Preparation of ζ-Carotene for Analysis.—All ζ-carotene samples except the one used for optical rotation data were prepared by the method outlined in detail in a previous publication.[3] Sample weights were estimated from the light absorption of a hexane solution at 3560 Å. After removal of the solvent under reduced pressure the sample and its container were introduced directly into the carbon–hydrogen train or into the solvents used in other chemical studies.

Molecular Weight.—Molecular weights were determined in camphor by the Rast micromethod. Melting points were observed microscopically with the microscopic hot stage described by Zscheile and White.[13] Using naphthalene, crystalline lycopene and crystalline β-carotene as known substances, the molal depression constant, k, of camphor was found to vary with concentration, a fact previously noted by Meldrum, Saxer and Jones.[14] Since at concentrations great enough to fall on the flat part of the calibration curve carotene solutions in camphor were too concentrated to permit ready observation of melting points, molalities were estimated using 536 as the molecular weight and the k-values corresponding to the estimated molalities were used in calculating the experimental molecular weights.

Analyses.—3.13 mg. of ζ-carotene in 34.8 mg. of camphor (k = 43.0); Δ = 6.65°; 3.13 mg. in 31.1 mg. of camphor (k = 41.2); Δ = 7.17; 3.76 mg. in 35.8 mg. camphor (k = 40.5); Δ = 7.81. Calcd. for $C_{40}H_{64}$: mol. wt., 544. Found: mol. wt., 582, 578, 543.

Carbon and Hydrogen.—Calcd. for $C_{40}H_{64}$: C, 88.15; H, 11.85. Found: C, 87.91, 87.27; H, 11.27, 11.17.

Hydrogenation.—9.98 mg. of substance added 4.04 ml. of hydrogen (0°, 760 mm.). 10.25 mg. added 4.04 ml. 10.98 mg. added 4.11 ml. hydrogen. Found: 9.78, 9.56 and 9.05 double bonds.

C-Methyl Groups.—C-Methyl groups were determined by the method of Kuhn and Roth[15] except that the larger number of distillations recommended by Ginger[16] was used. 13.4 mg. of substance required 17.54 ml. of 0.00865 N barium hydroxide for neutralization of the acetic acid formed. This corresponds, after subtraction of the blank, to 15.80 ml. of base. Found: 5.54 C-methyl groupings.

Isopropylidene Groups.—Isopropylidene groups were determined by the method of Kuhn and Roth[6]: 9.75 mg. of substance in 3 ml. of acetic acid, ozonized for four hours, yielded acetone equivalent to 3.12 ml. of 0.04998 N iodine solution. Expected for 2 isopropylidene groups: 2 moles acetone. Found: 1.45 moles. A control determination on β-carotene, which should yield no acetone, gave 0.22 mole; however, no iodoform precipitate was evident.

Identification of Acetone from Isopropylidene Determination.—Since methyl ketones other than acetone would yield iodoform in the isopropylidene determination, the dibenzylidene derivative of the ketone obtained on ozonolysis was prepared for identification: 55 mg. of substance was ozonized for ten hours in 6 ml. of acetic acid; 40 ml. of water, 32 ml. of 2 N sodium hydroxide and 10 ml. of 1 N potassium permanganate were added and the mixture refluxed for ten minutes; 15 ml. was then steam distilled from the solution and made alkaline with an excess of 0.5 ml. of 10% sodium hydroxide; 5 ml. of ethanol and 3 drops of benzaldehyde were added and the mixture boiled one minute. The precipitate was recrystallized from boiling ethanol: m.p. 112.2° using total immersion thermometer, m.p. of authentic sample of 1,3-dibenzylidene-acetone, 112.3°, mixed m. p. 112.3°.

Addition of 2,4-dinitrophenylhydrazine reagent to 30 ml. of additional distillate gave a very small amount of a precipitate melting above 200°.

Optical Rotation.—Since sodium ethylate might diminish optical activity, chilling to −70° in hexane solution was employed to remove the wax from a sample of ζ-carotene. The wax which precipitated did not evidence optical rotation in either hexane or carbon disulfide solution. 135 mg. of ζ-carotene in 13.8 ml. of hexane: $[\alpha]^{25}D$ 0°; in carbon disulfide: $[\alpha]^{25}D$ 0°. No test of the optical rotation of neo-isomers was attempted.

(13) Zscheile and White, *Ind. Eng. Chem., Anal. Ed.*, **12**, 436 (1940).

(14) Meldrum, Saxer and Jones, THIS JOURNAL, **65**, 2023 (1943).

(15) Kuhn and Roth, *Ber.*, **66**, 1274 (1933).

(16) Ginger, *J. Biol. Chem.*, **156**, 453 (1944).

Summary

Structural studies show that ζ-carotene possesses the typical carotenoid polyisoprenic structure and has an open chain with double bonds in the 1 and 1′ positions as does lycopene. Hydrogenation data show a total of either nine or ten double bonds and light absorption data indicate that seven of these are conjugated. The compound shows no optical activity. From these results it is deduced that the most probable structure of ζ-carotene is 5,6,7,8,5′,6′,7′,8′-octahydrolycopene ($C_{40}H_{64}$).

