LARGOMYCIN FII CHROMOPHORE COMPONENT 4, A NEW PLURAMYCIN ANTIBIOTIC

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The chromophore of the antitumor chromoprotein largomycin FII is a mixture of components belonging to the pluramycin class of antitumor antibiotics. Against most organisms tested, component 4 exhibited activity equal to or greater than the major chromophore components pluramycin A and deacetylpluramycin A. Data obtained from UV, IR, ^{1}H and ^{13}C NMR, and from fast atom bombardment mass spectrometry were used to determine the structure of component 4 as epoxykidamycin, a new member of the pluramycin class.

Largomycin FII (LM-FII) is an acidic 29,300 dalton chromoprotein produced by *Streptomyces pluricolorescens*[1,2]. In addition to possessing *in vitro* activity Gram-positive bacteria and fungi, LM-FII was shown to be cytotoxic *in vitro* and active *in vivo* against several transplantable tumors in mice[3,4]. The LM-FII holoprotein can be separated into its apoprotein and chromophore components using mild acidic conditions that denature the protein[2,3,5]. The chromophore of LM-FII, which possesses all of the antimicrobial activity associated with LM-FII, consists of a complex mixture of components separable by high performance liquid chromatography (HPLC). The chromophore components belong to the pluramycin group of anthraquinone antitumor agents. Two of the major components, designated 8 and 7, have been identified as pluramycin A and deacetylpluramycin A (rubiflavin A[6]), respectively[5]. Several of the other components are thought to be decomposition products of pluramycin A. The chromophore fraction designated component 4 does not appear to be a decomposition product of pluramycin A, and its *in vitro* activity seems comparable to or greater than that of the pluramycin A or deacetylpluramycin A components[5]. The antimicrobial activity and structure of component 4 are presented below.

Materials and Methods

Antibiotics

All largomycin samples were partially purified according to published procedures[3,7] by the Chemistry Section, Fermentation Program, National Cancer Institute, Frederick Cancer Research Facility, and were generously provided for our use. LM-FII lot 1B1 consisted of material that did not meet the criteria established for LM-FII use in animal testing. Hedamycin and rubiflavin complex were supplied by The National Cancer Institute. Largomycin FII chromophore components 7 and 8 (*i.e.*, deacetylpluramycin A and pluramycin A, respectively) were obtained as previously described[5]. Components 4, 7 and 8, and hedamycin were all greater than 90% pure as judged by HPLC and ^{1}H NMR. Rubiflavin was recently shown to be a complex mixture of components; rubiflavin A was identified as deacetylpluramycin A and is one of only two components of the complex fully characterized[6]. The drugs were handled under yellow lighting.

Chromophore Isolation and Initial Purification of Component 4

Isolation of LM-FII chromophore was accomplished by dissolving 20 g of LM-FII lot 1B1 in

100 ml of H_2O and adding to 750 ml of cold EtOH. The pH was adjusted to 5.3 with glacial acetic acid and the resulting suspension was centrifuged. (Further extraction of the pellet with ethanol did not remove significant quantities of component 4.) The pH of the supernatant was adjusted to 7.0 with 1 N NaOH. After removing the EtOH by evaporation under vacuum, the chromophore was converted to free base form by adjusting the pH of the remaining aqueous solution to 8.5. The solution was then extracted three times with equal volumes of CCl_4. The volume of the combined CCl_4 extracts was reduced to 25 ml, washed three times with alkaline water, and the CCl_4 removed. The dried chromophore extract was stored at −20°C. A total of 68 g of LM-FII lot 1B1 was extracted as described, and component 4 of the chromophore was further purified by HPLC.

