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Antitumor Proteins of *Streptomyces macromomyceticus*: Purification and Characterization of Auromomycin, Macromomycin A, and Macromomycin D†

Dale D. Vandrê and Rex Montgomery*

ABSTRACT: Macromomycin A and the two related proteins auromomycin and macromomycin D were isolated from the culture filtrates of *Streptomyces macromomyceticus* by chromatography on columns of DEAE-cellulose, Amberlite XAD-7, and decylagarose. Antibodies prepared against macromomycin A showed antigenic identity by Ouchterlony double diffusion between the three purified proteins. This similarity was further demonstrated by their behavior on disc gel electrophoresis, the amino acid compositions, and comparative peptide mapping of the aminoethylated derivatives. They differed, however, in other chemical and biological properties. Auromomycin and macromomycin A, p*I* 5.4, have antibiotic activity, which is absent in macromomycin D, p*I* 5.2. This antibiotic activity was associated with chromophore groups that were extractable by methanol. High-pressure liquid chromatography of the methanol extracts gave difference profiles for each of the purified proteins. The differences in the three proteins extended to their ultraviolet–visible spectra, fluorescence and circular dichroism, and the changes of these properties with heating. The heat denaturation, with auromomycin and macromomycin melting at 70.5 °C and macromomycin D at 57.0 °C, was reversible. Changes were noted in the spectra both during and following heating at 80 °C; the antibacterial activity was lost in auromomycin and only partially reduced in macromomycin A. The properties of the three proteins support the general similarities in their polypeptide structures, modifications in the properties of which are endowed by the differences in the associated nonprotein chromophores.

Several distinguishable but related proteins with antibiotic and antitumor activity have been isolated from the culture filtrates of *Streptomyces macromomyceticus*. Macromomycin (Chimura et al., 1968), purified to chemical homogeneity (Yamashita et al., 1976; Im et al., 1978), was cytotoxic to a variety of tumor cells both in vivo and in vitro. It inhibited the incorporation of thymidine into DNA (Beerman, 1978; Im et al., 1978; Suzuki et al., 1978; Vandrê et al., 1979) and caused single-stranded cleavage of cellular DNA in vivo (Beerman, 1978; Suzuki et al., 1978; Kappen et al., 1979; Vandrê & Montgomery, 1979). A second antitumor protein, auromomycin,[1] with comparatively greater biological activity, has been obtained from *S. macromomyceticus* (Yamashita et al., 1979). This protein shares several chemical and biological properties with macromomycin and can be converted into macromomycin by chromatography on Amberlite XAD-7, with the loss of a chromophoric material that absorbed at 355 nm. As with the antitumor antibiotic neocarzinostatin (Napier et al., 1979, 1980; Koide et al., 1980), a methanol-extractable chromophore, having both antitumor and antibiotic activity, is also associated with auromomycin (Suzuki et al., 1980; Woynarowski & Beerman, 1980; Kappen et al., 1980b).

Both macromomycin and auromomycin cleaved cellular DNA in vivo, but it is not clear whether both are capable of the same reaction in vitro. Although auromomycin and its chromophore cleaved DNA in vitro under a variety of experimental conditions (Kappen et al., 1979, 1980b; Suzuki et al., 1979b, 1980), the cleavage of DNA by macromomycin under similar conditions was not observed. However, cleavage of DNA by macromomycin in vitro has been demonstrated with high concentrations of protein after previous reduction with sodium borohydride (Suzuki et al., 1979a). Some of the observed differences in the DNA strand breakage by macromomycin may be related to the method of purification. Macromomycin, prepared by elution from Amberlite XAD-7 with water, is devoid of biological activity but blocks the cleavage of DNA by auromomycin (Kappen & Goldberg, 1979; Napier et al., 1980; Kappen et al., 1980b), and it has been suggested that the biological activity present in other macromomycin preparations may be the result of contaminating auromomycin (Kappen et al., 1980b). However, it

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[1] A new nomenclature is used in this paper for the purified proteins isolated from culture filtrates of *S. macromomyceticus*. Macromomycin D is a biologically inactive protein with an isoelectric point of 5.2. Biologically active protein having no absorbance at 355 nm is referred to as macromomycin A and has the same properties as macromomycin purified from auromomycin-free culture filtrates (Im et al., 1978; Yamashita et al., 1976; Vandrê et al., 1979). Auromomycin is the biologically active protein with an absorbance at 355 nm and has the same properties as previously reported (Yamashita et al., 1979). The macromomycin prepared by Kappen et al. (1980b), having no biological activity, is designated as apomacromomycin.

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should be noted that (a) in contrast with auromomycin the biologically active macromomycin used in several studies showed no absorption at 355 nm (Im et al., 1978; Yamashita et al., 1976; Vandrē et al., 1979), (b) macromomycin was only active in vivo while auromomycin was active both in vitro and in vivo (Kappen et al., 1979), and (c) two proteins designated MCR-1 and MCR-2 (Sawyer et al., 1979) and MCR-I and MCR-II (Woynarowski & Beerman, 1980) have been purified from the same crude culture filtrate, both having biological activity. The protein designated MCR-I contained a nonprotein chromophore, and both MCR-1 and MCR-I were more active than the MCR-2 or MCR-II peaks, respectively, suggesting that the more active proteins may be analogous to auromomycin.

The present study describes the purification and characterization of three related proteins, auromomycin, macromomycin A, and macromomycin D, from the same culture filtrate of *S. macromomyceticus*. Analysis of these proteins demonstrated that the biologically active proteins, auromomycin and macromomycin A, differed in the composition of their associated nonprotein chromophores. Macromomycin D is biologically inactive and differs from macromomycin A and auromomycin in both protein and chromophore content.

Materials and Methods

Macromomycin A, macromomycin D, and auromomycin were purified from the dried culture filtrates of *Streptomyces macromomyceticus*, NSC 170105, sample NF, obtained from Dr. John D. Douros, Natural Products Branch, Department of Toxicological Products, Division of Cancer Treatment, National Cancer Institute.

Amino Acid Analysis. The proteins were hydrolyzed with 6 N HCl at 110 °C for 24, 48, and 72 h. HCl was removed by rotary evaporation, and the samples were analyzed on an amino acid analyzer, Beckman Model 121 MB.

Isoelectric Focusing. Isoelectric points were determined with an LKB 8101 electrofocusing column containing 2% carrier ampholytes that covered the pH range 4–6.

Electrophoresis. The homogeneity of purified native proteins was examined by disc gel electrophoresis using 7.5% acrylamide gels as described by Davis (1965). Electrophoresis was carried out on 10-cm gels, at 2.5 mA/gel and approximately 200 V for 2.5–3.5 h. The initial pH of the electrode buffer was 8.9. Gels were stained for 30 min in 0.2% Coomassie brilliant blue R in 50% trichloroacetic acid (Cl_3CCOOH)[2] and destained with 7% acetic acid.

Absorption Spectra. Ultraviolet and visible absorption spectra were obtained on a Cary 17 spectrophotometer. Typically, protein solutions (1 mg/mL in 0.01 M Tris-HCl, pH 7.9) were scanned from 400 to 240 nm; a DXL Quartzline 650-W lamp was used.

Circular Dichroism. Circular dichroism was measured with a Cary Model 60 spectrophotometer. Aqueous protein solutions of 2 and 1 mg/mL concentration were used to fill cells of 1- and 0.01-cm path lengths, respectively. The longer path lengths were used for spectra from 430 to 235 nm and the 0.01-cm cells for spectra from 240 to 210 nm.

