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Largomycin: Preparation, Properties, and Structure†

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ABSTRACT: Largomycin has been purified to homogeneity by chromatography on hydroxylapatite whereby carbohydrate and protease impurities were removed. Largomycin is an acidic protein (p*I* 4.13, molecular weight 29 300) which forms a dimer in phosphate buffer. An N-terminal amino acid sequence analysis from the amino-terminal residue gave, for the first 32 residues, Asp-Ile-Leu-Ile-Ala-Gly-Ala-Thr-Gly-Asn-Val-Gly-Lys-Pro-Leu-Val-Glu-Gly-Leu-Leu-Ala-Ala-Gly-Lys-Pro-Val-Arg-Ala-Leu-Thr-Arg-Asn... The sequence from the carboxyl terminus was -Ala-Ala-Leu-Phe-OH with threonine, valine, and glutamic acid being released upon prolonged digestion. The same amino acid sequences were found for largomycin prepared from either the culture broth or the mycelium of *Streptomyces pluricolorescens*. The similarities extended to the other physical properties, the antimicrobial activity against *Staphylococcus aureus* and *Sarcina lutea*, and the antitumor activity against KB cells. Largomycin inhibits the biosynthesis of DNA and RNA. An iodinated derivative did not bind to KB cells. The antimicrobial activity was lost following ultraviolet irradiation, protection against which was not afforded by *p*-aminobenzoic acid.

One of more than fifty proteins reported to date with antitumor activity (Montgomery et al., 1981), largomycin is a fermentation product with antibiotic activity and antitumor activity against several tumors, including KB, P388, HeLa, Ehrlich ascites, and Sarcoma 180 (Yamaguchi et al., 1970a,b). Of three fractions isolated from the culture medium, largomycin II was purified further to a yellow, amorphous powder, with the properties of a chromoprotein. The protein was reported to contain carbohydrate and be similar to other an-

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titumor proteins, such as plurallin (Ogawara et al., 1966; Umezawa, 1967), in containing a prosthetic group with a pH-dependent chromphore. The proteins differed, however, in their amino acid composition.

Several proteins with antitumor activities have been described that have nonprotein chromophores. In such cases, the biological activity has been associated with the chromophores when these could be extracted from the apoproteins. Thus, auromomycin contains a yellow pigment that can be removed separately from a colorless material in which the biological activity resides (Kappen et al., 1980). Although it does not show antitumor activity, the yellow compound confers increased stability to auromomycin against ultraviolet irradiation, but is irreversibly dissociated by heating (D. D. Vandrē and R. Montgomery, unpublished experiments). Macromomycin, which can be derived from auromomycin by removing the yellow chromophore, contains a colorless nonprotein material that can be extracted with methanol; the methanol extract contains all the biologically active material (Vandrē & Montgomery, 1982). The same is true for neocarzinostatin (Napier et al., 1980), plurallin (Ogawara et al., 1966), and prunacetin A (Arai et al., 1967), while poracin contains a nondissociable chromophore (Schillings & Ruelius, 1968).

The present study reports on the further purification of largomycin, free from carbohydrate and protease contaminants, and the characterization of some physical and biological properties.

Materials and Methods

Materials

Crude largomycin was received from James C. Cradock, Division of Cancer Treatment, National Cancer Institute, and Dr. R. Stroshane, Frederick Cancer Research Center.

Methods

Purification of Largomycin. Crude largomycin (25–110 mg) was dissolved in 0.1 mM sodium phosphate buffer, pH 6.6, and applied to a column (1.5 × 21 cm) of fast-flow hydroxylapatite. The column was washed with starting buffer, resulting in the elution of a 280-nm-absorbing peak (HA-1). This was followed by a gradient from 0.1 mM sodium phosphate (400 mL) to 0.3 M sodium phosphate (200 mL), pH 6.6, at a flow rate of 15 mL/h. Three protein peaks were obtained with this gradient, a yellow peak eluting with 0.05 M sodium phosphate (HA-2), a brown peak eluting with 0.10 M sodium phosphate (HA-4), and a broad brown peak eluting with 0.15–0.3 M sodium phosphate (HA-5). The material between the peak at 0.05 and 0.065 M sodium phosphate was designated HA-3. The results are summarized in Figure 1. Peak HA-1 gave a positive phenol–sulfuric acid test; peaks HA-4 and HA-5 contained proteases; peaks HA-2 and HA-3 were active against *Sarcina lutea*.

Peak HA-2 was dialyzed against distilled water until free from inorganic phosphate, and the yellow solution was freeze-dried to give pure largomycin. The yield of HA-2 varied from 20 to 60% of the total material applied to the column from the crude largomycins of different culture filtrates. Similarly, purified largomycin was obtained from a partially purified extract of *Streptomyces pluricolorescens* mycelium.

Analytical Procedures. Protein was estimated by the method of Lowry et al. (1951) by using bovine serum albumin (BSA)[1] as the reference compound. Carbohydrate was estimated colorimetrically by the phenol–sulfuric acid method (Dubois et al., 1956). Inorganic phosphate was estimated by the method of Fiske & Subbarow (1925); organic phosphate was determined by using the method of Ames (1966).

