Synthesis and Bioactivity of Lincomycin-7-Monoesters

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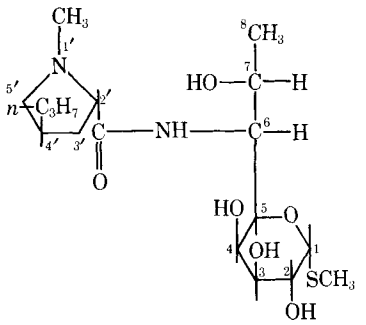
Abstract □ A series of linear acyl and alkoxy esters at Position 7 in the lincomycin molecule were synthesized. None of the esters possessed protective properties comparable to those of lincomycin when given orally to experimentally infected mice.

Keyphrases □ Lincomycin-7-monoesters—synthesis □ GLC—analysis □ TLC—separation, identification □ NMR spectroscopy—structure □ Bioactivity, *in vivo*, *in vitro*—lincomycin-7-monoesters

This paper is a continuation of the effort on the chemical modification of lincomycin (1, 2) for the purpose of enhancing the pharmaceutical acceptability of this antibiotic (taste, absorption) (3).

The lincomycin molecule (I) contains four potentially esterifiable hydroxyl groups. The ease of esterification of these hydroxyl groups has been discussed previously (3).

The objective of this project was to determine if lincomycin-7-monoesters would prove suitable as candidates for pediatric preparations of lincomycin.



I
lincomycin

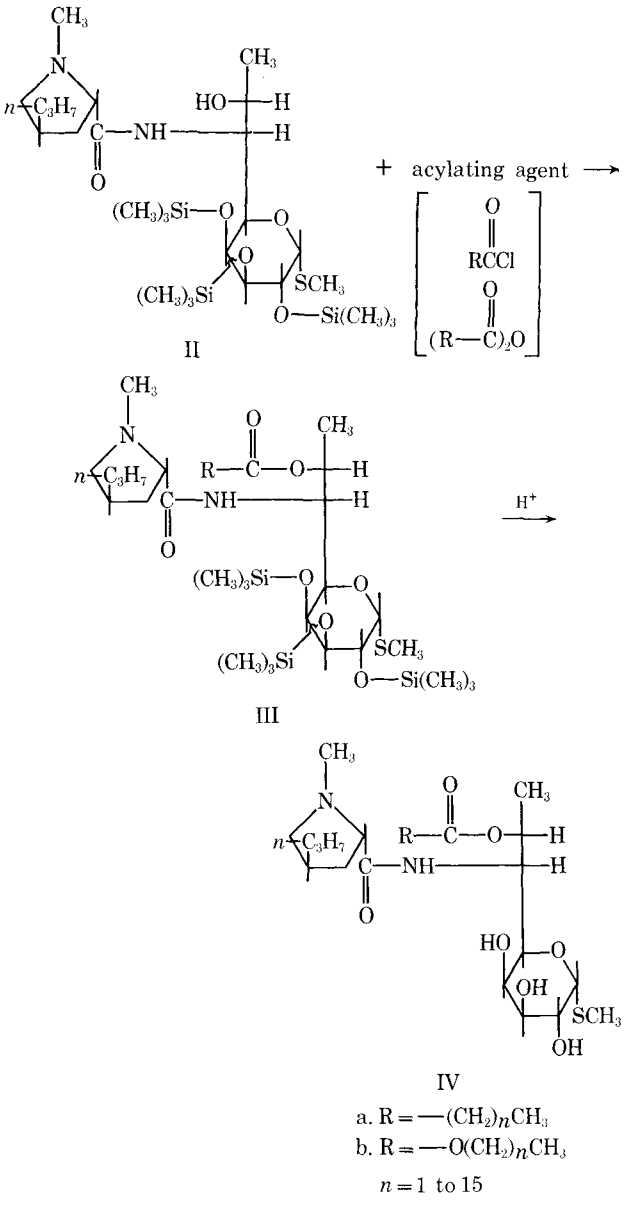
RESULTS AND DISCUSSION

Chemistry—The synthesis of lincomycin-7-monoesters is outlined in Scheme I. The reaction of 2,3,4-tris-*O*-trimethylsilyl lincomycin (II) (5) with an alkyl chlorocarbonate, an acid anhydride, or an acid chloride in the presence of pyridine afford the 2,3,4-*tris*-*O*-trimethylsilyl lincomycin-7-monoesters (III). Treatment of III with HCl or acetic acid at room temperature from 1–4 hr. yielded the desired lincomycin-7-monoesters (IV).