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Hitachi MPF-2A fluorometer. Samples were usually analyzed in 0.1 M Tris-HCl buffer, pH 7.9, at a concentration of 1 mg/mL. Emission spectra were recorded at excitation wavelengths of 280 and 360 nm. The temperature was regulated with a Laude K-2/R thermoregulator. Spectra were taken at 32, 42, 50, 60, 70, and 80 °C; the solutions were maintained at each temperature for 10 min prior to scanning. After the final spectrum at 80 °C was completed, the samples were cooled to 34 °C, and a second spectrum at this initial temperature was obtained.

Differential Scanning Calorimetry. Melting temperatures of the various purified proteins were determined by differential scanning calorimetry. Protein samples (2 mg/mL) were prepared in 0.01 M Pipes, pH 6.8. Calorimetry was preformed on a Microcal MC-1 scanning calorimeter. Protein and buffer samples were deaerated 15 min prior to loading in the dried sample cells, and 1 mL of sample was placed in each calorimetric cell. Sample cells were heated at 1 °C/min from approximately 10 to 85 °C, and the relative heat capacity was recorded on a 2-coordinate Omnigraphic 2000 recorder.

Antibody Preparation. Macromomycin A or D (0.5 mg) in 1 mL of phosphate-buffered saline was emulsified with 1 mL of Freund's complete adjuvant with a Mulsichurn. Initial subcutaneous footpad and abdominal immunization in rabbits was followed 30 days later by subcutaneous immunization with the same antigen mixed with Freund's incomplete adjuvant. Serum samples were collected weekly after the booster immunization. Ouchterlony immunodiffusion was carried out in 2% agar gels in 10 mM phosphate-buffered saline (140 mM), pH 7.4.

Aminoethylation. N-(Iodoethyl)trifluoroacetamide was used for the aminoethylation of cysteinyl residues, yielding the trypsin-susceptible protein containing 5-β-aminoethyl cysteinyl residues. Protein (1 mg/mL) was dissolved in 6 M guanidine hydrochloride buffered with 0.2 M *N*-ethylmorpholine, pH 8.1. The protein solution was placed in a water-jacketed flask. A 20-fold molar excess of dithiothreitol to cysteinyl residue was added, and the temperature of the reaction solution was adjusted to 50 °C. The solution was brought to pH 8.6 with 1 N NaOH, and the flask was flushed with nitrogen for 15 min. The nitrogen tubes were clamped, and the solution was maintained with stirring for 4–5 h to ensure complete denaturation. *N*-(Iodoethyl)trifluoroacetamide, dissolved in a minimal volume of methanol not to exceed a final 10% (v/v) mixture when added to the protein solution, was then added in two aliquots 1 h apart to a final 25-fold molar excess relative to the total thiol present. The reaction was stopped after a total of 6.5–7.5 h by the addition of acetic acid to pH 5.5. The solution was then extensively dialyzed against distilled water prior to lyophilization.

Peptide Mapping. Aminoethylated protein samples were digested with either trypsin (TPCK treated) or chymotrypsin in 0.1 M ammonium bicarbonate buffer, pH 8.1. Protein, present at 1–2 mg/mL, was incubated with enzyme (enzyme:protein ratio 1:100) for 18–24 h at 37 °C. The digests were lyophilized, redissolved in 0.1% (v/v) phosphoric acid at a concentration of 2 mg/mL, and analyzed by high-pressure liquid chromatography on an Altex system, using a 10-μm Lichrosorb C-18 column. The elution rate was 1.5 mL/min at a pressure of 2000–2500 psi; detection was at 210 nm with a full-scale absorbance range of 0.1, using 10–40 μg of polypeptide per injection. The program for elution of peptides used 0.1% phosphoric acid (v/v) (solvent A) and acetonitrile (solvent B). The sample was injected under column conditions of 100% solvent A; after 0.5 min, a linear gradient to 20% solvent B in 20 min was established; a second linear gradient was initiated at 20% solvent B to 75% solvent B during the

[2] Abbreviations: AUR, auromomycin; MCR-A, macromomycin A; MCR-D, macromomycin D; Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; Pipes, 1,4-piperazinediethanesulfonic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate.

next 20 min; after 75% solvent B was reached, the system was returned to 100% solvent A in 2 min and washed for 15 min prior to injection of the next sample. Total analysis time was 57.5 min. The phosphoric acid was filtered through a 0.2-μm filter and deaerated prior to use. The column was washed with methanol at the end of each day.

Extraction and Analysis of Chromophore. Macromomycin or auromomycin samples were placed in plastic centrifuge tubes. Methanol was added to give a fine suspension of 10 mg of protein/mL, formed by vortexing each sample. After the samples stood at room temperature for 1 h, the samples were placed at −20 °C for an additional 2 h. The insoluble protein was separated by centrifugation at 3000 rpm for 30 min, and the pellet was washed with methanol, centrifuged, and dissolved in 0.01 M Tris-HCl, pH 8.0. The methanol solutions were combined, evaporated under nitrogen, and stored at −20 °C. Prior to analysis by high-pressure liquid chromatography (HPLC), samples were redissolved in methanol. Briefly, 100 μL of redissolved methanol extract was injected onto a 5-μm Ultrasphere–ODS column (250 mm × 4.6 mm). The elution rate was 1.0 mL/min, and the sample was separated with a linear gradient from 48% methanol/water to 92% methanol/water over a 55-min period. Detection was either by absorption at 254 nm or by fluorescence excitation at 360 nm with a 418-nm emission cutoff filter, using a Schoeffel Model FS-970 detector. The amount of material in the injected sample was based on the equivalent dry weight of protein extracted.

Photoinactivation of methanol extracts was conducted in closed silica cells at a distance of 12 cm from a shortwave ultraviolet lamp ("Mineralite") providing 240 μW/cm^2 at 254-nm radiation. The antibacterial activity (minimum inhibitory concentration) was determined by sample diffusion from filter disks placed on Mueller-Hinton agar plates inoculated with *Sarcina lutea*.

Results

Purification of Proteins. A 4-g sample of the partially purified macromomycin (NSC 170105, sample NF) was dissolved in 10 mL of 0.01 M sodium phosphate buffer, pH 7.9, and applied to a column (30 × 4.5 cm) of DEAE-52 cellulose equilibrated with 0.01 M sodium phosphate buffer, pH 7.9. The active proteins were not retained on the ion-exchange resin, while the majority of a brown pigment remained bound. Two peaks, containing protein and showing biological activity, were partially separated on this column; the fractions collected were designated DEAE-1-1, yellow in color, DEAE-1-2, a shoulder to the first peak, and DEAE-1-3, a separate peak. After dialysis against water and lyophilization, fraction DEAE-1-3 was dissolved in 10 mL of 0.01 M Tris-HCl buffer and applied to a column (30 × 2.5 cm) of DEAE-cellulose equilibrated with 0.01 M Tris-HCl buffer, pH 7.9. The column was washed with starting buffer until no further protein was removed, requiring approximately 300 mL. This was immediately followed by elution with a linear gradient from 1000 mL of 0.01 M Tris-HCl buffer to 1000 mL of the same buffer, 0.16 M in sodium chloride. Two well-separated protein peaks were eluted from this column and designated DEAE-2-1 and DEAE-2-2. Fraction DEAE-2-1 contained all the biological activity. Peak DEAE-2-2 contained pure macromomycin D, based on polyacrylamide gel electrophoresis.

