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DNA Breakage Activity of the Methanol Extract of Auromomycin

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Summary. *The constituents of the antitumor agent auromomycin have been analyzed to determine their DNA-breakage activities. Spectral analysis showed that the methanol extract contained 70% of the non-peptide chromophore, whereas the residue contained 20%. Amino acid analysis of the methanol extract showed that it contained 21%–26% of the original auromomycin polypeptides.*

The DNA-degradation activity of the extract was 121% ± 28% of that of the untreated auromoycin, whereas that of the residue was only 22% ± 3.8%. Mixing of the residue and the methanol extract resulted in the loss of three-fourths of the total activity. Agarose gel electrophoretic analysis showed that the single-strand DNA breakage activity of the methanol extract was 6.5-fold greater than that of the double-strand DNA-breakage activity.

The difference in the total DNA-cleavage activity of the untreated, methanol-treated, and remixed auromomycin preparations may suggest the occurrence of certain non-peptide chromophore-polypeptide interactions in both the untreated and the remixed preparations. This is consistent with the fluorescent changes observed upon mixing of the extract and residue.

Fractionation of the methanol extract by Sephadex chromatography revealed that several column fractions which were enriched with non-peptide chromophore relative to the polypeptides contained in them still had significant DNA-degradation activity. These studies suggest that the non-peptide chromophore in the auromomycin preparation may contribute to most of the observed DNA breakage activity.

Introduction

Auromomycin is an antitumor antibiotic currently believed to be a complex between macromomycin and a non-peptide chromophore. Macromomycin is a polypeptide (12,500 mol. wt) originally isolated by Chimura et al. [1] from a culture filtrate of *Streptomyces macromomycetius*. Macromomycin has been shown to cause inhibition of DNA synthesis and DNA-strand breakage in cultured HeLa cells [20]. Auromomycin, isolated from the same culture broth as macromomycin, has been found to possess many chemical and physical properties similar to those of macromomycin [24]. However, Yamashita et al. [24] found by absorbance spectrophotometry and circular dichroism that the auromomycin preparation contains a non-peptide chromophore which is not present in the macromomycin preparation.

Previous studies [4, 20–22] suggest that auromomycin exerts its primary cytotoxic effect by degradation of DNA. Suzuki et al. [22] demonstrated that auromomycin inhibits DNA synthesis and causes DNA-strand scission in mouse lymphoblastoma L5178Y cells in tissue culture. A similar effect was also observed in intact cells of *Bacillus subtilis* [21]. The same workers [20] also studied the differences in the ability of both macromomycin and auromomycin to degrade PM-2 DNA in vitro.

The auromomycin preparation was able to induce DNA breakage without additional co-factors, while the macromomycin preparation required the presence of specific reducing agents such as sodium borohydride or dithiothreitol [20]. Kappen et al. [3] reported that purified macromomycin had minimal degradation activity on isolated DNA. These authors

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Abbreviations used in this paper: Tris, tris(hydroxymethyl) aminomethane; EDTA, ethylenediaminetetraacetic acid; EC_{50}, the concentration of an agent needed to cause 50% degradation of the DNA molecules present in a reaction mixture

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suggested that previous reports [15] on the DNA-breakage activity of macromomycin may have been caused by contamination of the particular preparations with auromomycin.

Both methanol extraction and column chromatography have been used to remove non-peptide chromophores from polypeptide antitumor antibiotic preparations. Recently Napier et al. [9] extracted and characterized the non-peptide chromophore from the antitumor antibiotic neocarzinostatin. A similar extraction procedure was utilized by Shoh [16] in the isolation and characterization studies on the carzinostatin complex. Yamashita et al. [24] were able to remove the auromomycin non-peptide chromophore from the auromomycin polypeptide(s) by column chromatography on Amberlite XAD-7.

In this paper we have characterized an auromomycin preparation with the aid of a methanol extraction procedure. In addition, the PM-2 DNA-breakage activities of the extract and the residue were investigated. The relationship of the polypeptide and the non-peptide chromophore to the observed DNA-breakage activity is discussed. Some of the results of this study have been reported in abstract form [2].

Materials and Methods

Chemicals and Reagents. Auromomycin (100 mg) was obtained as a lyophilized powder from Kanegafuchi Chemical Company (Japan). All other commercial chemicals and reagents were obtained from the following sources: methanol, reagent grade, from Fisher Scientific Co., Fairlawn, NJ, USA; Tris base, ethidium bromide, EDTA, and bromophenol blue from Sigma Chemical Co., St Louis, MO, USA; constant boiling 6 *N* hydrochloric acid from Pierce Chemical Co., Rockford, IL, USA; G-25 medium grade Sephadex from Pharmacia Fine Chemicals, Piscataway, NJ, USA; 2-mercaptoethanol from Mallinckrodt, St Louis, MO, USA; and Agarose ME from Miles Laboratories, Elkhart, IN, USA.

The PM-2 DNA used in this study was prepared by the method described by Salditt [13] and purified on a cesium chloride-ethidium bromide density gradient and dialyzed against sodium chloride ranging in concentration from 40 m*M* to 200 m*M*.