HPLC

Separations were carried out using a Waters Associates 6000A solvent delivery system and a Schoeffel SF770 variable-wavelength detector. A Waters Associates μBondapak C_{18} column (30 cm × 4.1 mm) was used for analytical purposes, while preparative separations were performed on a Whatman Partisil M9-1050 ODS-3 column (50 cm × 94 mm). The mobile phase consisted of MeOH - H_2O (55: 45) made 100 mM in ammonium acetate and adjusted to a pH of 5.1 with glacial acetic acid. The flow rate was adjusted to 1 ml/minute for the analytical separations and 3 ml/minute for the preparative separations. The chromophore components were detected at 254 nm. Component 4 was collected from the HPLC in an ice bath. The MeOH was removed under vacuum and the component converted to its free base form, extracted, dried, and stored at −20°C. From the 68 g of LM-FII lot 1B1 holoprotein, 1.98 mg of pure component 4 were obtained.

Antimicrobial Activity

Agars were made 0.01 M with Trizma Base (Sigma, St. Louis) and adjusted to pH 8.7 with 1 N NaOH before autoclaving. Antibiotic Medium 3 (Difco, Detroit) was used for *Staphylococcus aureus, Bacillus subtilis*, and *Escherichia coli*; Mueller-Hinton agar for *Micrococcus luteus*; Sabouraud dextrose agar for *Candida albicans* and *Penicillium notatum*; and Antibiotic Medium 1 for *Pseudomonas stutzeri*. Test samples were dissolved in 100 mM ammonium acetate and 15 μl were applied to 6.35-mm discs. The minimal inhibitory concentrations were calculated from a standard curve for each drug.

UV and IR Spectroscopies

The UV spectra of component 4 were taken in EtOH and recorded on a Perkin-Elmer Lambda 5 spectrophotometer. The IR spectrum was obtained by dissolving component 4 in $CHCl_3$, drying it under a stream of N_2 on a KBr salt pellet, and recording the spectrum on a Perkin-Elmer 180 spectrophotometer.

Fast Atom Bombardment Mass Spectrometry

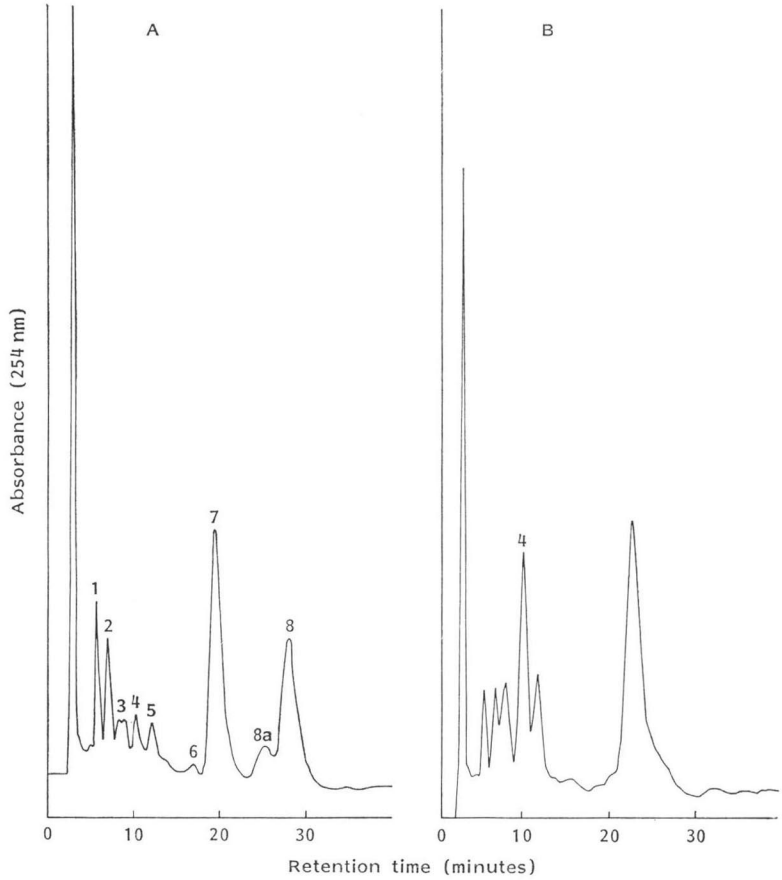
Fast atom bombardment mass spectra (FAB-MS) were obtained using a ZAB-2F (VG Analytical, Altrincham, UK) interfaced to a VG 2035 data system. Xenon atoms (8 KeV, 1 mA plasma discharge current; Ion Tech Ltd. gun, Middlesex, UK) were used as the ionizing particles. A mixture of glycerol - *N,N*-dimethylformamide (1: 1) was used as the supporting matrix. The scanning rate was 10 seconds/decade with a resolution of 1,000. A typical spectrum of glycerol was saved in the computer memory and was subsequently used for "background" subtraction. High resolution peak matching measurements were performed at 10,000 resolution (5% crossover definition) using $[Na_5I_4]$ (m/z 622.5667).

