MICROBIAL METABOLISM OF ANTHRACYCLINE ANTIBIOTICS DAUNOMYCIN AND ADRIAMYCIN

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It has been shown that the antitumor antibiotics daunomycin (**1**) and adriamycin (**4**) are metabolized by microorganisms in a fashion similar to their metabolism by mammalian cells. Both the fungus *Mucor spinosus* and its cell-free extracts reduce the 13-keto group of daunomycin to give daunomycinol (**2**) by a TPNH-dependent process. Cell-free extracts of *Streptomyces steffisburgensis* convert adriamycin and daunomycinol to their 7-deoxyaglycones (**5**) and (**3**) by DPNH-linked reductive glycosidic cleavage. Cell-free extracts of the latter organism convert 7-deoxyadriamycinone (**5**) to 7-deoxyadriamycinol aglycone (**6**) by TPNH-linked 13-keto reduction.

Adriamycin (**4**) and daunomycin (**1**) are antitumor agents which have been found to be remarkably effective in the treatment of leukemias and solid tumors[1,2]. However, improved drugs would be desirable both in effectiveness and in cardiotoxicity, the latter of which severely limits the utility of **1** and **4**[3]. Extensive microbial conversions of anthracyclinones have been carried out in our laboratories with the aim of obtaining improved antitumor activity[4,5]. Some of these studies have already been applied to **1**, and we have now continued this work with both **4** and **1** with a two-fold purpose. Most importantly it was deemed likely that biological modification might improve the antitumor properties of these agents, and secondly their bacterial metabolism was felt to be inherently interesting. Metabolic routes for mammalian metabolism of **1** and **4** have been presented[7,15], and metabolism of both **1** and **4** have proved to be essentially identical as would be expected because of their chemical similarity. Previous work[4,5,8,9] has indicated considerable similarity in mammalian and bacterial metabolism for **1** and **2**, and further studies of bacterial metabolism for comparison purposes seemed worthwhile.

Methods

1. Microbiological

S. steffisburgensis, UC®-5044, and *M. spinosus*, UC®-4356, were maintained on sterile soils in the culture collection of The Upjohn Company and were cultured in the seed media described by ARCAMONE *et al*[10]. Both organisms, when they were used as crude enzyme sources, were cultured aerobically at 28°C for 72 hours in their seed stages and were used to inoculate (5%) a medium (TYG) composed of tryptone, yeast extract, and glucose (5 g, 3 g, and 20 g, respectively, per liter of distilled H_2O). *S. steffisburgensis* and *M. spinosus* were grown aerobically at 25°C in the TYG medium for 72~96 hours. The mycelia of both organisms were sedimented by centrifuging at $10^4 \times g$ for 15 minutes, washed and stored as the frozen pellets.

In experiments where *M. spinosus* served as the agent for whole cell conversion of **1** to **2**, the organism was grown in a way identical to the procedure described previously to the point of 48 hours into the TYG stage. Here, **1** was added as an aqueous solution to a final concentration of 10 mg/liter.

Following drug addition, the fermentation was maintained as described and allowed to proceed up to 7 days.

2. Biochemical

Cell-free extracts (crude enzyme preparations) of *S. steffisburgensis* were made by suspending 10 g of the frozen mycelial preparations described in Section 1 in 10 ml of 100 mM potassium phosphate (pH 7.4) containing β-mercaptoethanol at a concentration of 10 mM. In the case of *M. spinosus*, 15 g of the frozen mycelial preparation was suspended in the previously described buffer - β-mercaptoethanol mixture in preparation for sonic disruption[5]. As all of the reactions studied increased linearly with added enzyme protein up to 1 mg/ml and their pH optima were *ca.* 7, one standard reaction mixture was employed for assay of all conversions. This was performed at 25°C in 1 ml volumes and contained the following components: anthracycline substrate, 1.5 mM; DPNH or TPNH, 1.5 mM; crude enzyme protein, 1 mg; β-mercaptoethanol, 10 mM; and potassium phosphate buffer (pH 7.4); 100 ml. The reactions were terminated by freezing the mixture in dry-ice - acetone.