Extraction of Auromomycin. All reactions and handling methods involving auromomycin were performed in minimal light. The extraction procedure used was a modification of the one described previously by Napier et al. [9]. Auromomycin samples (1–4 mg) were extracted with methanol (1 ml/mg auromomycin) by shaking continuously for 5 min at room temperature, and then centrifuged at 755 *g* for 30 min in a refrigerated centrifuge. The methanol supernatant was removed and the residue was re-extracted two additional times. The combined supernatants and the residue were dried by forced air evaporation.

Absorption and Fluorescence Studies. The absorption spectra of untreated auromomycin, the methanol extract, and the residue were determined on a Hitachi 110 double-beam spectrophotometer. The fluorescence emission and excitation spectra (obtained in 50 m*M* Tris-HCl, pH 8.5) were measured with an Aminco SPF-500 ratio spectrofluorometer equipped with an X-Y recorder. Uncorrected fluorescence spectra were reported.

Alkaline Ethidium Bromide Fluorescence Assay of PM-2 DNA Breakage. This DNA breakage assay was described by Strong and Crooke [18]. All samples were dissolved in 50 m*M* Tris-HCl pH 8.5) and incubated in total darkness. Triplicate 100-µl aliquots were removed and each one was separately added to 900 µl denaturation buffer which contained 40 m*M* sodium triphosphate, 150 m*M* sodium chloride, 10 m*M* EDTA, and 150 m*M* sodium hydroxide, pH 12.1. One hundred microliters of 22 µg ethidium bromide/ml denaturation buffer was then added to each of the above tubes. The fluorescence change at 590 nm versus a DNA control was determined on an Aminco-Bowman spectrofluorometer with an excitation at 530 nm. The reduction in the ethidium bromide fluorescence, when compared with control, indicates the extent of total DNA breakage, which includes single-strand breakage, double-strand breakage, and alkaline-labile damage.

Agarose Gel Electrophoretic Analysis of Single-strand and Double-strand DNA Breakage. This technique for analyzing single-strand and double-strand DNA breakage has been described previously [18]. Of the reaction mixture, 50 µl, containing 2.4 µg PM-2 DNA, extract, or untreated auromomycin, and 50 m*M* Tris buffer (pH 8.5) was added to an 80% glycerol-water solution containing 40 m*M* EDTA, 0.05% bromophenol blue, and 280 m*M* 2-mercaptoethanol. Samples (50 µl) were removed and placed on a 0.9% agarose slab gel and electrophoresed at 50 V for 8 h at room temperature, running buffer containing 40 m*M* Tris-HCl, 5 m*M* sodium acetate, and 1 m*M* EDTA (pH 7.8) being used. The gel was then stained with 0.5 µg ethidium bromide/ml in electrophoresis buffer and was then photographed with a Polaroid CU-5 Land Camera while being illuminated with a transilluminator (Ultra-violet Products, Inc.).

Densitometric scans of the photographic negative were performed on a Transidyne 2955 scanning densitometer equipped with a computing integrator.

In agreement with previous reports [6, 7], it was found that with the same amount of DNA, the ethidium bromide fluorescence intensity of form I DNA was only 70% that of form II or form III DNA, and thus this factor was used to normalize all observations. The densitometric readings were linearly proportional to the DNA concentration ranges in this study. This was similar to the results of Prunell et al. [12].

Preparation of Samples for Amino Acid Determination. Auromomycin (1.04 mg) was dissolved in 1.04 ml distilled water. We removed 100 µg auromomycin from this solution and lyophilized it to dryness for use as a control sample. The remaining 940 µg auromomycin was placed in a 15-ml Corex conical centrifuge tube, lyophilized, and then extracted with 940 µl methanol. Aliquots of the methanol extract (15, 30, and 50 µl ea) were removed and later assayed for their ability to degrade PM-2 DNA. The rest of the methanol extract (845 µg equivalent auromomycin) and the extraction residue were then dried and subjected to acid hydrolysis.

Amino Acid Analysis. Each sample was dissolved in 200 µl constant boiling 6 *N* hydrochloric acid or placed in 1.00 ml 6 *N* hydrochloric acid containing 40 µl 2-mercaptoethanol and hydrolyzed in sealed, evacuated, glass tubes for 22 h at 110° C [24]. Both methods of hydrolysis yielded consistent and comparable results. The hydrolyzed material was then cooled to room temperature and the residual hydrochloric acid was removed via vacuum dessication over sodium hydroxide pellets. The samples were then dissolved in 200 µl sample loading buffer and aliquots ranging from 20 to 60 µl

were subjected to amino acid analysis on a Durrum D-502 amino acid analyzer equipped with ninhydrin detection and the DOS-2 operation system. Those samples which contained Tris buffer did not interfere with the overall amino acid analyis. Control experiments demonstrated that only histidine was undetectable in the presence of Tris buffer. Neither cystine nor tryptophan was analyzed in these experiments, because both these amino acids are destroyed by acid hydrolysis [24].