NMR Spectroscopy

Spectra were obtained utilizing 1.98 mg of component 4 dissolved in 0.5 ml $CDCl_3$ (Merck) in a 5-mm tube. A Nicolet (GE Medical System) NT-300 spectrometer with a 1,280 data system was used in conjunction with tuned 5-mm carbon (75.46 MHz) and proton probes (300.04 MHz). Carbon spectra were broadband decoupled using Nicolet's implementation of Levitt (MLEV 16) decoupling. Homonuclear two-dimensional (2-D) proton correlation spectra were obtained using Nicolet's COSY sequence with 512 1K data points accumulated, sine bell apodized in both dimensions to give a 512 × 512 transformed data block.

Fig. 1. HPLC profile of LM-FII chromophore complex (2.0 mg equivalent weight of LM-FII dissolved in mobile phase) using reverse-phase paired-ion chromatography and a Waters Associates μBondapak C_{18} column as described in Materials and Methods.

(A) Chromophore extracted from lot KS-7G, (B) chromophore extracted from lot 1B1.



Results

Preparation of Component 4

Component 4 was originally identified as one of more than 8 HPLC separable components comprising the chromophore complex of LM-FII holoprotein[5]. While component 4 was normally not present in high concentrations in the chromophore mixture (Fig. 1A), one crude lot of LM-FII designated 1B1 was found to contain a substantial quantity of the component (Fig. 1B). Extraction of 68 g of LM-FII lot 1B1 yielded 1.98 mg of component 4.

Antimicrobial Activity

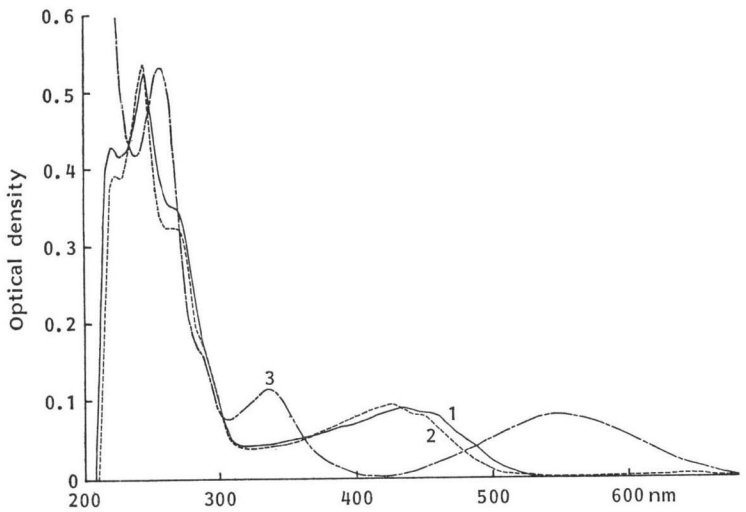
Table 1 presents the antimicrobial spectrum of component 4 in comparison with other active components of the LM-FII chromophore and known members of the pluramycin group of antibiotics. The activity of component 4 was comparable to that of hedamycin. Component 4 exhibited greater activity against Gram-positive bacteria than components 7 and 8 and slightly weaker activity against the fungus *P. notatum*. Rubiflavin complex was approximately 10 times less active than component 4.

Table 1. Antimicrobial activity of LM-FII chromophore component 4 compared with other pluramycins.

