MICROBIAL REDUCTION OF THE SIDE-CHAIN CARBONYL OF DAUNORUBICIN AND N-ACETYLDAUNORUBICIN

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Microorganisms reduced the side-chain carbonyl of daunorubicin to yield 13-dihydrodaunorubicin (daunorubicinol; daunomycinol). This microbial transformation occurred under aerobic conditions in agitated baffled shake flasks incubated at 37°C. The microorganisms were first grown in a medium which supported dense growth. Daunorubicin-HCl was then added. Following a period of incubation, broths were adjusted to pH 10.0 and extracted with chloroform. Daunorubicinol was recovered and purified from the chloroform extracts by preparative TLC. Identity of the daunorubicinol was established by TLC and spectroscopy (UV-vis, IR, NMR, MS, CD and ORD).

N-Acetyldaunorubicin was likewise reduced microbially to N-acetyldaunorubicinol. N-Acetyldaunorubicinol appears to be a new compound which is yet to be tested for antitumor activity.

WILEY and MARSHALL[1] have reported in a recent communication to this journal on anaerobic microbial reductive cleavage of several anthracycline antibiotics to yield 7-deoxy-anthracycline aglycones. Their work was undertaken because many anthracycline antibiotics exhibit antitumor activities, and microbial transformation is one method with potential for modifying anthracycline structures to produce new agents for antitumor testing. For the same reason, we too have been exploring microbial transformation of anthracycline antibiotics. During the course of our studies, we have observed under aerobic conditions the microbial reduction of the side-chain carbonyl of daunorubicin to yield 13-dihydrodaunorubicin (daunorubicinol; daunomycinol); structures are shown in Fig. 1. N-Acetyldaunorubicin is likewise reduced. A report of the findings of this aspect of our work follows.

Materials and Methods

Chemicals and Media. Daunorubicin-HCl was kindly supplied by Dr. JOHN D. DOUROS,

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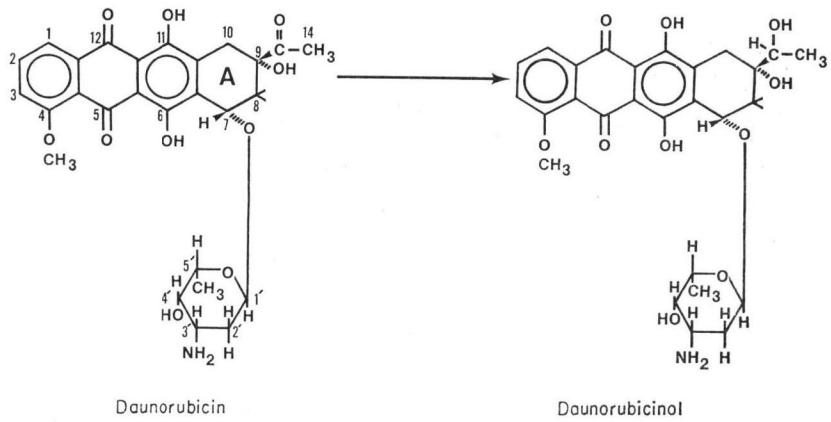
Fig. 1. Microbial transformation of daunorubicin to daunorubicinol



Daunorubicin Daunorubicinol

Division of Cancer Treatment, National Cancer Institute, U.S.A. N-Acetyldaunorubicin was chemically synthesized using procedures to be published. All other chemicals and media were standard commercially available grades.

Microorganisms. Unidentified actinomycete, yeast, and other bacterial and fungal cultures recently isolated from soils by our research section under Dr. A. L. GARRETSON were screened for ability to transform daunorubicin.