Chromatography of Methanol Extract. The methanol extract from 1.35 mg auromomycin was redissolved in 300 µl 50 m*M* Tris-HCl buffer, pH 8.5, and placed on a G-25 Sephadex column (1.0 × 18 cm) previously equilibrated with 50 m*M* Tris-HCl buffer, pH 8.5. Fractions were eluted at 4° C in the dark and collected as 1.10-ml aliquots. The absorbance of each fraction at 280 nm and 350 nm was determined. Aliquots of 15, 30, and 50 µl were removed from column fractions 4, 5, 20, 35, and 36 and were analyzed for DNA-breakage activity. The remaining solution in fractions 4, 20, and 35 was then lyophilized to dryness and subjected to acid hydrolysis for amino acid analysis.

Results

Absorption Studies of Components of Auromomycin Preparation

Absorption spectra were determined for the untreated auromomycin, the methanol extract, and the residue. These spectra are shown in Fig. 1. The spectrum of the untreated auromomycin (spectrum a) had a broad peak at 350 nm, a peak at 265–270 nm, and a strong band below 240 nm. The methanol extract (spectrum b) had a broad peak at 345–350 nm, a shoulder around 260 nm, and a strong absorption band below 240 nm. The residue (spectrum c) had a broad small peak from 330–350 nm, a peak at 275 nm, and a strong absorption band below 240 nm. The absorbance peak at 350 nm in the untreated auromomycin is beyond the absorbance range of typical proteins and is probably due to a non-peptide chromophore. The absorptions at 265–270 nm and below 240 nm are in the range of proteins. The methanol extract (spectrum b) contained 70% of the absorption at 350 nm of the untreated auromomycin preparation, whereas the residue contained 20%.

Fluorescence Spectral Characteristics of the Auromomycin Constituents

Figure 2a shows the excitation and emission fluorescence spectra of the untreated auromomycin preparation. On excitation at 340 nm, a broad fluorescence peak at 430 nm (spectrum a) was observed. The excitation spectrum obtained with the fluorescence at 430 nm (spectrum c) showed a peak at 363 nm and two shoulders at 270 nm and 240 nm. These excitation and fluorescent wavelength ranges are beyond those of typical proteins, indicating the possible existence of a non-peptide, fluorescent chromophore. Excitation at 285 nm resulted in an emission spectrum (spectrum b) with two peaks at 350 nm and 425 nm. The excitation spectrum with the fluorescence measured at 350 nm is shown in spectrum d, with a peak at 280 nm. The excitation-emission pair of 280–350 nm is typical of most proteins.

The residue remaining after methanol extraction had a fluorescence spectrum (Fig. 2b) that was characteristic of proteins, with an emission peak at 350 nm and an excitation peak at 285 nm. The extracted non-peptide chromophore (Fig. 2c) had a fluorescence spectrum beyond the normal protein fluorescence. It had a fluorescence peak at 425 nm (excitation at 340 nm) and a broad excitation spectrum (fluorescence at 435 nm) peaking at 350 nm with a shoulder at 310 nm. On excitation at 400 nm a weak fluorescence peak at 470 nm was observed. Thus, the characteristics of the fluorescence spectra of both the residue and the methanol extract could be found in those of the untreated auromomycin, as shown in Fig. 2a.

Possible Interactions Between Residue and Extract

Mixing experiments were performed to determine whether or not any interactions might occur between

