Lincomycin. II. Characterization and Gross Structure[1,2]

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Abstract: Physical and chemical characteristics of lincomycin, together with spectral data and examination of the products of hydrolysis and periodate oxidations, allow the postulation of a gross structure as a working hypothesis.

Lincomycin, as has been reported,[3-6] is a new antibiotic produced by an actinomycete designated *Streptomyces lincolnensis* var. *lincolnensis*, n.sp. Clinical use has shown it to be an effective agent for treatment of infections in humans.[7] This paper presents the general characteristics of the antibiotic and the preliminary degradations which led to the postulation of the gross structure as a working hypothesis.

Lincomycin is a basic compound in which the single amine group is tertiary with a pK_a' of 7.6. The free base is soluble in water and most organic solvents other than the hydrocarbons and has the empirical formula $C_{18}H_{34}N_2O_6S$. The crystalline hydrochloride salt, which is soluble in water and the lower alcohols, forms hydrates and was isolated as the hemihydrate, $C_{18}H_{34}N_2O_6S \cdot HCl \cdot 0.5H_2O$. Group analyses showed the presence of two C-methyl, one N-methyl, and no acetyl or methoxyl groups. The infrared absorption spectrum indicates multiple OH and NH groups and a monosubstituted amide function (amide I and II bands). Lincomycin shows no absorption maxima in the ultraviolet or visible regions.[4]

Although the nmr spectrum of lincomycin hydrochloride (Figure 1) is quite complex, with many overlapping areas, it provides some valuable information. An anomeric hydrogen is indicated by a doublet at 322 cps,[8] one C-methyl appears as a triplet at 53 cps, indicating two neighboring hydrogens, and the other C-methyl as a doublet at 70 cps, indicating one neighboring hydrogen. The N-methyl is readily recognized at 178 cps and, with no acetyl or methoxyl groups present, the remaining sharp singlet at 128 cps can be attributed to an S-methyl group. In addition, although the spectrum was not factorable, nor could complete assignments be made, area measurements to indicate the total number of hydrogens and their distribution were used to exclude certain otherwise possible structures.

These characterization data suggested a possible close structural similarity to celesticetin, an antibiotic isolated previously in these laboratories and on which some structure work had been done.[9] For this reason, initial experiments on lincomycin paralleled those on celesticetin, and results substantiated this similarity. Subsequent work elucidated the complete structural relationship.[10]

Vigorous acid hydrolysis of lincomycin was accompanied by considerable charring but afforded two products, methyl mercaptan, isolated and identified as its 2,4-dinitrophenyl thioether, and an amino acid, isolated as a crystalline hydrated hydrochloride. Analysis and titration of the amino acid indicated an empirical formula $C_9H_{17}NO_2 \cdot HCl \cdot 0.5H_2O$. No color was produced with ninhydrin or isatin. A similar C_6 amino acid from celesticetin had been identified as L-hygric acid (N-methylproline), and comparison of the nmr spectrum of the lincomycin moiety with that of a synthetic sample of DL-hygric acid[11] showed the new amino acid to be a *n*-propylhygric acid (Figure 2). The similarity of the spectra in the 100–300-cps region can be seen, together with the obvious differences in the lower frequency range. The presence in the spectrum of the lincomycin amino acid of a C-methyl group as a triplet at 45 cps and an area equivalent to four hydrogens in the 80-cps region representing the two methylene groups of the alkyl chain give a clear picture of a *n*-propyl group. The position and configuration of the alkyl group could not be determined from the spectrum, since the ring hydrogen multiplets could not be factored.