Biotransformation screen. Agitated (250 rpm) baffled shake flasks (50 ml) containing 10 ml of medium were employed. Cultures were inoculated from streak plates using an inoculating loop or bayonet. Typically, an isolate was incubated at 28°C and/or 37°C for 1~3 days in a medium which supported good growth (*e.g.*, BBL trypticase-soy broth [pH 7.5] for bacteria and yeasts, inorganic salts-starch broth [pH 7.5] for actinomycetes, and CZAPEK-DOX broth enriched with trypticase-soy broth [pH 7.3] for molds). A filter-sterilized aqueous solution of daunorubicin-HCl was then added (final concentration in medium, 0.12 mg/ml) and incubation was continued for 2~5 days. One control consisted of daunorubicin-HCl added to uninoculated medium and incubated for the same period. A second control was comprised of an inoculated fermentation unsupplemented with daunorubicin-HCl.

For biotransformation of N-acetyldaunorubicin, the powdered anthracycline was dispersed in filter-sterilized absolute ethanol and charged to fermentation flasks (0.25 ml ethanol containing 1.25 mg N-acetyldaunorubicin added to each 50-ml flask containing 10 ml broth).

Preparative biotransformation. Agitated (250 rpm) baffled shake flasks (1 liter) containing 200 ml of medium were used. Each flask was inoculated with a 1.0-ml aliquot of suspension from a slant washed with 5.0 ml of 0.9% sterile saline.

Extraction and thin-layer chromatography. After incubation the whole broths were adjusted to pH 10.0 with 2~4% aqueous sodium carbonate and were extracted with an aliquot of chloroform which had one-half the volume of the broth. In the case of the biotransformation screen, the chloroform extracts were directly spotted on silica gel plates (0.25 mm in thickness) containing fluorescent indicator and the plates were developed by one-dimensional thin-layer chromatography (TLC) with two solvent systems[2]: IA, $CHCl_3$-MeOH-H_2O (120:20:1); IB, $CHCl_3$-MeOH-H_2O (80:30:3). The developed TLC plates were examined under visible and UV light for evidence of daunorubicin transformation products. For preparative isolations, the chloroform extracts were concentrated at 40°C under vacuum and chromatographed in the dark on silica gel preparative plates (1.0 mm thick, no fluorescent indicator) with solvent system IA. The band containing transformation product was scraped off and suspended in solvent system IB for elution. The suspension was filtered with suction through paper, and the liquid eluate was washed in a separatory funnel with alkaline water (adjusted to pH 10 with sodium

carbonate). The resulting chloroform phase which remained after washing was vacuum-evaporated to dryness to yield the final product.

Spectroscopy. IR spectra (KBr pellet) were made with a Perkin Elmer 567 grating infrared spectrophotometer. UV-vis spectra (in methanol) were made with a Beckman model 25 instrument. Mass spectrometry was performed with a JEOL model JMS-01SG-2 double focusing mass spectrometer at an ionizing potential of 70 eV, trap current of 200μA, and accelerating voltage of 10 KV. Peracetylated derivatives, prepared as described elsewhere[3], were introduced on a solid probe and spectra taken at 250~300°C. ^{1}H-NMR studies were made with a Varian XL-100 instrument with tetramethylsilane as the reference standard (spectral conditions are given with spectra of compound A: spectral conditions for peracetylated compound A are the same as for compound A in $CDCl_3$). CD spectra (in methanol) were obtained on a Jasco J-40 instrument. The instrument was operated at 0.5 m°/cm sensitivity, four seconds-time constant, 1.0 nm/min scan rate, 1.0 nm slit width, and a path length of 1.0 cm. Sample concentrations were 0.003%. ORD measurements (in 1.0 mM HCl) were done with a Jasco J-20 automatic recording spectropolarimeter. The ORD instrument was operated at a sensitivity of 5.0 m°/cm, a chart speed of 1.0 cm/min, and a wave length expansion of 10 nm/cm. The cell path length was 1.0 cm and the temperature was 22°C.