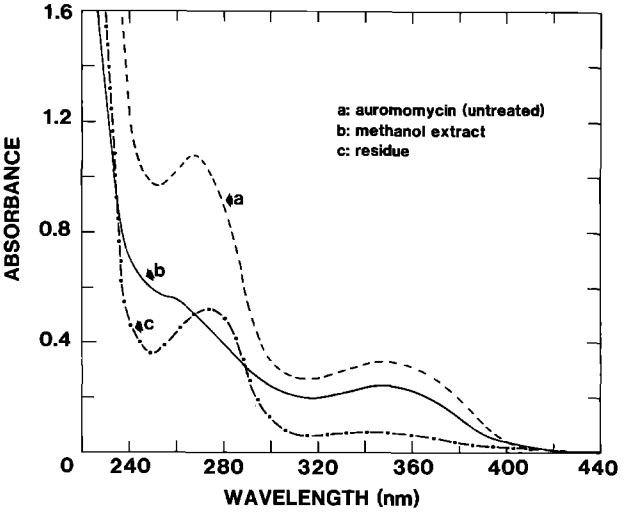
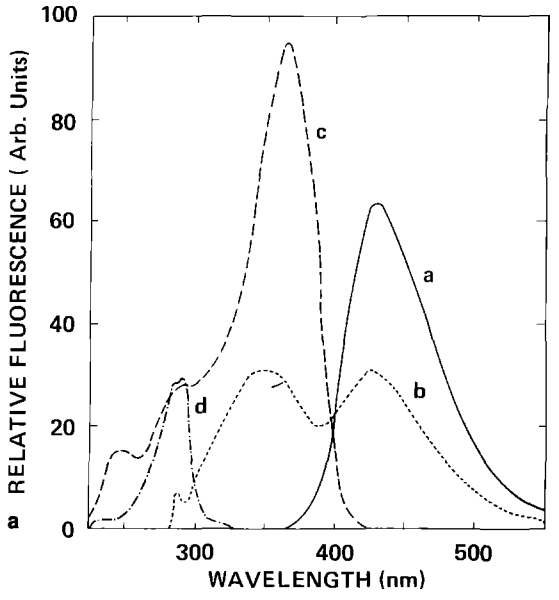
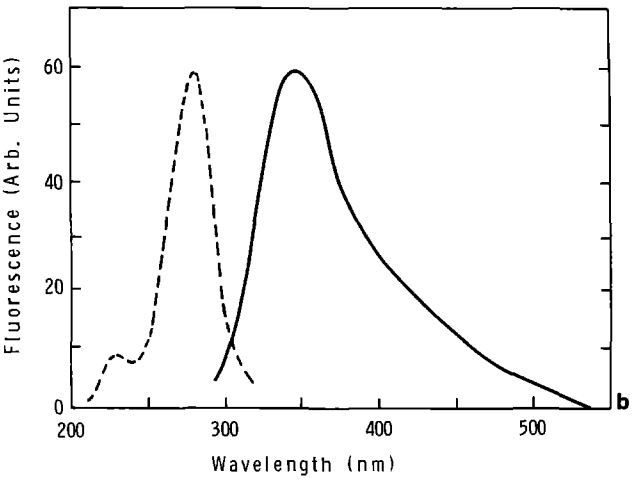


Fig. 1. The absorption spectra of the untreated auromomycin 1 mg/ml (spectrum a: – – –), the methanol extract (spectrum b: ———), and the residue (spectrum c: – · – · –) are shown as indicated. All samples were dissolved in 5 m*M* Tris-HCl, pH 8.5. The auromomycin was extracted by the procedure described in *Materials and Methods*





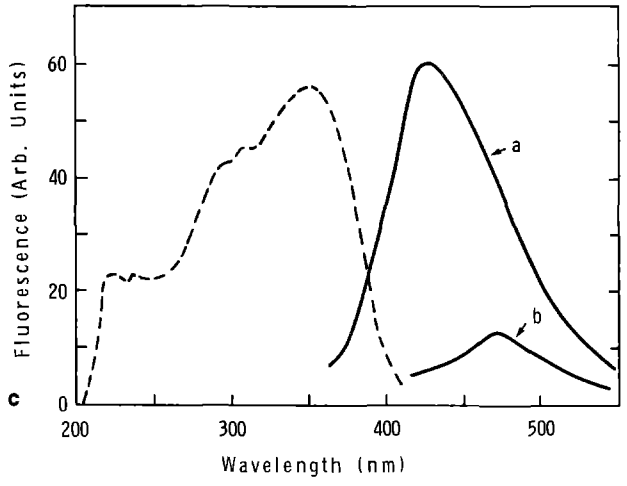


Fig. 2. a Excitation and emission spectra of the untreated auromomycin. Spectrum a: emission spectrum excited at 340 nm; spectrum b: emission spectrum excited at 285 nm; spectrum c: excitation spectrum with the fluorescence measured at 435 nm; spectrum d: excitation spectrum with the fluorescence measured at 350 nm. **b** The *solid curve* is the fluorescence spectrum of the residue (excitation at 285 nm) and the *dashed curve* is the excitation spectrum with fluorescence at 350 nm. **c** The *solid curves* are fluorescence spectra of the extracted chromophore excited at 340 nm (a) and 400 nm (b), respectively. The *dashed curve* is the excitation spectrum with fluorescence at 435 nm

the components present in the extract and the residue. Figure 3 depicts the fluorescence change as a result of the mixing of the residue and the extract at a concentration ratio equivalent to that of the untreated auromomycin. After subtraction of the very weak fluorescence of the residue, the mixing was found to enhance the 430 nm emission spectrum of, presumably, the non-peptide chromophore by 66%, with no apparent wavelength shift. This fluorescent enhancement suggests that the non-peptide chromophore in the extract may interact with some component(s) in the proteinaceous residue.

DNA Breakage Activity of the Untreated Auromomycin, the Methanol Extract and the Residue

Figure 4 shows the results of an alkaline ethidium bromide DNA fluorescence assay of the total DNA-breakage activity of the untreated auromomycin, the methanol extract, the residue, and the mixture of residue and extract in a weight ratio equivalent to the initial auromomycin preparation. The total DNA breakage activity includes single-strand breaks, double-strand breaks, and alka-

