ies suggest that a free terminal carboxyl is necessary for pressor action of angiotensin (Bumpus *et al.*, 1961).

A failure of the antibody to inhibit the biological effect of angiotensin on the isolated aortic strip suggests as one possibility that the binding affinity between the hormone and its physiologic site of action is considerably greater than that between the hormone and its antibody.

The availability of specific antibody directed against a peptide of small size and definite amino acid sequence, rather than a random copolymer, will facilitate study of the structure of antibody and the precise requirements and relationships of antigenic determinants. This type of copolymer allows for amino acid substitutions, inversions, and other specific modifications. Such flexibility of alteration is not possible in either random copolymers or natural protein antigens.

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U-21,669: A New Lincomycin-related Antibiotic*

A. D. Argoudelis, J. A. Fox, and T. E. Eble

ABSTRACT: U-21,699 is a new antibiotic very closely related to lincomycin. Both U-21,699 and lincomycin are produced by *Streptomyces lincolnensis* var. *lincolnensis*. Isolation of the activities from the fermentation broth has been achieved by carbon adsorption, followed by elution with aqueous acetone. U-21,699 has been separated from lincomycin by countercurrent distribution, followed by Florisil chromatography, and has been isolated in crystalline form as the hydrochloride salt. The structure of U-21,699 has been determined and the work supporting the proposed structure is discussed.

Antibiotic U-21,699 is a new lincomycin-related antibiotic, produced concomitantly with lincomycin[1] in fermentations of *Streptomyces lincolnensis* var. *lincolnensis*. The isolation and chemistry of lincomycin have been reported by Mason *et al.* (1962), Herr and Bergy (1962), and Hoeksema *et al.* (1964). The present paper describes the isolation and chemical properties of U-21,699. Biological properties of this new antibiotic have been described by Mason and Lewis (1964).

Experimental and Results

Isolation of U-21,699. Recovery from Fermentation Broth. A culture broth of lincomycin fermentation (24 kl) was adjusted to *p*H 3.0 with concentrated sulfuric acid, and filtered using filter aid. The filtered beer was adjusted to *p*H 8.0 with 50% aqueous sodium hydroxide solution. The alkaline clear beer was then passed through columns containing Pittsburgh Type CAL, 12–40 mesh granular carbon (109 kg). The carbon columns were

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[1] Lincocin is the trademark of The Upjohn Company for lincomycin hydrochloride.

washed first with water, followed by 99% aqueous acetone.

U-21,699 and lincomycin were eluted from the carbon by using 75% aqueous acetone at 50°. The acetone eluates (8.7 kl) were concentrated under reduced pressure to an aqueous concentrate of 0.3 kl. The aqueous concentrate was adjusted to *p*H 10.6 with 50% sodium hydroxide solution and extracted with methylene chloride (total volume of 0.4 kl). The methylene chloride extracts were mixed with water and the mixture was concentrated azeotropically to an aqueous solution (72 l), which was adjusted to *p*H 1.0 with concentrated hydrochloric acid. Acetone (0.7 kl) was added to the acidic aqueous concentrate and the mixture was allowed to stand overnight. Crude crystals of lincomycin and U-21,699 hydrochlorides were isolated by filtration and dried (30.75 kg).

This material was dissolved in water (32 l) and mixed with 0.3 kl of acetone. Lincomycin hydrochloride started precipitating almost immediately. The crystals of lincomycin hydrochloride were isolated by filtration and dried (24.3 kg). The mother liquors containing U-21,699 hydrochloride and residual lincomycin hydrochloride were concentrated to an aqueous solution (21 l). One liter of this solution was adjusted to *p*H 9.5 by using 2 N aqueous sodium hydroxide solution. The alkaline solution was then extracted with methylene chloride. The methylene chloride extract was concentrated to dryness to give 85.0 g of crude U-21,699 free base which was used as the starting material for the countercurrent distribution described in the next paragraph.