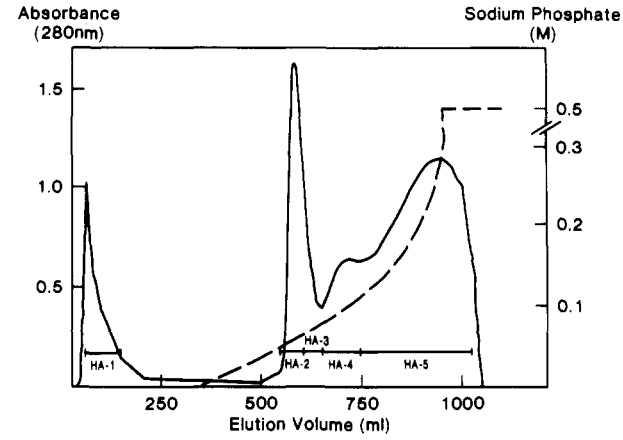

FIGURE 1: Hydroxylapatite chromatography of crude largomycin. Sample (110 mg) was dissolved in 0.1 mM sodium phosphate, pH 6.6, and applied to the column (1.5 × 21 cm) of hydroxylapatite. Contaminating carbohydrate was eluted in fractions HA-1 with starting buffer (0.1 mM sodium phosphate, pH 6.6). Purified largomycin (HA-2) was separated from contaminating proteases (HA-4 and HA-5) by elution with a concave sodium phosphate gradient.

Amino Acid Analysis. For amino acid analyses, 40 μg of protein was hydrolyzed in 400 μL of 6 N HCl for 24, 48, and 72 h at 110 °C with a norleucine internal standard. The samples were analyzed on a Beckman Model 121MB amino acid analyzer.

The analysis for each of the amino acids, with extrapolation to zero time for serine, threonine, and tyrosine, is 98.8 ± 1.6%, based on results with lysozyme as the model protein. The amino acid composition of largomycin was normalized to four residues of histidine, which except for methionine was in the smallest amount in the hydrolysate.

Polyacrylamide Gel Electrophoresis. Electrophoresis in $NaDodSO_4$ was carried out by the modified method of Shapiro et al. (1967). A resolving gel of 15% polyacrylamide containing 0.1 M sodium phosphate (pH 7.2), 0.1% $NaDodSO_4$, and 6 M urea and a stacking gel of 3.5% were used. The running buffer was 0.1 M sodium phosphate buffer (pH 7.2) and 0.1% $NaDodSO_4$. The gels were stained with 0.1% Coomassie Blue in 25% 2-propanol and 10% acetic acid containing 0.1% cupric acetate.

Isoelectrofocusing. The isoelectrofocusing of crude and purified largomycin was carried out for 72 h at pH 3.5–5 with an LKB Model 8101 column (110-mL capacity). The concentration of carrier ampholytes was 1%.

Sequence Analysis. The N-terminal amino acid sequence was determined by Edman degradation by using a Beckman 890C sequencer fitted with a cold trap and Sequamat P6 autoconverter. Typically, 30–50 nmol of protein was added to the cup without added Polybrene carrier. All reagents and

[1] Abbreviations: Leu-βNA, L-Leu-β-naphthylamide; BANA, N^{α}-benzoyl-DL-arginine-β-naphthylamide; Cbz-Ala-Arg-Arg-4MeOβNA, N^{α}-carbobenzyloxy-L-Ala-L-Arg-L-Arg-4-methoxy-β-naphthylamide; BSA, bovine serum albumin; TBS, Tris-buffered saline; LAR, largomycin; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; MIC, minimum inhibitor concentration; ID_{50}, concentration of largomycin causing 50% inhibition of thymidine incorporation into KB cells; a-ELISA, amplified enzyme linked immunosorbent assay; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid.

solvents were of Beckman sequencer grade. The Beckman 1 M Quadrol program (no. 111978) was followed with the only change being the use of 0.33 M Quadrol as coupling buffer and an appropriate change in its delivery time to compensate for the lower viscosity of this more dilute solution. The PTH derivatives were analyzed on an Altex high-pressure liquid chromatograph, using a 30-cm C_{18} reverse phase column of 5-μm size. The column was eluted with a gradient of 20–80% methanol in 0.01 M sodium acetate, pH 6.0.

C-Terminal Amino Acid Analysis. Largomycin samples were dissolved in 0.2 M ethylmorpholine acetate, pH 8.0, at a concentration of 2 mg/mL. Carboxypeptidase A was added to give a protein:enzyme ratio of 1:40. After various periods of incubation at 37 °C, samples were removed and acidified to pH 2.2 with 1 N HCl and centrifuged to remove any particulate material. The amino acids in the supernatant solutions were analyzed on a Beckman Model 121MB amino acid analyzer.

Molecular Weight Determinations. (i) Gel Chromatography. Columns (30 cm × 1.0 cm) of Bio-Gel P-60 and P-100 were equilibrated with 0.05 M sodium phosphate, pH 7.0, and calibrated with ferritin, BSA, ovalbumin, carbonic anhydrase, and cytochrome *c*. The unknown sample was chromatographed identically.

(ii) $NaDodSO_4$ Gel Electrophoresis. The procedure followed that given above for qualitative analysis, and the mobility of the unknown was compared with that of known proteins, ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme, cytochrome *c*, bovine trypsin inhibitor, and insulin (BRL protein molecular weight standards, molecular weight range 3000–43 000).

(iii) Analytical Ultracentrifugation. The sample was dissolved and equilibrated with 0.1 M sodium phosphate, pH 7.0, or 6 M guanidine and centrifuged at 40 000 rpm and 23 °C in a 12-mm double-sector cell. Centrifugation continued to equilibrium, and the Yphantis procedure of miniscus depletion was followed by using Rayleigh interference optics.

Absorbance Spectra. Ultraviolet and visible absorption spectra were obtained by using a Cary 17 spectrophotometer and solutions in water, 0.1 N HCl, and 0.1 N NaOH. Fluorescence spectra were obtained by using a Hitachi MPF-2A fluorometer.