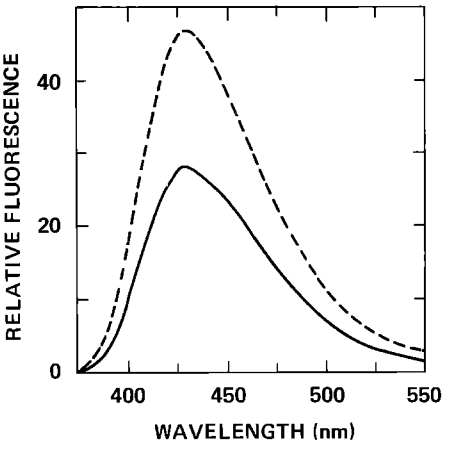


Fig. 3. Enhancement of the fluorescence of extract by residue. The excitation wavelength is 360 nm. *Solid curve* (———), extract alone (0.266 A_{350} units); *broken curve* (– – –), extract (0.266 A_{350} units) plus residue (1.82 A_{280} units) at concentration ratio equivalent to that of untreated auromomycin preparation. The weak fluorescence due to residue alone has been subtracted

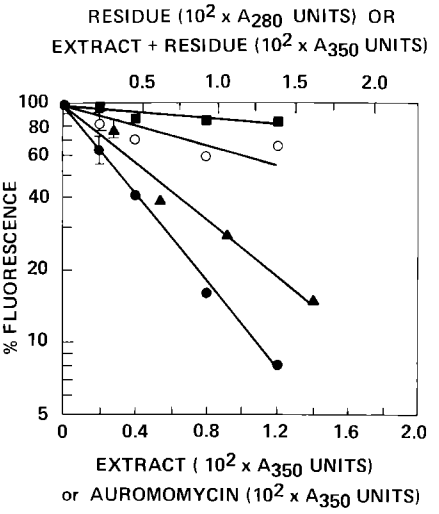


Fig. 4. DNA breakage activity of untreated auromomycin (– ▲ –), methanol extract (– ● –), residue (– ■ –), and residue plus methanol extract (– ○ –), as measured by ethidium bromide DNA fluorescence assay. Samples were incubated at room temperature, in total darkness, for 40 min in a total volume of 0.350 ml 50 m*M* Tris-HCl buffer, pH 8.5, plus 17.5 μg PM-2 DNA. Standard deviations for all graphed points are equal to or smaller than the points themselves unless indicated otherwise. To obtain actual A_{280} and A_{350} values plotted on the abscissas, divide each one by a factor of 100

line-labile damage. The results demonstrated that the methanol extract had the ability to induce DNA breakage. The activity of the extract was 121% ± 28% of that of the untreated auromomycin as determined by comparing the concentrations required to cause breakage of equivalent amounts of DNA molecules. These data are based on the A_{350} values of the extract and untreated auromomycin. Mixing of the methanol extract with the residue in a weight ratio comparable to that of the original auromomycin preparation resulted in a 78% ± 6.7% decrease in the total PM-2 DNA scission activity relative to the sum of the individual activities of the extract and residue. The residue had only 22% ± 3.8% of the DNA-breakage activity of the untreated auromomycin. The total DNA-degradation activities of both the residue and the extract plus residue were determined from the A_{350} and A_{280} values of the extract plus residue and the residue alone. Ultraviolet light exposure of the methanol extract resulted in a total loss in DNA degradation activity (data not shown).

The agarose gel electrophoretic patterns (Fig. 5) confirmed the concentration-dependent DNA-breakage activity of the methanol extract. Increasing amounts of the extract resulted in increased formation of form II (from single-strand breaks) and form III PM-2 DNA (from double-strand breaks) fragments. The patterns of DNA cleavage of both the methanol extract and the untreated auromomycin

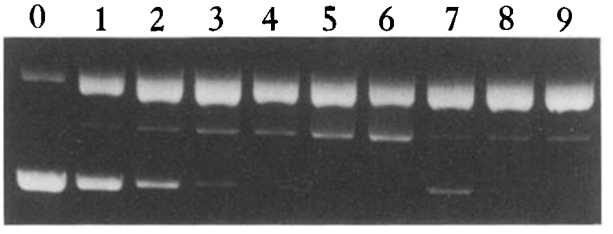


Fig. 5. Agarose gel electrophoretic patterns of 1.2 μg PM-2 DNA after treatment with methanol extract (lanes 1–6) and untreated auromomycin (lanes 7–9) for 40 min at room temperature in the dark. Lane 0 contained untreated DNA (control). Lanes 1–6 contained increasing amounts of methanol extract as follows: 1, $10^{-2} \times 0.2$ A_{350} units; 2, $10^{-2} \times 0.5$ A_{350} units; 3, $10^{-2} \times 0.8$ A_{350} units; 4, $10^{-2} \times 1.3$ A_{350} units; 5, $10^{-2} \times 2.2$ A_{350} units; 6, $10^{-2} \times 3.5$ A_{350} units. Lanes 7–9 contained increasing amounts of untreated auromomycin as follows: 7, 20 μg; 8, 40 μg; 9, 60 μg. The order of PM-2 DNA migration patterns from the top to the bottom of the gel is as follows: Top, form II open-circular PM-2 DNA; middle, form III linear PM-2 DNA; bottom, form I covalently closed superhelical PM-2 DNA

(also shown in Fig. 5) were similar. Densitometric measurements of the relative amounts of forms I, II, and III DNA generated from the reaction of the extract with PM-2 DNA were determined and plotted as shown in Fig. 6. At 50% breakage of the original

form I DNA, 43% of the total DNA was converted to form II and 8% to form III. Seventy-five percent breakage of the original form I DNA resulted in 61% generation of form II DNA and in 13% generation of form III DNA. Similar amounts of form II and form III DNA were also generated upon reaction of equivalent amounts of untreated auromomycin. Thus the methanol extract is able to degrade PM-2 DNA, causing both single- and double-strand breaks.

