Studies on the Biosynthesis of Lincomycin. I. Antibiotic U-11,921, an *S*-Ethyl Homolog of Lincomycin*

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ABSTRACT: Antibiotic U-11,921 is a new substance produced by *Streptomyces lincolnensis* var. *lincolnensis* when DL-ethionine is added in the culture media. The organism also produces lincomycin under the conditions of fermentation. Recovery of the antibiotic substances from the fermentation broth can be achieved by carbon adsorption followed by elution with aqueous acetone. Solvent extraction and countercurrent distribution separate U-11,921 from lincomycin. Antibiotic U-11,921 has been obtained in crystalline form as the hydrochloride salt. The structure of U-11,921 has been determined by nuclear magnetic resonance and degradative studies as being similar to lincomycin, with the exception that the $-SCH_3$ group present in lincomycin has been replaced by an $-SCH_2CH_3$ group in U-11,921.

The isolation of lincomycin,[1] a fermentation product of *Streptomyces lincolnensis* var. *lincolnensis*, has been reported by Herr and Bergy (1962). Chemical studies by Hoeksema and his co-workers (Hoeksema *et al.*, 1964) led to the elucidation of the complete structure of lincomycin, which is represented by compound Ib.

One of the characteristic structural features of lincomycin, the presence of *N*-CH_3 and *S*-CH_3 groups, was established very early in the structural studies. Preliminary precursor studies showed high incorporation of radioactivity in lincomycin when [*Me*-^{14}C]methionine was added in the fermentation media. This fact led to the hypothesis that the *N*-CH_3 and *S*-CH_3 functional groups are derived from methionine. Work reported from several laboratories (Whalley, 1963) shows that the origin of methyl groups attached to oxygen and nitrogen, and many of those attached to carbon, is the C_1 metabolic pool and that these methyl groups are attached to the appropriate receptor centers through transmethylation from C_1 donor systems (e.g., choline, methionine, tetrahydrofolic acid).

Evidence has been published that ethionine can participate in reactions analogous to transmethylation. *S*-Adenosylethionine has been isolated from yeast cells grown in the presence of ethionine (Schlenk and Tillotson, 1954). This substance participates in transethylation reaction with L-homocysteine to form ethionine in an *in vitro* enzyme system derived from *Torulopsis utilis* (Parks, 1958). Transethylation to form *N*-ethylglycocyamine was observed with pig liver homogenate (Tuppy and Dus, 1958). Dulaney and his co-workers (Dulaney *et al.*, 1962) were able to show transethylation in antibiotic biosynthesis with the formation of the *N*-methyl, ethyl derivative of oxytetracycline by *Streptomyces rimosus* when DL-ethionine was added in the fermentation media. Addition of DL-ethionine in culture media of *Pencillium griseofulvum* resulted in the formation of a 2′-ethoxy analog of griseofulvin (Jackson *et al.*, 1962). On the other hand, ethionine has been shown to serve as an inhibitor of transmethylation with *Streptomyces viridifaciens* at concentrations producing partial inhibition of growth of the streptomycete (Hendlin *et al.*, 1962). 6-Demethylchlortetracycline is produced in place of the normal metabolite, chlortetracycline.

The present study of the effect of DL-ethionine on lincomycin biosynthesis was initiated with the hope that a modification of the lincomycin molecule would be achieved biosynthetically. In view of the above-described work, no prediction could be made on the nature of any product produced by *S. lincolnensis* in the presence of DL-ethionine. This work supplements the studies done on the biosynthesis of lincomycin using radioactive precursors, in this laboratory (unpublished). The biological properties of U-11,921 have been described by Mason and Lewis (1964).

Experimental and Results

Fermentation Procedures. Seed cultures of *S. lincolnensis* var. *lincolnensis* were prepared in a medium consisting of glucose monohydrate (Cerelose), 10 g/liter; *N*-Z-Amine B, 5 g/liter; and Yeastolac, 10 g/liter. The cultures were incubated at 28° for 48 hours on a rotary shaker. A fermentation medium consisting of glucose monohydrate (Cerelose), 15 g/liter; starch, 40 g/liter; blackstrap molasses, 20 g/liter; Pharmamedia, 25 g/liter; and $CaCO_3$, 8 g/liter, was inoculated at a rate of 5% (v/v) with the 48-hour seed medium. The fermentations were incubated at 28° on a rotary shaker (250 rpm, 6-cm stroke). DL-Ethionine (2 g/liter) was added after the fermentation had progressed for 48 hours. Fer-

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

mentation beers were normally harvested after 120 hours.