Countercurrent Distribution. Ten g of crude U-21,699 free base obtained as described was dissolved in 75 ml of the lower phase of the solvent system consisting of equal volumes of 1-butanol and water. The *p*H was adjusted to 4.2 by addition of 1 N aqueous hydrochloric acid. This solution was then mixed with an equal volume of the same solvent system and the mixture was transferred in an all-glass Craig countercurrent distribution apparatus (10 ml/phase). The distribution was stopped when 1000 transfers had been completed. The distribution was then analyzed by the determination of solids (Figure 1) and thin-layer chromatography. Two peaks with *K* values of 0.09 and 0.15 were found. Thin-layer chromatography showed that tubes 60–90 contained U-21,699 as the only bioactive material. However, these fractions were found to contain impurities which were removed by the Florisil chromatography described in the next paragraph.

Florisil Chromatography. Fractions 60–90 from the countercurrent distribution described were combined and concentrated to an aqueous solution. The *p*H was adjusted to 10.5 and the solution was freeze-dried to give 900 mg of colorless amorphous material. This material (800 mg) was dissolved in 20 ml of acetone and the solution was added on the top of a 25-mm (i.d.) column containing 40 g of Florisil packed in Skellysolve B. The column was then eluted with Skellysolve-acetone mixtures of increasing acetone content. U-21,699 was eluted from the column with a mixture of Skellysolve-acetone in the volume ratio of 10:90. The eluates concentrated to dryness gave 400 mg of U-21,699 free base obtained as a colorless amorphous material; $[\alpha]_D^{25}$ +153° (*c* 0.87, water). The ultraviolet spectrum showed end absorption only. The infrared spectrum (Figure 4) showed absorption at the following frequencies: 3340, 2920, 2850, 2780, 1645, 1527, 1457, 1376, 1343, 1325, 1305, 1237, 1215, 1185, 1158, 1078, 1051, 988, 945, 902, 863, 802, 778, 720, and 692 cm^{-1}.

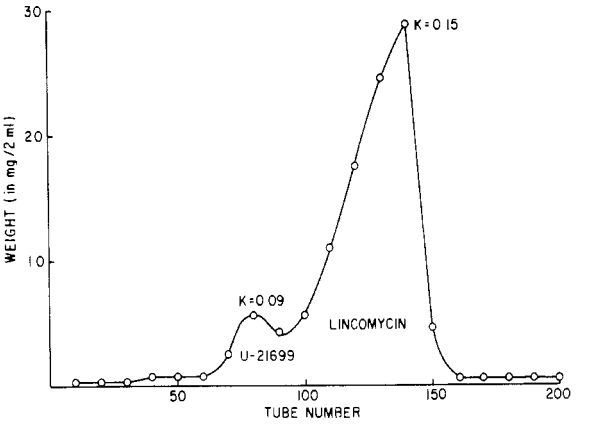


FIGURE 1: Countercurrent distribution of mixture of U-21,699 and lincomycin.

Anal. Calcd for $C_{17}H_{32}N_2O_6S \cdot \frac{1}{2}H_2O$: C, 50.92; H, 8.30; N, 6.99; S, 8.00; O, 25.84; mw 401. Found: C, 50.96; H, 8.52; N, 6.94; S, 7.89; O (diff), 25.69. Potentiometric titration showed the presence of one titratable group, pK_a' 7.68, equivalent weight 406.

U-21,699 Hydrochloride. U-21,699 free base (100 mg) was dissolved in 0.7 ml of 1 N aqueous hydrochloric acid. The solution was then mixed with 30 ml of acetone and 40 ml of ether, and the mixture was allowed to stand at room temperature for 15 hours. The crystalline colorless U-21,699 hydrochloride was isolated by filtration and dried (60 mg); $[\alpha]_D^{25}$ +147° (*c* 1, water). The infrared spectrum shows absorption at the following frequencies: 3470, 3360, 3240, 3180, 3090, 1690, 1680, 1590, 1580, 1305, 1270, 1235, 1205, 1160, 1145, 1125, 1100, 1085, 1050, 980, 905, 875, and 795 cm^{-1}.