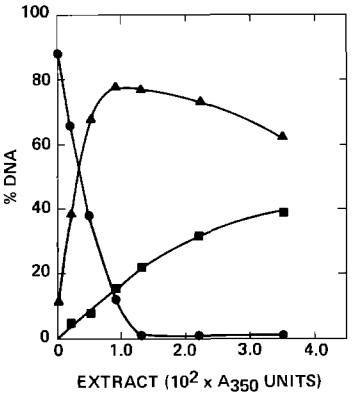


Fig. 6. Densitometry scan of photographic negative of electrophoretic gel patterns of DNA breakage induced by methanol extract of auromomycin. ●, form I superhelical covalently closed PM-2 DNA; ▲, form II open-circular PM-2 DNA (single-strand scission); ■, form III linear PM-2 DNA fragments (double-strand scission). To obtain actual A_{350} values plotted on the abscissas, divide each one by a factor of 100

Quantitative Determination of the Amount of Polypeptide in the Extract of Auromomycin

To study the amount and the type of polypeptide remaining in the methanol extract, the amino acid compositions of the extract, the residue, and the untreated auromomycin preparation were determined; these are summarized in Table 1. The peptides present in both the extract and the residue had virtually the same amino acid composition as the untreated auromomycin. Thus the major detectable polypeptide component(s) in both the extract and the residue was initial auromomycin polypeptides and not an external protein contaminant or degradation product(s) of auromomycin. Table 2 shows the distribution of peptides in the extract and the residue as determined from the data shown in Table 1. From 21%–26% of the original auromomycin peptide was found present in the methanol extract, while the rest of the detectable auromomycin peptide was found in the undissolved residue. The remaining 20% of the auromomycin polypeptide was undetected and probably lost during the extraction and separation process.

Table 1. Results of amino acid hydrolysis[a] of untreated auromomycin control, residue, and methanol extract

Amino acid	Macromomycin[b]	Auromomycin[b]	Untreated auromomycin	Residue	Methanol extract
Aspartic acid	8	8	8	8	8
Threonine	18	18	18	18	17
Serine	10	10	9	8	8
Glutamic acid	8	8	8	8	8
Proline	5	5	6	6	5
Glycine	20	20	18	18	18
Alanine	19	19	18	18	18
Half-cystine[c]	4	4	NA	NA	NA
Valine	16	16 or 17	15	15	15
Methionine	0	0	0	0	0
Isoleucine	3	3	3	3	3
Leucine	5	5	6	5	5
Tyrosine	1	1	1	1	1
Phenylalanine	2	2	3	3	3
Tryptophan[c]	1	1	NA	NA	NA
Lysine	3 or 4	3 or 4	4	3	3
Histidine	2	2	2	2	2
Arginine	0	0	0	0	0

[a] Samples hydrolyzed in sealed, evacuated, glass tubes at 110° C for 22 h

[b] Amino acid composition of auromomycin and macromomycin as reported by Yamashita et al. [24] and Sawyer et al. [14]

[c] Half-cystine and tryptophan residues were not analyzed for in these analyses

Column Chromatographic Fractionation of the Methanol Extract

To confirm the possibility that the DNA-degradation activity of the methanol extract was due to the non-peptide chromophore and not to the remaining polypeptide(s), the non-peptide chromophore was further fractionated by column chromatography. The elution profile of the methanol extract is indicated in Fig. 7. Comparison of the 280 nm and 350 nm absorptions of the various fractions reveals that the void volume (fractions 1–5) had the largest ratio of 280–350 absorption. In contrast, fraction 35 contained the largest amount of 350 nm-absorbing material. Those fractions which were assayed for DNA breakage activity are listed in Table 3, along with their EC_{50} values. EC_{50} is the amount of A_{280}- or A_{350}-absorbing material needed to cause degradation of 50% of the DNA molecules present in a given reaction mixture. All the column fractions had smaller EC_{50} values than the whole methanol extract, indicating that all have greater DNA-breakage activity than the methanol extract. Aliquots from fractions 20, 35, and 36 required smaller amounts of 280- and 350-absorbing material to produce 50% DNA breakage than fractions 4 and 5, and thus have increased degradation activity. As summarized in Table 3, the largest amount of peptide was found in the fraction which eluted at the void volume (fraction 4), while fractions 20 and 35 were substantially depleted of intact peptide, as indicated by their amino acid analysis. Thus there was a significant enrichment in

