STUDIES ON THE BIOSYNTHESIS OF LINCOMYCIN. V

EFFECT OF ETHIONINE ON FERMENTATION OF *S. LINCOLNENSIS*

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The effect of DL-ethionine on fermentations of *Streptomyces lincolnensis* depends on the composition of the fermentation media used (complex or synthetic), the levels of ethionine added, and the time of addition of the amino acid. Addition of ethionine into fermentations of *S. lincolnensis* grown in a complex medium resulted in the production of S-demethyl-S-ethyllincomycin. On the other hand, when ethionine was added into cultures of the organism grown in synthetic medium, S-demethyl-S-ethyllincomycin and a new antibiotic identified as N, S-didemethyl-N, S-diethyllincomycin were produced. The production of these two analogs of lincomycin by *S. lincolnensis* suggests that ethionine participates in transethylation reactions on both the nitrogen and the sulfur of the lincomycin molecule.

Streptomyces lincolnensis var. *lincolnensis* has been reported to produce the antibiotics lincomycin (I)[1] and 4′-depropyl-4′-ethyllincomycin (II)[2]. In the preceding communication in these series[3] it was also reported that the $-SCH_3$, the $-NCH_3$ and the terminal C-CH_3 group of the side chain of the amino acid moieties of lincomycin are derived from C_1 donor systems through transmethylation.

Evidence has been published that ethionine can participate in reactions analogous to transmethylation. The reactions occuring represents transethylation in place of normal transmethylation and the ethyl group is attached to either an oxygen[4] or a nitrogen[5] atom in the final product. On the other hand, ethionine has been shown to serve as an inhibitor of transmethylation with *S. viridifaciens* at concentrations producing partial inhibition of growth of the streptomycete[6].

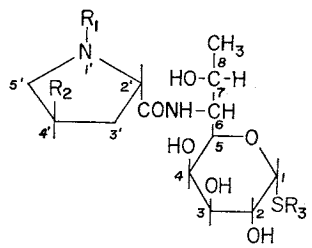
The present study of the effect of DL-ethionine on lincomycin biosynthesis was undertaken with the hope that modification of the lincomycin molecule would be achieved biosynthetically either by transethylation at one or all sites of methylation (carbon, nitrogen or sulfur) or by inhibition of methylation.

Discussion and Results

Effect of DL-Ethionine on Fermentations of *S. lincolnensis* Grown in a Complex Medium. Production of S-Demethyl-S-ethyllincomycin (III)

The fermentation medium (medium A) used in the initial stages of this work is shown in Table 1. Increasing amounts of DL-ethionine were added at the beginning

Fig. 1



I. $R_1=CH_3$; $R_2=CH_2CH_2CH_3$; $R_3=CH_3$
II. $R_1=CH_3$; $R_2=CH_2CH_3$; $R_3=CH_3$
III. $R_1=CH_3$; $R_2=CH_2CH_2CH_3$; $R_3=CH_2CH_3$
IV. $R_1=CH_2CH_3$; $R_2=CH_2CH_2CH_3$; $R_3=CH_2CH_3$

Table 1. Composition of fermentation media

Medium A (g/liter)		Medium B (g/liter)			
Cerelose	15.0	Glucose	30.0	NaCl	0.5
Starch	40.0	Sodium citrate	3.0	NH_4NO_3	2.0
Pharmamedia	25.0	$MgSO_4$	1.0	$ZnSO_4 \cdot 7H_2O$	0.001
$CaCO_3$	8.0	K_2HPO_4	2.5	$FeSO_4 \cdot 7H_2O$	0.001

of the fermentation (at the time of inoculation) or at 24, 48 or 72 hours after inoculation. Beers were harvested after a total fermentation time of 144 hours. The antibiotic titers were determined by a disc plate assay using *Sarcina lutea* as assay organism[7]. The bioactive components of the fermentation were extracted with methylene chloride and the obtained preparations were analyzed by thin-layer chromatography.