Assay of Protease Activity. Hydrolysis of azocasein by proteases present in largomycin samples was estimated by the method of Charney & Tomaretti (1947). The hydrolysis of substrates Leu-βNA, BANA, and Cbz-Ala-Arg-Arg-4MeOβNA was measured essentially as described by Barrett (1972) and modified by Otto & Riesenköning (1975) except that Tris-HCl buffer, pH 7.0, was used in the incubation mixture instead of phosphate buffer.

Antibiotic Activity. A standard amount of test organism, either *Staphylococcus aureus* or *Sarcina lutea*, was streaked on the surface of Mueller-Hinton agar. Test samples were applied to filter disks placed on the agar and incubated at 37 °C overnight. The minimal inhibitory concentration of drug was calculated from the zones of inhibition.

Antitumor Activity. The effect of largomycin on the incorporation of radiolabeled precursors of DNA, RNA, and amino acids in KB cells was examined as previously described for the antitumor proteins macromomycin and cesalin (Vandrē et al., 1979; Shepherd et al., 1980). The concentration of largomycin resulting in a 50% inhibition of incorporation following a 10-min pulse with [*methyl*-^{3}H]thymidine after a 5-h preincubation with largomycin was defined as the inhibitory dosage (ID_{50}).

Immunopotentiation Assay. Immunopotentiation activity was measured as described by Skurkovich et al. (1978) and Herberman (1977).

Antibody Preparation. Largomycin (0.5 mg) in 1 mL of phosphate-buffered saline was emulsified with an equal volume of Freund's complete adjuvant, and initial multiple-site abdominal and toe-pad subcutaneous injection into rabbits was followed in 30 days by subcutaneous injection in the neck with the same antigen mixed with Freund's incomplete adjuvant. Serum was collected weekly following the booster immunization. Ouchterlony immunodiffusion tests were carried out in 1% agar gels prepared in 8 mM phosphate-buffered saline (130 mM), pH 7.4.

Iodination of Largomycin with Chloramine-T. Purified largomycin (25 μg) was dissolved in 25 μL of 50 mM sodium phosphate buffer, pH 7.0, and added directly to 1 mCi of alkaline Na^{125}I in a microcentrifuge tube. Iodination was initiated by the addition of 1.5 μg of chloramine-T dissolved in water (15 μL). After 1 min at room temperature, the iodination was terminated by the addition of 20 μg of sodium metabisulfite in 80 μL of water. The iodinated largomycin was separated from the excess Na^{125}I by gel filtration at 4 °C on a column (1 × 53 cm) of Sephadex G 50 equilibrated with 0.01 M Hepes buffer, pH 7.2, containing 0.1% BSA and 0.15 M NaCl. Fractions from the iodinated largomycin peak were pooled and dialyzed overnight at 4 °C against 0.01 M Hepes buffer, pH 7.2, containing 0.1% BSA and 0.15 M NaCl following which the radioactivity was 344 000 cpm/10 μL and 84% of the counts were precipitated by Cl_3CCOOH.

Immunoassay. Competitive radioimmunoassay was set up by using a 1:800 dilution of antilargomycin serum as described below:

To each tube were added the following in turn: (i) 100 μL of ^{125}I-labeled largomycin (~10 000 cpm) diluted in TBS–BSA; (ii) 100 μL of a series of standard solutions of unlabeled largomycin, together with appropriate negative controls; (iii) 100 μL of a 1:800 dilution of largomycin antiserum diluted in TBS–BSA containing a 1:600 dilution of normal rabbit serum and normal rabbit serum controls. The final volume of each tube was made up to 0.3 mL with TBS–BSA as required, and antigen–antibody reaction proceeded overnight at 4 °C. A 2% (v/v) suspension of formalin-treated heat-killed *S. aureus* in TBS–BSA (250 μL) was added to each tube, and the tubes were gently agitated at room temperature for 30 min. After centrifugation, the pellets were washed with 1 mL of TBS–BSA and recentrifuged, and the pelleted immune complexes were counted in a Beckman Biogamma II counter.

Amplified enzyme linked immunosorbent assay (a-ELISA) followed the procedure of Butler et al. (1978).

Photoinactivation. Samples of largomycin (~400 μg) were dissolved in deaerated deionized water at a concentration of 1 mg/mL. Experiments were conducted in covered quartz cuvettes of 1-cm light path. Radiation (254 nm) was provided by a shortwave ultraviolet "Mineralight" lamp placed 4 cm away. Loss of antimicrobial activity with time, in both the presence and absence of 0.02% *p*-aminobenzoic acid, was followed by a standardized antimicrobial assay as previously described.

Binding of ^{125}I-Labeled Largomycin to KB Cells. KB cells were scraped from a T-25 falcon flask and washed with 10 mM Hepes, 0.15 M NaCl, and 0.1% BSA, pH 7.2, and aliquots (1.5 × 10^5 cells in 200 μL) were transferred to plastic centrifuge tubes. Iodinated largomycin (30 000 cpm) in 50 μL of the same buffer was added and incubated at 37 °C for various lengths of time, following which the cells were pelleted