Table 2. Quantitation of peptides contained in untreated auromomycin, residue, and methanol extract[a]

Sample	Peptide (nmoles)	Percent of initial peptide (%)
Auromomycin[b]	49.05 ± 0.240	100
Residue	29.30 ± 1.44	60
Methanol extract	10.21 ± 0.370	21
Total peptide recovered	39.51 ± 1.44	81

[a] Amount of peptide in all three fractions was calculated from nmoles of aspartic acid, glutamic acid, histidine, and isoleucine present in each fraction analyzed

[b] This is the total amount of auromomycin extracted

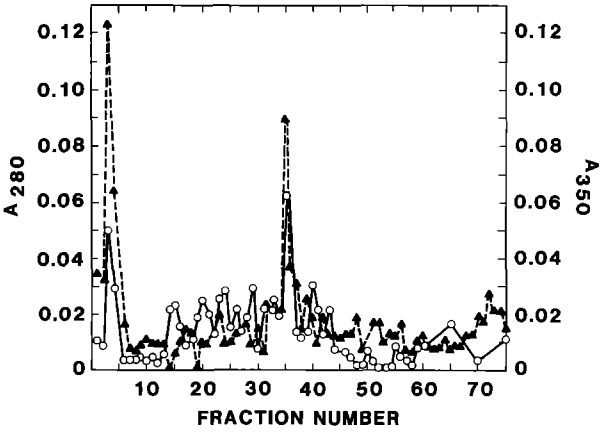


Fig. 7. Elution profile of methanol extract. V_0, 4 ml; V_t, 40 ml; V_i, 36 ml; bed volume, 10 ml. (▲– – –▲) A_{280}; (○———○) A_{350}

Table 3. DNA breakage activity[a] and nanomoles of intact peptides detected[b] in selected column fractions

Fraction	EC_{50} values[e] ($10^{-4} \times A_{350}$ values)	EC_{50} values[f] ($10^{-4} \times A_{280}$ values)	Peptide (nmoles)	Percent	Total
Methanol extract (control)	24.0	36.1	16.3	23%	
4	8.50	20.1	1.95 ± 0.333	2.76%[c]	12.0%[d]
5	7.20	16.3	–	–	
20	4.40	1.36	0.186 ± 0.0319	0.263%	1.14%
35	6.40	9.11	0.221 ± 0.0253	0.313%	1.36%
36	3.60	4.04	–	–	

[a] Aliquots (15, 30, and 50 µl ea) were removed from the selected column fractions. Samples were incubated with 13 µg PM-2 DNA in a total reaction volume of 0.350 ml for 40 min in the dark at room temperature

[b] The amount of peptides in each fraction was calculated from those amino acids which were similar quantitatively and qualitatively to those of untreated auromomycin. The total amount of auromomycin extracted was 70.7 nmoles. The total amount of auromomycin present in the methanol extract was 16.3 nmoles, which was 23% of the starting material

[c] Percentage is based on the amount of auromomycin extracted

[d] Percentage is based on the amount of auromomycin contained in the methanol extract prior to column chromatography

[e] EC_{50} is based on the amount of A_{350} absorbing material needed to induce breakage of 50% of the DNA molecules present in a given reaction mixture

[f] EC_{50} is based on the amount of A_{280} absorbing material needed to induce breakage of 50% of the DNA molecules present in a given reaction mixture

DNA-degradation activity in those fractions which were virtually devoid of intact polypeptides, suggesting that DNA degradation was effected by the non-peptide chromophore and was unrelated to the polypeptides remaining in the methanol extract after fractionation.

Discussion

Earlier studies [9, 16] have utilized methanol extraction as a means of removing non-peptide chromophores from polypeptide antibiotics. In addition to methanol, we have used in the present study a number of organic solvents including chloroform, benzene, hexane, acetone, 2-butanone, and diethyl ether; and none of these solvents proved to be as effective as methanol in achieving separation of the non-peptide chromophore from the polypeptide components of auromomycin (unpublished data). In the concentration range of auromomycin used in this study we found both the extract and the residue to be water-soluble.

Since the absorption and fluorescence spectra of the polypeptides and the non-peptide chromophore overlap, it became necessary to utilize other methods of analysis to determine the amount of polypeptide contained in the methanol extract. In addition, we also wanted to know the composition of the peptides detected in the extract. Both the Coomassie Blue dye binding method [11, 17] and the Folin-phenol method of Lowry et al. [8] were tried and found to have limitations. The Coomassie Blue dye binding procedure was ineffective in that we were unable to bind the dye to the auromomycin polypeptides and detect formation of the characteristic protein-dye complex which absorbs at 595 nm (data not shown). Pierce and Suelter [11] have shown that considerable variation occurs in the degree of dye binding to a number of proteins. Application of the method of Lowry et al. [8] resulted in formation of the expected complex, which has an absorption maximum at 750 nm. However, we were not able to determine whether this complex was a product of reaction with the polypeptides or the non-peptide chromophore contained in the methanol extract (unpublished data). The major disadvantage of the Folin-phenol method of Lowry et al. [8] is its lack of specificity with such compounds as phenol, which are known to cause strong interference [10]. Thus if the non-peptide chromophore contains a phenolic functionality, it could possibly give a false-positive test. It is for these reasons that amino acid analysis was employed as a method of determining the type and amount of auromomycin polypeptides contained in the methanol extract. The polypeptides detected in the extract had very similar amino acid composition to those found in the original auromomycin preparation. These peptides did not originate from degradation of the original auromomycin polypeptides or from external protein contamination.