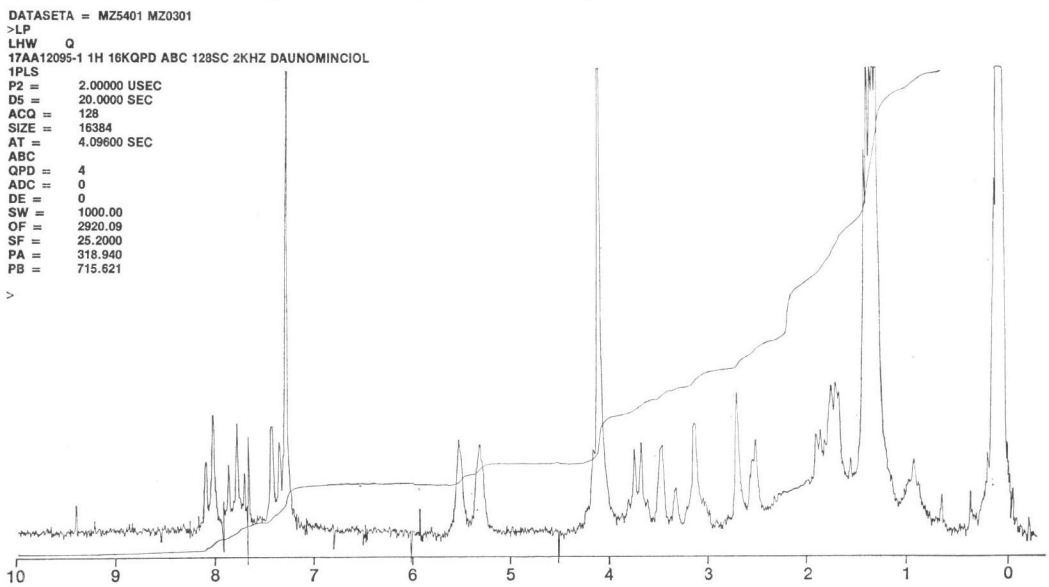
Results

Fermentation and Isolation of Daunorubicinol

When incubated in the presence of daunorubicin, many of the microbial isolates screened produced a product, designated compound A, which, in TLC, migrated with an Rf of 0.25 in solvent system IB, whereas daunorubicin itself displayed an Rf of 0.45. In solvent system IA the Rf of compound A was 0.04, whereas that of daunorubicin was 0.10. Compound A did not appear in incubated but uninoculated flasks containing daunorubicin in fermentation broth nor did it appear in inoculated broths incubated in the absence of daunorubicin.

With one of the soil isolates, FCRC 151 (identified as *Corynebacterium equi* by the American Type Culture Collection), milligram quantities of compound A were produced for structural

Fig. 2. NMR spectrum of compound A in $CDCl_3$



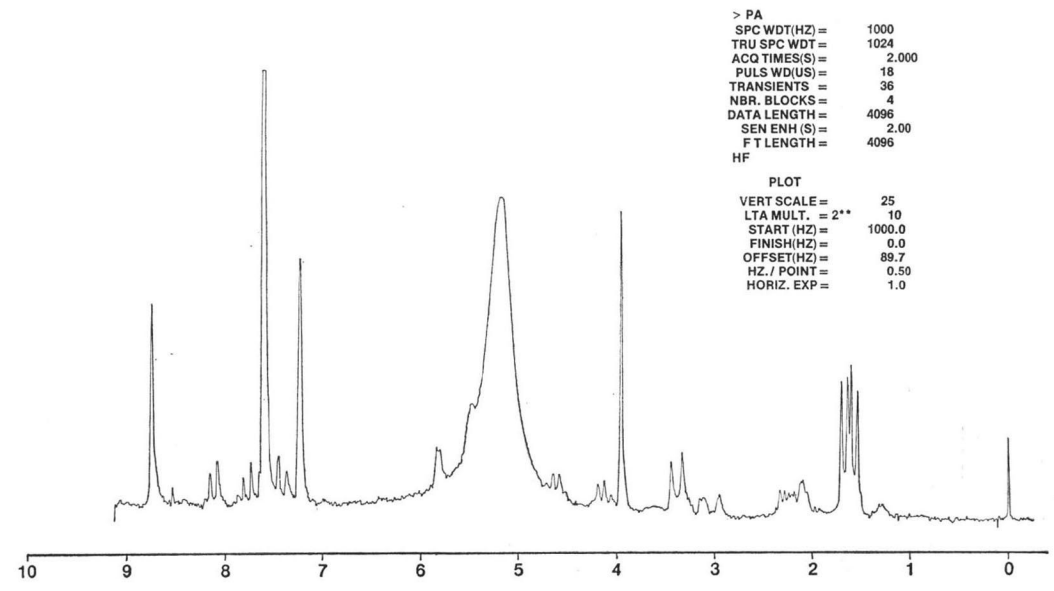
studies. FCRC 151 was grown in trypticase-soy broth at 37°C. Twenty-four hours after inoculation, daunorubicin-HCl was added and incubation was continued for three more days. The final pH of the broth was 8.4~8.7. As determined by TLC, virtually no daunorubicin remained at the end of incubation. The product recovered by extraction and preparative thin-layer chromatography ran as one spot in TLC on cellulose with solvent systems IA and IB. This product, which proved to be daunorubicinol, was identified as described below.