Isolation of U-11,921. Recovery from Fermentation Beer. Fermentation beer (235 liters) was filtered at harvest *p*H using filter aid. The mycelial cake was washed with water and the cake was then discarded. The combined filtered beer and water wash (275 liters) was stirred for 45 minutes with 12.5 kg of activated carbon and 2.5 kg of diatomaceous earth. The mixture was filtered and the filtrate was discarded. The cake was washed with 60 liters of water, followed by 70 liters of 20% aqueous acetone. The cake was then eluted with 90% aqueous acetone. The eluate was concentrated to an aqueous solution which was adjusted to *p*H 10.0 with 50% aqueous sodium hydroxide, and then extracted three times with 20-liter portions of methylene chloride. The methylene chloride extracts were concentrated to dryness to give a mixture of equal amounts of U-11,921 and lincomycin in the free-base form (7.14 g). This material was then dissolved in 100 ml of 1 N methanolic hydrogen chloride. This solution was mixed with 3.2 liters of ethyl ether with stirring. The precipitated colorless amorphous material, which was found to be a mixture of U-11,921 and lincomycin hydrochloride, was isolated by filtration and dried. This material was used as the starting material for the countercurrent distribution studies described in the next paragraph.

Countercurrent Distribution. Crude U-11,921 hydrochloride (7 g) was dissolved in 20 ml of water and 20 ml of 1-butanol. The *p*H was adjusted to 4.2 with 1 N aqueous hydrochloric acid, and the mixture was transferred in an all-glass Craig countercurrent distribution apparatus (10 ml/phase). The distribution was interrupted when 1000 transfers had been completed. The distribution was then analyzed by determination of solids (Figure 1) and by thin-layer chromatography. Two peaks with *K* values of 0.14 and 0.20 were found. Thin-layer chromatography showed that tubes 80–130 contained lincomycin. Tubes 135–190, which contained U-11,921 only, were combined, and the solution was concentrated to dryness *in vacuo* to give 2.44 g of colorless amorphous U-11,921 hydrochloride, which was used in the crystallization studies described in the next paragraph.

Crystallization of U-11,921 Hydrochloride. Isolation of Two Crystalline Forms. U-11,921 hydrochloride (500 mg) was dissolved in 2 ml of water, 1 ml of methanol, and 100 ml of acetone. This solution was mixed with ether until the first crystals appeared. The mixture was allowed to stand at room temperature for 1 hour. The precipitated crystals having the appearance of cubes (form II) were separated from the supernatant by decantation and recrystallized from water-methanol-acetone-ether to give 250 mg of U-11,921 hydrochloride (form II). The supernatant was allowed to stand at 5° for 4 hours. The crystalline U-11,921 hydrochloride (needles, form I) precipitated, was isolated by filtration, and was dried (150 mg).

Anal. Calcd for $C_{19}H_{36}N_2O_6S \cdot HCl \cdot H_2O$: C, 48.04; H, 8.26; N, 5.90; O, 23.59; S, 6.75; Cl, 7.46. Found: for form I crystals: C, 48.02; H, 8.35; N, 6.05; S, 7.03; Cl, 7.73; for form II crystals: C, 48.07; H, 8.30; N, 6.48; S, 6.94; Cl, 7.54.