The purification of these compounds is outlined in the *Experimental* section of this paper.

NMR Spectroscopy—The structures of the C_7 monoesters of lincomycin are readily determined *via* examination of the NMR spectral absorption pattern of the esterified carbinol hydrogen and the chemical shift of the C_8 methyl doublet. In all cases of C_7 monoesters (Table I), the esterified carbinol hydrogen absorption occurs in the 5.2–5.35 δ spectral region. The pattern observed is a doublet of quartets, $J_{7,8} = 6.7$ c.p.s. and $J_{7,6} = 3.0$ c.p.s., although not readily evident in the 60 Mc.p.s. spectra due to overlap with the anomeric hydrogen absorption. In all cases of C_7 monoesters, the C_8 methyl doublet is shifted downfield 0.2 p.p.m. from the corresponding lincomycin doublet.

At 100 Mc.p.s., the anomeric hydrogen and the esterified carbinol hydrogen absorptions are resolved.



II

III

IV

a. R = —$(CH_2)_nCH_3$
b. R = —$O(CH_2)_nCH_3$
$n = 1$ to 15

Scheme I

A detailed study of the NMR spectra of lincomycin and its degradation products has been published (6).

Bioactivity—*In Vitro Test Methods*—The method used for determining *in vitro* bioactivity of the 7-monoesters of lincomycin was the standard-curve plate bioassay described by Hanka *et al.* (7). The test organism was *Sarcina lutea* (UC-130) and the assay sensitivity was 0.31 mcg./mg. The free base of lincomycin had an assigned potency of 1000 mcg./mg. The activities of all the esters were calculated and reported as the free base equivalents per milligram of antibiotic.

In Vivo Test Method—The *in vivo* protection studies reported here were performed in CF-1 male albino mice. Groups of 10 mice (18–20 g.) were selected randomly from an animal pool and experimentally infected by injecting intraperitoneally approximately 100 median lethal doses (LD_{50}'s) of a standardized suspension of

Table I—NMR Spectral[a] Data of 7-Monoesters of Lincomycin

Compound	Solvent	Instrument Mc.p.s.	Anomeric H (δ)	Esterified Carbinol H (δ)	C_3 Methyl Doublet (δ)
Lincomycin (I)	D_2O[b]	60/100	5.34	—	1.14
I-7-methylcarbonate·HCl	D_2O[b]	60	5.37	5.31	1.29
I-7-hexylcarbonate·HCl	D_2O[b] 85°	100	5.41	5.315	1.36
	d_7DMF/D_2O	100	5.28	5.19	1.34
I-7-acetate·HCl	D_2O[b]	60/100	5.39	5.195	1.28

[a] Chemical shift in delta (δ) units, p.p.m. downfield from internal reference line. [b] SDSS calibration. Organic solvent calibration with TMS.

Staphylococcus aureus (UC-76). Maintenance of the culture and the method of inducing experimental infections have been described in detail previously (8).

Treatment of the infected mice with the various monoesters was initiated immediately with increment doses of the antibiotic either subcutaneously with 0.2 ml. or orally by gavage with 0.5 ml. The esters were suspended in 0.25% methylcellulose.[1] The mice were treated once per day for 4 days. Evaluation of antibiotic activity was based after 7 days on the median protective dose (CD_{50}) of the ester compared to the CD_{50} calculated for a lincomycin-treated control group. Calculations were program-computed on the IBM 360 digital computer according to the method of Spearman and Karber (9).