The specific rotation of the amino acid hydrochloride in water, $[\alpha]D$ −153°, and the slight positive rotational shift of the free amino acid from $[\alpha]D$ −67° in water to $[\alpha]D$ −62° in 5 *N* hydrochloric acid suggested the L configuration, although this was not definite due to the presence of the second asymmetric center.[12] The position of the propyl group and the complete stereochemistry of the amino acid was determined by subsequent work.[13]

Although the sugar portion of the molecule is destroyed during acid hydrolysis, much could be deduced from other chemical and nmr data. The ease of removal of the S-methyl under acid conditions, plus the clear indication of an anomeric hydrogen by nmr, placed this as a glycosidic group. Acetylation of lincomycin showed the presence of four hydroxyls by formation

(1) This work was presented in part at the 148th National Meeting of the American Chemical Society, Chicago, Ill., Aug 1964.

(2) Part I of this series was a preliminary communication: H. Hoeksema, B. Bannister, R. D. Birkenmeyer, F. Kagan, B. J. Magerlein, F. A. MacKellar, W. Schroeder, G. Slomp, and R. R. Herr, *J. Am. Chem. Soc.*, **86**, 4223 (1964).

(3) D. J. Mason, A. Dietz, and C. DeBoer, *Antimicrobial Agents Chemotherapy*, 554 (1962).

(4) R. R. Herr and M. E. Bergy, *ibid.*, 560 (1960).

(5) L. J. Hanks, D. J. Mason, M. R. Burch, and R. W. Treick, *ibid.*, 565 (1962).

(6) C. N. Lewis, H. W. Clapp, and J. E. Grady, *ibid.*, 570 (1962).

(7) G. K. Daikos, *et al.*, *ibid.*, 197 (1963); W. J. Holloway, *et al.*, *ibid.*, 200 (1963); J. Harnecker, *et al.*, *ibid.*, 204 (1963); E. W. Walters, *et al.*, *ibid.*, 210 (1963); J. C. Trakas and H. E. Lind, *ibid.*, 216 (1963).

(8) Frequencies in water solutions are measured from sodium 4,4-dimethyl-4-silapentanesulfonate (SDSS); in chloroform solution from tetramethylsilane (TMS).

(9) H. Hoeksema and J. W. Hinman, *J. Am. Chem. Soc.*, **86**, 4979 (1964).

(10) H. Hoeksema, Abstracts, 148th National Meeting of the American Chemical Society, Chicago, Ill., Aug–Sept 1964, p 385.

(11) Furnished by Dr. W. Schroeder; obtained by acid hydrolysis of the methyl ester, which was prepared by dry distillation of stachydrin. See G. Trier, *Z. Physiol. Chem.*, **67**, 324 (1910).

(12) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1961, p 83.

(13) B. J. Magerlein, R. B. Birkenmeyer, R. R. Herr, and F. Kagan, *J. Am. Chem. Soc.*, **89**, 2459 (1967).

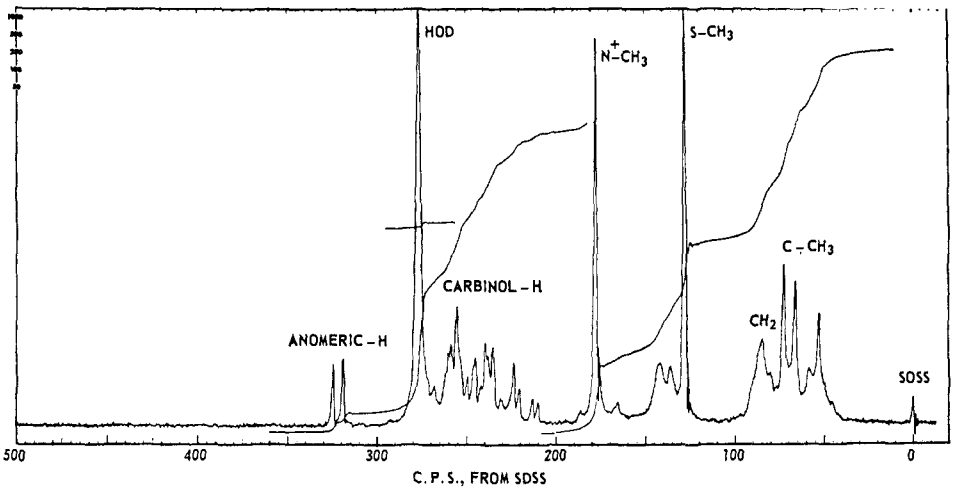
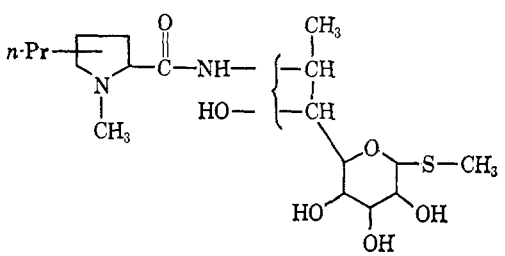