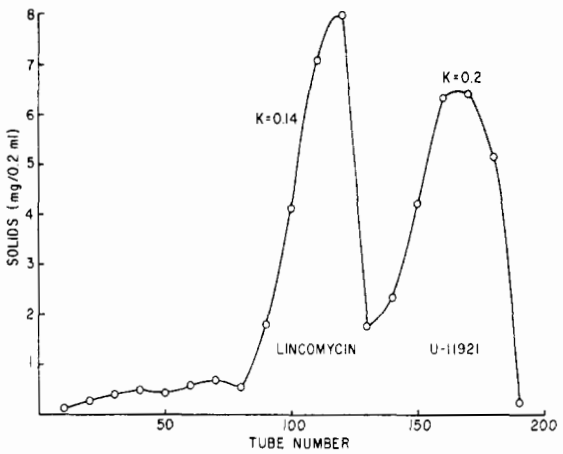


FIGURE 1: Countercurrent distribution of crude U-11,921.

The specific rotation ($[\alpha]_D^{25}$) of form I and II crystals was found to be +140.5° (*c* 0.427, water) and +143° (*c* 0.620, water), respectively. Thin-layer and paper chromatography failed to separate the two forms of U-11,921 hydrochloride. NMR[2] of forms I and II of U-11,921 hydrochloride were identical. The two crystalline forms can be easily distinguished by infrared spectra (Figure 2). Tabulation of the absorption bands of the infrared spectrum (Nujol mull) of both forms follows:

Form I: 3300, 3060, 2920, 2850, 2720, 2340, 1680, 1568, 1456, 1375, 1363, 1333, 1318, 1300, 1262, 1230, 1210, 1140, 1094, 1073, 1048, 1000, 990, 965, 932, 900, 890, 865, 795, 708, 675, and 660 cm^{-1}.

Form II: 3550, 3440, 3340, 3210, 3070, 3020, 2920, 2850, 2720, 1673, 1650, 1610, 1560, 1555, 1535, 1450, 1420, 1400, 1370, 1360, 1345, 1320, 1290, 1260, 1225, 1200, 1145, 1140, 1095, 1070, 1040, 1000, 990, 975, 960, 935, 900, 865, 800, 730, 715, 705, and 660 cm^{-1}.

Hydrazinolysis of U-11,921. Isolation of Ethylthiolincosaminide (Compound IIa). The procedure described for the hydrazinolysis of U-21,699 (Argoudelis *et al.*, 1965) was used for the degradation of 1.8 g of U-11,921 free base. Material insoluble in acetonitrile was recrystallized from dimethylformamide to give 750 mg of crystalline ethylthiolincosaminide, mp 191–195°, $[\alpha]_D^{25}$ +258° (*c* 0.76, water).

Anal. Calcd for $C_{10}H_{21}NO_5S$: C, 44.98; H, 7.93; N, 5.25; S, 12.01; O, 29.96; mw, 267. Found: C, 44.09; H, 7.91; N, 5.24; S, 11.32.

Potentiometric titration showed the presence of one basic group with pK_a' of 7.17; equivalent weight 271. The infrared spectrum (Nujol mull) showed absorption bands at 3340, 3275, 2950, 2920, 2850, 2710, 1689,