DEAE-1-2, containing primarily macromomycin A, was dissolved in ammonium sulfate (8% of saturation) and applied to a column of Amberlite XAD-7 (2.5 × 27 cm), also equilibrated in 8% saturated ammonium sulfate. The column was washed with 300 mL of 8% ammonium sulfate followed by 30% ethanol, which resulted in immediate elution of protein. The first half of the single broad protein peak (sample XAD-1-1) was orange in color, while the last portion of the peak (sample XAD-1-2) was colorless. Each of the two pooled samples was evaporated in vacuo to remove ethanol, and the remaining solution was dialyzed against water. Sample XAD-1-2, containing pure macromomycin A, was lyophilized. The orange color was removed from sample XAD-1-1 by a second chromatography on a column (1.8 × 28 cm) of DEAE-cellulose, equilibrated with 0.01 M Tris-HCl, pH 7.9, with a linear gradient from 0 to 0.16 M sodium chloride. A protein peak, eluting at 0.04 M sodium chloride (DEAE-3-1), also contained purified macromomycin A. The second peak (DEAE-3-2) was orange and contained a chromophore of auromomycin that had been separated during its conversion to macromomycin A on Amberlite XAD-7. Fractions XAD-1-2 and DEAE-3-1 were shown to have identical biological and spectral properties.

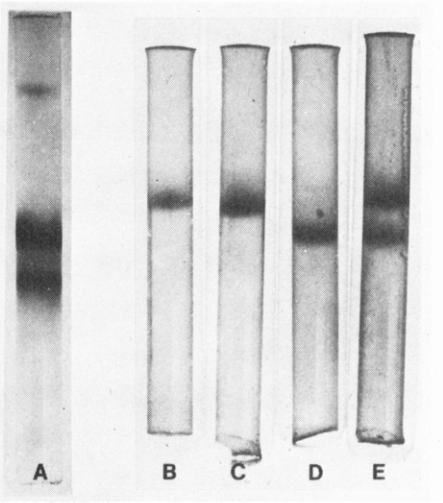

FIGURE 1: Davis polyacrylamide gels of proteins purified from the culture filtrate of *S. macromomyceticus*. (A) Crude culture filtrate NSC 170105 sample NF, 50 μg; (B) purified auromomycin, 25 μg; (C) purified macromomycin A, 25 μg; (D) purified macromomycin D, 25 μg; (E) mixture of purified macromomycin A, auromomycin, and macromomycin D, 10 μg of each protein.

Pure auromomycin was obtained by chromatography of peak DEAE-1-1 on a column (1.8 × 30 cm) of decylagarose, equilibrated with 0.1 mM sodium phosphate, pH 6.7, and made 25% of saturation with ammonium sulfate. A single broad yellow colored protein peak (DA-1) was eluted with equilibration buffer. Biologically inactive protein remained bound to the decylagarose and was eluted with water (DA-2). The pooled fractions were extensively dialyzed prior to lyophilization.

Each of the purified proteins gave a single protein band on Davis gel electrophoresis (Figure 1). Auromomycin and macromomycin A showed identical mobility, while macromomycin D migrated more to the anode. The altered mobility of macromomycin D shown by polyacrylamide gel electrophoresis correlated with the different retention it demonstrated on DEAE-52 columns with respect to macromomycin A and auromomycin. The isoelectric point of macromomycin D was 5.2, which is lower than that for both macromomycin A and auromomycin, p*I* 5.4 (Yamashita et al., 1979; Im et al., 1978). Denaturing $NaDodSO_4$–polyacrylamide gels, however, indicated identical molecular weights for the three proteins.

The amino acid composition of macromomycin D is similar to that of macromomycin A and auromomycin (Table I).

Table I: Amino Acid Composition of Auromomycin, Macromomycin A, and Macromomycin D[a]

amino acid	no. of residues		
	AUR	MCR-A	MCR-D
aspartic acid	7-8	7	7
threonine	17	17	17
serine	9	9-10	9-10
glutamic acid	8	8	8
proline	6	6	6
glycine	17-18	17-18	17-18
alanine	18-19	18-19	19-20
half-cystine	4	4	4
valine	19	18	18-19
methionine	0	0	0
isoleucine	3	3	3
leucine	4-5	4	4-5
tyrosine	1	1	1
phenylalanine	2	2	2
lysine	3	3	3
histidine	2	2	2
tryptophan	1	1	1
arginine	0	0	0

[a] All labile residues were obtained after extrapolation to zero time of hydrolysis. Tryptophan was determined spectrophotometrically in 0.1 N alkali.

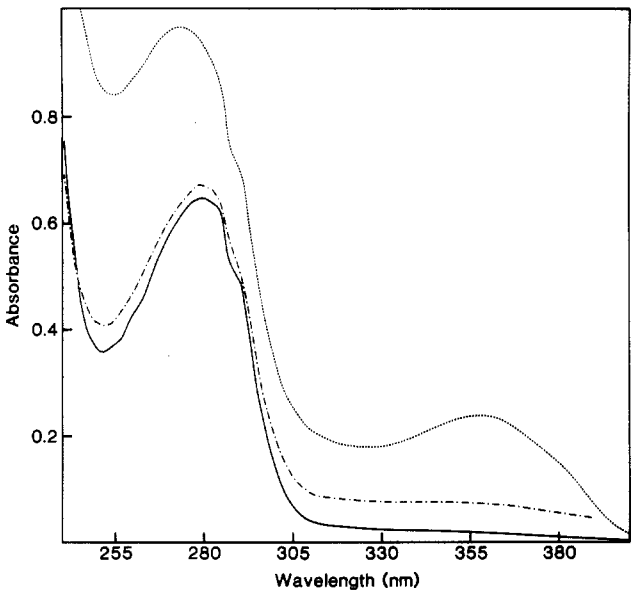

FIGURE 2: Ultraviolet and visible absorbance spectra of macromomycin A, macromomycin D, and auromomycin. Proteins (1 mg/mL) were dissolved in 0.01 M Tris-HCl, pH 7.9. Macromomycin A (—); macromomycin D (-···-); auromomycin (···).

Together with their similar behavior on ion-exchange chromatography, gel filtration, and polyacrylamide gel electrophoresis (Figure 1), the similarities between the three proteins were further established by immunological procedures. Antisera to macromomycins A and D were prepared in rabbits, and each of them demonstrated immunological identity with the three protein antigens when examined by Ouchterlony double diffusion.

Spectral Properties. The visible and ultraviolet absorbance spectra of macromomycins A and D were similar (Figure 2). Neither showed significant absorption in the visible region of the spectrum, but both had a similar maxima at 278 nm, a shoulder at 290 nm, and a trough between 245 and 255 nm. The auromomycin, however, showed a visible absorption with a maximum at 354 nm, and an ultraviolet maximum at 274 nm (Figure 2). The trough in the 250-nm region was also significantly different from the macromomycins, giving an

Table II: Ultraviolet and Visible Spectral Properties of Auromomycin and Macromomycins A and D

wavelength (nm)	absorption ($E^{1\%}_{1cm}$)		
	AUR	MCR-A	MCR-D
274	9.7		
278		6.5	6.3
354	2.4		
280/255	1.15	1.73	1.70

