gave 12.3 mg of materials which, on the basis of tlc, contained 35~50% **3** and a slower moving component which was not **2**. Fractions A, B, and C all had mass spectra showing molecular ions of 400 (calcd. for **3**, 400). Each of these fractions was compared to authentic 7-deoxysteffimycinol (**4**) by tlc in $CHCl_3$ - CH_3OH (9: 1, Rf 0.68), $CH_3COOC_2H_5$ - CH_3OH (95: 5, Rf 0.62), and $CH_3COOC_2H_5$ - C_2H_5OH - H_2O (92: 5: 3, Rf 0.72). Each showed a single spot moving with **3**. Yield 72 mg (75%).

Results and Discussion

Scheme 1.



The use of steffimycinone (**1**) as a substrate for the anthracycline-producing organisms *S. nogalater* and *S. peucetius* var. *caesius* has been found to result in reduction of the ketonic carbonyl group at C–10 to a hydroxyl group with the formation of steffimycinol (**2**, Scheme 1). Cell-free extracts of *S. nogalater* were found to bring about the same reaction.

The conversion product was isolated by extraction with organic solvents and purified by column chromatography. Identification as **2** was by comparison with authentic material, prepared by reduction of **1** with $NaBH_4$, using tlc in several solvent systems, melting point, analysis, and mass and ^{13}C NMR spectra. It seemed likely that the reduction was stereospecific as only one isomer was isolated, but the configuration at C–10 was not established. The two microorganisms gave essentially the same yield which was rather low in both cases, 32~34% although it compared favorably with the chemical yield[4]. The cell-free extract gave a somewhat lower yield of 22%.

The optimal conditions for conversion of **1** to **2** with respect to pH and added protein enzyme were studied using *S. nogalater* cell-free extracts. The pH was regulated with potassium phosphate buffer over the range 5.0 to 8.5. Fig. 1 presents these data as a roughly bell-shaped curve with pH optimum of the reaction being 6.5~7.0. Fig. 2 demonstrates the direct proportionality of reaction rate as a function of added enzyme protein. Data presented in Figs. 1 and 2 were used to establish the standard reaction conditions employed in the assay of the keto reductase.

As would be expected from previous studies[3,6,7] it was found that *S. nogalater* cell-free extracts require TPNH (Fig. 3) for reduction of the ketonic carbonyl of **1**. Because of endogenous levels of TPNH present in the cell-free extracts, dialysis was required to determine the enzyme's requirement for reduced pyridine nucleotide. Following dialysis, the cell-free extract was added to the previously described reaction mixture in the absence of reduced pyridine nucleotide. Either DPNH or TPNH, in addition to cell-free extract, were added in other experiments. Conversion of **1** was evident only in the reaction mixture containing exogenously supplied TPNH, thus demonstrating its requirement as a cofactor for conversion of **1** to **2**.

We have had considerable interest in preparing new aglycones of anthracyclines since they offer possibilities for conversion to new antibiotics. We have also been interested in the reductive cleavage of benzylic C–O bonds in anthracyclines and anthracyclinones[2]. Steffimycinol (**2**) offers a compound

Fig. 1. Effect of pH on keto reductase activity

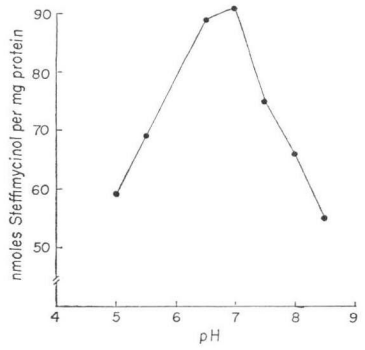
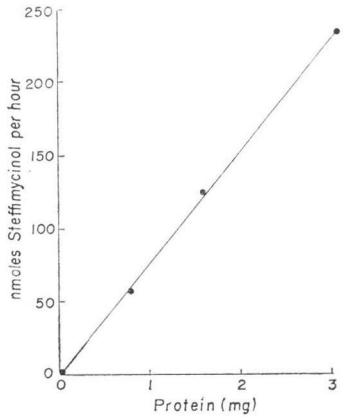
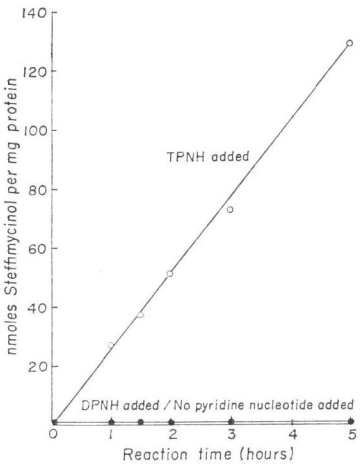