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

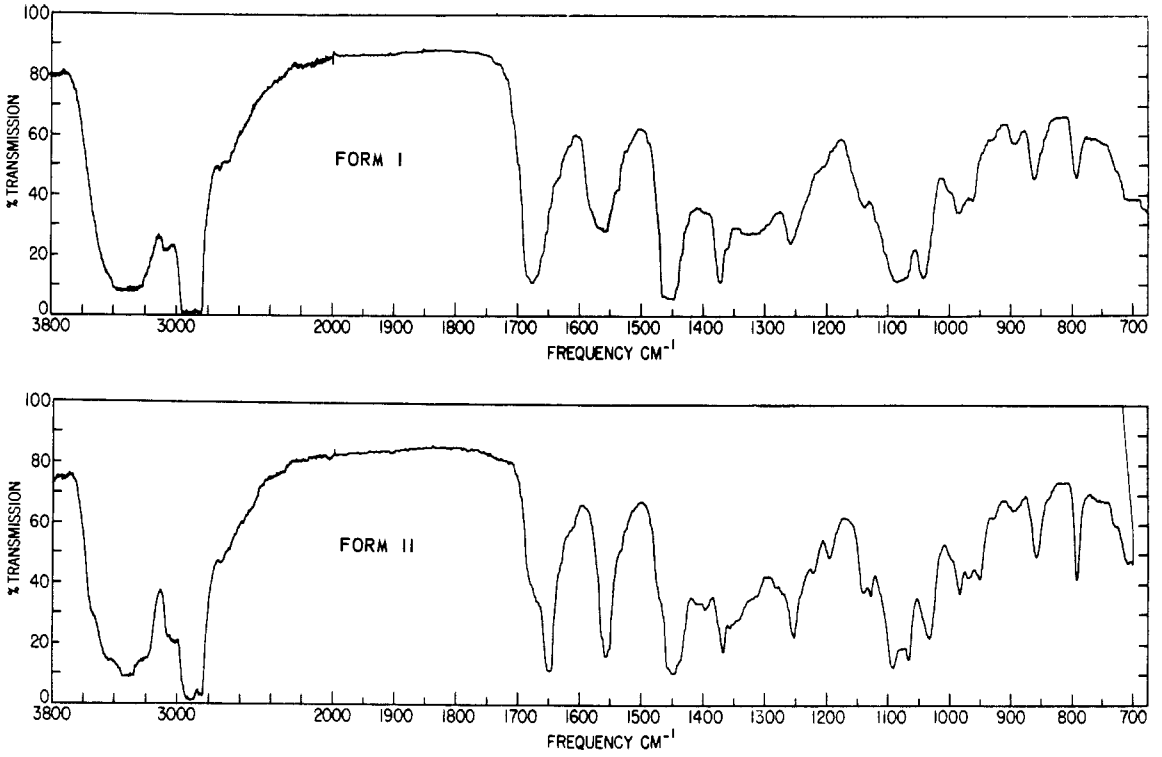


FIGURE 2: Infrared spectra of U-11,921 hydrochloride (in mineral oil suspension). Upper curve: form I; lower curve: form II.

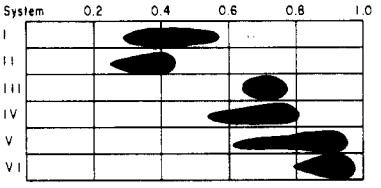


FIGURE 3: Paper chromatography of U-11,921 hydrochloride. Solvent systems: (I) 1-butanol-water (84:16), developed 16 hours; (II) 1-butanol-water (84:16) plus 0.25% *p*-toluenesulfonic acid, developed 16 hours; (III) 1-butanol–acetic acid–water (2:1:1), developed 16 hours; (IV) 1-butanol-water (84:16) 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.

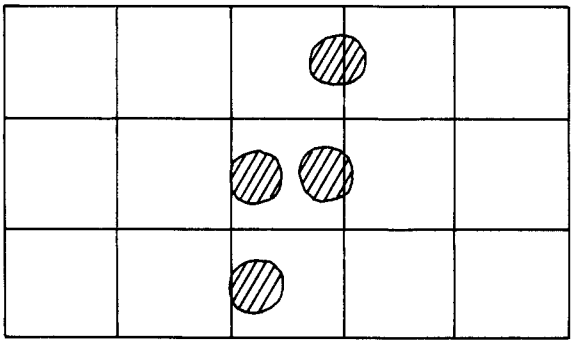


FIGURE 4: Thin-layer chromatography of U-11,921 hydrochloride. Upper, U-11,921 hydrochloride; middle, U-11,921 and lincomycin hydrochloride; lower, lincomycin hydrochloride. Thin-layer plates were prepared from Silica Gel G (Merck Darmstadt). Thickness of the film was 0.4 mm. The solvent system consisted of 150 ml of methyl ethyl ketone, 50 ml of acetone, and 20 ml of water. Detection systems used: periodate-permanganate spray and bioautography on *Sarcina lutea* seeded agar.

1654, 1605, 1513, 1453, 1400, 1375, 1345, 1309, 1264, 1239, 1214, 1199, 1170, 1154, 1129, 1094, 1074, 1045, 989, 955, 924, 904, 860, 843, 804, 778, 758, 704, 692, 679, and 674 cm^{-1}.