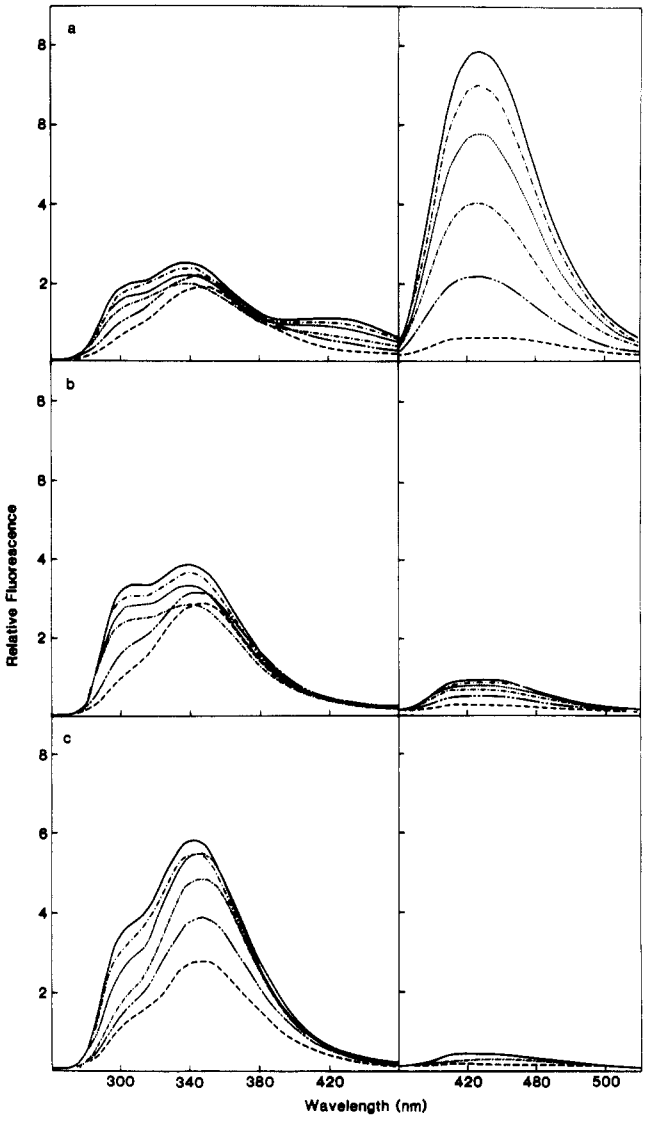

FIGURE 3: Thermal denaturation fluorescence emission spectra of (a) auromomycin, (b) macromomycin A, and (c) macromomycin D. A sample of protein (1 mg/mL) in 0.01 M Tris-HCl, pH 7.9, was heated by 10 °C increments to 80 °C. Fluorescence emission spectra were obtained after each sample had been at the specified temperature for at least 15 min. The first spectrum was obtained at 32 °C (—), followed by spectra at 42 (-···-), 50 (···), 60 (-·-), 70 (-··-), and 80 °C (---). Left panels, excitation at 280 nm; right panels, excitation at 360 nm.

A_{280}/A_{260} ratio of 1.15. The pertinent ultraviolet–visible absorbance data are summarized in Table II.

The fluorescence emission spectra of the three proteins were significantly different. Fluorescence emission of auromomycin (Figure 3a) was observed at an excitation of both 280 and 360 nm. Each protein had emission maxima at 305 and 340 nm at 30 °C by using an excitation of 280 nm (Figure 3a–c). The auromomycin showed a third smaller emission centered around 430 nm. Both macromomycin A (Figure 3b) and macro-

Table III: Fluorescence Emission Spectral Properties of Auromomycin and Macromomycins A and D

wavelength (nm)	temp of sample (°C)	fluorescence emission (rel intensity) AUR	MCR-A	MCR-D
304	32	2.00	3.35	3.70
	42	1.85	3.10	3.30
	50	1.70	2.80	2.80
	60	1.50	2.50	2.00
	70	1.20	1.90	1.70
	80	0.80	1.20	1.30
	32[a]	2.05	3.20	3.40
340	32	2.50	3.85	5.80
	42	2.40	3.60	5.50
	50	2.20	3.35	5.50
	60	1.95	2.85	4.90
	70	2.15	3.15	3.80
	80	1.85	2.90	2.70
	32[a]	3.20	4.40	6.80
427	32	7.90	0.80	0.30
	42	7.05	0.70	0.30
	50	5.80	0.65	0.25
	60	4.05	0.55	0.20
	70	2.20	0.40	0.15
	80	0.60	0.15	0.10
	32[a]	2.50	0.45	0.20

[a] Sample was cooled to 32 °C.

momycin D (Figure 3c) showed much reduced emission at 430 nm and significantly higher emissions at 305 and 340 nm than the auromomycin at 30 °C (Figure 3a). Macromomycin D could be distinguished from macromomycin A by the increased emission at 340 nm. The relative intensity of the emission peaks is summarized in Table III.

The excitation wavelengths of 280 and 360 nm were then used to examine each protein at different temperatures ranging in 10 °C intervals from 30 to 80 °C. A thermal transition in the 280-nm fluorescent emission spectra of auromomycin and macromomycin A (panels a and b of Figure 3, respectively) occurred between 60 and 70 °C. This transition was characterized by a decrease in the emission maximum at 305 nm and an increase in the maximum at 340 nm. Both of these changes deviated significantly from the expected linear decrease in emission peak intensity usually observed in model proteins with increasing temperature (Steiner & Edelhoch, 1963). A similar transition occurred in the macromomycin D spectrum, but between 40 and 50 °C (Figure 3c). In all three protein spectra, the emission maximum at 340 nm shifted to 345 nm after the thermal transition, and the 360-nm emission maximum at 430 nm was essentially absent at the higher temperatures. The changes in fluorescence emission intensity due to temperature are also summarized in Table III.

After the final spectrum at 80 °C was recorded, the protein samples were cooled to 30 °C, and a second spectrum at this temperature was taken (Figure 4a–c). Comparing the two spectra at 30 °C, it was obvious that the original spectra were not recovered upon cooling. In all cases, the emission maxima shift to 345 nm was retained, but the intensity of this peak was significantly higher than that in the original spectra before heating. Most significantly, the 430-nm emission maximum in auromomycin did not recover its initial intensity.

Since the emission maximum at 430 nm in auromomycin appeared to correlate with the presence of the yellow chromophoric group on this protein, the fluorescence spectra suggested that this group might have been modified after heating, seen also in the visible and ultraviolet absorbance spectra (Figure 5) in which the absorbance maximum at 354 nm in the native protein was shifted to shorter wavelengths

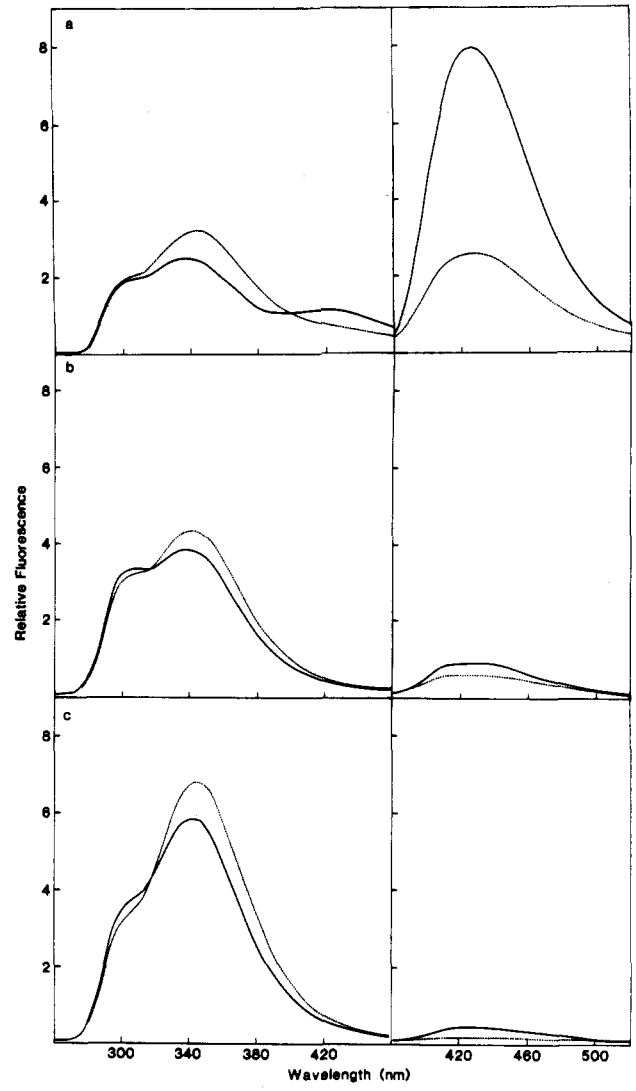

FIGURE 4: Comparative fluorescence emission spectra of (a) auromomycin, (b) macromomycin A, and (c) macromomycin D following heating and cooling. A sample of protein (1 mg/mL) in 0.01 M Tris-HCl, pH 7.9, was heated by 10 °C increments over a period of 2.5 h to 80 °C. The sample was rapidly cooled to 32 °C, and the fluorescence emission spectrum was obtained. Protein prior to heating (—) and protein after heating and cooling (···). Left panels, excitation at 280 nm; right panels, excitation at 360 nm.