3. Analytical

The reaction mixtures thawed in the presence of $CHCl_3$ were extracted with $CHCl_3$, and the combined extracts were evaporated to dryness. The extract was reconstituted in a measured aliquot of $CHCl_3$ and a sample was applied to an E. Merck silica gel 60 tlc plate using solvent system A (Section 4) for glycosides or solvent system G for aglycones. Separated materials were then quantitated *in situ* by scanning absorbance densitometry, a modification of the method used by WATSON and CHAN[11], using a Schoeffel SD-3000 spectrodensitometer in the reflectance mode. The monochromator was set at 240 nm. Standards were prepared for each substance spanning the range of concentrations encountered in the samples. The standards were spotted and developed in the same fashion as the samples. Standard curves of peak height *vs.* amount spotted for each substance were linear and no interference from co-extracted materials occurred.

Protein concentrations were determined by the LOWRY method[12] using bovine serum albumin as a standard.

4. Chemical

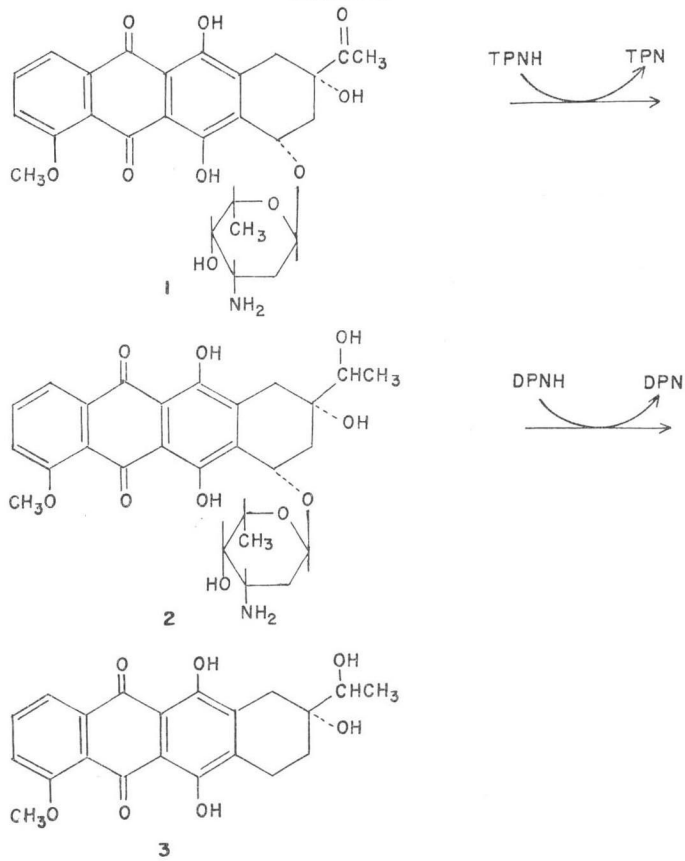
Thin-layer systems

A. $CHCl_3$ - CH_3OH - H_2O - CH_3COOH (80: 20: 3: 7)
B. $CHCl_3$ - CH_3OH - H_2O (78: 20: 2)
C. CH_3OH - H_2O - CH_3COOH (55: 40: 5)
D. C_2H_5OH - CH_3OH - H_2O (50: 45: 5)
E. $CHCl_3$ - CH_3OH (9: 1)
F. $CH_3COOC_2H_5$ - C_2H_5OH - H_2O (92: 5: 3)
G. $CHCl_3$ - CH_3OH (92.5: 7.5)
H. $CHCl_3$ - CH_3OH (95: 5)
I. C_6H_{12} - $CH_3COOC_2H_5$ - 95% C_2H_5OH (5: 3: 2)