Test organism	MIC (μg/ml)[a]				
	Component 4	Component 7 (deacetyl-plura-mycin A)	Component 8 (plura-mycin A)	Hedamycin	Rubiflavin
Staphylococcus aureus ATCC 6539P	2.5	5	5	1.0	25
Bacillus subtilis ATCC 6633	0.5	1.5	2.5	0.5	10
Micrococcus luteus ATCC 9341	0.005	0.5	0.5	0.01	2.5
Escherichia coli ATCC 10536	10.0	25	50	5	100
Pseudomonas stutzeri ATCC 11607	2.5	25	25	5	50
Candida albicans ATCC 10231	>100	>100	>100	>100	>100
Penicillium notatum ATCC 9478	50	25	10	25	100

[a] Compounds were dissolved in 100 mM ammonium acetate. Concentrations were calculated from optical density measurements at 430 nm normalized to a reference sample of hedamycin having an $E^{1\%}_{1cm}$ of 87 in the same solvent.

Fig. 2. UV visible spectra of component 4 from LM-FII chromophore complex.
Spectra were obtained in EtOH (1), EtOH made 0.01 N in HCl (2), and EtOH made 0.01 N in NaOH (3).



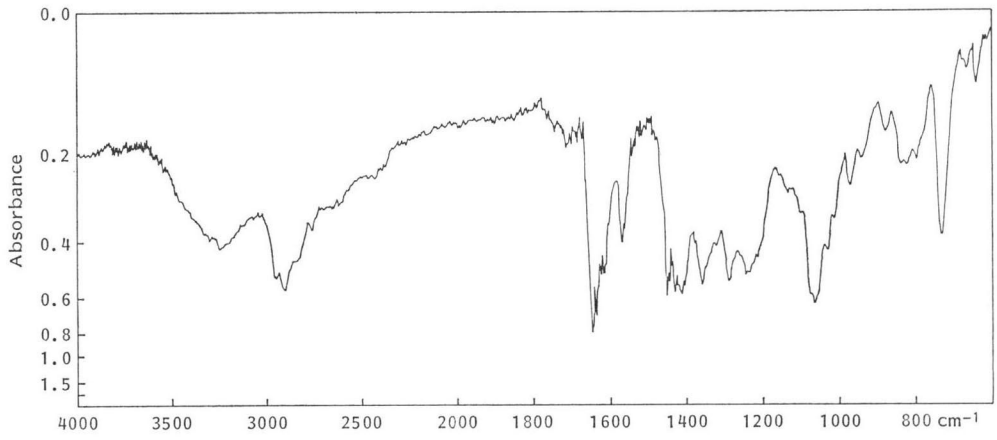
Spectrometry

The UV spectra of component 4 (Fig. 2) were characteristic of the pluramycin class of antibiotics, including the spectral shift that appears in base: UV λ^{EtOH}_{max} nm ($E^{1\%}_{1cm}$) 246 (474), 268 (sh 316), 288 (sh 153), 434 (82); $\lambda^{0.01N\ HCl-EtOH}_{max}$ 245 (482), 268 (sh 293), 288 (sh 153), 428 (87); $\lambda^{0.01N\ NaOH-EtOH}_{max}$ 257 (480), 283 (sh 172), 335 (105), 555 (73).

The IR spectrum of component 4 is shown in Fig. 3. The bands occurring at 2960, 2900, 2760, 1650, 1620, 1570, 1455, 1430, 1415, 1360, 1290, 1250, and 1070 cm^{-1} are characteristic of compounds in the pluramycin class. A notable feature of the spectrum is the absence of a major band at 1730 cm^{-1}, a carbonyl-stretching frequency characteristic of the naturally occurring pluramycins acetylated at the 4″ position.

As described previously[5], the technique of FAB-MS is well suited for the study of pluramycins.

Fig. 3. IR spectrum of component 4 from LM-FII chromophore complex.