with no definitive maximum. A second spectrum, determined after the heated sample had been dialyzed overnight, showed a decrease in the absorbance by the chromophore. This pointed not only to modification of the chromophore but also to its dissociation from the protein of auromomycin (Figure 5).

Differential Scanning Calorimetry. The observed thermal transitions demonstrated a change in the conformation of the proteins at elevated temperatures. Differential scanning calorimetry showed that the fluorescence transitions correlated with protein denaturation. As can be seen (Figure 6), macromomycin A and auromomycin had similar thermal-stability profiles with the peak of the transition occurring at 70.5 °C. Macromomycin D, however, showed less thermostability, melting at 57.0 °C. The calorimetric transitions occurred at the same temperatures as those observed in the fluorescence emission spectra. When the samples were cooled and reheated, all three proteins showed thermal-stability profiles identical with their respective initial profiles.

Circular Dichroism Spectroscopy. The circular dichroism spectra were essentially identical for macromomycins A and D (Figure 7). In the region of the peptide absorbance

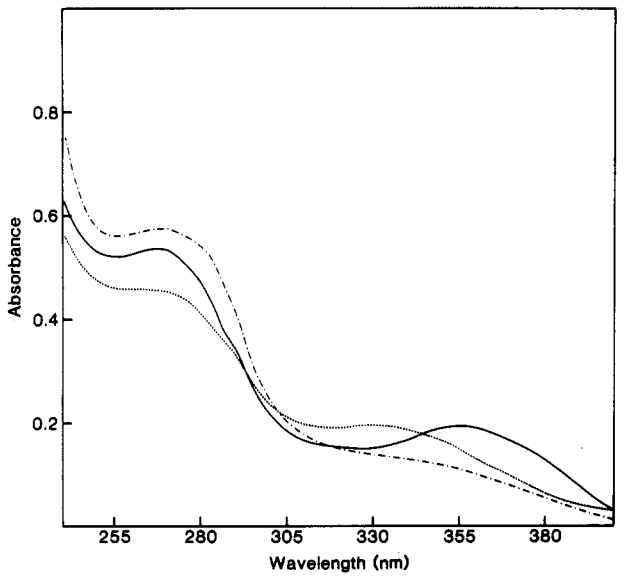

FIGURE 5: Ultraviolet and visible absorbance spectra of auromomycin following heating. A sample of auromomycin (1 mg/mL) in 0.1 M Pipes, pH 6.5, was heated over a 60-min period to 85 °C. The ultraviolet and visible spectra were obtained prior to heating (—), after heating and cooling to room temperature (···), and after dialysis of the heated and cooled sample (-·-·-).

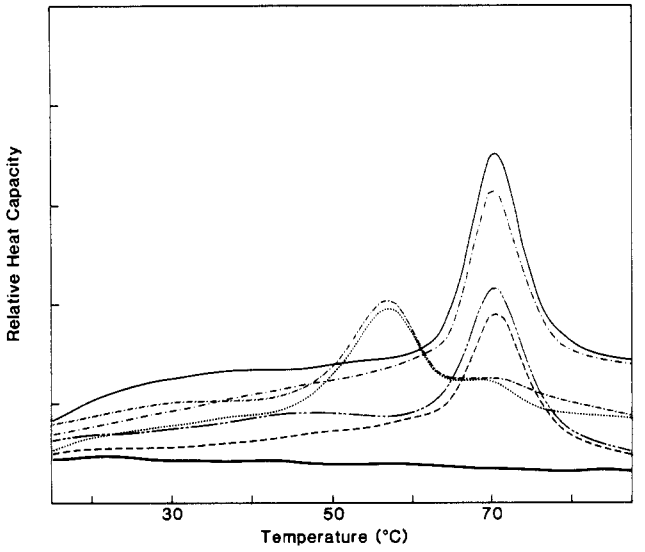

FIGURE 6: Differential scanning calorimetry of auromomycin and macromomycins A and D. Protein samples (2 mg/mL) in 0.01 M Pipes, pH 6.8, were heated at 1 °C/min from 4 to 85 °C: macromomycin A (—); macromomycin B (-·-·-); and auromomycin (-·-·-). The samples were then cooled and remelted: macromomycin A (-·-); macromomycin D (···); and auromomycin (-·-).

(210–245 nm), the spectrum of auromomycin was also identical, but significant differences were observed at the longer wavelengths (Figure 7). Specifically, a negative peak of ellipticity present in auromomycin was twice that in the macromomycins. Also auromomycin showed a positive peak centered around 375 nm that was absent in either the macromomycin A or the macromomycin D spectra. The calorimetric analysis indicated that the polypeptide chain resumed a native configuration following cooling after thermal denaturation. This was consistent with the same circular dichroism before and after heating.

The proteins have four cysteinyl residues per mole and no free sulfhydryl groups, indicating the presence of two disulfide bonds (Im et al., 1978). This, and a negative ellipticity at 210 nm indicative of a large percentage of β-pleated sheet structure,

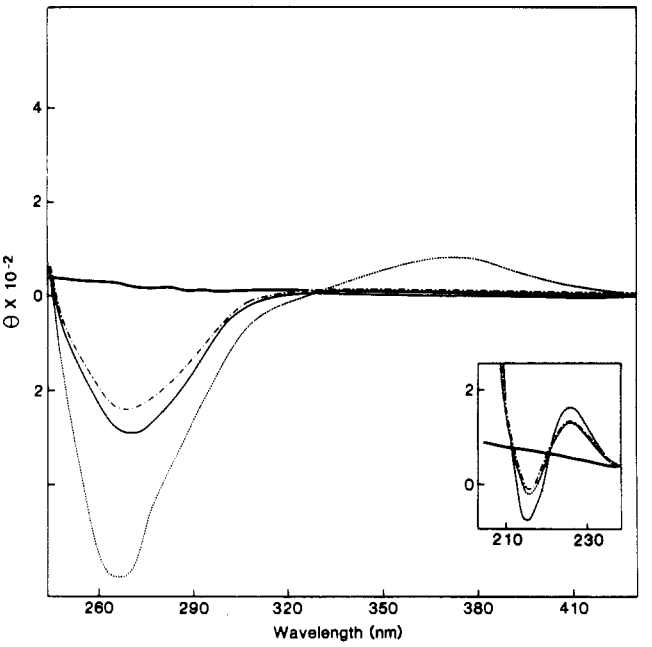

FIGURE 7: Circular dichroism spectra of auromomycin and macromomycins A and D. Aqueous protein solutions were deaerated and scanned from 430 to 210 nm. The spectra from 430 to 235 nm were of a 2 mg/mL solution in a cell of 1-cm path length. The spectra from 240 to 210 nm (inset) were of a 1 mg/mL protein solution in a cell of 0.01-cm path length: auromomycin (···); macromomycin A (—); and macromomycin D (-·-·-).