Identification of Daunorubicinol

The TLC results already given indicated that compound A is more polar than daunorubicin. The IR spectrum indicated the absence of a carbonyl function at 1710 cm^{-1} which was attributed to the disappearance of the ketone function at C-13[4]. Quantitative UV-vis spectroscopy suggested that the chromophore in compound A is the same as in daunorubicin[5] and that the molecular weight of compound A is very close to that of daunorubicin.

NMR studies of compound A and its peracetylated derivatives were made with a 100 MHz instrument using D_5-pyridine and $CDCl_3$ as solvents. The spectrum of compound A in $CDCl_3$ is shown in Fig. 2. Significant differences in this spectrum as compared to the published results of ARCAMONE *et al.*[6] on N-acetyldaunorubicin (in $CDCl_3$ as solvent) are as follows. Beside the obvious absence of the N-acetyl at δ 1.93 in the spectrum of compound A, the three-proton singlet at δ 2.41 present in the spectrum of N-acetyldaunorubicin due to the side chain, $-CH_3$(C-14 in Fig. 1), cannot be observed. Instead, a doublet at δ 1.28 (appears as quartet due to the 6′-CH_3 at the same place; see spectrum in D_5-pyridine for better resolution) is present. Also, a new quartet at δ 3.78 is apparent, and irradiation at δ 3.78 collapses the doublet at δ 1.28. These results indicate that the side chain protons, $-CH_3$, are coupled to a proton which is not present in N-acetyldaunorubicin. No other major differences are evident between the spectra of compound A and N-acetyldaunorubicin in chloroform. Chemical shifts, however, are slightly different and the NH_2 protons could not be observed in our spectrum. The phenolic

Fig. 3. NMR spectrum of compound A in D_5-pyridine



protons at δ 13.15 and 13.85 were observed but are not shown in Fig. 2.

The 100 MHz spectrum of compound A in D_5-pyridine is shown in Fig. 3. As mentioned above, the side chain, $-CH_3$, appears as a doublet at δ 1.62 separate from the 6'-CH_3. No additional gross characteristic information can be obtained from this spectrum. Shift assignments and coupling constants are shown in Table 1, and they are in agreement with those found for β-rhodomycinone by BROCKMANN *et al.*[7,8]