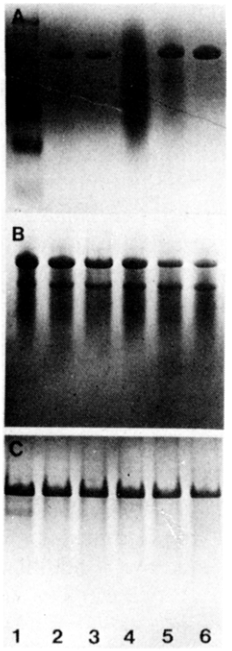

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of largomycin. Electrophoresis was carried out as described under Methods. (A) (Lane 1) Standard proteins: ovalbumin, 43 500; α-chymotrypsinogen, 25 700; β-lactoglobulin, 18 400; lysozyme, 14 300; cytochrome *c*, 12 300; bovine trypsin inhibitor, 6200; insulin, 3000. Note that lysozyme and cytochrome *c* were not separated. (Lane 2) Crude largomycin (10 μg); (3) purified largomycin; (4) HA-4 (25 μg); (5) crude largomycin (25 μg); (6) purified largomycin (25 μg). (B) (Lane 1) Crude largomycin in H_2O (control, 25 μg); (2–6) at 37 °C for 6, 12, 24, 48, and 72 h (25 μg of protein each) in water. (C) (Lane 1) Crude largomycin in 0.1 M phosphate buffer, pH 7.0 (control, 25 μg); (2–6) at 37 °C for 6, 12, 24, 48, and 72 h (25 μg of each) in 0.1 M phosphate buffer, pH 7.0.

and washed with buffer. The amount of cell-bound radioactivity was determined in a Beckman Biogamma II counter.

Results

The original crude largomycin from culture filtrates was brown and contained carbohydrate, 14.9% glucose by phenol–sulfuric acid colorimetric analysis and gas–liquid chromatography of an acid hydrolysate. The sample of largomycin was heterogeneous with respect to protein as indicated by gel electrophoresis of the original material (Figure 2A) and contained significant protease activity.

Similar properties were noted for the crude largomycin that had been isolated from the mycelium. After solutions of crude largomycin were incubated in distilled water at 37 °C (Figure 2B), smaller molecular weight fragments are produced with loss of antibiotic activity against *S. lutea*. The loss of this activity was reduced in the presence of phosphate (Figure 2C); the concentration of phosphate was not related in any simple way to the change in electrophoretic pattern after 70 h at 37 °C or the antibiotic activity after this time.

A strong protease activity was detected in crude largomycin from culture filtrates using azocasein as substrate whereas that isolated from the mycelium showed less than 10% of this protease activity. The crude largomycin hydrolyzed the specific substrates BANA, Leu-βNA, and Cbz-Ala-Arg-Arg-4MeOβNA, showing thereby both endopeptidase and aminopeptidase activities. No carboxypeptidase activity was demonstrated with hippurylarginine as substrate. The unexplained stabilizing effect of inorganic phosphate on the antitumor activity of the crude largomycin samples was observed in these studies on synthetic substrates. In buffers of 0.1 M phosphate most commonly used for such studies, the proteases in the crude largomycin showed no activity even by prolonging the incubation times. The protease activities noted above were demonstrated in Tris buffer. The optimum pH range for the protease activity was broad with a maximum above pH 7.0. The protease activity was totally inhibited by the addition of 0.5 mM $HgCl_2$. Phenylmethanesulfonyl fluoride inhibited 76% of the protease activity at 1.0 mM.

Table I: Physicochemical Properties of Largomycin

color	yellow
mol wt	30 000, gel electrophoresis 58 000, ultracentrifugation in PBS 29 300, ultracentrifugation in 6 M guanidine hydrochloride
isoelectric point	4.13 (pH gradient 3.5–5.0)
UV absorption maxima	
in 0.1 N HCl	272 nm, $E^{1\%}_{1\,cm}$ 16.4; 420 nm, $E^{1\%}_{1\,cm}$ 1.8
in water	274 nm, $E^{1\%}_{1\,cm}$ 14.9; 425 nm, $E^{1\%}_{1\,cm}$ 1.6
in 0.1 N NaOH	288 nm, $E^{1\%}_{1\,cm}$ 15.6 (shoulder); 525 nm, $E^{1\%}_{1\,cm}$ 2.0
amino acid composition (normalized to four histidine residues)[a]	
lysine	12.55 ± 0.57
histidine	4
arginine	10.40 ± 0.22
aspartic acid	20.34 ± 0.16
threonine	19.90[a]
serine	16.30[a]
glutamic acid	36.91 ± 0.42
proline	16.07 ± 0.18
glycine	23.88 ± 0.47
alanine	28.31 ± 0.43
half-cystine	
valine	27.47[b]
methionine	1.87 ± 0.27
isoleucine	9.70[b]
leucine	20.39 ± 0.39
tyrosine	3.66[b]
phenylalanine	9.77 ± 0.20
tryptophan	4[c]
antibiotic activity (MIC) (mg/mL)	
S. aureus	0.04
S. lutea	0.01
antitumor activity (ID_{50}) (μg/mL)	8×10^{-3} against KB cells

[a] Values extrapolated to zero time of hydrolysis. [b] Values based on 72-h hydrolysis. [c] Obtained from UV spectra.

Purification of Largomycin on Hydroxylapatite. Chromatography of partially purified culture filtrates from *S. pluricolorescens* on hydroxylapatite resulted in the removal of carbohydrate and protease present in the crude material from largomycin. The purified largomycin gave a single protein band on $NaDodSO_4$ gel electrophoresis (Figure 2A) and was free from carbohydrate and protease activity. The carbohydrate impurity had eluted in faction HA-1 (Figure 1) and the protease activity in fractions HA-4 and HA-5. The amino acid composition (Table I), normalized to four histidyl residues to give a molecular weight corresponding to the results of ultracentrifugation in 6 M guanidine hydrochloride, is notable for the relatively high proportion of hydrophobic and acidic residues and the absence of cysteine as was also reported by Yamaguchi et al. (1970a). The absence of cysteine contrasts with macromomycin, which has four residues (Im et al., 1978) and served as the control for the amino acid analysis. The acidic isoelectric point, 4.13 (Figure 3), indicates that the