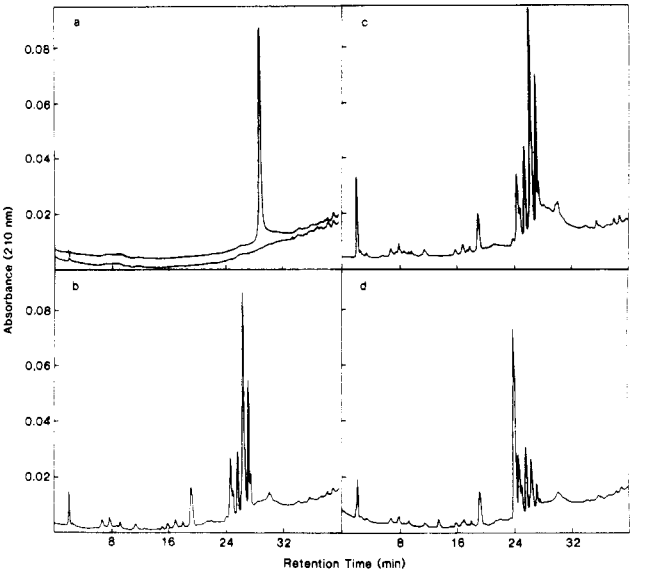

FIGURE 8: Chymotryptic peptide maps determined by HPLC. Aminoethylated protein was digested overnight with chymotrypsin at 37 °C. The sample was centrifuged, and 10 μL of the supernatant containing approximately 30 mg of protein was injected onto a Lichrosorb C_{18} reverse-phase column. The sample was eluted with a 0.1% phosphoric acid–acetonitrile gradient. (a) Undigested macromomycin D and base line; (b) auromomycin; (c) macromomycin A; (d) macromomycin D.

was consistent with a tightly folded native configuration, which contributed to the general resistance of the proteins to trypsin or chymotrypsin.

Peptide Mapping. The native proteins were aminoethylated to expose sites sensitive to proteolysis. Peptide maps were obtained by high-pressure liquid chromatography on a column of Lichrosorb C_{18}. Aminoethylated macromomycin D was the only intact protein that could be eluted from the Lichrosorb C_{18} column (Figure 8a); the aminoethylated derivatives of

Table IV: Antibacterial Activity of Auromomycin and Macromomycins A and D against *S. lutea*

protein	minimum inhibitory concn (mg/mL)
auromomycin	0.03
methanol-extracted protein	>2.0
methanol extract[a]	0.08
heated to 70 °C for 20 min	0.03
heated to 80 °C for 10 min	>2.0
macromomycin A	0.02
methanol-extracted protein	>2.0
methanol extract[a]	0.08
heated to 70 °C for 20 min	0.02
heated to 80 °C for 10 min	0.03
macromomycin D	>10.0
apomacromomycin[b]	>10.0

[a] Activity present in the methanol extract is based on the equivalent weight of protein from which the extract was obtained. The HPLC peak eluting between 37 and 38 min contained all the biological activity present in the extract. [b] Apomacromomycin was prepared by elution of protein with water from Amberlite XAD-7 when previously loaded with purified auromomycin or macromomycin A in 8% ammonium sulfate. In each case, biologically active protein was obtained by elution with 30% ethanol following elution with water.

macromomycin A or auromomycin remained bound to the column (Figure 8a).

The tryptic and chymotryptic digests of the proteins gave complex elution patterns. The chymotryptic digests of auromomycin and macromomycin A (panels b and c, respectively, of Figure 8) both showed the same seven major peaks and several minor peaks; the most intense peak had a retention time of 26.5 min. Seven major peaks are also present in the chymotryptic digest of macromomycin D (Figure 8d); however, the most intense peak has a retention time of 24.0 min. The other major and minor peaks have comparable retention times to those of auromomycin and macromomycin A (panels b and c, respectively, of Figure 8).

Similarly, the tryptic digests of auromomycin and macromomycin A have major peaks with identical retention times. Again, the tryptic digest of macromomycin D has a major peak with a shorter retention time (24.5 min) than the major peak in the auromomycin and macromomycin A profiles (28.2 min); the other peaks have similar retention times.

Chromophore. Auromomycin and macromomycins A and D were extracted with glacial acetic acid, methanol, 1-butanol, and *tert*-butyl alcohol in an attempt to remove the nonprotein chromophoric groups. Only extraction with methanol led to the removal of an ultraviolet-absorbing material. Thin layer chromatography of the proteins on silica gel H plates ($CHCl_3$–MeOH–acetic acid–H_2O, 25:15:4:2 v/v) showed the separation of fluorescent material from only auromomycin.

The chromophoric material present in the methanol extract of auromomycin was separated by HPLC into five major peaks absorbing at 254 nm, two of which were also highly fluorescent (Figure 9a). The peak eluting from the column between 37 and 38 min contained all the antibiotic activity (Table IV). The chromophore from macromomycin A (Figure 9b) was not as complex; three major peaks were detected, none of which were significantly fluorescent. As with the auromomycin, the peak with detectable antibiotic activity eluted between 37 and 38 min and represented 5–8% of the amount of the active peak from auromomycin per milligram of dry protein.

The methanol extracts of macromomycin D and apomacromomycin contained no components on HPLC chromatography (panels c and d, respectively, of Figure 9) with retention times similar to those of the active peak from auromomycin or macromomycin A. Finally, the biologically inactive protein eluting from decylagarose with water after the auromomycin had been removed was examined for chromophore content. As with macromomycin D, several peaks having 254-nm absorbance were obtained, but the peak corresponding to the biologically active peak from auromomycin and macromomycin A was absent.

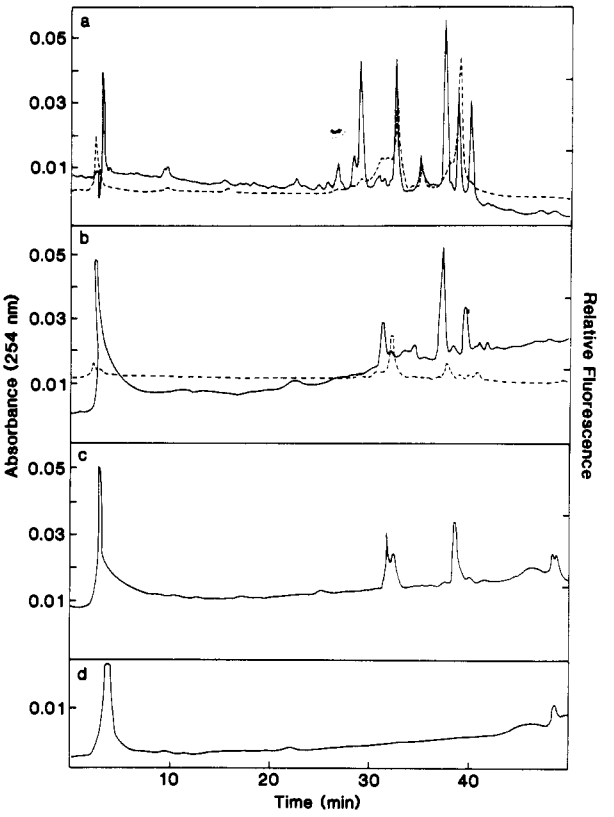

FIGURE 9: Reverse-phase HPLC separation of methanol extract obtained from (a) 1 mg equiv wt of auromomycin, (b) 10 mg equiv wt of macromomycin A, (c) 10 mg equiv wt of macromomycin D, and (d) 10 mg equiv wt of apomacromomycin. Ultraviolet-absorbing peaks were detected at 254 nm (—), and fluorescent emitting peaks were detected at an excitation wavelength of 360 nm (---).