As shown in Table 2, the amount of antibiotics produced decreased with increasing levels of DL-ethionine. The effect of ethionine was more evident at the early stages of the fermentation. For example, addition of 1,600~3,200 mcg/ml at 0 or 24 hours resulted in poor growth of the organism and no production of antibiotics.

Low levels of DL-ethionine (100~400 mcg/ml) added at 24, 48 or 72 hours after inoculation did not have appreciable effect on the total antibiotic activity produced. Thin-layer chromatographic (TLC) analysis showed that lincomycin was the main bioactive component. Increased amounts of ethionine (800~3,200 mcg/ml) resulted in decrease of antibiotic titers. However, a new bioactive component, in addition to lincomycin, was produced at these high ethionine levels. The isolation of this new antibiotic (U-11,921) and identification as S-demethyl-S-ethyllincomycin (III) has been described in a preliminary report[8] of the present study. In large scale fermentations, highest yield of S-demethyl-S-ethyllincomycin was obtained when 2,000 mcg/ml of DL-ethionine was added 48 hours after inoculation.

Table 2. Antibiotics[a,b] produced[c] by *S. lincolnensis* grown in medium A[d] in the presence of DL-ethionine

Amount of DL-ethionine (μg/ml)	DL-Ethionine added at							
	0 hour		24 hours		48 hours		72 hours	
	Total[e] activity	Antibiotics produced	Total[e] activity	Antibiotics produced	Total[e] activity	Antibiotics produced	Total[e] activity	Antibiotics produced
100	1,400	I	1,590	I	2,000	I	——	I
200	1,350	I	1,880	I	1,740	I	——	I
400	250	I	1,350	I	1,340	I (III, traces)	1,490	I, III (traces)
800	0	None	430	I, III	1,240	I, III	1,380	I, III
1,600	0	None	0	None	860	I, III	1,200	I, III
3,200	0	None	0	None	890	I, III	1,000	I, III

a) Antibiotics were identified by thin-layer chromatography. b) Small amounts of 4′-depropyl-4′-ethyllincomycin (II) were always produced under conditions permitting lincomycin production.
c) Fermentation beers were harvested after a total fermentation of 144 hours. d) See Table 1.
e) Total activity determined by disc plate activity using *S. lutea* (HANKA *et al.*, 1962) is expressed in μg/ml of lincomycin.

The effect of DL-ethionine on the fermentations of *S. lincolnensis* could be reversed by addition of L-methionine. As shown in Table 3, DL-ethionine added at the beginning of the fermentation and once every 24 hours thereafter (24, 48, 72 and 96 hours after inoculation) partially inhibited the growth of *S. lincolnensis* and resulted in poor production of both lincomycin and S-demethyl-S-ethyllincomycin. On the other hand, addition of equal levels of DL-ethionine and L-methionine in the fermentation, at the same time intervals as above, resulted in production of high antibiotic titers. Lincomycin was the only antibiotic detected by TLC. These results indicate that L-methionine not only reversed the effect of DL-ethionine on the growth of the organism but also prevented the ethionine-stimulated production of S-demethyl-S-ethyllincomycin.

Table 3. Antibiotics[a,b] produced[c] by *S. lincolnensis* grown in the presence of DL-ethionine and L-methionine

Amount of DL-ethionine (μg/ml)	Amount of L-methionine (μg/ml)	Total antibiotic activity[d]	Antibiotics produced
100	0	460	I and III
200	0	<460	I and III
400	0	traces	I and III
100	100	1,410	I
200	200	1,040	I
400	400	1,260	I

a) Antibiotics were identified by thin-layer chromatography. b) Small amounts of 4′-depropyl-4′-ethyllincomycin (II) were always produced under conditions permitting lincomycin production.
c) Fermentation beers were harvested after a total fermentation time of 144 hours. d) Total activity determined by disc plate activity using *S. lutea* (HANKA *et al.*, 1962) is expressed in μg/ml of lincomycin.