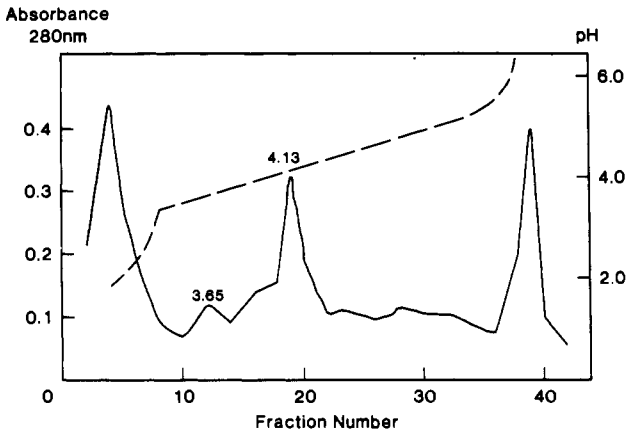

FIGURE 3: Isoelectric focusing of purified largomycin. Experimental conditions are as described under Methods.

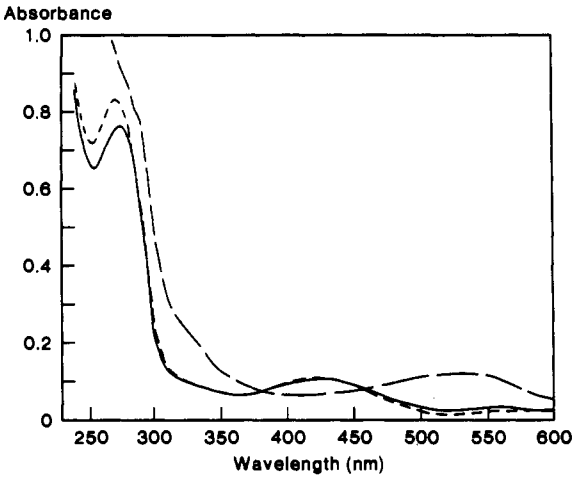

FIGURE 4: Ultraviolet and visible absorbance spectra of purified largomycin. Protein was dissolved (1 mg/mL) and spectra were obtained in water (—), 0.1 N HCl (- - -), and 0.1 N NaOH (– –). The visible absorbance shift from 420 nm in water or 0.1 N HCl to 525 nm in 0.1 N NaOH corresponded to a change in the color of the protein solution from yellow to purple.

majority of the aspartic and glutamic acid residues are in the form of free acids.

Physical Properties. The molecular size of largomycin, studying either crude or pure material, by gel electrophoresis or gel chromatography was around 30 000 daltons. The values contrasted dramatically with the behavior of an aqueous solution of crude largomycin (lot VIII-III-200) in the ultracentrifuge where the principal sedimenting component corresponded to a molecule of 12 900 daltons. With the identification of proteases in the crude preparations, it would appear that the 12 900-dalton component was a fragment of proteolysis; this hydrolysis was significantly reduced in phosphate where the protein behaved in part as a dimer, molecular weight 58 000 by ultracentrifugation. In 6 M guanidine, the molecular weight of largomycin by sedimentation equilibrium was 29 300.

The isoelectric point of largomycin was 4.13, determined by free-solution isoelectric focusing (Figure 3). The fraction that moved into the alkaline compartment of the column was purple and appeared to be a degradation product of largomycin from this procedure since it appeared in all refocusing analyses and in the analysis of all crude largomycins.

The ultraviolet and visible absorption spectra of pure largomycin (Figure 4) were indicative of the presence of an associated pH-indicator chromophore. In water or 0.1 N HCl, the protein solution was yellow with a broad absorbance

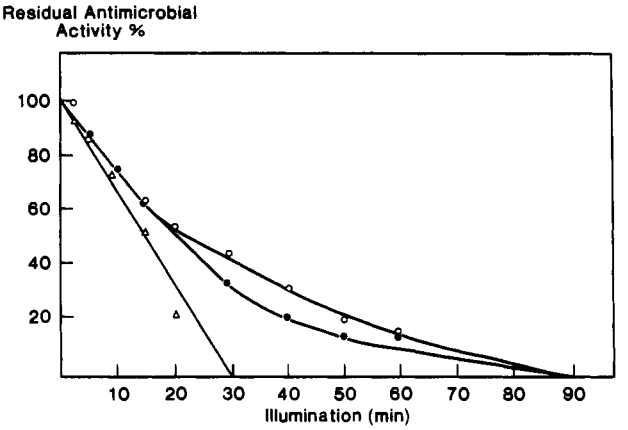

FIGURE 5: Photoinactivation of largomycin. The antibacterial activity of largomycin was tested against *S. lutea* following ultraviolet irradiation in the presence (○) or absence (●) of 0.02% *p*-aminobenzoate. This is compared with similar photoinactivation of macromomycin (△).

maximum at 420–425 nm. This absorbance maximum shifted to 525 nm in 0.1 N NaOH, the purple color being similar to the alkaline fraction from isoelectric focusing. Associated changes in absorbance at 272 and 288 nm were consistent with the amino acid composition of largomycin for tyrosine and tryptophan. The fluorescence emission spectra of largomycin were typical for tryptophan residues, showing a maximum at 340 nm when excited at 288 nm.

Photoinactivation. The antibacterial activity of largomycin was completely destroyed after 90 min of irradiation with ultraviolet light from a lamp placed 4 cm away, as shown in Figure 5. The loss of antibacterial activity was slower than that observed for macromomycin under identical conditions. Also, the presence of 0.02% *p*-aminobenzoic acid did not protect the antibacterial activity of largomycin as had been observed for macromomycin (Im et al., 1978).

The antigenic activity of largomycin was not affected by ultraviolet irradiation. Samples that had been irradiated for 18 h in the presence or absence of 0.02% *p*-aminobenzoic acid each gave only one precipitin line of identity with the unirradiated control by Ouchterlony double diffusion analysis with antilargomycin serum.