Antibacterial Activity. Antibacterial activity was present in auromomycin and macromomycin A. The minimal inhibitory concentration of each protein to *Sarcina lutea* is listed in Table IV. Macromomycin D and apomacromomycin were devoid of any such activity, and pretreatment of assay plates with macromomycin D for up to 60 min had no effect on the activity of auromomycin or macromomycin A.

After being heated to 70 °C for 20 min, both macromomycin A and auromomycin retained their original antibacterial activity. However, when heated to 80 °C for 10 min, the auromomycin sample lost all activity, while macromomycin A showed only a partial loss of activity even after 30 min (Table IV).

Photoinactivation. Macromomycin A was inactivated by ultraviolet irradiation in a time-dependent fashion and was protected from photoinactivation by free-radical scavenging agents (Im et al., 1978). Auromomycin was less sensitive to the effects of ultraviolet irradiation than macromomycin A; however, the biological activity present in the methanol extracts of each protein was lost rapidly upon irradiation (Table V). The loss in antibiotic activity of the methanol extract of auromomycin correlated with a time-dependent loss in peak area of the biologically active component as separated by reverse-phase HPLC (data not presented).

Table V: Effect of Ultraviolet Irradiation on the Antibiotic Activity of Auromomycin and Macromomycin A[a]

time of irradiation (min)	% of activity remaining			
	AUR	AUR-MeOH extract	MCR-A	MCR-A-MeOH extract
0	100	100	100	100
2.5	97	44	79	83
5	94	32	72	44
10	91	18	61	18
20	83	0	37	0
30	83	0	6	0
45	77	0	0	0
60	67	0	0	0

[a] Samples were irradiated for various periods of time at a distance of 12 cm from a shortwave ultraviolet lamp (Mineralite). The area of the zone of inhibition at zero time of irradiation was set at 100% for each sample, and the activity remaining was based on comparison of the inhibition zone area after appropriate times.

Discussion

Three related polypeptides in the culture filtrate of *S. macromomyceticus*, auromomycin, macromomycin A, and macromomycin D, show acidic isoelectric points, amino acid composition, and mode of biological action similar to those for a group of acidic polypeptides isolated from other actinomycetes, including actinoxanthin [*Actinomyces globisporius* (Khokhlov et al., 1969)] and neocarzinostatin [*Streptomyces carzinostaticus* (Ishida et al., 1965)].

Separation of macromomycin D from macromomycin A and auromomycin took advantage of the difference in their isoelectric points by chromatography on DEAE-cellulose and of the difference in hydrophobicity by the purification of auromomycin on decylagarose. Alternatively, macromomycin A was purified by chromatography on Amberlite XAD-7 during which the small amount of contaminating auromomycin is converted to macromomycin A, as reported earlier by Yamashita et al. (1979). In all studies, macromomycin A prepared as above was identical with that from culture filtrates containing no auromomycin (Im et al., 1978). The polarity of the solvent used for the conversion of auromomycin to macromomycin A on Amberlite XAD-7 was a critical step in the purification process. Both the present studies and those of Yamashita et al. (1979) resulted in the isolation of active macromomycin A from the Amberlite XAD-7 column by elution with 30% ethanol. However, elution of the purified macromomycin A from the Amberlite XAD-7 column with water gave an inactive product, apomacromomycin (Kappen et al., 1980b).

The polypeptides of auromomycin, macromomycin A, and macromomycin D were comparable in amino acid composition and antigenicity. None of the three proteins contain methionine or arginine, and the other amino acids were present in the same relative quantities. The partial amino acid sequence of macromomycin A determined through residue 45 (Sawyer et al., 1979) demonstrated that a high degree of sequence homology existed between macromomycin A, neocarzinostatin, and actinoxanthin (Montgomery et al., 1981). As with the macromomycins, an analogue of neocarzinostatin, preneocarzinostatin, has also been reported (Kikuchi et al., 1974; Maeda & Kuromizu, 1977). It is more acidic than native neocarzinostatin by a single charge, p*I* 3.26 and 3.34, respectively, and is devoid of antibacterial activity. The relationship between macromomycin D and macromomycin A is not as clearly defined. However, samples of pure macromomycin A have been shown by gel electrophoresis to contain small amounts of macromomycin D after a long period of storage at −20 °C. The single charge difference in macromomycin D may be the result of a deamidation or other molecular degradation in macromomycin A as noted for neocarzinostatin and preneocarzinostatin.

Both macromomycin A and auromomycin showed identical thermal stability (Figure 6); a lower degree of thermal stability observed with macromomycin D suggests that the more negative charge, as evidenced by the isoelectric focusing and gel electrophoresis, destabilized the folded protein.

The fluorescence emission spectra of macromomycins A and D and auromomycin reflected the same transition that occurred at the denaturation temperatures as determined by calorimetry. The intensity of fluorescence emission of tyrosyl (304-nm emission) and tryptophanyl (330–350-nm emission) residues decreased linearly as expected with increasing temperature to the point of denaturation. The deviation from linearity in the change of the fluorescence emission in macromomycin A and auromomycin occurred between 60 and 70 °C, while that of macromomycin D was between 40 and 50 °C. This change was not reversible, differing from the comparable calorimetric behavior. It would appear that such an environmental change occurred at a tryptophan residue in macromomycin and auromomycin, leading to both a shift in emission maxima from 340 to 345 nm and greater intensity of fluorescence.

Fluorescence emission studies of 21 different proteins led Teale (1960) to propose that proteins containing both tryptophan and tyrosine demonstrate only emission characteristics of tryptophan. Two other antitumor proteins, neocarzinostatin (Napier et al., 1980) and largomycin (D. D. Vandré et al., unpublished results), contain both tyrosine and tryptophan residues but show fluorescence emission characteristic of tryptophan only. Tyrosine fluorescence emission can be detected in proteins that contain tyrosine but not tryptophan. Auromomycin and macromomycins A and D contain one tyrosyl residue and one tryptophanyl residue but show fluorescence emission spectra with some characteristics of both residues. To this uncommon property may be added the pH dependence of fluorescence emission spectra of macromomycin A. At pH 2.5–4.0, the intensity of the emission maximum at 304 nm increased approximately 2-fold over that in neutral solutions, while at pH >10 the intensity was unchanged. In free tyrosine solutions, or di- and tripeptides containing a tyrosyl residue, the fluorescence intensity of the 304-nm peak is quenched at both high and low pH values (Russell & Cowgill, 1968). A similar decrease in the intensity of tyrosine fluorescence in ribonuclease (containing six tyrosine and no tryptophan residues) was noted in alkali, but it increased sharply below pH 4, where a change in the difference spectra of RNase has been ascribed to the loss of hydrogen bonding between "abnormal" tyrosyl residues and carboxyl groups in the molecule (Cowgill, 1964; Scheraga, 1957). The fluorescence emission behavior in auromomycin, macromomycin A, and macromomycin D at 304 nm may be indicative of unusual environmental conditions in which the residue is involved in hydrogen bonding to another residue in the protein or to the associated prosthetic group. The absence of any fluorescence in the nonprotein chromophore of macromomycin A tends to exclude this group from giving rise to the 304-nm emission.