Figure 1. Nuclear magnetic resonance spectrum (60 Mc) of lincomycin hydrochloride in water.

of a tetra-O-acetate, as shown by group analyses and nmr. The nmr spectrum of the tetraacetate in chloroform solution showed four resolved acetyl methyl absorptions at 114, 119, 124, and 127 cps (Figure 3).

In periodate oxidations about 4 moles of periodate was consumed per mole of lincomycin. The quantitation, however, was somewhat unreliable because of fading end points due to the influence of the oxidized sulfur group.[14] Replacement of the S-methyl group by hydrogen on treatment with Raney nickel gave a product designated anhydrolincomycitol, which cleanly consumed 2 moles of periodate/mole. As expected, anhydrolincomycitol showed no S-methyl group or anomeric hydrogen in its nmr spectrum (Figure 4). On a preparative scale, periodate oxidation of lincomycin followed by brief acid hydrolysis allowed isolation of glyoxal as its 2,4-dinitrophenylhydrazone. Also, titration with alkali following periodate oxidation showed the formation of 1 mole of formic acid. Based on these data, plus the proton distribution data obtained from the nmr spectrum, structure 1 was proposed as a possible partial structure for lincomycin.



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Periodate results require the three adjacent hydroxyls in the ring, with the glyoxal derived from carbons 1 and 2 and the formic acid from carbon 3. The nmr spectrum (Figure 1) shows that, other than the methyl hydrogens, all the remaining hydrogens in the sugar portion of the molecule are on carbons bearing negative groups. Thus, the methyl group represented by the doublet at 70 cps is attached to a carbon holding one hydrogen plus either an oxygen or nitrogen function. These facts require the pyranose ring since any furanose ring structure would either contain a tertiary hydroxyl

(14) W. A. Bonner and R. W. Driski, *J. Am. Chem. Soc.*, 73, 3699 (1951).

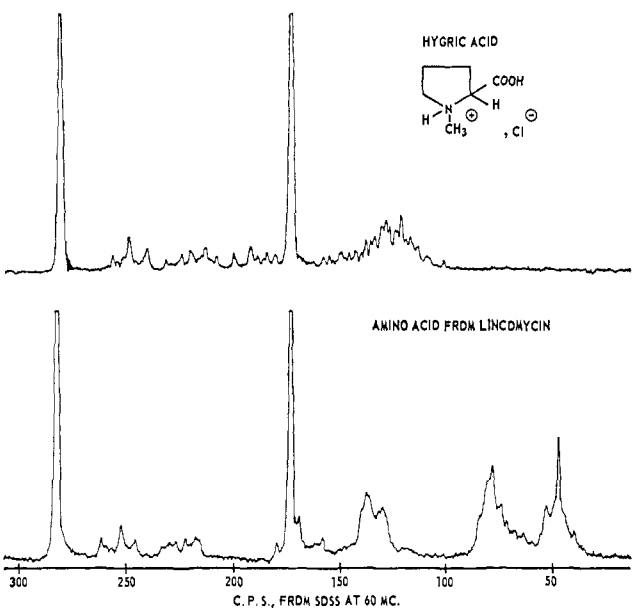

Figure 2. Nuclear magnetic resonance spectra of DL-hygric acid hydrochloride (synthetic) and the amino acid hydrochloride from lincomycin, in water.