The bioactivities of lincomycin-7-monoesters as they were measured *in vitro* and in *Staphylococcus aureus* infected mice are summarized in Table II. The data show the esters to be of low acivity in the standard-curve plate assay. The amount of activity measured as lincomycin in this assay (< 10% of ester) could be accounted for as lincomycin base released by hydrolysis of the ester by the assay organism.

It was also found that these esters were more slowly hydrolyzed *in vitro* by certain ester-hydrolyzing enzymes than their corresponding 2-esters and this may be the cause of the lower bioactivity exhibited by the 7-monoesters (4).

Most of the esters in this series that were in the mouse-protection test possessed less activity than lincomycin when administered *via* the subcutaneous route and none was as active when administered *via* the oral route. Three of the esters possessed protective properties statistically equivalent to that of lincomycin when given subcutaneously. These were the laurate, hexylcarbonate, and hexadecylcarbonate esters. This observation agreed well with that of the *in vivo* activity of lincomycin-2-palmitate (3) but the *in vivo* therapeutic responses of the 2-esters and 7-esters with the palmitate and stearate did not parallel.

The bioactivity of the 7-esters as it related to the substituent chainlength is shown in Table I. These data show the subcutaneously administered esters were more active as the chain length increased until an optimum of 12 carbon atoms was reached. The activity decreased with esters containing more than 12 carbon atoms.

EXPERIMENTAL

Chromatography—Gas chromatography was run on the silanized lincomycin-7-alkyl esters. The silyl ethers were made by mixing a pyridine solution containing 1% of the ester to be silanized, 20% hexamethyldisilazane, and 20% trimethylchlorosilane. One microliter of this solution was injected into an F & M Model 700 gas chromatograph (flame ionization). The column was 0.31 × 43.2 cm. (0.125 × 17 in.) stainless steel containing 1% OV-1 on diatomaceous earth (100–120 mesh).[2] Oven temperatures ranged from 235 to 300°.

Thin-layer chromatography (TLC) of the alkoxy esters was conducted on 500-μ layers of Silica Gel G [5.1 × 20.3-cm. (2 × 8-in. plates)]. These esters were detected on the plate at $R_f \sim 0.8$ using PMPI[3] spray reagent as spot developer and bromophenol blue spray reagent[3] as spot fixative. The solvent system consisted of methylethyl ketone–acetone–water (140:40:22).

A second TLC system was also used for both acyl and alkoxy esters and proved to be the best suited for determination of the progress of the reaction and for purity. The chromatographic support again was 500-μ layers of Silica Gel G. The solvent system was hexane–ether–methylpropyl ketone–methanol–ammonia (60:20:20:9:1). The spot developer was 50% aqueous H_2SO_4, the plate being heated after spraying to char any organic material present.

NMR Spectra—All spectra were examined as 0.15 *M* solutions in D_2O and are calibrated to the internal reference sodium-2,2-dimethyl-2-silapentane-5-sulfonate (SDSS). The 60 Mc.p.s. spectra were calibrated with internal 1% SDSS, the 100 Mc.p.s. spectra were calibrated *via* frequency difference between pattern and lock signal, and corrected for internal SDSS 0.25 p.p.m. downfield from the lock signal. The 100 Mc.p.s. lock signal was provided *via* hexamethyldisilazane in a sealed capillary inserted into the D_2O solutions.

Synthetic—Acid anhydrides were preferred for synthesis of acyl esters because less color formation occurs in the reaction media. Typical examples of reaction conditions are illustrated. Elemental analyses are outlined in Table III.