A. Daunomycinol (2)

(1) From *M. spinosus*: A 1-liter fermentation of *M. spinosus* (pH 8.1) utilizing 10 mg of **1** as

Scheme 1.



the substrate was filtered, and the mycelia were washed with 250 ml of H_2O. Both the filtrate and the mycelia were extracted with four 250-ml portions of $CHCl_3$. All of the extracts were combined and concentrated under reduced pressure, first on a rotary evaporator and then under oil pump pressure. The residue weighed 77 mg. Chromatography on 16 g of silica gel in solvent system B combining 2.5-ml fractions 62~90 gave 3.6 mg of **2**. The product had Rf values of 0.29, 0.19, 0.67, and 0.06 in solvent systems A, B, C, and D respectively. In all cases, the Rf was the same as that of **2**. Mass spectrum, *m/e* 400 (the expected value for daunomycinol aglycone).

(**2**) From *M. spinosus* Cell-free Extract: A 60-ml cell-free conversion in which 10 mg of **1** had been subjected to the effect of *M. spinosus* cell-free extract was adjusted to pH 8.3 with 2 N NaOH. The system was extracted with 15-ml portions of $CHCl_3$. The extracts were combined and evaporated under reduced pressure, yield 58.8 mg. The residue was chromatographed on 12 g of silica gel using solvent system B. A total of ninety-five 2.5-ml fractions were collected. Two color maxima were present in the fractions. On the basis of tlc in system B fractions 44~75 were combined and evaporated to dryness under reduced pressure. The residue was dissolved in $CHCl_3$-CH_3OH, and the solution was filtered and reevaporated. The residue weighed 13 mg. In tlc in systems A and B, it had the same Rf values as **2**. Mass spectrum, *m/e* 400 (the expected value for daunomycinol aglycone).

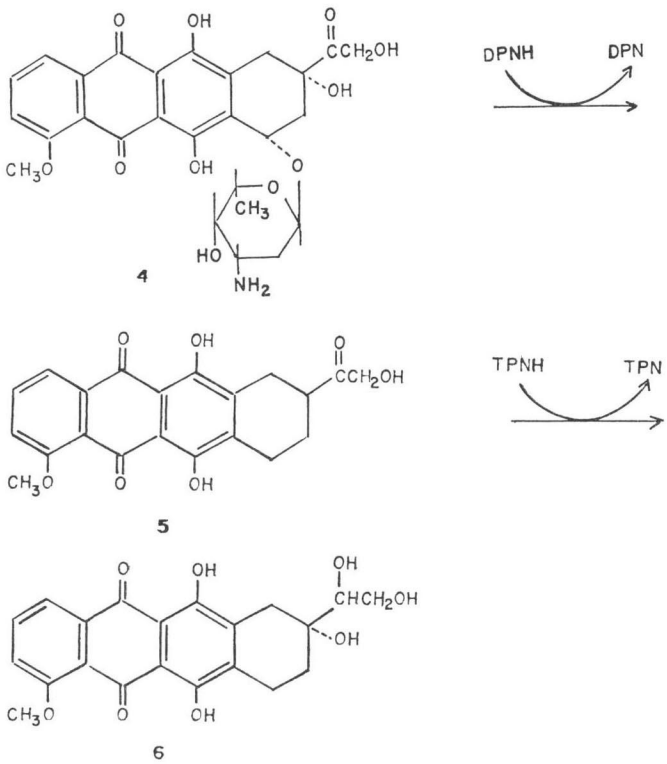
B. 7-Deoxydaunomycinol Aglycone (**3**)

A 4.8 ml *S. steffisburgensis* cell-free extract conversion of 0.2 mg of **2** was extracted with $CHCl_3$. The combined extracts were concentrated and compared by tlc in solvent systems B, F, G, H, and I with authentic **3**. The Rf values were 0.72, 0.51, 0.44, 0.32, and 0.37, respectively, and in all cases were the same as those for **3**.