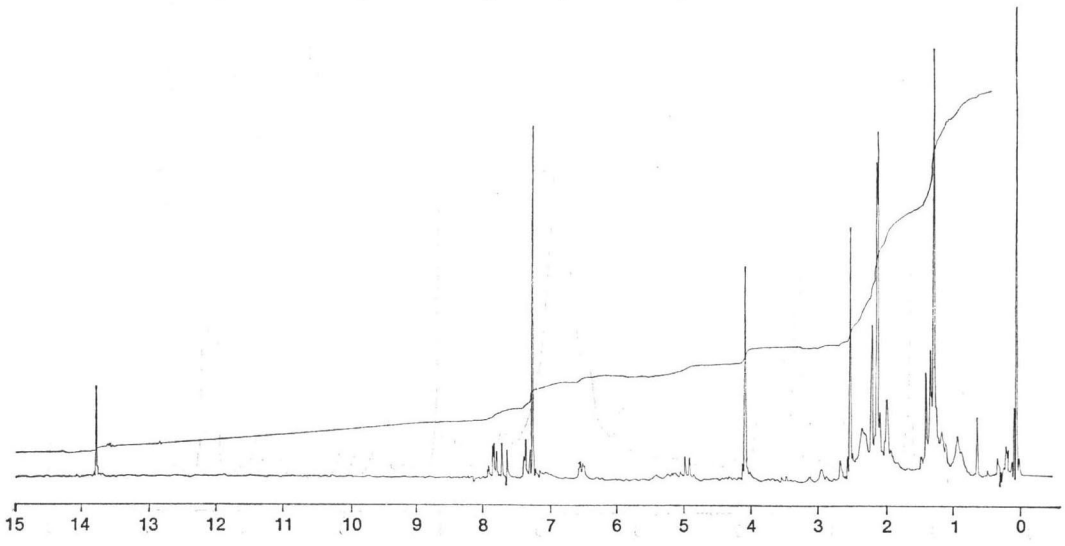
The 100 MHz spectrum of peracetylated compound A in $CDCl_3$ is shown in Fig. 4. Integration can account for 5 acetyl groups: the N-acetyl at δ 1.93 and the -OH acetyl at δ 2.13,

Table 1. 100 MHz Proton magnetic resonance spectrum of compound A in D_5-Pyridine

Protons	Chemical shift*	No. of H	Coupling constants
1	8.12 (d)	1H	$J_{1,2}=8$ Hz
2	7.78 (t)	1H	$J_{1,2}=J_{2,3}=8$ Hz
3	7.41 (d)	1H	$J_{2,3}=8$ Hz
1'	5.81 (d, d)	1H	—
7	5.48 (d, d)	1H	$J_{7,8\,eq}=4$ Hz, 2 Hz
8	3.04 (br, d, d)	2H	$J_{8A,8B}=16$ Hz
10	3.38 (d)	2H	$J_{10A,10B}=11$ Hz
2'	2.22 (c)	2H	—
3'	2.12	1H	—
4'	—	—	—
6' CH_3	1.68 (d)	3H	$J_{CH_3,H_5}=6.5$ Hz
14 CH_3	1.57 (d)	3H	$J_{CH_3,H_{13}}=6.5$ Hz
13	4.16 (q)	1H	$J_{13,CH_3}=6.5$ Hz
5'	4.62 (q)	1H	$J_{CH_3,H_{5'}}=6.5$ Hz
4 OCH_3	3.98 (s)	3H	—

* (d)-doublet, (t)-triplet, (d, d)-double doublet, (br, d, d)-broad double doublet, (c)-quintet, (q)-quartet, (s)-singlet.

Fig. 4. NMR spectrum of peracetylated compound A in $CDCl_3$

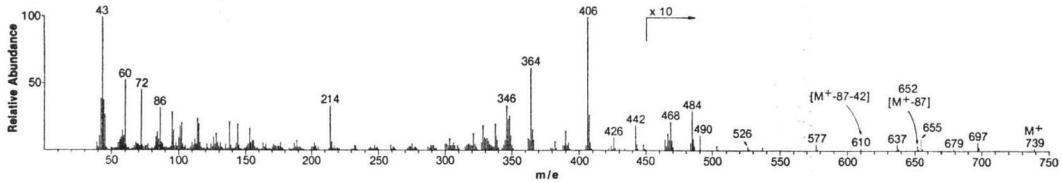


2.19, 2.20 and 2.53. One phenolic -OH could not be acetylated in compound A as evidenced by the signal at δ 13.85[6]. Daunorubicin, the parent compound, can be acetylated at all -OH positions as found and described by ROLLER *et al.*[3] Apparently the change introduced by biotransformation, perhaps in combination with acetylation, prevented the acetylation of one phenolic -OH under the experimental conditions employed (steric hindrance, for example, might have blocked the C-11 phenolic OH).