Acid Anhydride Method—*Lincomycin-7-Acetate·HCl*—A solution of 1.71 g. II in 5 ml. of dry pyridine was treated with 2 ml. of acetic anhydride. After 16 hr. at R.T. the solution was concentrated to a viscous liquid *in vacuo* at 45°. The residue was dissolved in 8 ml. of glacial acetic acid and 2 ml. of water added. After 2.5 hr. at R.T. the solvent was removed *in vacuo* at 60° and the residue dissolved in 25 ml. of water. The solution was adjusted to pH 8 by addition of 5% aqueous sodium carbonate. The resulting precipitate of lincomycin-7-acetate was extracted with four 25-ml. portions of chloroform. The chloroform fractions were combined, dried, and the solvent removed *in vacuo*. The residue was dissolved in 100 ml. of ether and addition of gaseous HCl gave 1.27 g. (87%) of white amorphous powder. The compound was dissolved in 6 ml. of hot isopropyl alcohol and the solution diluted with 100 ml. of acetone. After standing 24 hr. at 4°, 1.09 g. (91% recovery) of lincomycin-7-acetate·HCl was obtained.

Table II—Antibacterial Activity of Lincomycin-7-Monoesters[a]

Lincomycin-7-Ester·HCl	*In Vitro* Activity, μg./mg.[b]	Relative Median Protective Dose (CD_{50})[c] Subcutaneous	Oral
Acetate	10 (1.0)	0.08	—
Butyrate	30 (3.0)	0.32	0.36
Hexanoate	45 (4.5)	0.34	0.33
Valerate	30 (3.0)	0.55	0.52
Octanoate	40 (4.0)	0.59	0.55
Laurate	59 (5.9)	1.06	0.55
Palmitate	20 (2.0)	0.01	0.05
Stearate	9 (0.9)	0.01	0.1
Methylcarbonate	4 (0.4)	[d]	[d]
Hexylcarbonate	32 (3.2)	0.71	0.64
Hexadecylcarbonate	42 (4.2)	0.86	0.28

[a] Activities calculated as lincomycin base equivalents. [b] As measured on a standard-curve agar assay *versus Sarcina lutea*. Results expressed as mcg. of lincomycin base activity per mg. of ester and as percent of lincomycin base activity (in parentheses). [c] Median protective dose relative to that of lincomycin (lincomycin = 1.0). [d] Data not available.

[1] Methocel, Dow Chemical Co.
[2] Gas Chrom Q, Applied Science Laboratories.
[3] PMPI spray reagent: sodium bicarbonate, 10 g; sodium metaperiodate, 8 g.; potassium permanganate, 1 g. The salts were dissolved in 500 ml. H_2O and allowed to stand for 12 hr. The resulting white precipitate was filtered. Bromophenol blue spray reagent: 0.1 g. of bromophenol blue was dissolved in 7.45 ml. of *N*/50 NaOH and diluted with H_2O to 250 ml.