The positive and negative ion FAB spectra of component 4 are shown in Fig. 4. Component 4 shows a characteristic pseudomolecular ion $[M+H]^+$ (*m/z* 705) and a molecular anion $[M]^-$ (*m/z* 704). The elemental composition of the pseudomolecular ion cluster was determined to be $C_{39}H_{49}N_2O_{10}$ (705.3376, *i.e.*, +1.7 ppm) using peak matching. Fragment ions corresponding to the two sugar rings (*m/z* 158 and *m/z* 172) were detected in the positive ion FAB spectrum, as well as fragment ions from the probable loss of C_2H_2 (26 mass units) from both of the sugar moieties (*m/z* 132 and *m/z* 146). Lower molecular weight fragment ions, such as *m/z* 70, 88, 102 and 114, are probably due to sugar fragmentations.

NMR

Carbon and proton chemical shifts, proton multiplicities and proton *J*-connectivities for component 4 are shown in Table 2. Carbon and proton assignments were made by conventional procedures using the assignments of deacetylpluramycin A[5] as a starting point, and with considerable use of the connectivities in the 2-D homonuclear correlation spectrum. Fig. 5 shows the most heavily overlapped region of the 2-D spectrum (1~6 ppm). In this presentation, the off-diagonal cross peaks demonstrate *J*-couplings between horizontally and vertically projected resonances on the central diagonal. Thus, A in Fig. 5 shows a strong connection between resonances 2″ (4.08 ppm) and 2″-CH_3 (1.51 ppm). The cross peak at B shows the power of 2-D in a crowded spectrum. A methyl singlet at 3.00 ppm completely obscures a resonance (4′) centered beneath it, but the shape of the cross peak (from 3′ at 3.21 ppm) quite readily reveals the presence of 4′. Hence, the chemical shift of 4′ is determined.

Detailed comparison of carbon and proton shift data for component 4 with the data for deacetylpluramycin A indicates that the only differences occur in the side chain from C-14 to C-17. The coupling of the quartet at 3.46 ppm with the doublet at 1.54 ppm in the proton spectrum requires the presence of a methyl at C-17, adjacent to an oxygen-bearing methine carbon at C-16. The lack of further coupling at C-16 requires the next carbon along the chain (C-14) to be quaternary. Consideration of the molecular formula then requires the singlet methyl (1.83 ppm) be attached at C-14 and assigned as C-15 and, further, that the oxygen at C-16 be attached through an epoxide bond to C-14. The existence of an epoxide is consistent with the two side chain carbon resonances observed at 62.0 ppm and 57.6 ppm in the carbon spectrum. A comparison of these data with reported values for epoxy side chains of pluramycin analogues[8] supports this structural assignment. On the basis of

Fig. 4. FAB-MS of component 4 from LM-FII chromophore complex.

(A) Positive ion; (B) negative ion.

The ion appearing at m/z 421 in the negative spectrum is due to a contaminant which was observed in the spectra of several unrelated compounds.