and additional paraffin hydrogens which are not seen in the nmr spectrum or would not account for the formation of formic acid on oxidation. Another pyranose structure with a branched sugar skeleton is allowed by these data, but structure 1 was used as a working hypothesis, and the straight chain skeleton was confirmed by subsequent examination of degradation products.[15]

The relative positions of the amide function and the last hydroxyl group were uncertain. The fact that lincomycin and some of its degradation products give negative iodoform tests led us to postulate the positions shown in **1**, but later nmr data was in better agreement with the reverse arrangement.[16] Subsequent chemical work showed conclusively that their true positions are the latter.[15]

(15) W. Schroeder, B. Bannister, and H. Hoeksema, *ibid.*, 89, 2448 (1967).

(16) G. Slomp and F. A. MacKellar, *ibid.*, 89, 2454 (1967).

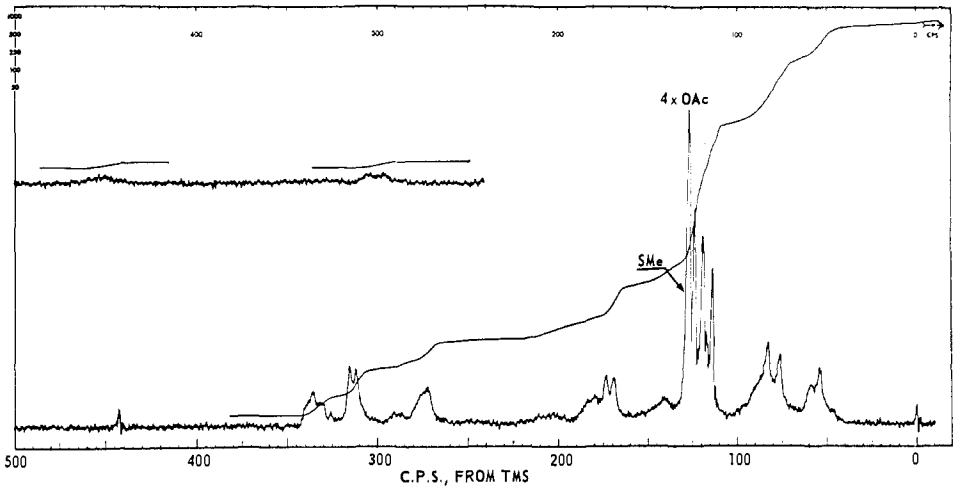

Figure 3. Nuclear magnetic resonance spectrum (60 Mc) of tetraacetyllincomycin in chloroform.

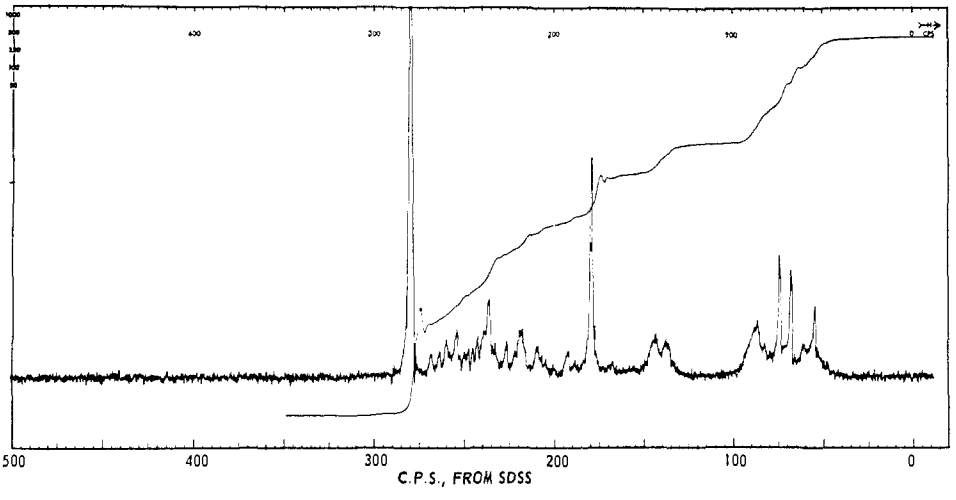

Figure 4. Nuclear magnetic resonance spectrum of anhydrolincomycin hydrochloride in water.