C. 7-Deoxyadriamycinone (**5**) and 7-Deoxyadriamycinol Aglycone (**6**)

A 200-ml reaction mixture in which 100 mg of **4** had been subjected to conversion by *S. steffisburgensis* cell-free extract was mixed with 10 g of filter-aid and filtered, washing with 50 ml of H_2O. The filtrate was extracted with four 100-ml portions of CH_2Cl_2, and the filter cake was extracted with five 100-ml portions of CH_2Cl_2. All of the extracts were combined and evaporated under reduced pressure to give 114 mg of red solid. The residue was chromatographed on 11.4 g of silica gel using $CHCl_3$-CH_3OH (98: 2) for 620 ml (124 fractions), $CHCl_3$-CH_3OH (95: 5) for 280 ml (54 fractions), and $CHCl_3$-CH_3OH (3: 1) for 575 ml (125 fractions). Fractions 8~55 were combined as pool 1 (color maximum), fractions 56~134 as pool 2 (intermediate) and fractions 135~303 as pool 3 (color maximum). Evaporation under reduced pressure gave, respectively, 31 mg, 17 mg, and 33 mg. A similar run twice as large gave 100 mg from pool 1, 20 mg from the intermediate pool, and 28.9 mg from the last pool. The material from the last two pools from each run

Scheme 2.



was combined and chromatographed on 20 g of silica gel in $CHCl_3$-CH_3OH (96: 4) collecting one hundred and sixty-eight 5-ml fractions. On the basis of tlc in solvent B, fractions 16~31 were combined as pool 1 and fractions 39~65 as pool 2. Evaporation of pool 1 under reduced pressure gave 13 mg, and pool 2 gave 16.9 mg. These materials were compared with authentic samples of **5** and **6** by tlc using solvent systems B, E, and F. The faster moving material gave Rf's 0.78, 0.65, and 0.41, respectively, which were the same as those of **5**. The slower moving material gave Rf's 0.52, 0.21, and 0.32, respectively which were the same as those of **6**.

Mass spectra: Faster moving *m/e* 398.1009 (calcd. for **5** 398.1002)
Slower moving *m/e* 400.1170 (calcd. for **6** 400.1178)

A similar workup of the two faster moving pools from the initial chromatography gave 19.5 mg of material identified as **5** by tlc in systems B, E, and F and *m/e* 398.

Results and Discussion

It was found that the mold *M. spinosus* and its cell-free extract both reduce the ketonic carbonyl group at C-13 in **1** to a hydroxyl group. In both cases the conversion product was isolated, and its identity as **2** was established by tlc in several solvent systems and by mass spectra. The mass spectra did not give the molecular ion, but instead the ion was that of the aglycone and had *m/e* 400. However, such an ion could only arise from the compound in which the C-13 carbonyl was reduced. The yield was somewhat better with the cell-free extract being in fact higher than theoretical, but it is probable that a slight error was made in weighing substrate. The reduction of **1** to **2** by microbial fermentation has already been reported[8,9] using one organism of the genus *Corynebacterium*, two organisms of the genus *Streptomyces*, and an unidentified bacterium.

The rates of disappearance of **1** and appearance of **2** are shown in Fig. 1. The half-life of **1** was about 2 days with total disappearance in 7 days. The rates measured were not in complete agreement in that the rate of appearance of **2** always lagged behind disappearance of **1**, suggesting either further conversion of **2** or a second metabolic pathway for **1**. Fig. 2 shows the results of studies demonstrating

Fig. 1. C-13 keto reduction of daunomycin by fermentations of *M. spinosus*.
The organism was grown aerobically at 25°C using the tryptone, yeast extract, and glucose medium described in the text.