Amino Acid Sequence. With 37.7 nmol of largomycin, the following sequence was obtained with an average repetitive yield of 96 ± 7% (see Table II) to the 30th cycle:

$$\overset{1}{\text{Asp}}\text{-Ile-Leu-Ile-}\overset{5}{\text{Ala}}\text{-Gly-Ala-Thr-Gly-}\overset{10}{\text{Asn}}\text{-}\overset{11}{\text{Val}}\text{-Gly-Lys-Pro-}\overset{15}{\text{Leu}}\text{-Val-}$$
$$\text{Glu-Gly-Leu-}\overset{20}{\text{Leu}}\text{-}\overset{21}{\text{Ala}}\text{-Ala-Gly-Lys-}\overset{25}{\text{Pro}}\text{-Val-Arg-Ala-Leu-}\overset{30}{\text{Thr}}\text{-}\overset{31}{\text{Arg}}\text{-Asn}$$

The initial yield calculated for all residues over the first 30 cycles was 32%, as compared to 43% calculated for the leucine residues. These yields were comparable to those obtained in test runs of the sequenator with sperm whale apomyoglobin, where the initial yield calculated for valine and leucine residues was 44%. The analysis of the PTH-amino acids showed little or no background, suggesting that the sample was free of contaminating peptides. Furthermore, the largomycin purified from the mycelium was sequenced through residue 15 with similar results and was shown to be homologous. The amino-terminal residue of largomycin derived from the mycelium was clearly aspartic acid, whereas the largomycin purified from culture filtrates gave some other peaks. Acid hydrolysis of the PTH derivative from the first cycle of the sequence confirmed the aspartate residue but also showed some glutamate, glycine, alanine, and α-aminobutyric acid. The second cycle of the sequence, and each additional cycle up to the first prolyl

Table II: Sequence Analysis of Largomycin

cycle	amino acid assigned[a]	yield (nmol)[b]	repetitive yield (%)
1	Asp	4.9 (13.8)[c]	
2	Ile	13.7	
3	Leu	14.7	
4	Ile	11.1	90
5	Ala	9.7	
6	Gly	9.0	
7	Ala	10.4	103
8	Thr	8.6	
9	Gly	11.6	
10	Asn		
11	Val	12.4	
12	Gly	10.6	97
13	Lys		
14	Pro	18.8	
15	Leu	9.7	97
16	Val	7.1	89
17	Glu		
18	Gly	6.2	97
19	Leu	7.9	96
20	Leu	7.9	96
21	Ala	6.7	98
22	Ala	6.7	98
23	Gly	10.3	96
24	Lys		
25	Pro	14.5	97
26	Val	8.9	102
27	Arg		
28	Ala	5.9	98
29	Leu	6.2	97
30	Thr	5.4	95
31	Arg		
32	Asn		

[a] Amino acid assignment is based upon HPLC identification of the PTH-amino acid derivatives separated by high-pressure liquid chromatography. The first residue was also identified after hydrochloric acid hydrolysis of the PTH derivative. [b] Absolute yields of PTH-amino acids from 37.7 nmol of largomycin placed in the cup were determined by conversion of peak heights from high-pressure liquid chromatographs into nanomoles of each derivative by using values obtained from standard PTH-amino acids. [c] The equivalent yield of PTH-Asp from the largomycin isolated from mycelium is given in parentheses.

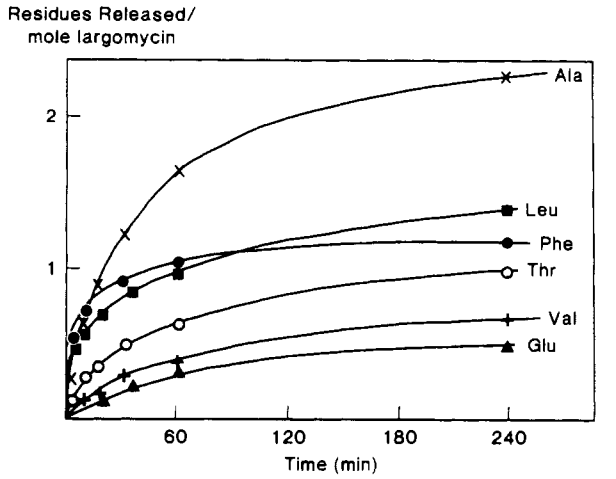

FIGURE 6: Hydrolysis of largomycin with carboxypeptidase A. The amount of amino acids released from the carboxy terminus of largomycin after various periods of incubation with carboxypeptidase A was determined in relation to the amount of largomycin present (see Materials and Methods).

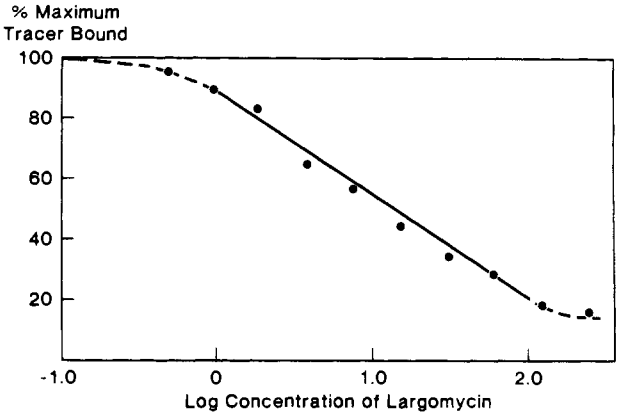

FIGURE 7: Radioimmunoassay of largomycin. The standard curve for a largomycin radioimmunoassay was found to be linear over a concentration of 1–100 mg of competing purified largomycin.

residue of largomycin, gave a single identifiable PTH-amino acid peak upon high-pressure liquid chromatography (HPLC) analysis.