Experimental Section[17]

Acid Hydrolyses. (a) For Methyl Mercaptan. A solution of lincomycin hydrochloride (1 g) in 5 *N* sulfuric acid (10 ml) was refluxed and the evolved gases were passed through a mixture of 0.3 ml of 3.3 *N* sodium hydroxide (aqueous) and 3 ml of ethanol. To the resulting solution was added a solution of 1-chloro-2,4-dinitrobenzene (200 mg) in warm benzene (3 ml). The yellow crystals which formed immediately were collected and recrystallized from ethanol to give 58 mg of shinyl yellow plates, mp 127–128; lit. for methyl 2,4-dinitrophenyl thioether, mp 128.[18] The infrared spectrum was identical with that of an authentic sample.

Anal. Calcd for $C_7H_6N_2O_4S$: C, 39.25; H, 2.83; N, 13.08; S, 14.97. Found: C, 39.03; H, 2.67; N, 12.84; S, 14.95.

(b) For Propylhygric Acid. A solution of lincomycin hydrochloride (2 g) in 6 *N* hydrochloric acid (50 ml) was refluxed for 30 min and then distilled to dryness. The dry residue was dissolved in water (20 ml) and extracted twice with chloroform (two 10-ml portions). The aqueous solution was diluted with 60 ml of water and extracted twice with butanol (two 20-ml portions). The aqueous solution was then evaporated to dryness and the dry residue was dissolved in ethanol (20 ml). The ethanolic solution was decolorized with carbon, and ether (100 ml) was added to the filtrate. A gummy precipitate which separated was removed by filtration and discarded. The filtrate was again treated with carbon, and additional ether (1000 ml) was added to the filtrate. After standing several weeks in the refrigerator crystals formed; yield 650 mg. A sample for analysis was recrystallized several times from ethanol–ether. On heating, the crystals softened at about 123° and finally melted with decomposition at 160–170°. The pK_a's in water were 2.4 and 10. The infrared spectrum in mineral oil suspension suggested a tertiary amino acid hydrochloride, hydrated, and had bands at 3530, 3460, 2720 (broad), 1740, 1225, 1210, 1190, 1150, 1120, 1105, 1040, 1025, 980, 925, 845, and 810 cm^{-1}.

Anal. Calcd for $C_9H_{17}NO_2 \cdot HCl \cdot 0.5H_2O$: C, 49.87; H, 8.84; N, 6.46; Cl, 16.36; mol wt, 216.7. Found: C, 49.35; H, 8.55; N, 6.75; Cl, 16.96; mol wt (electrometric titration), 216.

Lincomycin Free Base. A solution of lincomycin hydrochloride (100 g) in deionized water (1700 ml) was adjusted to pH 10 by the addition of 5 *N* sodium hydroxide and extracted three times with chloroform (three 1700-ml portions). The extracts were combined and dried over sodium sulfate, and the chloroform was evaporated under reduced pressure while deionized water was added. The resultant aqueous solution was freeze dried to yield 85 g of white amorphous lincomycin base. Karl Fischer determination showed this material to contain 1.50% water. Analytical values shown are corrected for the water content, with actual values shown in parentheses. The optical rotation was $[\alpha]D$ +158° (156°) (*c* 1, water). The nmr spectrum was very similar to that of the hydrochloride except that the N-methyl absorption of the free base was shifted 38 cps upfield as a result of the removal of the charge from the nitrogen.