Anal. Calcd for $C_{17}H_{32}N_2O_6S \cdot HCl \cdot \frac{1}{2}H_2O$: C, 46.72; H, 7.38; N, 6.41; S, 7.34; Cl, 8.11; O, 23.77. Found: C, 46.77; H, 8.03; N, 6.36; S, 7.46; Cl, 7.98; O (diff), 23.40.

Hydrazinolysis of U-21,699. Isolation of α-Methylthiolincosaminide (Compound II). U-21,699 free base (500 mg) was dissolved in 10 ml of hydrazine hydrate. The solution was kept at reflux for 24 hours. It was then concentrated to dryness *in vacuo*. The residue was triturated three times with 10-ml portions of acetonitrile. Material insoluble in acetonitrile was isolated by filtration and dried (250 mg). Recrystallization from dimethylformamide afforded 172 mg of crystalline α-methylthiolincosaminide, mp 213–215° (uncorr); mixed mp 214–216° (uncorr); mp of authentic sample obtained from lincomycin, 216–217° (uncorr).

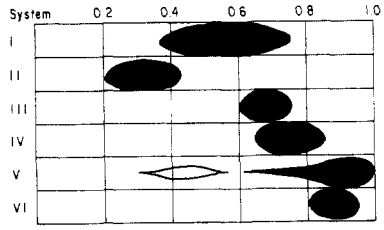


FIGURE 2: Paper chromatography of U-21,699 hydrochloride. Solvent systems: (I) 1-butanol-water (84:16), developed 16 hours; (II) 1-butanol-water (84:16) plus 0.25% *p*-toluenesulfonic acid, developed 16 hours; (III) 1-butanol–acetic acid–water (2:1:1), developed 16 hours; (IV) 1-butanol-water (84:16) plus 2% piperidine, developed 16 hours; (V) 1-butanol-water (4:96), developed 5 hours; (VI) 1-butanol-water (4:96) plus 0.25% *p*-toluenesulfonic acid, developed 5 hours. The antibiotic was detected by bioautography on *Sarcina lutea* seeded agar.

Anal. Calcd for $C_9H_{19}NO_5S$: C, 42.72; H, 7.57; N, 5.54; S, 12.67. Found: C, 42.27; H, 7.65; N, 5.53; S, 12.40. The specific rotation of the crystalline material was found to be +274° (*c* 0.782, water); reported for α-methylthiolincosaminide, +274° (Hoeksema *et al.*, 1964). Infrared and NMR[2] spectra of the crystalline material obtained from U-21,699 and α-methylthiolincosaminide were identical.

The second fragment of the U-21,699 molecule, obtained by hydrazinolysis as the hydrazide, is soluble in acetonitrile and was found in the filtrate obtained as described. This material was not isolated, but it was converted to 4-ethylhygric acid hydrochloride by acid hydrolysis described in the next paragraph.

Acid Hydrolysis. Isolation of Ethylhygric Acid Hydrochloride (Compound IIIa). The acetonitrile filtrate, obtained as described, was concentrated to dryness. The residue was dissolved in 15 ml of 6 N aqueous hydrochloric acid and the solution was kept at reflux for 6 hours. The hydrolysate was then concentrated to dryness. The residue was dissolved in 30 ml of water. The *p*H was adjusted to 7.0 by addition of solid silver carbonate. Insoluble material was separated by filtration and the filtrate was decolorized with Darco G-60. The clear solution was then concentrated to dryness. The residue was dissolved in 3 ml of 1 N methanolic hydrogen chloride, 5 ml of methanol, and 10 ml of acetone. Ether was added until the solution became cloudy. The precipitated crystalline material was isolated by filtration and dried. The infrared spectrum, which shows the following absorptions, suggested an α-amino acid: 3520, 3450, 2710, 2660, 2580, 2500, 1745, 1700, 1600, 1225, 1185, 1150, 1115, 1090, 1020, 850, 840, 805 cm^{-1}. $[\alpha]_D^{25}$ −46° (*c* 0.48 in water, *p*H 4.5) and −64° (*c* 0.48, water, *p*H 11).