Effect of DL-Ethionine on Fermentations of *S. lincolnensis* Grown in a Synthetic Medium. Production of N, S-Didemethyl-N, S-diethyllincomycin (IV)

Lincomycin and S-demethyl-S-ethyllincomycin were the only bioactive compounds detected in small scale fermentations of *S. lincolnensis* grown in medium A in the presence of DL-ethionine. However, crude preparations obtained from large scale fermentations contained trace amounts of a third bioactive compound, designated antibiotic U-25,468, in addition to lincomycin and S-demethyl-S-ethyllincomycin. This new antibiotic was found to be produced in significant amounts when DL-ethionine was added in fermentations of *S. lincolnensis* grown in synthetic medium B (Table 1).

Table 4 summarizes our studies on the effect of DL-ethionine on *S. lincolnensis* fermentations in the synthetic medium B. Addition of DL-ethionine in the early stages of the fermentation (0 or 24 hours) completely inhibited the growth of the organism. Low levels (100~400 mcg/ml) of DL-ethionine added 48 hours after inoculation resulted in partial growth of *S. lincolnensis* and poor production of lincomycin and S-demethyl-S-ethyllincomycin ; higher levels (800~3,200 mcg/ml) were found inhibitory to the organism. When DL-ethionine (100~200 mcg/ml) was added 72 hours after inoculation lincomycin and S-demethyl-S-ethyllincomycin were produced. However, higher levels (400~3,200 mcg/ml) afforded U-25,468 in addition to lincomycin and S-demethyl-S-ethyllincomycin.

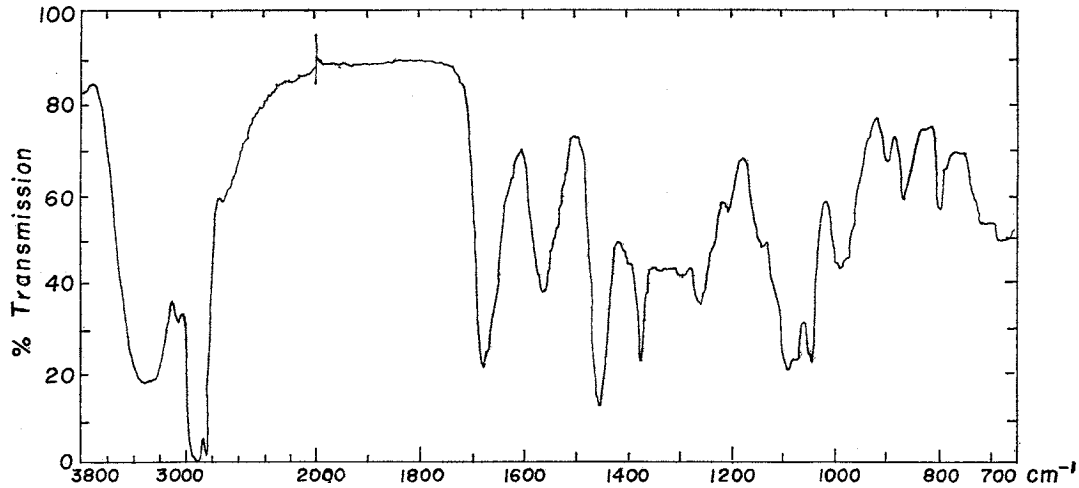
For the production of U-25,468, 500 mcg/ml of DL-ethionine was added in fermentations of *S. lincolnensis* grown in synthetic medium B, 72 hours after inoculation. The beers were harvested after a total fermentation time of 144 hours. The antibiotics

Table 4. Antibiotics[a,b] produced[c] by *S. lincolnensis* grown in medium B[d] in the presence of DL-ethionine