Fig. 2. Effect of concentration of crude enzyme protein on rate of reduction



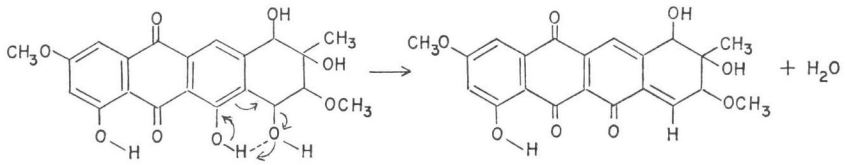
in which these two interests coincide in that it could be used as a substrate for *A. hydrophila* catalyzed microaerophilic benzylic cleavage to form a new aglycone. Furthermore, conversion of **2** to **3** by reductive cleavage is of interest from a mechanistic standpoint. YESAIR *et al.*[19] have proposed a mechanism whereby a *peri* hydroxyl group at C–6 is necessary for such reduction. We had independently conceived of a very similar reaction mechanism except that we considered that the proton on the C–6 hydroxyl would attack the oxygen at C–7, thus facilitating formation of the intermediate **4** (Scheme 2) or an intermediate electrondelocalized form.

Fig. 3. Dependence of the reductase activity on TPNH as a cofactor



The proposal of YESAIR *et al.*[19] implies that a proton is furnished to **4** directly from TPNH to give **3**. Our preliminary experiments with TPNT using steffimycin as a substrate suggest that this is not the case and that the transfer must occur through a more extended system even though only one

Scheme 2.



2 **4**

enzyme is involved[20]. In **2** two benzylic hydroxyl groups are present, and *a priori* it might be expected that either or both could be removed. If, as proposed, an aromatic hydroxyl group must be *ortho* to the benzylic C–O bond being cleaved, then reduction should occur only at C–7 to give 7-deoxysteffimycinol (**3**). When **2** was used as a substrate for the microaerophilic fermentation of *A. hydrophila*, **3** was isolated as a pure solid in a yield of 75%. A mixture of **3** and two less-polar compounds was obtained, and a mixture of **3** and a more polar material was isolated. We never succeeded in purifying these mixtures and isolating their components. On the basis of tlc analyses of the mixtures, it was estimated that they contained at least 10 mg of **3**. Thus, the yield of **3** must be well over 80% showing that the reduction is at least greatly facilitated by a *peri* hydroxyl at C–6 and suggesting that, in accordance with the above mechanism, such a group is necessary.

Acknowledgment

This work was supported in part by Contract NO1-CM-43753 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare. We wish to thank Dr. LUBOMIR BACZYNSKYJ and his associates for mass spectral data. Also, appreciation is extended to Mr. LESTER REINEKE for assistance with HPLC and to Miss ALMA DIETZ and Mrs. GRACE LI for furnishing the *Streptomyces* species employed.