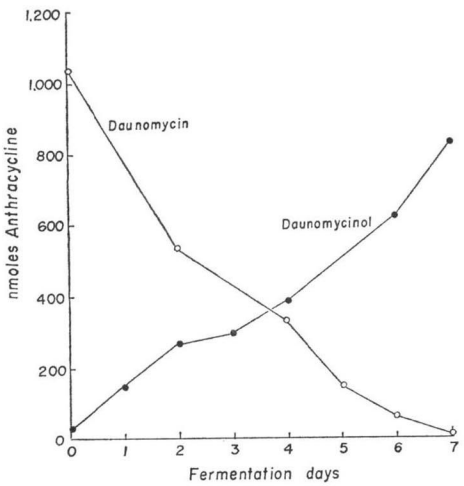
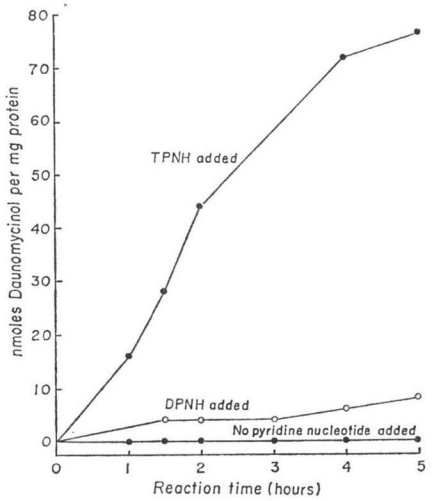


Fig. 2. Reduced pyridine nucleotide dependent C-13 keto reduction of daunomycin catalyzed by cell-free preparations of *M. spinosus*.
Reaction conditions are described in the text.



that cell-free conversion of **1** to **2** by crude enzyme preparations of *M. spinosus* are TPNH-linked as would be expected from previous work[4]. In addition, a minimal reaction rate was detected in the presence of DPNH. In the case of the corresponding reaction in mammals, the required cofactor was also TPNH[13]. Further study of the reaction showed that the pH requirement was not very rigid (Fig. 3) with substantial reaction occurring over the range of 5.5 to 8.0 but with highest activity at pH 7.0. The reaction rate studied as a function of crude enzyme protein concentration was approximately linear to 1 mg of protein added per ml of reaction mixture (Fig. 4).

The reductive cleavage of **2** to 7-deoxydaunomycinol aglycone (**3**, Scheme 1) was found to occur in the presence of cell-free extract of *S. steffisburgensis*. The presence of **3** was demonstrated by

Fig. 3. Cell-free conversion of daunomycin to daunomycinol studied as a function of buffer pH. Cell-free preparations of *M. spinosus* were prepared and assayed as described in the text.

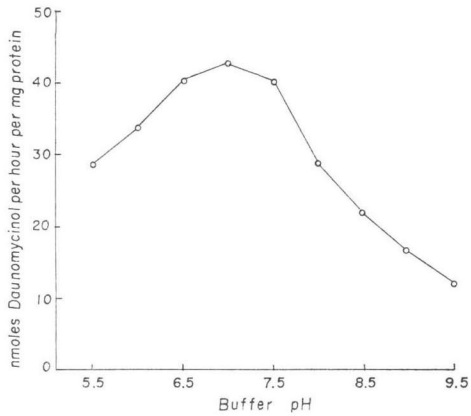


Fig. 4. Conversion of daunomycin to daunomycinol studied as a function of *M. spinosus* cell-free extract addition.
Experimental conditions are described in the text.

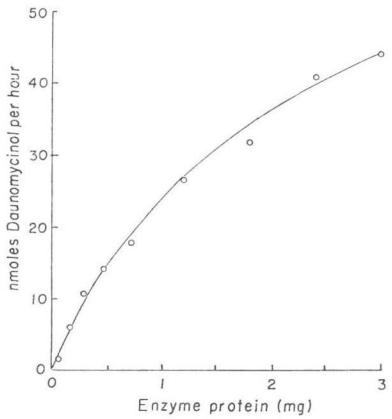


Fig. 5. Reduced pyridine nucleotide dependent reductive glycosidic cleavage catalyzed by cell-free preparations of *S. steffisburgensis*.
Experimental procedures are described in the text.