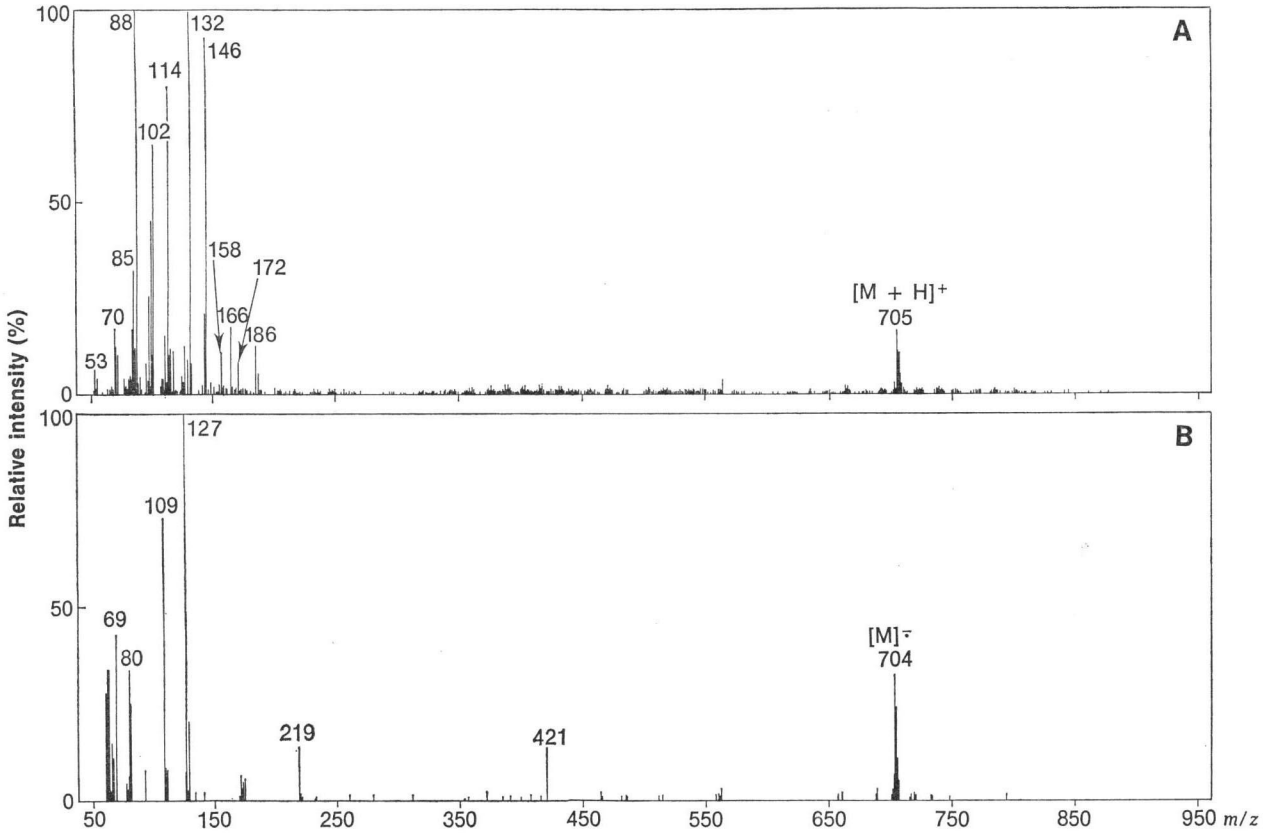


Table 2. ^{1}H and ^{13}C NMR values and 2D connectivities of LM-FII chromophore component 4[a].

Position	^{13}C NMR (chemical shifts)		^{1}H NMR (component 4)	
	Component 4	Deacetyl-pluramycin A[5]	Chemical shift	Connections (2D)
2	168.0	167.3	—	—
3	109.7	109.8	6.48 s	—
4	178.9	178.8	—	—
4a	125.9*	126.1	—	—
5	149.9	149.7	—	—
6	125.8	125.7	8.00 d	13
6a	137.0	137.1	—	—
7	183.2	183.1	—	—
7a	126.3*	126.1	—	—
8	139.7	140.0	—	—
9	132.9	132.8	8.32 d	6′, 6″
10	137.8	138.2	—	—
11	159.7	159.7	14.07 (OH) s	—
11a	116.1	115.9	—	—
12	187.9	187.9	—	—
12a	119.1	119.1	—	—
12b	156.0	155.9	—	—
13	24.3	24.1	3.00 s	6
14	57.6	59.0	—	—
15	13.8	14.3	1.83 s	—
16	62.0	61.5	3.46 q	17
17	14.1	123.0	1.54 d	16
18	—	134.0	—	—
19	—	14.0	—	—
2′	77.2	77.1	3.58 dq	3′, 2′-CH_3
3′	71.3	71.6	3.21 dd	2′, 4′
4′	67.8	67.3*	2.98 m	3′, 5′a, 5′b
5′a	28.8	28.2	2.27 m	4′, 5′b, 6′
5′b	—	—	1.40 m	6′, 4′, 5′a
6′	74.8	74.9	5.45 m	5′a, 5′b
2′-CH_3	18.8	18.8	1.45 d	2′
4′-$N(CH_3)_2$	40.3	40.3	2.41 s	—
2″	67.5	66.9*	4.08 qd	3″, 2″-CH_3
3″	70.3	70.6	3.42 s, br	2″, 5″a
4″	57.6	57.3	—	—
5″a	33.3	33.1	2.58 dd	3″, 6″, 5″b
5″b	—	—	2.29 m	5″a, 6″
6″	69.4	69.6	5.45 m	5″a, 5″b
2″-CH_3	17.4	17.6	1.51 d	2″
4″-CH_3	13.1	12.2	0.78 s	—
4″-$N(CH_3)_2$	36.9	36.6	2.31 s	—