Anal. Calcd for $C_{18}H_{34}O_6N_2S$: C, 53.18; H, 8.43; N, 6.89; S, 7.89; mol wt, 406.5. Found: C, 53.11 (52.31); H, 8.40 (8.44); N, 6.95 (6.83); S, 7.98 (7.86); mol wt (electrometric titration), 408 (415).

Lincomycin Tetraacetate. Lincomycin base (1.7 g) was acetylated with pyridine (50 ml) and acetic anhydride (25 ml) at room temperature. After 24 hr the excess reagents were removed under

(17) Melting points were determined on a Kofler hot-stage apparatus.

(18) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," 2nd ed, John Wiley and Sons, Inc., New York, N. Y., 1940, p 225.

reduced pressure and the residue was dissolved in methylene chloride and water. The mixture was neutralized with saturated sodium bicarbonate, phases were separated, and the aqueous layer was extracted with methylene chloride. The combined methylene chloride extracts were washed until they were neutral and dried over sodium sulfate, and the solvent was evaporated under reduced pressure. The glassy residue did not crystallize and was therefore distributed countercurrently for 500 transfers using the solvent system Skellysolve B–acetone–water in the ratio 7:16:3. The distribution was analyzed by the determination of solids and showed a single peak with the center at tube 323, $K = 1.8$, which fit well with the theoretical curve. Tubes 310–350 were pooled and the solvents evaporated under reduced pressure. The residue, a glass which did not crystallize, was converted to the hydrochloride salt by dissolving in ether and bubbling in hydrogen chloride. The hydrochloride which precipitated was collected by filtration and dissolved in a small amount of chloroform, which was then layered with ether and chilled in the refrigerator. Crystallization began at the interface, more ether was added gradually, and finally the layers were mixed. The crystals were collected, recrystallized by the same procedure, and air dried, mp 226–233°; $[\alpha]D$ +149° (*c* 1, water). The infrared spectrum was consistent with the formation of a tetraacetate; the NH absorption at 3200 cm^{-1} remained but the OH absorption at 3400–3600 cm^{-1} had disappeared. Analyses indicated the crystalline hydrochloride as prepared to be a hemihydrate.

Anal. Calcd for $C_{26}H_{42}N_2O_{10}S \cdot HCl \cdot 0.5H_2O$: C, 50.35; H, 7.15; N, 4.52; O, 27.09; S, 5.17; Cl, 5.72; H_2O, 1.45; acetyl (4), 27.8; equiv wt, 620. Found: C, 49.83; H, 7.57; N, 4.52; O, 27.10; S, 4.99; Cl, 5.81; H_2O, 1.72; acetyl, 25.53; equiv wt, 605.

Periodate Oxidation of Lincomycin Hydrochloride. (a) **Titrations.** Titration of lincomycin hydrochloride by the Fleury–Lange procedure using 0.23 *M* sodium metaperiodate solution showed consumption of periodate varying from 3.5 to 5 moles because of fading end points. Titration of the products of oxidation was carried out as follows. Two 45-mg samples of lincomycin hydrochloride were each dissolved in 20 ml of water. Five milliliters of a 50-mg/ml solution of sodium metaperiodate in water was added to each sample and the beakers were wrapped with foil. A blank, using 20 ml of water and 5 ml of the periodate solution, was similarly treated. After standing at room temperature for 45 min, ethylene glycol (2 ml) was added to each beaker; the mixture was again let stand for 1 hr. The solutions were then titrated with 0.02 *N* sodium hydroxide. A second blank containing 47.6 mg of lincomycin hydrochloride in 20 ml of water was also titrated with the sodium hydroxide. Comparison of the results indicated 1.2 moles of acid, $pK_a' = 3.5$, formed per mole of lincomycin (lit. for formic acid is $pK_a' = 3.75$).