Amount of DL-ethionine (μg/ml)	DL-Ethionine added at									
	0 hour		24 hours		48 hours		72 hours		96 hours	
	Total[e] activity	Antibiotics produced	Total[e] activity	Antibiotics produced	Total[e] activity	Antibiotics produced	Total[e] activity	Antibiotics produced	Total[e] activity	Antibiotics produced
100	0	none	0	none	65	I, III	410	I, III	—	—
200	0	none	0	none	traces	I, III	440	I, III	—	—
400	0	none	0	none	traces	I, III	325	I, III, IV	—	—
800	0	none	0	none	0	none	350	I, III, IV	375	I, III, IV
1,600	0	none	0	none	0	none	325	I, III, IV	410	I, III, IV
3,200	0	none	0	none	0	none	300	I, III, IV	478	I, III, IV

a) Antibiotics were identified by thin-layer chromatography. b) Small amounts of 4′-depropyl-4′-ethyllincomycin (II) were always produced under conditions permitting lincomycin production. c) Fermentation beers were harvested after a total fermentation time of 144 hours. d) See Table 1. e) Total activity determined by disc plate activity using *S. lutea* (HANKA *et al.*, 1962) is expressed in μg/ml of lincomycin.

Fig. 2. Infrared spectrum of N, S-didemethyl-N, S-diethyllincomycin in Nujol mull



produced were extracted by methylene chloride. Lincomycin (I) and small amounts of 4′-depropyl-4′-ethyllincomycin (II) were separated from S-demethyl-S-ethyllincomycin (III) and U-25,468 by counter double current distribution using 1-butanol - water (1 : 1 v/v) as the solvent system. Antibiotic U-25,468 was separated from S-demethyl-S-ethyllincomycin by silica gel chromatography.

Antibiotic U-25,468 was isolated as the crystalline hydrochloride, $C_{20}H_{38}N_2O_6S \cdot HCl \cdot H_2O$. The calculated molecular weight of the hydrochloride salt monohydrate is 448; potentiometric titration showed the presence of a basic group (pKa′ 7.7, water) and equivalent weight of 501. The specific rotation, the infrared spectrum (Fig. 2), and the antibacterial spectrum (Table 5) of U-25,468 hydrochloride indicated a lincomycin-like material.

Comparison of the nmr spectra of U-25,468 and lincomycin (Fig. 3) showed that the sharp singlets at δ 2.96 (NCH_3) and δ 2.14 (SCH_3), which characterize the spectrum

of lincomycin, are absent in the spectrum of U-25,468. Instead, absorptions characteristic of an $-NCH_2CH_3$ group (quadruplet at δ, 3.05, 3.17, 3.29, 3.41 ; triplet at δ, 1.09, 1,21, 1.33) and of an $-SCH_2CH_3$ group (quadruplet at δ, 2.40, 2.51, 2.62, 2.73 ; triplet at δ, 1.09, 1.21, 1.33) are present in the spectrum of U-25,468. Both spectra contain an unsymmetrical triplet at *ca.* δ, 0.70 to 0.95 assigned to the $\underline{CH_3}$-C of the *n*-propyl group and absorption peaks assigned to hydrogens of the aminoacid moiety. Furthermore both nmr spectra are identical in the region from δ 3.5 to δ, 5.6 suggesting identical stereochemistry in the aminosugar moiety of both U-25,468 and lincomycin.

Table 5. Antibacterial spectra of N, S-didemethyl-N, S-diethyllincomycin (IV) and lincomycin (I)

Test organism	Minimum inhibitory concentration (μg/ml)	
	N, S-Didemethyl-N, S-diethyllincomycin hydrochloride (IV)	Lincomycin hydrochloride (I)
S. aureus 76	0.2	0.2
S. aureus 552	0.4	0.8
S. aureus 70	0.2	0.2
St. hemolyticus 152	0.4	0.4
St. faecalis 157	0.4	0.4
St. faecalis 3235	25.0	12.5
E. coli 51	50.0	>200.0
P. vulgaris 93	>200.0	>200.0
K. pneumoniae 57	12.5	50.0
S. schottmuelleri 126	50.0	>200.0
Ps. aeruginosa 95	>200.0	>200.0
B. subtilis 564	50.0	25.0

Test method: Two-fold dilution endpoints in brain-heart infusion broth; read at 20 hours.