Table III—Analytical Data Obtained with Lincomycin-7-Monoesters

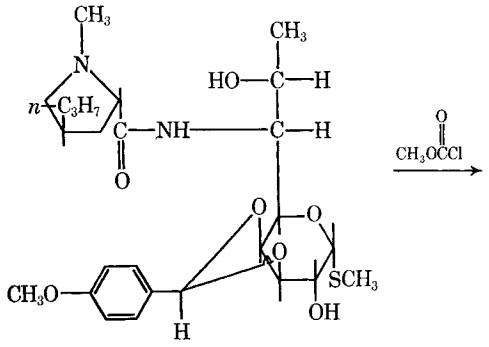
Lincomycin-7-Ester·HCl	Empirical Formula	Mol. Wt.	Analysis (Corr. for Water Content), % Calcd.	Found	H_2O, %[a]	Yield, %	M.p., °C.[b]
-Acetate	$C_{20}H_{37}ClN_2O_7S$	485.06	C, 49.52 H, 7.69 N, 5.77 Cl, 7.31 S, 6.61	C, 49.29 H, 7.88 N, 5.50 Cl, 7.51 S, 6.60	3.74	87	
-Butyrate (C_4)	$C_{22}H_{41}ClN_2O_7S$	513.09	C, 51.50 H, 8.10 N, 5.46 S, 6.25	C, 51.61 H, 8.10 N, 5.65 S, 6.26	5.99	83	137–160
-Valerate (C_5)	$C_{23}H_{43}ClN_2O_7S$	527.13	C, 52.41 H, 8.22 N, 5.31 Cl, 6.73 S, 6.08	C, 52.60 H, 8.14 N, 5.62 Cl, 6.87 S, 6.14	5.59	90	169–175
-Hexanoate (C_6)	$C_{24}H_{45}ClN_2O_7S$	541.16	C, 53.27 H, 8.38 N, 5.18 Cl, 6.55 S, 5.93	C, 52.94 H, 8.29 N, 4.92 Cl, 6.66 S, 6.08	4.14	85.4	165–169
-Octanoate (C_8)	$C_{26}H_{49}ClN_2O_7S$	569.22	C, 54.86 H, 8.68 N, 4.92 S, 5.63	C, 54.81 H, 8.96 N, 5.24 S, 5.71	5.22	85	151–165
-Laurate (C_{12})	$C_{30}H_{57}ClN_2O_7S$	625.27	C, 57.62 H, 9.19 N, 4.48 S, 5.13	C, 57.46 H, 8.99 N, 4.80 S, 5.23	4.16	84.4	161–164
-Palmitate (C_{16})	$C_{34}H_{65}ClN_2O_7S$	681.43	C, 59.93 H, 9.62 N, 4.11 Cl, 5.20 S, 4.71	C, 59.70 H, 9.94 N, 4.06 Cl, 5.33 S, 5.00	4.38	81	168–170
-Stearate (C_{18})	$C_{36}H_{69}ClN_2O_7S$	709.43	C, 60.95 H, 9.80 N, 3.95 S, 4.52	C, 61.15 H, 9.62 N, 4.25 S, 4.53	4.68	72	168–174
-Methyl Carbonate	$C_{20}H_{37}ClN_2O_8S$	501.04	C, 47.94 H, 7.44 N, 5.59 Cl, 7.07	C, 48.88 H, 7.59 N, 5.63 Cl, 7.46	1.93	19	98–101
-Hexyl Carbonate	$C_{25}H_{47}ClN_2O_8S$	571.18	C, 52.57 H, 8.29 N, 4.90 Cl, 6.21 S, 5.61	C, 52.15 H, 8.59 N, 5.36 Cl, 6.26 S, 5.72	2.78	70	133–135
-Hexadecyl Carbonate	$C_{35}H_{67}ClN_2O_8S$	711.45	C, 59.09 H, 9.49 N, 3.94 Cl, 4.98 S, 4.51	C, 58.28 H, 9.48 N, 4.20 Cl, 4.85 S, 4.47	3.99	46	159–160

[a] Percent water determined by Karl Fischer method. [b] Melting points are of the hydrated samples and were determined on a Thomas-Hoover melting point apparatus and are uncorrected.

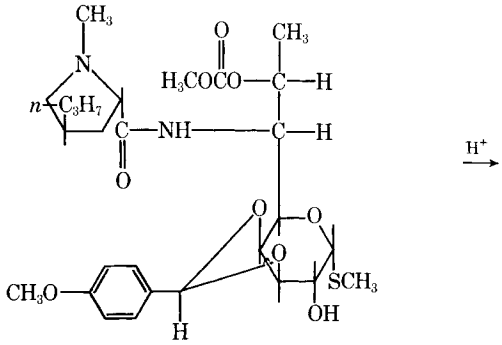
Acid Chloride Method—*Lincomycin-7-Palmitate·HCl*—A solution of 2.89 g. II in 10 ml. of pyridine was treated with a solution of 1.45 g. of palmitoyl chloride in 5 ml. of chloroform. After 1 hr. at R.T. the reaction was complete. The pyridine solution was concentrated *in vacuo* at R.T. to a viscous residue. The residue was dissolved in 15 ml. of chloroform and this solution extracted with 50 ml. of 0.05 *N* hydrochloric acid. The organic phase was separated and the solvent removed *in vacuo* at R.T. The resulting viscous residue was dissolved in 40 ml. of 80% aqueous acetic acid and stirred at R.T. for 2 hr. The solvent was removed *in vacuo* at 60° and the residue dissolved in 50 ml. of water. This solution was treated with 5 g. of sodium carbonate dissolved in 10 ml. of water. The precipitate was extracted with 50 ml. of chloroform and the chloroform layer washed with 150 ml. of 0.05 *N* hydrochloric acid. The chloroform was removed *in vacuo* and the residue dissolved in 200 ml. of ether. The ether solution was dried with sodium sulfate and the addition of gaseous HCl afforded 2.57 g. (94%) of crude lincomycin-7-palmitate·HCl. A solution of 2.20 g. of this compound in a mixture of 2 ml. of water and 15 ml. of acetone warmed to 40° was diluted with 50 ml. of acetone to give 1.78 g. (81% recovery) of crystalline lincomycin-7-palmitate·HCl.