References

1) WILEY, P. F. & V. P. MARSHALL: Microbial conversion of anthracycline antibiotics. J. Antibiotics 28: 838~840, 1975
2) MARSHALL, V. P.; E. A. REISENDER, L. M. REINEKE, J. H. JOHNSON & P. F. WILEY: Reductive microbial conversion of anthracycline antibiotics. Biochem. 15: 4139~4145, 1976
3) MARSHALL, V. P.; E. A. REISENDER & P. F. WILEY: Bacterial metabolism of daunomycin. J. Antibiotics 29: 966~968, 1976
4) KELLY, R. C.; I. SCHLETTER, J. M. KOERT, F. A. MACKELLAR & P. F. WILEY: The structures of steffimycin and steffimycin B. J. Org. Chem. In Press
5) WILEY, P. F.; R. B. KELLY, E. L. CARON, V. H. WILEY, J. H. JOHNSON, F. A. MACKELLAR & S. A. MIZSAK: Structure of nogalamycin. J. Am. Chem. Soc. 99: 542~549, 1977
6) ASBELL, M. A.; E. SCHWARTZBACH, F. J. BULLOCK & D. W. YESAIR: Daunomycin and adriamycin metabolism via reductive glycosidic cleavage. J. Pharmacol. Exp. Ther. 182: 63~69, 1972
7) FELSTED, R. L.; M. GEE & N. BACHUR: Rat liver daunorubicin reductase. J. Biol. Chem. 249: 3672~3679, 1974
8) BACHUR, N. R.: Cytoplasmic aldo-keto reductases: A class of drug metabolizing enzymes. Science 193: 595~597, 1976
9) CULP, H. W. & R. E. MCMAHON: Reductase for aromatic aldehydes and ketones. J. Biol. Chem. 243: 848~852, 1968
10) LEIBMAN, K. C.: Reduction of ketones in liver cytosol. Xenobiotica 1: 97~104, 1971
11) BACHUR, N. R. & R. L. FELSTED: Drug Metab. Dispos. 4: 299~243, 1976
12) MORELAND, T. A. & D. S. HEWICK: Studies on a ketone reductase in human and rat liver and kidney soluble fraction using warfarin as a substrate. Biochem. Pharmacol. 24: 1953~1957, 1975
13) NINET, L.; J. FLORENT, J. LUNEL, A. ABRAHAM, B. LOMBARDI & R. TISSLER: Duborimycin (20798 R. P.)-preparation by biochemical reduction of daunorubicin. Abstract No. 17.06, 5th International Fermentation Symposium, Berlin, 1976
14) ASZALO, A. A.; N. R. BACHUR, B. K. HAMILTON, A. F. LANGLYKKE, P. P. ROLLER, M. Y. SHEIKH, M. S. SUTPHIN, M. C. THOMAS, D. A. WAREHEIM & L. H. WRIGHT: Microbial reductions of the side-chain carbonyl of daunorubicin and N-acetyldaunorubicin. J. Antibiotics 30: 50~58, 1977
15) KARNETOVÁ, J; J. MATEJU, P. SEDMERA, J. VAKOUN & Z. VANEK: Microbial transformation of daunomycin by *Streptomyces aureofaciens* B-96. J. Antibiotics 29: 1199~1202, 1976
16) HAMILTON, B. K.: Personal communication.
17) MARSHALL, V. P.; E. A. REISENDER & P. F. WILEY: A bacterial model of mammalian adriamycin and daunomycin metabolism. 77th Annual Meeting of the American Society of Microbiology, New Orleans, 1977
18) ARCAMONE, F.; G. CASSINELLI, G. FANTINI, A. GREIN, P. ONEZZI, C. POL & C. SPALLA: Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *S. peucetius* var. *caesius*. Biotech. Bioeng. 11: 1101~1110, 1969
19) YESAIR, D. W. & S. MCNITT: Proposed mechanism for the metabolism of daunoruficin and adriamycin via a reductive glycosidic cleavage. Z. Physiol. Chem. 357: 1066~1067, 1976
20) MCCARVILLE, M. & V. MARSHALL: Partial purification and characterization of a bacterial enzyme catalyzing reductive cleavage of anthracycline glycosides. Biochem. Biophys. Res. Comm. 74: 331~335, 1977