Isolation of 4-n-Propyl-L-hygric Acid Hydrochloride (Compound III). The procedure described for the isolation of ethylhygric acid hydrochloride (Argoudelis *et al.*, 1965) was used. The crystalline hydrochloride obtained was identified as 4-*n*-propyl-L-hygric acid hydrochloride by comparison of $[\alpha]_D$ values and NMR and infrared spectra with those of an authentic sample of 4-*n*-propyl-L-hygric acid obtained from degradation of lincomycin.

Acid Hydrolysis of U-11,921. Isolation of Ethyl Mercaptan. U-11,921 hydrochloride (1 g) was dissolved in 10 ml of 5 N aqueous sulfuric acid solution. The solution was kept at reflux for 45 minutes under a continuous stream of nitrogen. Volatile substances were carried by nitrogen and passed through a solution of 0.3 ml of 10% sodium hydroxide and 3 ml of ethanol. The ethanolic solution was then mixed with a solution of 200 mg of 2,4-dinitrochlorobenzene in 3 ml of absolute methanol. The yellow crystalline material, which precipitated instantly, was isolated by filtration and dried; yield 220 mg. Recrystallization from 6 ml of 95% ethanol afforded long needles of 2,4-dinitrophenyl ethyl sulfide. The mp and mixed mp were 117–118° (reported 115°).

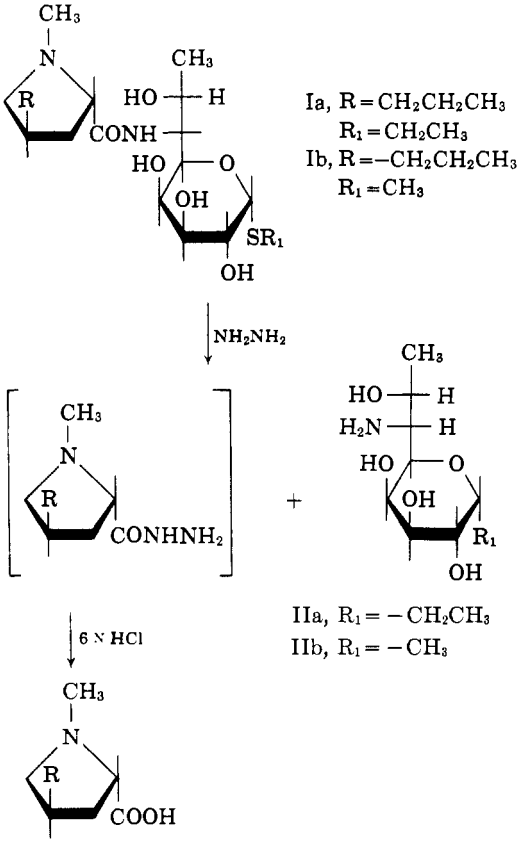
Anal. Calcd for $C_8H_8N_2O_4S$: C, 42.14; H, 3.54; N, 12.29; S, 14.06. Found: C, 41.91; H, 3.20; N, 12.27; S, 14.52.

Discussion

DL-Ethionine was added in fermentations of *S. lincolnensis* var. *lincolnensis* at levels of 0.5–4 g/liter on day 0, 1, 2, or 3 of the fermentation. Best results were obtained when 2 g/liter of DL-ethionine was added on the second day (48 hours after inoculation). Details on the fermentation conditions are presented in the experimental part. Under the conditions used, lincomycin and a second bioactive substance designated U-11,921 were produced in almost equal amounts. The paper chromatographic pattern of U-11,921, shown in Figure 3, is identical to that of lincomycin. However, the two antibiotic substances could easily be separated by thin-layer chromatography. A typical thin-layer chromatogram is presented in Figure 4.

U-11,921 was recovered from the fermentation broth by a carbon process similar to that used for extraction of U-21,699, a recently described lincomycin-related antibiotic (Argoudelis *et al.*, 1965). Separation of U-11,921 from lincomycin was achieved by countercurrent distribution of the hydrochloride salts. A typical run is presented in Figure 1. Antibiotic U-11,921 hydrochloride has been crystallized in two crystal forms designated form I (needles) and form II (cubes). The two crystal forms showed identical biological properties. Their physical and chemical properties are also identical with the exception of the infrared spectra, which are shown in Figure 2.