The yellow chromophore remained associated with the dried protein in the sequenator cup through solvent extraction with ethyl acetate and benzene, treatment with heptofluorobutyric acid, and coupling with phenyl isothiocyanate. Following the first coupling step, a second solvent extraction resulted in removal of colored material. The protein remained in the sequenator cup and underwent a second coupling step prior to the first cleavage reaction. Further study revealed that organic extraction under acidic conditions similar to those that the protein was exposed to in the sequenator cup also removed the chromophoric group.

The carboxy-terminal amino acids were determined by carboxypeptidase A digestion of largomycin for various times (Figure 6). Phenylalanine and leucine were released rapidly, closely followed by alanine. The amount of phenylalanine released at various times through the first 60 min of digestion was always slightly higher than the amount of leucine released, and the leucine only exceeded the alanine at early time points due to the sequence of two continuous alanine residues. Threonine, valine, and glutamic acid were formed after significantly longer incubation with the carboxypeptidase A. Taken together with the relative rates of hydrolysis of model compounds, these results are consistent with an amino acid sequence from the carboxy terminus of -Ala-Ala-Leu-Phe-OH. The same results were obtained for the amino- and carboxy-terminal residues of largomycin whether purified from culture filtrates or mycelial extracts.

Homogeneity of Largomycin. Largomycin, purified from the culture filtrate or the mycelium of *S. pluricolorescens*, behaved identically and as a homogeneous polypeptide in chromatography on hydroxylapatite, in $NaDodSO_4$ gel electrophoresis, in the sequencing cycles before the first prolyl residue whereby all the PTH residues were single peaks with little background, in ultracentrifugation as a single sedimenting species, and in Ouchterlony immunodiffusion where each preparation gave a single precipitin line of identity.

Immunoassay of Largomycin. Antibody against largomycin was raised in rabbits. The largomycin antibody did not cross-react with fractions HA-1, HA-4, or HA-5 or the other antitumor proteins, macromomycin, auromomycin, or cesalin. This serum antibody permitted the development of an a-ELISA assay and radioimmunoassay for largomycin, within the ranges 0–15 and 0.5–100 ng, respectively. The standard curve for the radioimmunoassay is shown in Figure 7.

Biological Activities of Largomycin. Pure largomycin showed antibiotic activity against *S. aureus* (MIC 0.04 mg/mL) and *S. lutea* (MIC 0.01 mg/mL), and these activities were used as indicators of the antitumor activity in vitro against KB cells. The latter activity, ID_{50} 8×10^{-3} μg/mL, was

Table III: Inhibition by Largomycin of DNA, RNA, and Protein Biosynthesis in KB Cells

largomycin concn (μg/mL)	inhibition (% of control)		
	DNA	RNA	protein
1×10^{-5}	0		
1×10^{-4}	11		
1×10^{-3}	35		
1×10^{-2}	72	24	4
1×10^{-1}	92	39	19
1.0	97	91	29

determined by the inhibition of incorporation of [*methyl*-^{3}H]thymidine into DNA. The inhibition was less for the incorporation of [5,6-^{3}H]uridine into RNA or ^{14}C-labeled amino acids into protein (Table III). The development of P388 cells in mice was inhibited at 1 mg of largomycin per kg body weight.

By a plaque reduction assay, largomycin was demonstrated to be inactive when compared to interferon. Similarly, it showed no immunopotentiation of lymphocytes. In these respects, it is similar to macromomycin and cesalin.

Discussion

Largomycin is a yellow chromoprotein containing a single polypeptide of molecular weight 29 300, which is consistent with its behavior in $NaDodSO_4$ gel electrophoresis. The protein behaves as a dimer in phosphate buffer, in which it is resistant to proteolysis by enzymes present in the culture fluid. To the polypeptide is complexed a yellow, biologically active chromophore that cannot be removed by extraction with organic solvents unless the protein has been denatured by such agents as urea or acid. In this regard, it differs from macromomycin, auromomycin, and neocarzinostatin, where the chromophores can be extracted entirely with methanol. However, similar to the proteins of auromomycin, neocarzinostatin, and plurallin, hydrophobic amino acid residues are in the majority, which may explain the difficulty with which these proteins are eluted from reverse-phase columns in HPLC.

Purification of largomycin takes advantage of both its acidic character, by chromatography on DEAE-cellulose (Yamaguchi et al., 1970a), and also its binding to phosphate, by chromatography on hydroxylapatite, whereby the crude largomycin can be separated from impurities of carbohydrate and proteolytic enzymes.

One of the problems of the isolation of proteinaceous materials from cells, tissues, or tissue fluids is the degradation by associated proteases. Precautions, such as the addition of protease inhibitors or working at reduced temperatures, are usually taken. The presence of proteases in the culture fluids is frequently refractory to inhibition under circumstances that permit a continuation of the fermentation, with the result that the proteinaceous compounds of interest may copurify during isolation with the proteases. This has been demonstrated in the present study with largomycin and was also found with macromomycin. It is possible, therefore, that the products finally isolated may be degraded proteins, the extent of proteolysis varying somewhat between preparations. Such a possibility was investigated with largomycin isolated from the mycelium before it is released into the fermentation liquids. This crude largomycin contained less than 10% of the protease activity present in preparations from the culture fluid. Both N-terminal and C-terminal amino acid sequences of the largomycins from both sources were compared. The hydrolysis of largomycin by carboxypeptidase A rapidly released phenylalanine, leucine, and alanine consistent with the sequence -Ala-Ala-Leu-Phe-OH. The first 15 amino acids from the amino-terminal residue and those at the carboxyl terminus were the same for largomycin from mycelium and culture filtrate, suggesting that the products isolated by either process were not artifacts of proteolysis of a native protein. It was also noted that crude preparations of macromomycin contained proteases, as determined by hydrolysis of azocasein, but the purified product was resistant to further proteolysis.