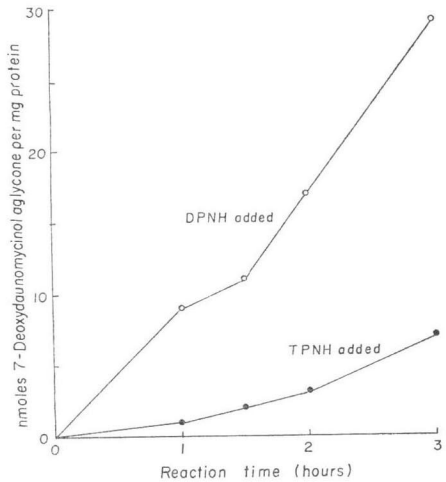
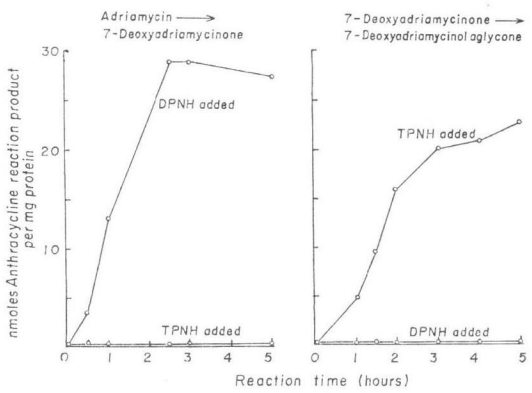


Fig. 6. Reduced pyridine nucleotide dependent metabolism of adriamycin and 7-deoxyadriamycinone catalyzed by cell-free preparations of *S. steffisburgensis*.
Experimental procedures are described in the text.



extraction from the reaction mixture and identification by tlc comparison in several solvent systems with an authentic sample. A very similar reaction has previously been reported[4] to occur with such enzyme preparations. Again, the cofactor requirement was studied, but in this case it was found to be DPNH with much lower activity in the presence of only TPNH (Fig. 5). Such a cofactor requirement has also been shown in the reductive cleavage of **1** by *Aeromonas hydrophila*[5,14].

The conversion of **1** to 7-deoxydaunomycinone and **3** by cell-free extracts of *S. steffisburgensis* has already been reported[4]. An exactly analogous series was found to occur with adriamycin (**4**, Scheme 2). In the presence of the crude enzyme preparation, both 7-deoxyadriamycinone (**5**) and 7-deoxyadriamycinol aglycone (**6**) were formed. The products were removed by extraction, purified by column chromatography, and identified by tlc comparison with authentic samples and by mass spectra. The yields were rather poor being 15% for **5** and 7.7% for **6** of isolated, purified material, but the difficult separation and purification undoubtedly caused considerable loss of material already present. The DPNH and TPNH requirements of these conversions were studied in the same fashion as was done with **1**[4], and the results were the same. The benzylic cleavage occurring with **4** to give **5** was found to require DPNH while the C-13 keto reduction (**5**→**6**) required TPNH. These results are shown in Fig. 6. The mammalian counterpart of the keto reduction also requires TPNH[6].

The major pathway for the human metabolism of both **1** and **4** is that shown for **1** in Scheme 1[7]. We have demonstrated that the microorganism *M. spinosus* has a partially identical pathway for **1**. A minor pathway for the human metabolism of both **1** and **4** is that shown for **4** in Scheme 2[15]. In our previous work[4] it has been found that *S. steffisburgensis* cell-free extracts modify **1** by a pathway identical to the minor human metabolic pathway, and in this work the same conversion has been shown for **4**.

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Authentic daunomycinol (Rhône-Poulenc) was furnished by Drs. B. K. HAMILTON and A. F. LANGLYKKE while samples of 7-deoxyadriamycinone and 7-deoxyadriamycinol aglycone were provided by Dr. D. W. YESAIR.

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