As seen by the results of our studies, we can conclude that the methanol extract causes DNA-strand scission. However, the role of the non-peptide chromophore and the polypeptides to the observed DNA-breakage activity of the methanol extract is less certain. Since the methanol extract contained the largest amount of extracted non-peptide chromophore as well as the greatest amount of DNA-breakage activity, it may be possible to attribute the enhanced activity of the extract to enrichment in non-peptide chromophore. In contrast, the residue which contained the least amount of non-peptide chromophore also had the lowest amount of total DNA-breakage activity. This suggests that the non-peptide chromophore may be essential in the ability of auromomycin to degrade DNA.

Significant differences were observed in the total DNA degradation activity of the untreated auromomycin, the methanol extract, and the remixed extract plus residue. These results may have been affected by interactions between the non-peptide chromophore and the polypeptide components. The increased breakage activity of the extract when compared with the untreated auromomycin may be caused by decreased inhibition resulting from depletion of polypeptides. A further possible indication of this trend occurred upon fractionation of the methanol extract by column chromatography, which resulted in enhanced DNA-breakage activity in those column fractions which were further depleted in polypeptides. As seen in this study, the EC_{50} value (based on A_{350} readings) for the original extract was greater than for fractions 4, 20, and 35. This increased activity may be caused by decreased peptides, which may result in decreased inhibition in the breakage activity of the non-peptide chromophore.

Methanol extraction may lead to changes in the interactions between the peptides and the non-peptide chromophore. This may explain the decrease in total DNA-degradation activity when the residue and extract were recombined. Such interactions may have been observed by fluorescence studies (Fig. 3). Addition of increasing amounts of residue resulted in enhanced fluorescence of the methanol extract. A similar fluorescent enhancement could possibly occur in the interaction of the non-peptide chromophore with the polypeptides contained in the original auromomycin preparation. Perturbation in the interactions of the peptides and non-peptide chromophore

may also lead to similar changes in the DNA-breakage activity of the original auromomycin preparation which are comparable to the changes observed in the DNA-cleavage activity of the remixed extract plus residue.

The inhibition of the breakage activity of the extract by the residue and the possible inhibitory effect of peptides on the breakage activity of the non-peptide chromophore may be analogous to the results reported by Kappen et al. [3]. They found that inactivated neocarzinostatin blocked the DNA-strand scission activity of native 'untreated' neocarzinostatin. The inactivated neocarzinostatin was produced by heat, ultraviolet irradiation, and removal of the non-peptide chromophore. The DNA-strand scission activity of auromomycin was also found to be inhibited by macromomycin [4].

Both auromomycin and neocarzinostatin are polypeptide antitumor antibiotics which contain non-peptide chromophores. The fluorescence properties of the non-peptide auromomycin chromophore are very similar to the non-peptide neocarzinostatin chromophore [9], except that the 465 nm fluorescence peak of the neocarzinostatin non-peptide chromophore (which is probably equivalent to the 470 nm peak shown in Fig. 2c) is more intense. Thus the two non-peptide chromophores (from neocarzinostatin and auromomycin) may be related structurally.

Experiments performed by Kappen et al. [4] suggest that the non-peptide chromophore causes DNA-strand scission through a free radical mechanism, since auromomycin activity was decreased in the presence of α-tocopherol. Similar effects were also observed [4, 5] with neocarzinostatin and the isolated non-peptide neocarzinostatin chromophore. Neither the untreated auromomycin preparation nor the methanol extract require reducing agents for their breakage activity, while both the initial neocarzinostatin preparation and the non-peptide neocarzinostatin chromophore require 2-mercaptoethanol for maximum activity [5]. This implies a potential difference in the mechanism of DNA-strand scission of these agents.

The role of the polypeptide components of auromomycin and their relationship to the non-peptide chromophore is not known. We speculate that there may be specific peptide linkages which may contribute to the activity of the methanol extract and which may be essential for maximum activity. Changes in peptide conformation may also play an important role in the activity of the non-peptide chromophore of auromomycin. The polypeptide components may stabilize the chromophore or may even contribute to the potential DNA site-binding specificity, which may be altered or absent in the extract and the chromatographed extract. It may be possible that inhibition of the total DNA-degradation activity of auromomycin represents an increase in site specificity of DNA degradation caused by interactions of the DNA with the polypeptide components of auromomycin.

During the preparation of this paper, a report has been published by Suzuki et al. [23] on the DNA-breakage activities of the non-peptide chromophores of both neocarzinostatin and auromomycin, and this suggests that these workers have made similar observations.

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