The above NMR results, taken together with the TLC, UV-vis, and IR data, indicate that the only primary structural difference between daunorubicin and compound A is that the side chain ketone is reduced to an alcohol.

Further evidence for this proposed change comes from mass spectroscopic studies on the peracetylated derivative of compound A (see Fig. 5). As shown above by NMR studies on

Fig. 5. Mass spectrum of peracetylated derivative of compound A

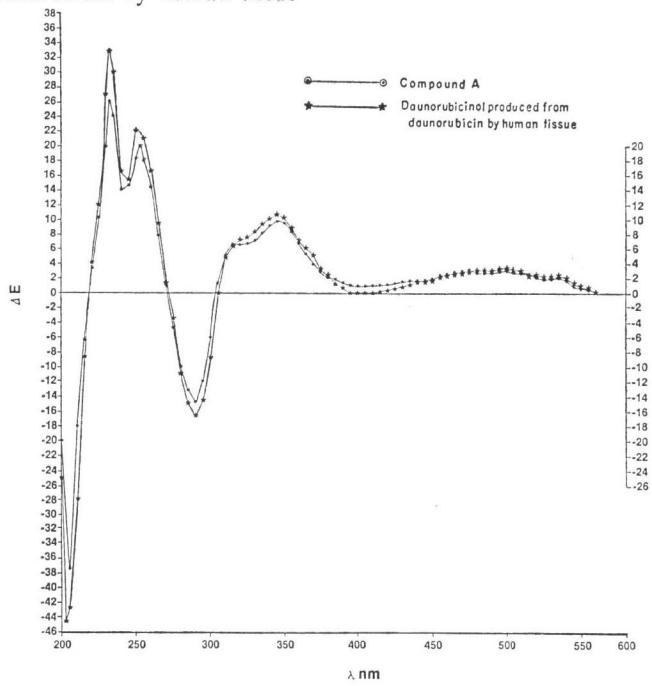


the peracetylated compound A, one phenolic -OH was not acetylated. The expected M^+ is at *m/e* 739 and was observed in the mass spectrum. The fragmentation pattern of the peracetylated compound A was very similar to that of the peracetylated daunorubicin, which is described elsewhere[8], and largely results from the loss of ketene and acetyl functions from the molecule, and from the aglycone and the sugar. In addition, a group of mass 87 split off in the spectrum of peracetylated compound A, indicating that the side chain is the acetylated form of the reduced ketone function.

More evidence for the identity of compound A was obtained by comparison with daunorubicinol obtained from daunorubicin through reduction by human tissue as described by BACHUR[4]. Furthermore, it was of interest to determine whether the daunorubicinols produced by human tissue and by microorganism FCRC 151 have the same stereochemistry at the newly created asymmetric carbon. The two daunorubicinols were compared as follows: TLC in 3 solvent systems indicated identity (IA, Rf=0.04; IB, Rf=0.25; *n*-propanol-H_2O-MeOH-pyridine [8:2:1:1], Rf=0.87). The IR, 100 MHz NMR ($CDCl_3$), UV-vis, and CD (see Fig. 6) spectra were superimposable. The optical rotation of each compound as a base was $[\alpha]_D^{20}=170°$ (*c* 0.05, EtOH-HCl). The ORD spectra showed a trough at 298 nm with equal intensity for both biologically produced daunorubicinols and were virtually identical between 500 and 240 nm, indicating the same stereochemistry in both compounds. The absolute configuration of the newly created asymmetric carbon remains to be determined.