Crystalline U-11,921 hydrochloride is soluble in water and methanol. It is moderately soluble in 95% or absolute ethanol, and insoluble in acetone, ethyl acetate, and chlorinated and saturated hydrocarbon solvents. Analytical data obtained on both crystalline forms of U-11,921 hydrochloride suggest the molecular formula $C_{19}H_{36}N_2O_6S \cdot HCl \cdot H_2O$. Potentiometric titration showed the presence of one basic group with a pK_a' of 7.73 and an equivalent weight of 490 (calcd 475). The specific rotation of U-11,921 hydrochloride was found to be $[\alpha]_D^{25} = +143°$ (*c* 0.620, water). The infrared spectra of the two crystalline forms of U-11,921 hydrochloride are shown in Figure 2. Tabulation of the absorption bands is presented in the experimental part of this paper.



III, R=$CH_2CH_2CH_3$

The physical and chemical characteristics of U-11,921 hydrochloride resemble those reported for lincomycin (Herr and Bergy, 1962). In addition, comparison of the nuclear magnetic resonance spectra of the hydrochloride of U-11,921 and lincomycin hydrochloride shows that these antibiotics have several common structural features. Specifically, the spectra of both compounds contain an unsymmetrical triplet at 40–60 cps assigned to a CH_3—C of an *n*-propyl group. A doublet at 65, 75 cps has been assigned to CH_3CHO grouping. A sharp singlet at *ca.* 175 cps present in both spectra is due to —NCH_3 group. In addition, absorption peaks assigned to hydrogens of the "hygric acid nucleus" (compound III) are present in both spectra. Furthermore, the NMR spectra of the hydrochloride of U-11,921 and lincomycin hydrochloride are identical in the region from 200 to 325 cps, strongly suggesting identical stereochemistry in the sugar moiety of both antibiotics.[3]

[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 are gratefully acknowledged.