The nmr data strongly suggested that U-25,468 is N, S-didemethyl-N, S-diethyl-

Fig. 3. Nuclear magnetic resonance spectra of lincomycin hydrochloride (I) and N, S-didemethyl-N, S-diethyllincomycin hydrochloride (IV). Spectra were observed with a Varian A-60 spectrometer on solutions (*ca.* 0.4 ml, *ca.* 25 M) of the compounds in deuterium oxide.

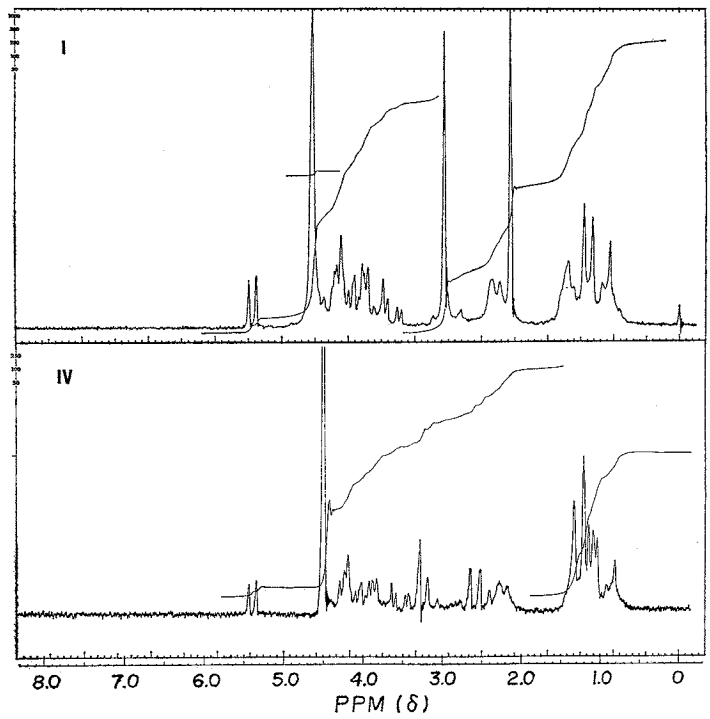
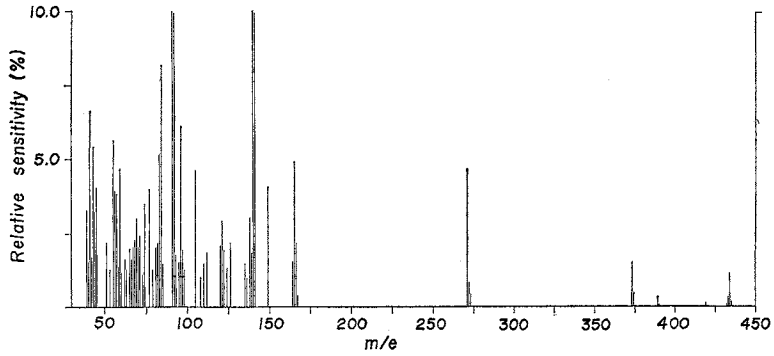
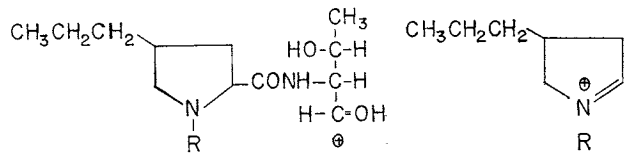


Fig. 4. Mass spectrum of N, S-didemethyl-N, S-diethyllincomycin



lincomycin (IV). This conclusion was substantiated by mass spectroscopy. The mass spectrum of U-25,468 (Fig. 4) showed molecular ion peak at m/e 434 (calcd. mol. weight U-25,468 free base, 434). A fragment peak at m/e 373 is assigned to M-SCH_2CH_3. A peak at m/e 271 is assigned to ion **Va** (Fig. 5). Finally the base peak at m/e 140 is due to ion **VIa** (Fig. 5) resulting from the aminoacid moiety. The mass spectrum of lincomycin contained molecular ion peak at m/e 406 ($C_{18}H_{34}N_2O_6S$) and peaks at m/e 359 (M-SCH_3), 257 (ion **Vb**, Fig. 5) and 126 (ion **VIb**, Fig. 5)[9]. These results conclusively establish the structure of U-25,468 as N, S-didemethyl-N, S-diethyllincomycin (IV).

