MICROBIAL TRANSFORMATIONS OF NATURAL ANTITUMOR AGENTS
13. CONVERSIONS OF 13-BLOCKED ANTHRACYCLINONES

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Microbial transformation experiments were performed using 13-ketone-blocked daunomycinone derivatives which were converted into the corresponding carminomycinone derivatives by microbial O-demethylation.

Microbial transformation studies have been conducted with a wide variety of antitumor compounds[1~3] for the purposes of preparing useful derivatives of antitumor agents; and for elaborating novel pathways for their metabolism[4]. The anthracyclines are highly-active antitumor antibiotics, and their metabolism has been studied in humans and also in microorganisms. Reduction of the 13-ketone functional group is the major mammalian and microbial biotransformation reaction[5~10] although reductive cleavage of the 7-oxygen position also occurs[8,11,12] and microorganisms achieve N-acetylation reactions of the amino sugar[13]. In mammals, 4-O-demethylation reactions occur, but the same reaction has not yet been reported for microorganisms. The cleavage of aryl-ethers has been an excellent fermentation type-reaction in our hands[3], and we examined the possibility of converting compounds like daunomycinone (**1**) into carminomycinone (**5**) using microorganisms. Screening experiments revealed that O-demethylation could occur, but that reaction mixtures were always complicated by 13-ketone reduction as the major metabolic conversion. We attempted to prevent 13-ketone reduction by using 13-blocked daunomycinone derivatives as substrates in order to direct biotransformations to other parts of the anthracycline nucleus. This report describes our results with daunomycinone-13-ethylene ketal (**4**), and daunomycinone-13-benzoylhydrazone (**3**).

Materials and Methods

Sources of authentic standards used in this work were as follows: daunomycinone (**1**), Parke Davis Laboratories, Detroit, Mich., U.S.A.; daunomycin and daunomycinol, National Cancer Institute, Bethesda, Md., U.S.A.; carminomycinone (**5**) and dihydrocarminomycinone (**6**), Rhône-Poulenc, 13-quai Jules-Guesde, F 94400 Vitry-sur-Seine, France.

Physical Methods

Melting points were determined on a Thomas Hoover apparatus in open ended capillaries and are uncorrected. IR spectra were determined on a Perkin-Elmer 267 grating infrared spectrophotometer or Beckman IR 4240. NMR spectra were determined on a Varian Associates T-60 spectrometer or Bruker B-90C spectrometer using tetramethylsilane as an internal standard. Low resolution mass spectral data were obtained on a Finnigan Model 3200 GC mass spectrometer. Field desorption mass spectra were provided by Prof. KENNETH RINEHART, Jr., School of Chemical Sciences, University of Illinois, Urbana, Ill., U.S.A. The high resolution mass spectra were provided by the Mass Spectrometry Laboratories of the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A. Ultraviolet spectra in methanol were obtained on a Philips Pye-Unicam SP1800 spectrophotometer.

Chromatography

Thin-layer chromatography (TLC) was performed on 0.25 mm layers of pH 6.5 phosphate buffered silica gel GF_{254} (Merck) plates which were prepared by slurrying 25 g of silica gel GF_{254} in 50 ml of pH 6.5 phosphate buffer (1/15 M), and spreading the mixture on glass plates with a Quickfit Industries spreader. After air drying, plates were activated in an oven at 110°C for 30 minutes before use. Excellent resolution of all compounds was obtained using a solvent system of CH_2Cl_2 - isopropanol (15 : 1) and developed TLC plates were visualized by fluorescence quenching under 366 nm UV light (Table 1). Daunomycinone derivatives presented an orange color under these conditions, while carminomycinone derivatives were yellow.

Column chromatography was performed on buffered Baker Silica gel (3405). Silica gel for column chromatography was prepared by slurring 100 g of the powder with 200 ml of pH 6.5 phosphate buffer (1/15 M), and drying the slurry to a powder in an oven at 110°C. The dry buffered silica gel was then slurry-packed into glass columns for preparative scale separations.