Anal. Calcd for $C_8H_{16}NO_2Cl$: C, 49.65; H, 8.34; N, 7.24; O, 16.54; Cl, 18.32. Found: C, 48.54; H, 8.35; N, 7.05; Cl, 18.52.

[2] Abbreviation used in this work: NMR, nuclear magnetic resonance.

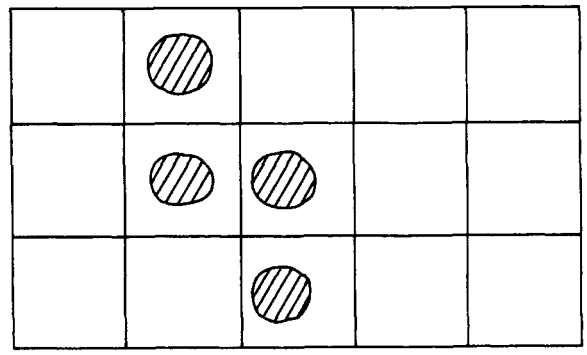


FIGURE 3: Thin-layer chromatography of U-21,699 hydrochloride. Upper, U-21,699 hydrochloride; middle, U-21,699 and lincomycin hydrochloride; lower, lincomycin hydrochloride. Thin-layer plates were prepared from Silica Gel G (Merck Darmstadt). Thickness of the film was 0.4 mm. The solvent system consisted of 150 ml of methyl ethyl ketone, 50 ml of acetone, and 20 ml of water. Detection systems used were periodate-permanganate spray, and bioautography on *Sarcina lutea* seeded agar.

Discussion

Fermentation conditions for the production of U-21,699 are identical to those reported for lincomycin by Mason *et al.* (1962). Under these fermentation conditions both U-21,699 and lincomycin are produced, the former being a minor component. The exact amount of U-21,699 produced is not known because of the lack of a differential assay.

The paper chromatographic pattern of U-21,699 (Figure 2) is so similar to that of lincomycin that U-21,699 was not detected in early stages of production. It was first observed in thin-layer chromatograms of impure lincomycin preparations. A typical thin-layer chromatogram of U-21,699 and lincomycin is shown in Figure 3.

U-21,699 as the free base is soluble in water and also in most organic solvents other than the hydrocarbons. Results of elemental analyses best fit the molecular formula $C_{17}H_{32}N_2O_6S \cdot \frac{1}{2}H_2O$ (mw 401). Potentiometric titration showed the presence of one basic group with a pK_a' of 7.68 and equivalent weight of 406. Specific rotation measurements showed that U-21,699 is strongly dextrorotatory ($[\alpha]_D^{25}$ +153°). The infrared spectrum of the free base (Figure 4) shows absorptions in the —OH, NH region and at 1645, 1527 cm^{-1} indicating the presence of an amide group.

Crystalline U-21,699 hydrochloride is very soluble in water, soluble in methanol and ethanol, and relatively insoluble in less polar organic solvents. Analytical values, combined with potentiometric titration data, suggest the molecular formula $C_{17}H_{32}N_2O_6S \cdot HCl \cdot \frac{1}{2}H_2O$. The infrared spectrum of U-21,699 hydrochloride