Alkyl Chlorocarbonate Method—*Lincomycin-7-Hexylcarbonate·HCl*—Lincomycin-2,3,4-tris-*O*-(tri methylsilyl) ether (15 g., 0.024 mole) was dissolved in 100 ml. of pyridine (AR). The reaction mixture was cooled to −35° and 5 g. (~0.03 mole) of *n*-hexyl chlorocarbonate (Eastman White Label) was added dropwise. An additional 3 g. of *n*-hexyl chlorocarbonate was added after 1 hr. The reaction mixture was stirred for an additional hour. The mixture was poured into 1 l. of ice-water acidified to pH 2 with concentrated HCl. The aqueous suspension was immediately extracted with 750 ml. of ether, dried with anhydrous $MgSO_4$, and the ether removed by warming on a steam bath. The resulting orange syrup was dissolved in 150 ml. of absolute methanol, 75 ml. of water added, and 2 ml. of concentrated HCl added slowly with good stirring. The mixture was stirred for 45 min. and the pH of the solution was increased to 3 with sodium bicarbonate. All of the solvent was removed and the resulting gum dissolved, with warming, in 500 ml. of acetone. The insoluble NaCl was removed by filtration and the filtrate concentrated to 200 ml. Ether was added to the cloud point and the solution placed overnight in a refrigerator. A yield of 9.56 g. (69.7%) of lincomycin-7-hexylcarbonate·HCl was obtained.

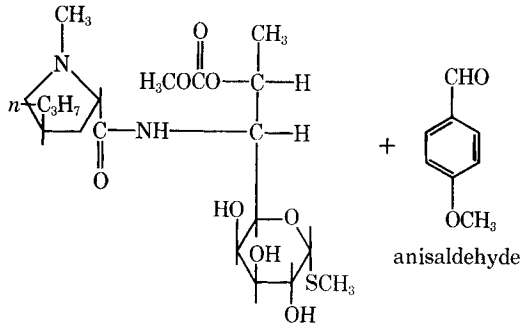
Partial Blocking Group Method—*Lincomycin-7-Methylcarbonate·HCl*—3,4-O-Anisylidene Lincomycin-7-Methylcarbonate-3,-4-*O*-anisylidene lincomycin base (15 g., 0.029 mole) was dissolved in 375 ml. of acetone. This solution was stirred well and 158 ml. of distilled water was added slowly. Methylchloroformate (48 ml., 0.5 mole) was then added in 5-ml. increments over the period of 1 hr. The pH of the reaction was monitored with a Beckman Zero-



3,4-*O*-anisylidene lincomycin



3,4-*O*-anisylidene lincomycin-7-methylcarbonate



lincomycin-7-methylcarbonate

Scheme II—Partial Blocking Group Method

matic pH meter standardized at pH 4 prior to use. The pH of the reaction mixture was allowed to fluctuate between 5 and 7. Sodium hydroxide (2 *N*) was used to maintain the pH within these limits. Final pH of the solution was 6.8. Most of the acetone was removed under vacuum at room temperature. The remaining aqueous portion was extracted with three 300-ml. portions of ether. The ether extracts were combined and washed with two 100-ml. portions of water. Extracts were then dried over anhydrous magnesium sulfate and the ether removed under reduced pressure affording 20 g. of amorphous white powder. TLC (alumina-neutral grade, benzene–acetone, 4:1) showed the presence of four spots, the major spot being anisylidene lincomycin-7-methylcarbonate. Minor spots included some dicarbonate and starting material. One minor spot was unidentified (see Scheme II).