The 100 MHz NMR spectrum of compound A in D_5-pyridine (Table 1) also supplied information on the conformation of the *A* ring (see Fig. 1) in the aglycone portion of the molecule. The similarity of the conformations of the *A* rings in daunomycinone and β-rhodomycinone was deduced by BROCKMANN *et al.*[7,8] based in part on NMR spectra. Comparison of data in Table 1 with data given by BROCKMANN *et al.* indicated that the *A* ring in compound

Fig. 6. CD spectra of compound A and daunorubicinol produced from daunorubicin by human tissue



A has the same conformation as the *A* ring in daunomycinone and β-rhodomycinone, that is, half chair and 7H equatorial. In particular, in the spectrum of compound A, the 7H proton, δ 5.48 (double doublet, J=4 Hz, 2 Hz), is split due to equatorial coupling to 8H. This finding compares well to that of BROCKMANN and NIEMEYER[7] for β-rhodomycinone in D_5-pyridine (7H: δ 5.55, J=4 Hz, 2 Hz). In addition, in the spectrum for compound A, both 8H protons (A: δ 3.12, J_{7-8}=4 Hz; B: δ 2.96, J_{7-8}=2 Hz; J_{8e-8a}=16 Hz) are split into double doublets due to ABX coupling. This finding also compares well to that of BROCKMANN and NIEMEYER (A: δ 2.55, J_{7-8}=4 Hz; B: δ 2.44, J_{7-8}=2 Hz; J_{8e-8a}=14 Hz). Moreover, the 10H's which appeared at δ 3.33 and δ 3.44 (J=11 Hz) were not split due to long range coupling to 8H in compound A and β-rhodomycinone in D_5-pyridine, while such splitting was found by ARCAMONE *et al.*[6]

N-Acetyldaunorubicinol

FCRC 151 was grown by applying the procedure employed for daunorubicin biotransformation, and N-acetyldaunorubicin was added 24 hours after inoculation. Incubation was continued for three more days. The final pH of the broth was 8.4. In TLC, the Rf's of N-acetyldaunorubicin extracted into chloroform from uninoculated broth were 0.58 with solvent system IA and 0.82 with solvent system IB. TLC of the chloroform extract from inoculated broth yielded a product with Rf's of 0.44 with solvent system IA and 0.73 with solvent system IB; only transformation product appeared, and no N-acetyldaunorubicin remained. Identity of the transformation product as N-acetyldaunorubicinol was secured by IR spectroscopy (absence of the carbonyl function at 1710 cm^{-1}) and NMR spectroscopy in $CDCl_3$ (spectrum of N-acetyldaunorubicinol has the characteristic N-acetyl signal at δ 1.93 for 3 protons, but otherwise is identical to that of daunorubicinol). Elemental analysis indicated the presence of 2.45% N

(theoretical 2.46 %).

Discussion

Microbial reduction of daunorubicin to daunorubicinol has also been observed by other workers[9] who found that the transformation can be carried out by *Streptomyces lavendulae* ATCC 8644, *S. roseochromogenes* ATCC 13400, *Corynebacterium simplex* ATCC 6946, and *Bacterium cyclooxydans* ATCC 12673. In addition, daunorubicinol can be prepared by direct fermentation[10,11] or by chemical reduction of daunorubicin[12]. BACHUR[13] has reported that daunorubicinol is a major metabolite of daunorubicin appearing in rats and humans, and DIMARCO and ARCAMONE[14] observed that daunorubicinol is a metabolite of the daunorubicin producing strain of *Streptomyces peucetius*. These observations which indicate that mammals and microorganisms reduce daunorubicin to daunorubicinol reinforce the concepts recently reviewed by SMITH and ROSAZZA[15] on the parallelism between mammalian and microbial metabolism of drugs.

The antitumor activity of daunorubicinol has been studied[10,16,17]. N-Acetyldaunorubicinol is to our knowledge a new compound which is yet to be tested for antitumor activity.

Acknowledgements

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