Although auromomycin and macromomycin A were denatured at 70.5 °C, the antibacterial activity was not affected after the protein was heated for short periods of time at this temperature. Raising the temperature to 80 °C for 10 min destroyed the antibacterial activity present in auromomycin

without a similar effect on the biological activity of macromomycin A. The loss of biological activity corresponded to changes in the visible absorption spectrum of auromomycin, in which the visible peak maximum at 354 nm was shifted to shorter wavelengths after heating and, following dialysis of this sample, its intensity was decreased. Unlike the fluorescence emission peaks at 304 and 340 nm after heating and cooling, the fluorescence maximum at 430 nm of auromomycin was only 39% of the intensity observed in native auromomycin. These spectral results, in conjunction with the observed loss of biological activity, indicate a change in conformation, together with dissociation of a nonprotein component of auromomycin with heating.

Comparative peptide maps of auromomycin, macromomycin A, and macromomycin D were obtained on the aminoethylated derivatives of the reduced proteins. The tryptic and chymotryptic maps of these derivatives of auromomycin and macromomycin A further supported the identity between their respective polypeptide components. The chymotryptic digests of aminoethylmacromomycin D differed from those of aminoethylmacromomycin A and aminoethylauromomycin by the presence of a single peak of shorter retention time (24 min) and the absence of a single peak that corresponded to one found in the digests of the other proteins (26.5 min). Since separation of peptides is based upon both hydrophobic and ionic interactions, the observed difference in retention times probably indicates a charge difference between the amino acid residues on this peptide.

The chromophore material in each protein that was extracted with methanol showed differences by HPLC. Auromomycin, which is 10 times more active against KB cells than macromomycin, yielded a complex chromatograph in which the active material elutes between two strongly fluorescent peaks (Figure 9a). Similar results were noted by Kappen et al. (1980a). The same active component, but at a concentration of 8% of that in the corresponding auromomycin extract, was found in macromomycin A with, however, the absence of any fluorescent material (Figure 9b). These differences in the amount of the fluorescent peaks present in the two methanol extracts indicate the purified macromomycin A obtained here is not contaminated by auromomycin. It does demonstrate, however, that during chromatography on Amberlite XAD-7 the various nonprotein components are removed with varying efficiencies. No peak corresponding to the active component was observed in the chromatograph of the methanol extracts of the biologically inactive macromomycin D or apomacromomycin. It is noted that the chromophore of neocarzinostatin was similarly extracted by methanol and removed by Amberlite XAD-7 (Napier et al., 1980). The chromophore contained a residue of methyl 2-hydroxy-5-methoxy-7-methyl-1-naphthalenecarboxylate (Edo et al., 1980) attached to a dideoxygalactose moiety through a C_{15} residue of unknown structure (Albers-Schönberg et al., 1980). A carbonyl group present in the C_{15} residue is required for biological activity. A carbonyl group may be present in the chromophores of auromomycin and macromomycin A since antibacterial activity is lost upon sodium borohydride reduction of the protein or reaction with 5 mM phenylhydrazine (D. D. Vandrē and R. Montgomery, unpublished results). A similar compound, 3-methoxy-5-methyl-1-naphthalenecarboxylate, was isolated from the antitumor protein carzinophilin, produced by *Streptomyces sahachiroi* (Onda et al., 1969).

The properties of auromomycin and the macromomycins support a molecular model in which auromomycin is composed of a polypeptide complexed to two chromophoric compounds, one of which (C_1) is removed by XAD-7 chromatography to give macromomycin A. As a result of this loss of C_1, the activity of macromomycin A is more sensitive to ultraviolet irradiation but is more resistant to heat. Chromophore C_1 is not active as an antibiotic or antitumor compound, these activities residing in the second chromophore, C_2, removal of which from macromomycin A by methanol extraction leaves the inactive apomacromomycin. The polypeptide of the apomacromomycin is different from macromomycin D, which is inactive and produced from macromomycin A on long standing at −20 °C; macromomycin D has a more acidic p*I* and may therefore result from loss of C_2 and hydrolysis of an amide group, which, however, has not been demonstrated experimentally. The polypeptides in auromomycin and macromomycin A are reversibly denatured by heat, as demonstrated by differential calorimetry and circular dichroism, but the fluorescence and visible spectrum of auromomycin are changed, indicating an irreversible reordering of C_1 in the complex after heating. The protective effect of C_1 on C_2 in the ordered complex of auromomycin is further demonstrated by the relative losses of activity following irradiation with ultraviolet of the methanol extracts of both auromomycin and macromomycin A, in the presence or absence of the apoprotein.

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Hydrodynamic Determination of Molecular Weight, Dimensions, and Structural Parameters of Pf3 Virus†

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ABSTRACT: Measurements of the translational, D_T, and rotational, D_R, diffusion coefficients of Pf3 virus by low-angle polarized intensity fluctuation spectroscopy and field-free transient electric birefringence, respectively, give a length of 720 ± 25 nm and diameter of 6.5 ± 1.5 nm upon simultaneous solution of the Broersma equations for rigid rods. Sedimentation coefficient and density increment values obtained under solvent conditions identical with those of D_T give a molecular weight of (13.4 ± 0.8) × 10^6 g mol^{-1}, which gives a mass per length of 18 600 ± 1300 g mol^{-1} nm^{-1}. Combining these results with the molecular weight of Pf3 DNA yields a number of protein subunits of 2500 ± 160 and 2.38 ± 0.14 nucleotides/protein subunit. Sedimentation coefficient and density increment values of Xf virus when combined with a value for the Xf translational diffusion coefficient [Chen, F. C., Koopmans, G., Wiseman, R. L., Day, L. A., & Swinney, H. L. (1980) *Biochemistry 19*, 1373] yield a molecular weight of (17.9 ± 1.0) × 10^6 g mol^{-1}, a number of protein subunits of 3590 ± 230, 2.07 ± 0.15 nucleotides/protein subunit, and a mass per length of 18 300 ± 1200 g mol^{-1} nm^{-1}. Thus, despite major differences in the DNA–protein packing between these viruses, as well as fd virus, the mass per lengths are surprisingly similar.

Structural data that have been gathered on a group of filamentous viruses, all of which have Gram-negative bacteria as hosts and all of which contain a circular single-stranded DNA molecule packed in a helical sheath of protein subunits, indicate considerable differences in the DNA structures maintained in the viruses, in the nature of chemical contacts between DNA and protein, and in the way differing symmetries of DNA and protein are meshed [Day & Wiseman (1978) Day et al. (1979), Marzec & Day (1980), Banner et al. (1981) Makowski & Caspar (1981), Nave et al. (1981), and Casadevall & Day (1982) are recent papers describing comparative data and current ideas]. The structural differences are striking in view of close similarities in overall morphology and in mass per length and the common function of packing single-stranded circular DNA. One of the main structural tools is X-ray fiber diffraction, but proper interpretation of fiber patterns is critically dependent on external input such as the size of the structural units and the mass per length. Indeed the currently accepted 5-fold rotational symmetry for fd (Makowski & Caspar, 1978, 1981) became apparent after the mass per length of fd was accurately determined (Newman et al., 1977). X-ray fiber studies are now underway on Pf3 virus (W. Winter, personal communication), and mass per length values are needed for that study. In addition, currently available data suggest that Pf3, like fd, may have a noninteger number of nucleotides per subunit, and establishment of this is also critical for structure studies. For these reasons an attempt was made to obtain independent measures of the mass, the mass per length and, through them, the chemical stoichiometry of Pf3 virus by carrying out measurements of its hydrodynamic properties. We have also gathered density and sedimentation velocity data on Xf virus, which we combine with the diffusion coefficients and hydrodynamic dimensions established by Chen et al. (1980). The

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