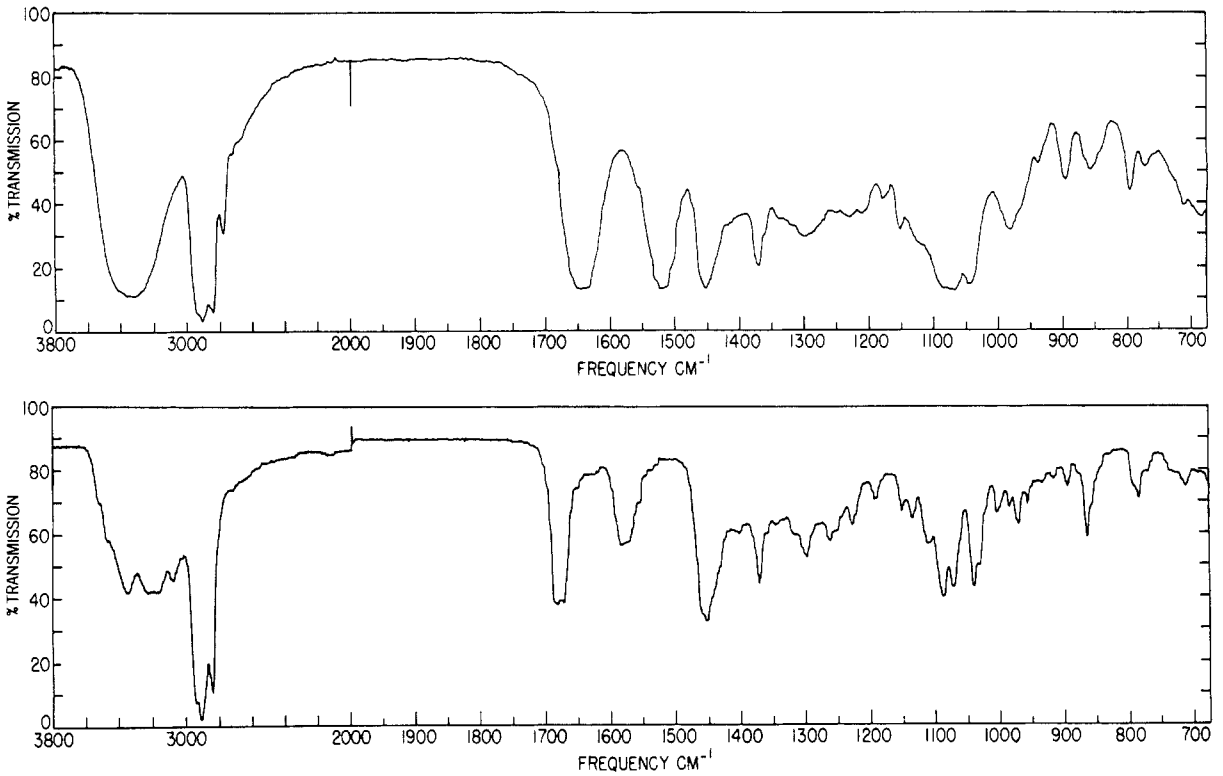


FIGURE 4: Infrared spectra of U-21,699 (in mineral oil suspension). Upper curve, U-21,699, free base; lower curve, U-21,699, hydrochloride.

(Figure 4) is similar to that of the free base with the exception that the amide absorption occurs at 1690–1680, 1590 cm^{-1}.

Table I contains some of the properties of U-21,699 and lincomycin, the structure of which has been elucidated recently (Hoeksema *et al.*, 1964) and is represented by compound Ib. Comparison of the data tabulated reveals striking similarities between the two antibiotics. In addition, the NMR spectrum[3] of U-21,699 (Figure 5) is identical to that of lincomycin in the region from 100 to 400 cps, indicating the presence of $—NCH_3$ and $—SCH_3$ functional groups, as well as the presence of α-methylthiolincosaminide (compound II) moiety and the hygric acid nucleus (compound IIIb) in the U-21,699 molecule. In the region of 30–90 cps there are absorption peaks of total relative area of 8 hydrogens while the lincomycin spectrum shows the presence of 10 hydrogens in the same area. Both spectra contain the doublet at 67, 75 cps (3 H) assigned to the $\underline{CH_3}—CHO$ grouping present in methylthiolincosaminide. They also contain a triplet at 45, 52, 59 cps (3 H) assigned to the $\underline{CH_3}—CH_2—$ of the side chain on the hygric acid nucleus. The presence of the triplet, well defined in the case of U-21,699, combined with the presence of two hydrogens in the $—CH_2—$ region of the spectrum indicates the presence of an ethyl group instead of the propyl group present in lincomycin, i.e., the structure of U-21,699 is pictured by compound Ia.

TABLE I: Properties of U-21,699 and Lincomycin.