High performance liquid chromatography was performed with a Waters ALC/GPC 202 instrument equipped with an M6000 solvent delivery system, an U6K universal injector, and a 254 nm differential UV detector. Separations were best achieved with a micro-porasil column (0.4×30 cm, Waters) using $CHCl_3$ - AcOH (95: 5) at an average flow rate of 1.3 ml/minute at a pressure of 1,900 psi. Both solvents used were reagent grade in quality. Samples were dissolved in $CHCl_3$ - MeOH (9: 1) (1 mg/ml) and 4 μl samples were injected for analysis. Mixtures of authentic standards were injected singly to establish individual retention volumes, and mixtures of metabolites were well resolved (Table 1). The identities of individual peaks in microbial extracts were confirmed by "spiking" with analytical standards.

Daunomycinone

Daunomycinone (**1**) was provided by Parke Davis, Detroit and was fully characterized (m.p., nmr, uv, ir) before being used in these experiments.

Preparation of Daunomycinone Benzoylhydrazone (**3**)

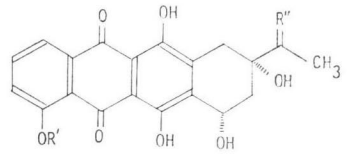
A 100 ml round-bottomed flask was charged with 2 g (5 mmol) of daunomycinone, 0.82 g (6 mmol) of benzoylhydrazine, 0.5 ml of glacial acetic acid and 60 ml of absolute ethanol[16]. The mixture was stirred at room temperature for 44 hours, chilled in an ice bath, and the resulting precipitate was collected by filtration. After washing with cold ethanol, the solid was dried *in vacuo* to give 2.5 g of red

Table 1. Chromatographic properties of various anthracyclinone derivatives.

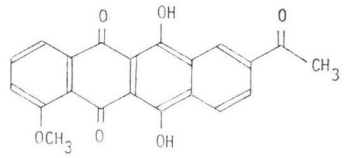
Compound	TLC Rf value*	HPLC Retention volume** (ml)
1	0.78	6.2
2	0.24	21.5
3	0.55	—
4	0.68	7.8
5	0.90	4.9
6	0.42	15.6
7	0.86	5.4
8	0.95	—

* Silica gel GF_{254} buffered at pH 6.5 using a solvent system of CH_2Cl_2 - isopropanol, 15: 1.

** Microporasil column (0.4×30 cm, waters) using $CHCl_3$ - AcOH, 95: 5 at a flow rate of 1.3 ml/min at a pressure of 1,900 psi.



Compounds	R′	R″
1	CH_3	=O
2	CH_3	H, OH
3	CH_3	N-NH-CO-C_6H_5
4	CH_3	O-CH_2CH_2-O
5	H	=O
6	H	H, OH
7	H	O-CH_2CH_2-O



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solid: mp 197~200°; IR (KBr disc) cm^{-1}, 1670, 1640, 1615 and 1585; mass spectrum (Field Desorption), *m/e* (% relative abundance) 516 (M^+, 100), 498 (30), 480 (17); UV, λ max 234 (42,312), 253 (39,732), 480 (12,900), 497 (13,416), 532 (7,740).

Preparation of Daunomycinone Ethylene Ketal (**4**)

A 500-ml round-bottomed flask was charged with 1.5 g (3.7 mmol) of daunomycinone, 2 g (33 mmol) of ethylene glycol, 150 mg of *p*-toluenesulfonic acid and 250 ml of dry benzene. The above mixture was refluxed for 16 hours and the water formed was removed by use of a Dean Stark separator. Benzene was removed by rotary evaporation, and the resulting red residue was dissolved in a minimum volume of $CHCl_3$-MeOH (9: 1). The reaction mixture was purified by column chromatography over buffered silica gel, pH 6.5 (150 g, 2.5×52 cm, slurry packed in $CHCl_3$) which was eluted with $CHCl_3$ to provide 100 mg of totally aromatized compound **8**, 1.3 g of the desired ketal **4**, and a small amount of unidentified material with low TLC mobility. The characterization of these compounds is as follows: compound **8**: mp 298~304°C; IR (KBr disc) cm^{-1} 1685, 1610, and 1575; mass spectrum *m/e* (% relative abundance) 362 (M^+, 100), 344 (76), 319 (15), 301 (28); UV MeOH, λ max (ε) 265 (15,385), 500 (4,706), 534 (3,800).