Fig. 5



Va. R=CH_2CH_3 **VIa.** R=CH_2CH_3
Vb. R=CH_3 **VIb.** R=CH_3

The production of the S-ethyl and the S-ethyl, N-ethyl analogs (compounds III and IV) of lincomycin by *S. lincolnensis*, when ethionine was added in the fermentation media, suggest that ethionine participates in transethylation reactions on both the nitrogen and the sulfur of the lincomycin molecule. These reactions most probably occur by mechanisms identical to those involved in the biosynthesis of lincomycin, since addition of methionine in the media prevented the ethionine-stimulated production of the ethyl analogs.

Ethylation on nitrogen undoubtedly occurs by a transethylation process involving S-adenosylethionine. This mechanism has been well described in the literature[10]. However, the biosynthesis of the -SCH_3 group of lincomycin or of the -SCH_2CH_3 group of III and IV can occur either by a transalkylation process (involving S-adenosylmethionine or S-adenosylethionine) or by a "transthioalkylation" process in which the -SCH_3 group of methionine or the -SCH_2CH_3 of ethionine are transferred intact to the appropriate acceptor. The latter mechanism has been also considered by PATTERSON *et al.*[11] in their studies related with formation of the S-ethyl analog of lincomycin by *Streptomyces umbrinus*. It must be pointed out, however, that such a biosynthetic mechanism, which involves the intact transfer of the -SCH_3 group of methionine with the subsequent formation of methylthio compounds, has not been described in the literature.

Experimantal

A. Effect of DL-Ethionine on Fermentations of *S. lincolnensis*

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°C for 48 hours on a rotary shaker. Fermentation media A or B (Table 1) were inoculated at a rate of 5 % (v/v) with the 48-hour seed medium. The fermentations were incubated at 28°C on a rotary shaker (250 rpm, 6-cm stroke). DL-Ethionine was added at the desired time and the beers were harvested after total fermentation time of 144 hours. Antibiotic titers were measured by disc plate activity using *S. lutea* as assay organism[7].

Extraction Procedures. The fermentation broth was filtered using filter aid. The filtrate was adjusted to pH 10.0 and extracted three times with one third of its volume of methylene chloride. The extract was concentrated to dryness *in vacuo*. The residue obtained was analyzed for antibiotic content by thin-layer chromatography.

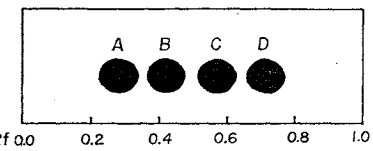
Thin-Layer Chromatography. The purification and separation of the different antibiotic components was followed mainly by TLC on silica gel using methyl ethyl ketone - acetone - water (140 : 40 : 22 v/v) as the eluting solvent.

B. Production and Isolation of N,S-Didemethyl-N,S-diethyllincomycin (IV)

Fermentation Procedures. Seed cultures of *S. lincolnensis* were prepared as described above. Fermentation medium B (Table 1) was inoculated at a rate of 5 % (v/v) with the 48-hour seed medium. The fermentations were incubated at 28°C on a rotary shaker (250 rpm, 6-cm stroke). DL-Ethionine (0.5 g/liter) was added after the fermentation had progressed for 62 hours. Fermentation beers were harvested after 144 hours.

Extraction of Antibiotic Activities from Fermetation Beer. Fermentation beer (250 liters) was filtered at harvest pH using filter aid. The mycelial cake was washed with water and the cake was then discarded. The combined filtered beer and water wash (240 liters) was then adjusted to pH 10 (with aqueous sodium hydroxide) and extracted three times with 60 liters of methylene chloride each time. The methylene chloride extracts were combined and the solution was concentrated to an oily material (74.8 g). Thin-layer chromatography showed the presence of lincomycin (I), 4′-depropyl-4′-ethyllincomycin (II), S-demethyl-S-ethyllincomycin (III) and of a new activity which was designated as U-25,468. A typical thin-layer chromatogram is presented in Fig. 6.