	U-21,699	Lincomycin
Infrared	Contains amide I + II bands	Contains amide I + II bands
$[\alpha]_D^{25}$		
(1) Base	+153°	+156°
(2) Hydrochloride	+147°	+147°
Titration		
pK_a'	7.68	7.60
Functional groups	$—NCH_3$ $—SCH_3$ two $C—CH_3$	$—NCH_3$ $—SCH_3$ two $C—CH_3$
Molecular formula	$C_{17}H_{32}N_2O_6S$	$C_{18}H_{34}N_2O_6S$
Antibacterial spectrum	Identical	

[3] Spectra were calibrated in cps units at 60 Mc, downfield from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Spectra were observed with a Varian A-60 spectrometer on solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in deuterium oxide. The helpful discussions with Messrs. F. A. MacKellar and J. F. Zieserl of The Upjohn Company are gratefully acknowledged.

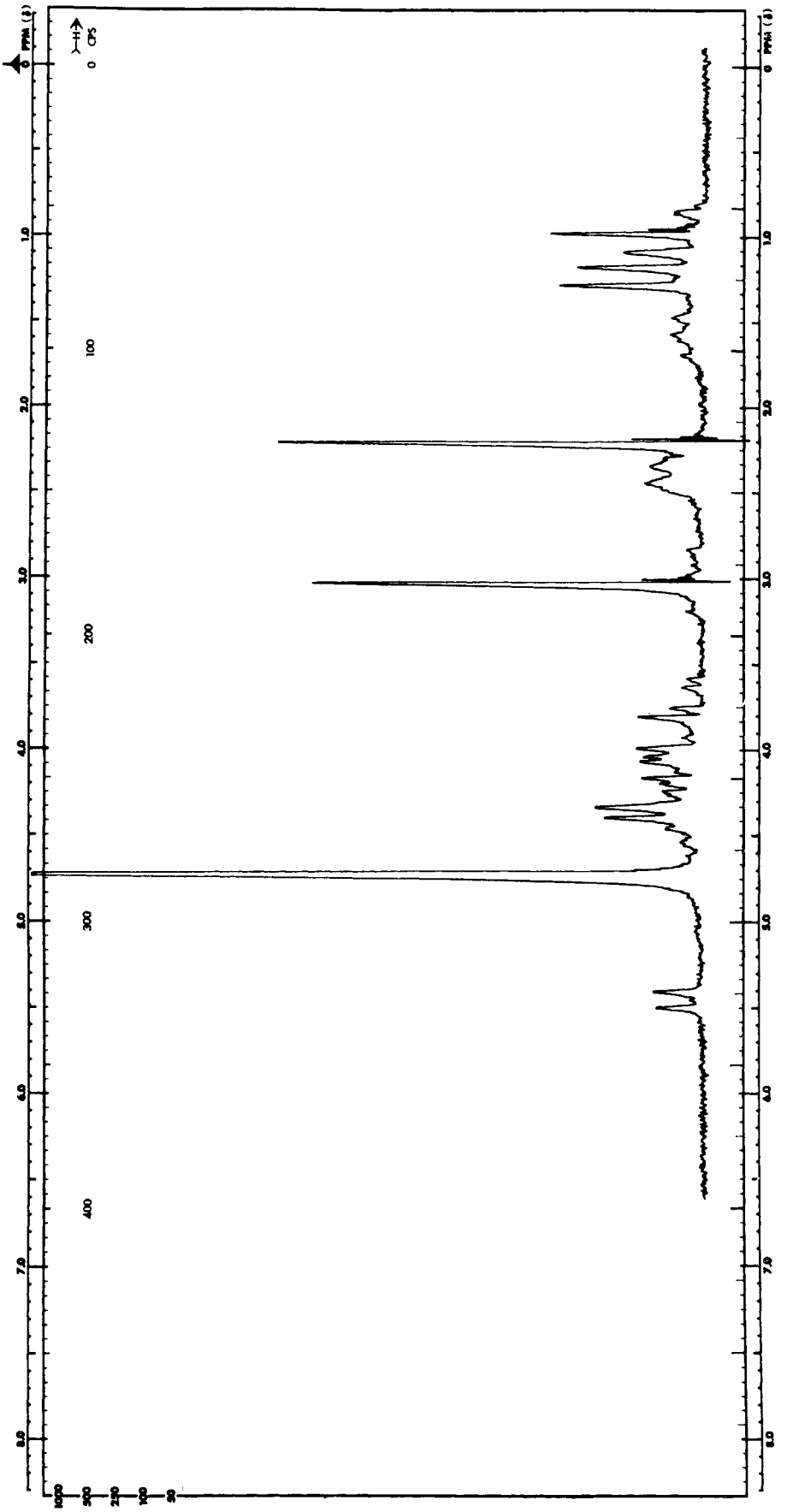
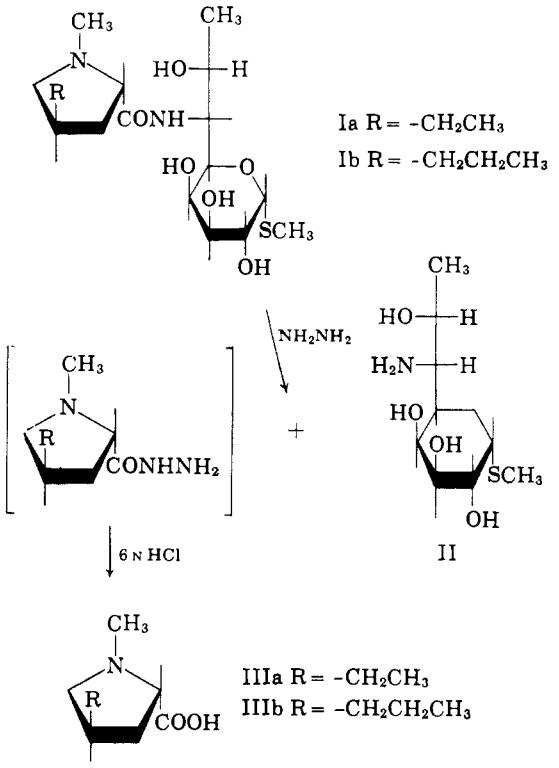