Compound **4**: mp 245~247°C (CH_3CN); IR (KBr disc) cm^{-1} 1610 and 1575; NMR ($CDCl_3$) ppm, 1.49 (s, 3H), 4.09 (s, 7H, methoxy and ethylene ketal), 5.32 (m, 1H, H-7), 7.41 (d, 1H, H=8 Hz, H-3), 7.75 (t, J=8 Hz, H-2), 8.27 (d, J=8 Hz, H-1), 13.36 and 13.98 (phenolic OH); mass spectrum, *m/e* (% relative abundance), 442 (M^+, 7), 406 (76), 391 (100), 319 (17); UV MeOH, λ max (ε), 234 (43,316), 252 (34,476), 290 (10,829), 480 (15,690), 496 (16,266), 531 (9,280). Elemental anal. for $C_{23}H_{22}O_9$, calcd. C 62.44, H 5.01; found C 62.67, H 5.44.

Fermentation Procedures

Cultures used in this work are maintained in the University of Iowa, College of Pharmacy culture collection and are stored at 4°C in sealed screw-cap culture tubes on agar slants. Organisms were grown according to a previously described two-stage process[2,3]. Stainless-steel capped Delong culture flasks holding one-fifth of their volumes of medium were sterilized in an autoclave at 1.05 kg/cm^2 for 15 minutes at 121°C. Cultures were incubated on rotary shakers (New Brunswick Scientific, Model G-25, 1″ stroke) operating at 250 rpm and 27°C. Stage I cultures were incubated in this manner for 24 hours and then used as inoculum (10%) for Stage II cultures. Substrates were added to 24 hours old Stage II cultures either as a suspension in dimethylformamide, or emulsified with Emulphor (polyoxyethylated vegetable oil) EL-620 (GAF Corporation, New York). The progress of microbial transformation reactions was monitored by withdrawing 4-ml samples of fermentations at various time intervals, extracting these with 1 ml of ethyl acetate, and 30 μl of the extracts were spotted on thin-layer chromatography plates.

Controls were used to verify that metabolites observed in fermentations were produced enzymatically, and that they were not simply artifacts arising from physical exposure of substrates to changing fermentation conditions. These controls consisted of substrates added to sterile medium, and to buffers at pH 3.0 (0.1 M potassium phthalate), pH 6.9 (0.1 M phosphate) and pH 9.5 (0.1 M sodium borate); and also cultures containing no substrate. TLC analysis of daunomycinone benzoylhydrazone (**3**) indicated that the compound was completely hydrolyzed to daunomycinone (**1**) within 4 hours at pH 3.0 and nearly 50% of this compound was hydrolyzed in pH 6.9 and 9.5 incubation conditions within one week. The ethylene ketal derivative of daunomycinone (**4**) was not decomposed in any of the conditions examined over a period of one week.

Microbial Transformation of Daunomycinone Benzoylhydrazone (3)

Sepedonium chrysospermum (ATCC 13378) was grown according to the usual fermentation procedure using a medium containing: glucose 10 g, corn-steep liquor 5 g, KH_2PO_4 3 g, $CaCO_3$ 3.5 g, soybean oil 2.2 ml, yeast extract 2.5 g, distilled water 1,000 ml and adjusted to pH 7.0 with 10% NH_4OH. A total of 1.7 g of **3** was suspended in 17 ml of dimethylformamide and distributed evenly among 17-liter flasks holding 3.4 liters of 24-hour Stage II culture. Formation of metabolites was evident in incubation mixtures within 24 hours, and after 96 hours, estimated TLC yields of **2** and **6** were 15% and 20% of the substrate used respectively. The fermentation beers were pooled, and exhaustively extracted with $CHCl_3$ - MeOH (9: 1), and the combined extracts were filtered through Celite, dried over anhydrous Na_2SO_4 and concentrated to 2.8 g of red solid. The solid material was dissolved in the extracting solvent, mixed with 20 g of pH 6.5 buffered silica gel, and dried in a rotary evaporator to a red powder. The powdered sample was applied to the top of a pH 6.5 phosphate buffered silica gel column (280 g, 5×38 cm) which had been slurry-packed in $CHCl_3$, and the column was eluted with $CHCl_3$ - MeOH (99: 1) to afford 50 mg of **2** and 80 mg of **6**. Besides these two metabolites unreacted starting material **3** and daunomycinone (**1**) were recovered. The metabolites were identified as follows:

Compound **2**: IR (KBr disc) 1585 cm^{-1} and 1616 cm^{-1} (Quinone carbonyls), and the band at 1700 cm^{-1} of daunomycinone was absent; mass spectrum for $C_{21}H_{20}O_8$, Field Desorption 401 (100%, M+1) and 365 (99%, M+1−$2H_2O$); EI 70 eV identical to that published by KARNETOVA *et al.*[2], *m/e* (% relative abundance) 400 (4), 364 (25%, −$2H_2O$), 346 (13%, −$3H_2O$), 338 (15%, $M^{+\cdot}-H_2O$, −C_2H_4O), 303 (25%, $M^{+\cdot}-3H_2O$, −C_2H_3O), 321 (25%, $M^{+\cdot}-2H_2O$, −C_2H_3O), 306 (21%); UV (MeOH) was identical to that of daunomycinone.

Compound **6**: IR (KBr disc) 1595 cm^{-1} (overlapped quinone and C=C)[17]; UV (MeOH) λ max (ε) 235 (27,483), 255 (23,172), 294 (6,948), 493 (12,740), 527 (9,264); mass spectrum, EI, 70 ev, *m/e* (% relative abundance) 386 ($M^{+\cdot}$, 24%), 368 (11), 350 (100), 335 (20), 324 (79), 307 (72), 296 (33); chromatographically, TLC and HPLC identical to authentic dihydrocarminomycinone (**6**).

Conversion of Daunomycinone-13-Ethylene-Ketal (4) to Carminomycinone-13-Ethylene Ketal (7) by *Beauveria sulfurescens* ATCC 7159

B. sulfurescens was grown according to the usual fermentation procedure in a medium of the following composition: soybean meal 30 g, glycerol 20 g and distilled water 1,000 ml. A total of 360 mg of **4** was suspended with the minimum amount of 0.1% Emulphor EL-620 solution, and distributed evenly among fifty six, 125-ml Erlenmeyer flasks each holding 25 ml of 24 hours Stage II culture. Metabolite formation was monitored by TLC, and **7** was evident in incubation mixtures within 24 hours of substrate addition. After 6 days, the TLC estimated yield of **7** was 20%, and the fermentation beers (pH 7.0) were pooled and exhaustively extracted with $CHCl_3$ - MeOH (9: 1). The combined extract was washed with water and dried over anhydrous sodium sulfate before being evaporated to dryness. The red solid extract was dissolved in $CHCl_3$, mixed with 5 g of pH 6.5 buffered silica gel, and dried to a powder using a rotary evaporator. The sample was added to the top of a pH 6.5 buffered silica gel column (120 g, 2.5×35 cm, slurry packed in $CHCl_3$) and eluted with $CHCl_3$ - MeOH (199: 1). After a 250 ml forerun, samples of 15 ml were collected to provide 30 mg of **7** from combined fractions 18~20, and the metabolite was identified as follows: IR (KBr disc) cm^{-1} 1605 (superimposed quinone C=O and C=C[17]); UV (MeOH) λ max (ε) 234 (35,780), 254 (31,158), 290 (10,229), 492 (13,782), 531 (9,116); mass spectrum, for $C_{22}H_{20}O_9$. Field desorption *m/e* 428 (100%), 411 (17%, M^+-OH) and 384 (20%, $M^+-C_2H_4O$ ketal);

high resolution mass spectrum, 70 eV electron impact, *m/e* 392.08744 found for $C_{22}H_{16}O_7$, $M^+ - 2\ H_2O$ - calculated 392.08960; NMR, $CDCl_3$ - CD_3OD (10 : 1), ppm, 1.47 (s, 3H, 13-CH_3), 4.07 (s, 4H, –O–CH_2CH_2–O–). Hydrolysis of 1 mg of **7** to **5** was accomplished with *p*-toluenesulfonic acid in 1 ml H_2O at 40°C for 30 minutes. Identification of **5** was based on TLC and HPLC comparison with authentic **5**.