(b) Isolation of Glyoxal 2,4-Dinitrophenylhydrazone. A solution of lincomycin hydrochloride (1 g) in 25 ml of water was added to a solution of sodium metaperiodate (2 g) in 75 ml of water. After standing at room temperature for 1 hr, barium hydroxide solution was added until no more precipitate formed. The mixture was filtered and the filtrate was extracted with methylene chloride (three 50-ml portions). The extracts were combined and concentrated under reduced pressure. The almost colorless oily residue was dissolved in ethanol (25 ml) and added to a hot solution of 2,4-dinitrophenylhydrazine (1 g) in ethanol (75 ml). Concentrated hydrochloric acid (2.5 ml) was added and heating continued. The remaining undissolved reagent went into solution and almost immediately a precipitate began to separate. The product was collected, washed with ethanol, and air dried; mp 319–325° (lit.[19] for the glyoxal derivative 328°). It was identified as the 2,4-dinitrophenylhydrazone of glyoxal by comparison of its infrared and ultraviolet spectra with those of an authentic sample.

Anhydrolincomycitol. Raney nickel (W-3) was washed with water until the washings were neutral, and about 40 g of this nickel was added to a solution of lincomycin hydrochloride (5 g) in water (125 ml). The mixture was stirred and refluxed for 1 hr, at which time about 25 g of fresh, washed Raney nickel was added. Again the mixture was stirred and refluxed for 1 hr, and again 25 g of fresh nickel was added. After a third hour of stirring and reflux, the mixture was cooled and the nickel was removed by filtration. The filtrate was adjusted to pH 9.2 with sodium hydroxide and extracted exhaustively with chloroform (six 250-ml portions, then six 500-ml portions). The chloroform extracts were combined and the solvent was removed under reduced pressure. The glassy residue (2.6 g) was distributed for 300 transfers using the solvent system methyl isobutyl ketone–water–ethanol in the ratio 5:5:2. The distribution was analyzed by the determination of solids and showed a single peak, peak tube 82, $K = 0.38$, with an excellent fit to the theoretical curve. Tubes 65–100 were pooled and concentrated to an aqueous solution (10 ml) under reduced pressure. This concentrate was acidified with concentrated hydrochloric acid (ten drops) and acetone was added slowly while stirring until the solution became cloudy (140 ml of acetone). Another 60 ml of acetone was added, and scratching the flask induced crystallization. The crystals were collected and dried in a vacuum desiccator; 1.52 g. Addition of 150 ml of acetone to the mother liquor gave a second crop of crystals, 214 mg; total yield of anhydrolincomycitol hydrochloride, 1.73 g (38%). A sample for analysis was recrystallized from acetone–water, mp 206–210°. The nmr spectrum was consistent with the proposed removal of a thiomethyl glycosidic group; both the anomeric hydrogen doublet at 314 cps and the S-methyl singlet at 120 cps had disappeared. Analyses indicated the crystalline hydrochloride to be a hemihydrate. The compound showed no measurable optical rotation at the sodium D line.

Anal. Calcd for $C_{17}H_{32}N_2O_6 \cdot HCl \cdot 0.5H_2O$: C, 50.29; H, 8.43; N, 6.90; O, 25.62; Cl, 8.73; H_2O, 2.22; equiv wt, 406. Found: C, 50.35; H, 8.40; N, 6.77; O, 25.79; Cl, 8.94; H_2O, 2.43; equiv wt, 396.

Periodate Oxidation of Anhydrolincomycitol Hydrochloride. Anhydrolincomycitol hydrochloride was titrated by the Fleury–Lange procedure using 0.23 *M* sodium metaperiodate solution. The consumption of periodate was, in hours (moles): 0.5 (2.00), 1.5 (2.06), 3.5 (2.00), 19 (2.00).

Acknowledgments. The authors wish to thank Mr. Forrest MacKellar for technical assistance and Mr. Raymond Anderson and his associates for the analytical results.

(19) Reference 18, p 188.