Fig. 6. Thin-layer chromatography of a mixture of:
A: 4′-Depropyl-4′-ethyllincomycin (II)
B: Lincomycin (I)
C: S-Demethyl-S-ethyllincomycin (III)
D: N,S-Didemethyl-N,S-diethyllincomycin (IV)
Thin-layer plates were prepared from silica gel G (Merck-Darmstadt). The solvent system consisted of 140 ml of methyl ethyl ketone, 40 ml of acetone, and 22 ml of water. Detection systems used : periodate-permanganate spray and bioautography on *Sarcina lutea* seeded agar.



Separation of Lincomycin (I) and 4′-Depropyl-4′-ethyllincomycin (II) from S-Demethyl-S-ethyllincomycin (III) and U-25,468 (IV) by Counter Double Current Distribution

Forty grams of the oily material, obtained as described above, was dissolved in 240 ml of absolute methanol. This solution was clarified by filtration and mixed with 8 liters of ethyl ether. Methanolic hydrogen chloride (1 N 160 ml) was then added under continuous stirring. The precipitated material (hydrochloride salt of the antibiotics) was isolated by filtration and dried; yield 19.5 g. This material was then distributed in an all-glass counter double current distribution apparatus (25 ml per phase) using the system consisting of equal volumes of 1-butanol and water. The distribution was analyzed after 130 transfers by thin-layer chromatography. 4′-Depropyl-4′-ethyllincomycin (II) (K=0.1) and lincomycin

(I) (K=0.14) were separated from S-demethyl-S-ethyllincomycin (III) (K=0.2) and U-25,468 (IV) (K=0.25). Fractions containing S-demethyl-S-ethyllincomycin and U-25,468 were combined and concentrated to dryness; yield 4.33 g.

Separation of U-25,468 (IV) from S-Demethyl-S-ethyllincomycin (III) by Silica Gel Chromatography

Three grams of the mixture of U-25,468 and S-demethyl-S-ethyllincomycin, obtained as described above, was chromatographed over 900 g of silica gel (Merck-Darmstadt No. 7734) using methyl ethyl ketone - acetone - water (100 : 30 : 5 v/v) as the solvent. TLC showed that early fractions contained U-25,468. Later fractions contained a mixture of U-25,468 and S-demethyl-S-ethyllincomycin followed by fractions containing S-demethyl-S-ethyllincomycin. Concentration of the eluates containing U-25,468 afforded 270 mg of crystalline U-25,468 hydrochloride.

Anal. Calcd. for $C_{20}H_{38}N_2O_6S \cdot HCl \cdot H_2O$:
C 49.12, H 8.47, O 22.95, N 5.74, S 6.57, Cl 7.27.
Found : C 48.14, H 8.93, N 5.76, S 6.44.

Potentiometric titration in water showed the presence of one titratable group, pKa' 7.7, equivalent weight 501 (calc. mol. weight for $C_{20}H_{38}N_2O_6S \cdot HCl \cdot H_2O$ 448.5). Mass spectrum (Fig. 4) showed molecular ion peak at 434 mass units (calcd. for $C_{20}H_{38}N_2O_6S$, 434). The specific rotation was found to be $[\alpha]_D^{25}$ +144.5° (*c* 0.4, water). The infrared spectrum in Nujol is presented in Fig. 2. The nmr spectrum of U-25,468 hydrochloride is presented in Fig. 3.

Concentration of the eluates containing S-demethyl-S-ethyllincomycin gave 1.4 g of crystalline hydrochloride which had infrared and nmr spetra as well as chromatographic (paper and TLC) behavior identical to those reported for S-demethyl-S-ethyllincomycin (III) hydrochloride[8].

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