Discussion

Microbial transformation experiments required the development of sensitive methods for the detection and isolation of anthracyclinones and their metabolites. High-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and open column chromatographic procedures were designed specifically for these experiments. Phosphate buffered (pH 6.5) silica gels were far superior separation media when compared to all other stationary chromatographic phases examined including those previously reported in the literature. All compounds were well-resolved, and columns for preparative scale isolations were highly efficient in resolving closely related anthracycline derivatives.

Initial microbial transformation screening experiments were performed with microorganisms selected from a broad taxonomic range. Many of the cultures examined first were those with known O-demethylase activity[3]. When daunomycin or daunomycinone were used as substrate, the major and most commonly observed reaction was 13-ketone reduction, a result which confirmed earlier findings by others[5~10]. Ketone-blocked derivatives of daunomycinone were then examined as substrates in microbial transformation reactions. This approach was taken in order to "direct" biotransformations to other parts of the anthracycline nucleus.

Experiments with the benzoylhydrazone derivative of daunomycinone (**3**) were disappointing since this compound was highly unstable in aqueous fermentation medium, and it was extremely insoluble in all solvents including water. Although **3** gave no EI mass spectrum, the field desorption mass spectrum confirmed the molecular weight of the derivative. When **3** was used as substrate with *Sepedonium chrysospermum*, products isolated included daunomycinone (**1**), dihydrodaunomycinone (**2**), and dihydrocarminomycinone (**6**), all of which were identified by chromatographic comparisons with authentic standards, or with literature values. Dihydrodaunomycinone (**2**) was identified by mass, UV and IR spectroscopy, all of which were identical to values in the literature[17]. Pertinent features included the aliphatic carbonyl band at 1700 cm^{-1} found in daunomycinone, but absent in **2**; and electron impact and field desorption mass spectra both of which provided patterns identical to those for **2** in the literature. Dihydrocarminomycinone (**6**) was identified by spectral analyses (IR, UV, mass spectrum) and was compared with authentic **6** by TLC and HPLC. The value of this experiment lay in the demonstration of the O-demethylation reaction with anthracyclinones.

To bypass the difficulty encountered with unstable 13-ketone derivatives like **3**, the ethylene-ketal (**4**) was prepared and used as substrate. From screening experiments several cultures demonstrated the ability to achieve cleavage of the ethylene-ketal protecting group, and also to achieve O-demethylation. *B. sulfurescens* (ATCC 7159) was selected for preparative scale work based on its ability to achieve O-demethylation and not additional complicating side-reactions. The major metabolite produced by *B. sulfurescens* was identified as **7** by IR, UV and mass spectral analyses. The NMR spectrum of **7** contained a peak at 4.07 ppm diminished by 3 protons *vs.* the starting material **4**. High resolution and field desorption mass spectral data provided the molecular ion, the ion minus water, and a major peak at *m/e* 384 indicating the loss of the ketal moiety from the molecular ion. Final proof of structure was obtained by the smooth *p*-toluenesulfonic acid hydrolysis of **7** to carminomycinone (**5**) which was identified by TLC and HPLC comparisons with authentic **5**.

This work clearly demonstrates the potential for microbial O-demethylation of anthracyclines to occur with microorganisms. It is unique in that protecting groups are employed to direct microbial transformation reactions to sites other than the 13-ketone of the anthracycline nucleus. This is also an excellent example of the capability of microorganisms to mimic mammalian metabolic transformations within the anthracyclines[4] and is the first microbial O-demethylation reaction reported for this class of antibiotics.

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