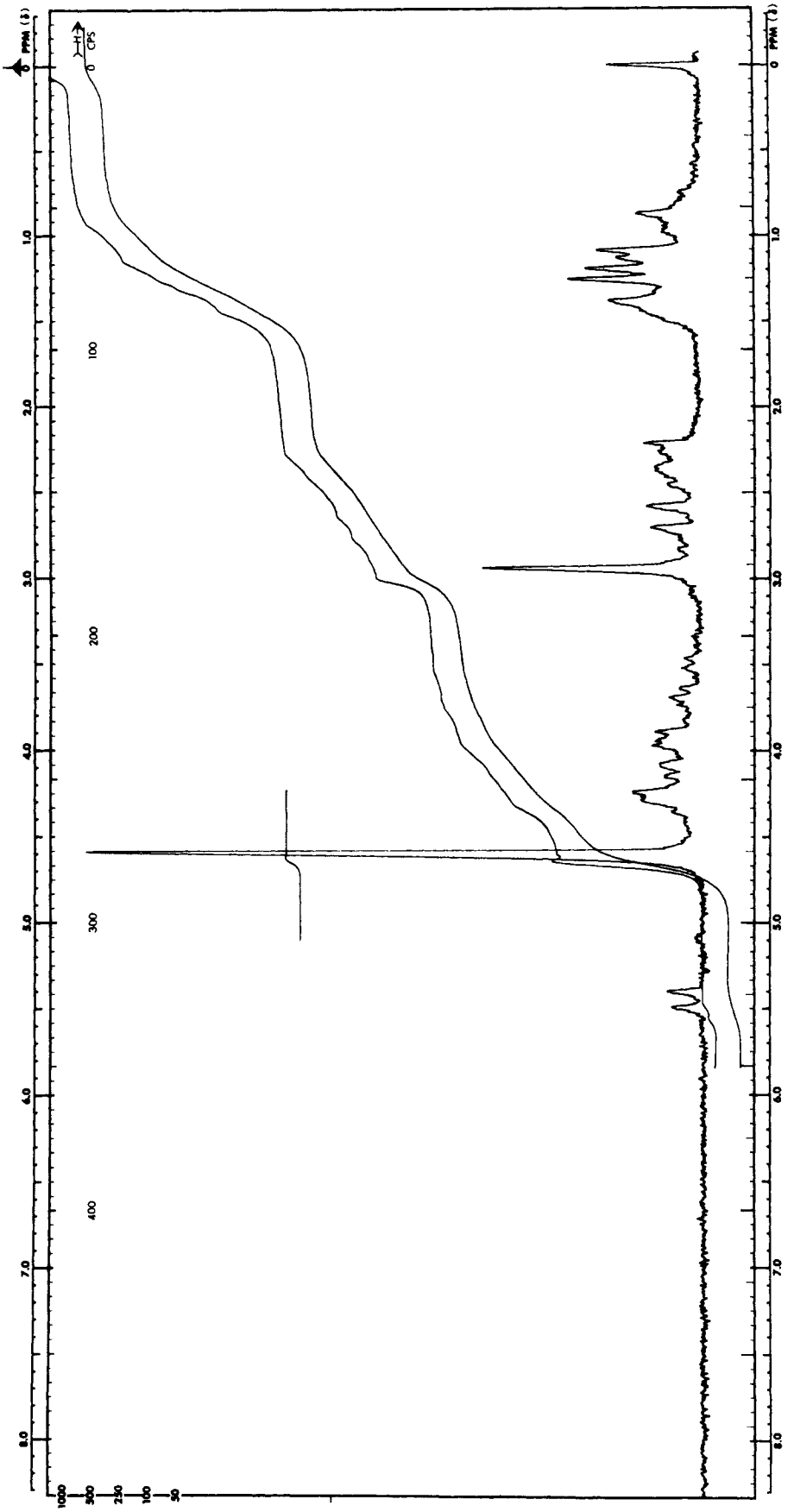


FIGURE 5: Nuclear magnetic resonance spectrum of U-11,921 hydrochloride.

The only difference between the NMR spectra of U-11,921 and lincomycin hydrochloride is that the sharp singlet at 125 cps of relative area 3 (due to an S—CH_3 group), which is present in the NMR spectrum of lincomycin hydrochloride, is absent in the spectrum of U-11,921. Instead, a quadruplet at 147, 155, 162, 170 cps and a triplet at 65, 75, 85 cps are present in the spectrum of U-11,921 and have been assigned to an —S—CH_2CH_3 group.

The NMR data in combination with the analytical data, infrared spectra, titration, and specific rotation, suggest Ia as the structure of antibiotic U-11,921.

This conclusion has been substantiated by degradative studies. Acid hydrolysis of U-11,921 yielded ethyl mercaptan isolated as the 2,4-dinitrophenyl ethyl sulfide derivative. On the other hand hydrazinolysis of the antibiotic afforded the hydrazide of an amino acid which was converted to the crystalline hydrochloride, $C_9H_{17}NO_2 \cdot HCl$ (III), by acid hydrolysis. Comparison of infrared and NMR spectra revealed the identity of III to 4-*n*-propyl-L-hygric acid hydrochloride isolated by Hoeksema and his co-workers from lincomycin (Hoeksema, *et al.*, 1964). The remainder of the U-11,921 molecule was isolated as a crystalline colorless material, $C_{10}H_{21}NO_5S$ (IIa). Properties of IIa (specific rotation, infrared spectra, solubilities) resemble those of methyl thiolincosaminide (IIb).

In addition, comparison of the NMR spectra of compound IIa, for which the name ethyl thiolincosaminide is proposed, and compound IIb shows that the main difference in the spectra of these compounds is that the sharp singlet at 131 cps (3 H), due to an *S*—CH_3 group, which is present in the spectrum of compound IIb, is absent in the spectrum of compound IIa. Instead, a quadruplet at 150, 157, 164, and 171 cps and a triplet at 71, 78, 85 cps are present in the spectrum of compound IIa and have been assigned to an —SCH_2-CH_3 group.

These results suggest that ethyl thiolincosaminide (compound IIa) has structure, including stereochemistry at all asymmetric centers, identical to that of methyl-thiolincosaminide, with the exception that the —SCH_3 group present in compound IIb has been substituted with an —SCH_2CH_3 in compound IIa.

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