[a] All numerical values are chemical shifts in ppm vs. internal TMS. Proton values are ±0.05 ppm; carbon values are ±0.1 ppm.

* Assignments may be interchanged. (a) and (b) designate separate protons of a methylene pair where exact stereochemistry is not assigned; (a) refers to the lower field member of the pair.

the spectroscopic data presented, the structure of LM-FII chromophore component 4 is determined to be epoxykidamycin, the 14,16-epoxy analogue of kidamycin (Fig. 6) and a new member of the pluramycin class of antitumor antibiotics.

Fig. 5. The 2D homonuclear ^1H NMR spectrum of component 4 from LM-FII chromophore complex showing the expanded 1~6 ppm range.

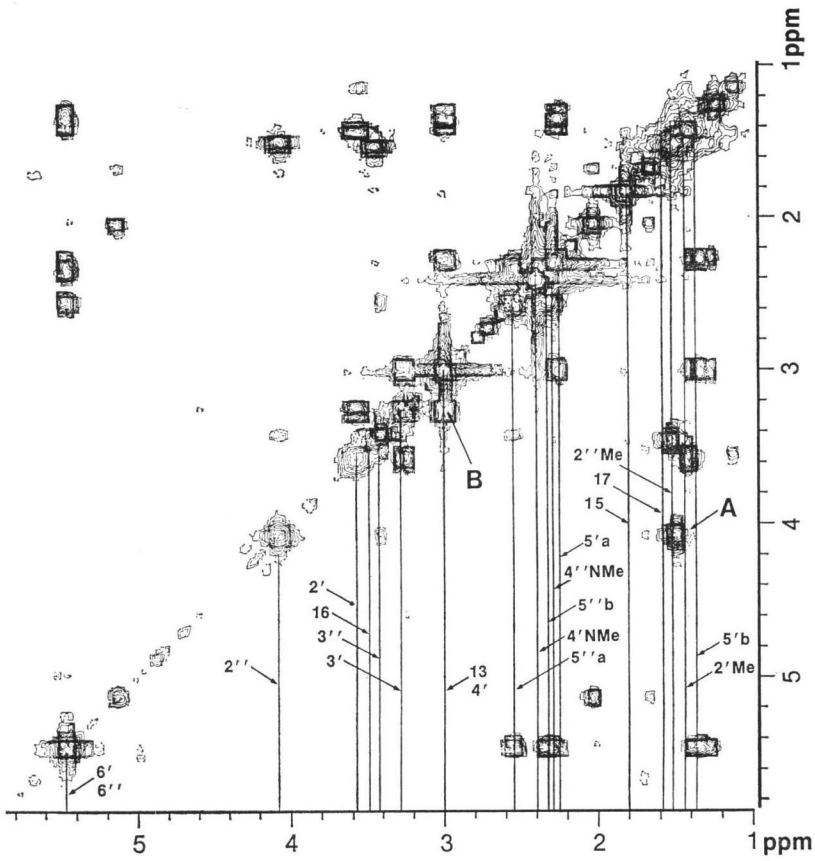
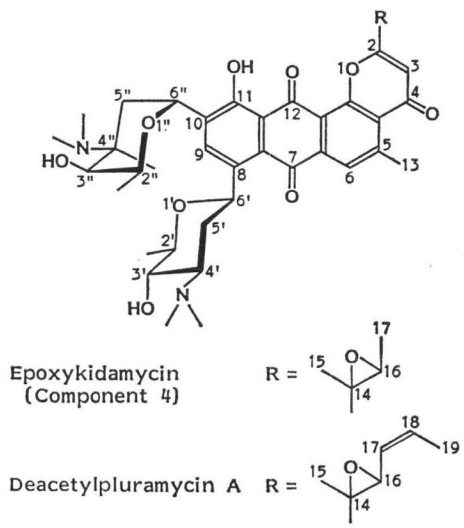


Fig. 6. Structure of component 4 and deacetylpluramycin A.