A 2.8 cm. × 121.9 cm. (1.1 in. × 48 in.) F and P chromatography tube was packed with 275 g. of Florisil (TLC mesh) using a benzene slurry. A linear gradient elution apparatus was set up using acetone (2000 ml.) and benzene (2000 ml.) as the eluting solvents. A Technicon automatic fraction collector containing a 200-tube rack was used to collect 12–15 ml. of eluate per tube. Ten grams of crude carbonate powder was dissolved in 200 ml. of benzene and passed through the column. Elution was started using pure benzene as initial solvent. The final eluate composition was acetone–benzene (3:2). A total of 250 tubes was collected. Fractions 115–170, containing pure monocarbonate (as determined by TLC), were pooled and evaporated to dryness under vacuum. The resulting amorphous powder (8 g.) was dissolved in 100 ml. of methanol, filtered and cooled to 5–10°. Distilled water was then added dropwise to the cloud point and the solution maintained at 5–10°. Crystals that formed were collected and the procedure repeated.

Anal.—Calcd. for $C_{28}H_{42}N_2O_9S$: C, 57.71; H, 7.26; N, 4.81; H_2O, 0; Eq. wt. 583. Found (corrected for H_2O): C, 57.88; H, 6.96; N, 5.00; H_2O, 5.87; Eq. wt. 577. λ_{max}^{EtOH} 226 mμ (ϵ 13,385).

3,4-*O*-Anisylidene lincomycin-7-methylcarbonate (10 g.) was dissolved in 25 ml. of absolute methanol. A 0.2 *N* HCl solution was added dropwise to this methanolic solution to the cloud point. More methanol and HCl were added until a total of 100 ml. of 0.2 *N* HCl was added. The reaction mixture was allowed to stir for 2 hr. The pH of the solution was adjusted to 4 with sodium bicarbonate. Most of the methanol was removed under vacuum at room temperature and the pH again adjusted to 2 with sodium bicarbonate. This aqueous solution was freeze-dried and the reulting white powder washed with 200 ml. of absolute ether to remove the anisaldehyde formed. The crude lincomycin-7-methylcarbonate (6.1 g.) was distributed in a 500 transfer countercurrent distribution apparatus, using butanol–water (1:1) as the partition system. The material from tubes 60–120 contained the pure carbonate (as determined by solids analysis) and was recovered by evaporation of the solvent under vacuum and freeze drying. It displayed a K-value of 0.25. Recovery was 3.5 g. of "Craig-pure" lincomycin-7-methylcarbonate.

Vapor phase chromatography of lincomycin-7-methylcarbonate indicated that a small amount of impurity remained in the sample. This impurity was thought to be *p*-methoxybenzoic acid resulting from oxidation of anisaldehyde during one or both of the previous synthetic sequences. NMR also indicated a mixture of compounds as evidenced from the 5.4 p.p.m. region. Thus, to 550 mg. of lincomycin-7-methylcarbonate was added 24 ml. of 20% w/v sodium carbonate solution and the resulting mixture placed in a separator. Four 8-ml. portions of methylene chloride were used to extract the aqueous portion. The combined methylene chloride extracts (32 ml.) were then shaken with 96 ml. of sodium carbonate solution in a separator. Anhydrous sodium sulfate was used to dry the methylene chloride extract. After the solvent was removed, the residue was dissolved in 25 ml. of dry ether and anhydrous hydrogen chloride bubbled through the solution. The resulting white flocculent precipitate was filtered and washed with 50 ml. of dry ether. Recovery of pure lincomycin-7-methylcarbonate was 380 mg.

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