FIGURE 5: Nuclear magnetic resonance spectrum of antibiotic U-21,699 hydrochloride.



This conclusion derived from interpretation of NMR spectra was substantiated by degradative studies. Hydrazinolysis of U-21,699 afforded α-methylthiolincosaminide, $C_9H_{19}NO_5S$ (compound II), identified as such by direct comparison with an authentic sample obtained from lincomycin. The second fragment of the U-21,699 molecule was isolated as the crystalline hydrochloride $C_8H_{16}NO_2Cl$ (compound IIIa). Infrared spectrum combined with the positive rotational shift at lower *p*H suggests an α-L-amino acid. The NMR spectrum of the crystalline hydrochloride is identical to that of *n*-propylhygric acid (compound IIIb) (obtained from lincomycin) in the region from 120 to 300 cps. This indicates that compound IIIa contains a N-CH_3 group and the "hygric acid nucleus" present is *n*-propylhygric acid. The difference in the NMR spectra of compounds IIIa and IIIb is located in the area from 40 to 120 cps. A well-defined triplet of relative area 3 and a broad peak of area 2 are present in the spectrum of compound IIIa and are assigned to the $\underline{CH_3}CH_2$— and —$\underline{CH_2}$— of the ethyl side chain. The NMR spectrum of compound IIIb shows the presence of a triplet (area 3) and a broad peak (area 4) assigned to the $CH_3CH_2\underline{CH_2}$— side chain. The assignment of the *trans*-4-ethyl configuration is based on the close specific rotation values of compounds IIIa and IIIb (−46° and −46.8°, respectively) and on the similarity of their NMR spectra. The stereochemistry of compound IIIb has been established both by degradation to D(+)-propylsuccinic acid and by synthesis from 4-hydroxy-L-proline (Hoeksema *et al.*, 1964).

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