Discussion

Two of the major LM-FII components were identified earlier as pluramycin A and deacetylpluramycin A[5]. Component 4 was chosen for study because it had demonstrated greater antimicrobial activity than the other chromophore components and because it was not thought to be a decomposition product of pluramycin A. The antimicrobial activity of purified component 4 is approximately five times greater than was observed in our previous study using less purified material[5]. In comparison with the other two LM-FII chromophore components of major biological importance, pluramycin A and deacetylpluramycin A, component 4 exhibits between 2 and 100 fold better antibacterial activity. None of the components are active against the yeast *C. albicans*, and component 4 has slightly less activity than components 7 and 8 against *P. nota-*

tum. Component 4 exhibits a spectrum of activity quite similar to that of hedamycin.

The UV and IR spectra firmly place component 4 in the pluramycin class of antitumor antibiotics. The absence of a strong absorption peak at 1730 cm^{-1} in the IR spectrum eliminates the possibility that any of the sugar positions are acetylated. Data obtained from FAB-MS establish the molecular weight of component 4 as 704 and the elemental composition of the $[M+H]^+$ pseudomolecular ion as $C_{39}H_{49}N_2O_{10}$. The positive ion FAB-MS also indicates the presence of sugars with the same mass as those present in deacetylpluramycin A. The intense signals at *m/z* 132 and 146 are characteristic of C_2H_2 (26 mass unit) losses from each of the two C-glycosidic dimethylamino sugar moieties, as observed in the FAB-MS of deacetylpluramycin A[5]. Interestingly, this unusual loss of a 26 mass unit fragment also occurs in the positive ion FAB-MS fragmentation of ravidomycin, another antitumor antibiotic that contains a C-glycosidic dimethylamino sugar linked to an aromatic ring system[9] (G. T. CARTER, personal communication).

The ^1H and ^{13}C NMR spectra clearly show that the only structural differences between component 4 and deacetylpluramycin A reside in the side chain at C-2. The side chain of component 4 is easily identified as a 1,2-epoxy-1-methylpropyl group from the proton couplings and chemical shifts, the carbon chemical shifts, knowledge of the molecular weight, and the elemental composition. Thus, component 4 is unambiguously established as epoxykidamycin, a new pluramycin antibiotic.

The nature of the relationship between the pluramycin chromophore and the LM-FII apoprotein remains a topic for further study. Earlier studies have shown that the chromophore benefits from the association by gaining stability towards light and heat[5]. The protein may also play a role *in vivo* by transporting or targeting the chromophore to its site of action. The precise nature of the chromophore-protein binding is not known. The presence of the two basic dimethylamino groups on the C-glycosidic sugar rings, together with the observation that the chromophore is most easily extractable when the pH approaches the isoelectric point of the acidic holoprotein [pI 4.1[3]], suggests that ionic binding may be involved. This hypothesis agrees with the finding that LM-FII is not the only chromoprotein present in the fermentation broth[2]. In fact, the yellow color and prophage induction capability characteristic of the pluramycin antibiotics is found in the fermentation broth associated with proteins ranging in size from several thousand to over 100,000 daltons (M. E. GUSTAFSON, personal communication). This suggests that the chromophore may nonselectively associate with many acidic proteins during the course of fermentation. Such a nonselective binding might also explain why all of the other known members of the pluramycin class of antibiotics except epoxykidamycin were originally discovered as free antibiotics[10~15] rather than as chromoproteins.

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