The partial amino acid sequence of largomycin was found to be unique among those proteins with antitumor and antibiotic activities (Montgomery et al., 1981). By comparison, there is a high degree of sequence conservation in specific regions of macromomycin, neocarzinostatin, and actinoxanthin; on the average (excluding deletions), 0.752 base change per triplet codon in the DNA sequence would be required to convert the amino acid sequence of neocarzinostatin to that of actinoxanthin. Any suggestion of a structure–function relationship for these proteins would not extend to largomycin.

The fluorescence emission spectrum of largomycin demonstrated excitation arising from the tryptophan residues without any apparent contribution from the tyrosyl residues or the chromophore. This contrasts with auromomycin in which a strong fluorescence was noted from the yellow chromophore as well as from the tyrosyl residue. Like auromomycin, macromomycin, and neocarzinostatin, largomycin looses its biological activity when exposed to light. However, the rate of photoinactivation of largomycin was slower than that for macromomycin. The biologically inactive product from photoinactivation showed no change in molecular weight, as determined by $NaDodSO_4$ gel electrophoresis, and was immunologically identical with the native protein. As in the case of macromomycin, the biological activity of largomycin resides in a nonprotein chromophore, which is degraded by the ultraviolet irradiation without any change being detected in the polypeptide. Similar to other chromophore-containing antitumor proteins, it appears that the protein moiety of largomycin serves to protect and carry a biologically active component that is unstable in free solution.

Largomycin is cytotoxic to KB cells in vitro and to P388 in mice. It also inhibits the growth of Gram-positive (*S. lutea* and *S. aureus*) organisms with approximately the same facility. With the demonstration of antitumor and antibacterial activities, it is perhaps not surprising that largomycin does not act by initiating interferon formation and has no antiviral activity. The cytotoxicity against KB cells, 2.7×10^{-10} M, compares with cesalin, ID_{50} 1×10^{-14} M, and macromomycin, ID_{50} 2×10^{-8} M, the mechanism of action for each of them being in part due to inhibition of DNA synthesis, which occurs at significantly lower concentrations of largomycin than for the equivalent inhibition of RNA or protein biosynthesis (Montgomery et al., 1981; Shepherd et al., 1980; Vandrê et al., 1979). Detailed studies of the mechanism of action of largomycin on tumor cells have not been completed, but preliminary studies indicated that like cesalin and macromomycin the protein inhibits $(Na^+ + K^+)$-ATPase. Unlike cesalin, however, the iodinated largomycin used for the radioimmunoassays did not bind to KB cells (Shepherd & Montgomery, 1980).

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Calorimetric Evidence for Phase Transitions in Spin-Label Lipid Bilayers†

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ABSTRACT: Dispersions of pure, spin-label phosphatidylcholines in aqueous buffer have been investigated with the Privalov high-sensitivity differential scanning calorimeter. The lipids studied are mixed-chain ones in which C-2 of glycerol bears a spin-label derivative of stearic acid and the fatty acid group at C-1 is palmitate. A well-defined phase transition is observed at 30.3–30.7 °C for the phosphatidylcholine labeled near the polar end of the stearate chain (label at C-5). A sharp transition (32–34 °C) is also observed for the lipid spin-labeled near the terminal methyl of stearate (label at C-16), but the thermodynamic parameters for this lipid depend strongly on the history of the sample. Calorimetric evidence for hysteresis in the phase transition of the C-16-labeled lipid is presented. In contrast to the above spin-label lipids, the lipid labeled at C-12 does not show a sharp transition in the region 5–35 °C. In general, therefore, the thermal behavior of the spin-label phosphatidylcholines resembles that of phosphatidylcholines bearing double bonds or branched methyl groups at similar locations on acyl chains. During synthesis of mixed-chain lipids, migration of acyl chains occurs. Methyl esterification procedures which are compatible with the acid-labile spin-label group are described. Gas chromatographic analysis of methyl esters shows that chain migration during synthesis gives 15–20% of the spin-label fatty acid at the glycerol C-1 position.

Considerable insight into the physical chemistry of phospholipid bilayers, with and without protein components, has been obtained in studies which employ lipid probes. These probes may contain paramagnetic groups for electron paramagnetic resonance (EPR)[1] studies (Seelig, 1970; Hubbell & McConnell, 1971; Gaffney & McConnell, 1974), chromophores for fluorescence measurements (Sklar et al., 1975; Radda, 1975), or nuclei, such as deuterium (Seelig, 1977) or fluorine (Sturtevant et al., 1979), suitable for NMR investigation. In some cases, the physical properties of the probe molecule may have important consequences for the course of the experiment. For instance, the α- and β-paranaric acids, which are fluorescent lipid probes, partition between fluid and solid lipid phases to differing extents (Sklar et al., 1975), and

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[1] Abbreviations: PSPC, 1-palmitoyl-2-stearoylphosphatidylcholine; P16MSPC, 1-palmitoyl-2-(16-methylstearoyl)phosphatidylcholine; 1-palm-2-(*m*,*n*)PC, a spin-label derivative of PSPC bearing the 1-oxy-2,2-dimethyloxazolidine group on the $n + 2$ carbon of the 2-stearoyl chain; DSC, diffferential scanning calorimetry; EPR, electron paramagnetic resonance; GC, gas chromatography; TLC, thin-layer chromatography.

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