LAFAYETTE, INDIANA RECEIVED FEBRUARY 28, 1948

[CONTRIBUTION FROM THE DIVISIONS OF ORGANIC CHEMISTRY AND PHARMACOLOGY, THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH]

Streptomycin. VIII. Isolation of Mannosidostreptomycin (Streptomycin B)[1]

BY JOSEF FRIED AND ELWOOD TITUS

In the routine purification of streptomycin concentrates by means of flowing chromatography, essentially as described by Carter, *et al.*,[2] it was observed that the more firmly adsorbed fractions of low bioactivity[3] yielded about two to three times as much maltol per unit of activity on treatment with dilute alkali as the more readily eluted highly active fractions. The isolation from such fractions of a streptomycin-like substance designated Streptomycin B has been reported in a preliminary communication from this laboratory.[4]

The streptomycin concentrates employed in this work were obtained by growing *Streptomyces griseus* in submerged culture in a medium containing soybean meal, glucose and sodium chloride, treating the filtered broth with activated charcoal, and eluting the streptomycin with warm

(1) The nomenclature of streptomycin preparations used in this paper follows the recent suggestion of Prof. S. A. Waksman (*Science*, **107**, 233 (1948)).

(2) H. E. Carter, R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell and W. A. Strong, *J. Biol. Chem.*, **160**, 337 (1945).

(3) All bioassays reported in this paper were carried out with *K. pneumoniae* as the test organism as described by R. Donovick, D. Hamre, F. Kavanagh and G. Rake, *J. Bact.*, **50**, 623 (1945).

(4) J. Fried and E. Titus, *J. Biol. Chem.*, **168**, 391 (1947).

mineral acid. The resulting eluates were precipitated with phosphotungstic acid, and the streptomycin phosphotungstate fractionally decomposed with barium hydroxide. The material thus obtained, which as a rule assayed at about 400–500 γ/mg. and contained between 30 and 40% mannosidostreptomycin, was chromatographed over sulfuric acid-washed alumina from 80% methanol using 80% methanol and finally water as the eluting agents. The effluent fractions were freed from methanol *in vacuo* and the water was removed by lyophilization. The resulting solids were bioassayed and their capacity to form maltol[5] was measured.

The procedure used here differed somewhat from that described recently by Boxer, Jelinek and Leghorn.[6] Whereas these authors utilized the colors produced by the action of ferric ion or of the phenol reagent of Folin and Ciocalteu on maltol, we determined the latter directly by measuring the extinction coefficient of the alkali-treated streptomycin solution at 325 mμ. The ultraviolet absorption curve of such a solution (curve A, Fig. 1) actually represents the sum of two curves, namely, that given by maltol alone in alkaline solution (curve B), and that of a water-soluble substance

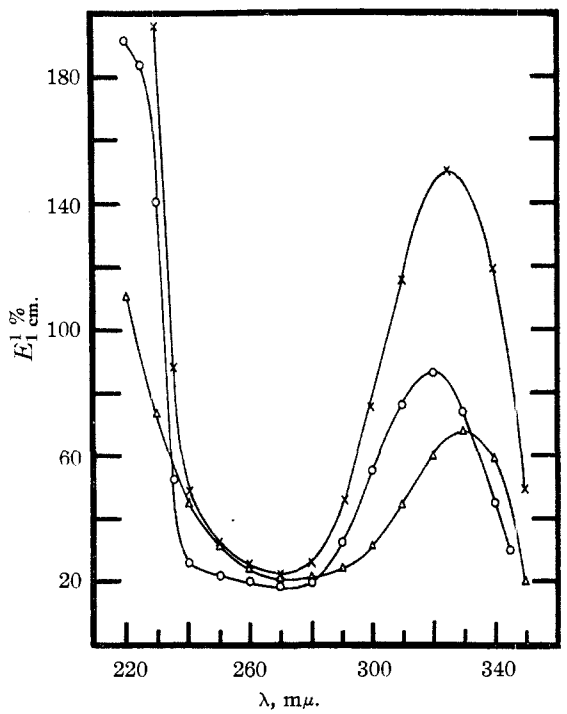

Fig. 1.—Ultraviolet absorption spectra: curve A, X-X, streptomycin heated in 0.1 *N* alkaline solution; curve B, O-O, maltol in 0.1 *N* alkaline solution; curve C, Δ-Δ, alkaline streptomycin solution after removal of maltol by extraction with chloroform.

(5) J. R. Schenck and M. A. Spielman, THIS JOURNAL, **67**, 2276 (1945).

(6) G. E. Boxer, V. C. Jelinek and P. M. Leghorn, *J. Biol Chem.*, **169**, 153 (1947).

of unknown nature (curve C) which remains in the aqueous phase after extraction of the maltol with chloroform. It has been demonstrated[7] for a large number of streptomycin samples over a wide range of potencies and concentrations that the height of the peak at 325 mμ in curve A is directly proportional to the height of the peak at 320 mμ for pure maltol alone. Therefore, to simplify matters, the maltol-forming power of a streptomycin sample has been expressed in terms of the difference in the extinction coefficients at 325 mμ of a 1% aqueous solution of that sample in a cell of one centimeter thickness before and after boiling with dilute alkali under standard conditions. Evaluated on this basis pure streptomycin trihydrochloride showed a "$\Delta E^{1\%}_{1\ cm.}$" of 153.

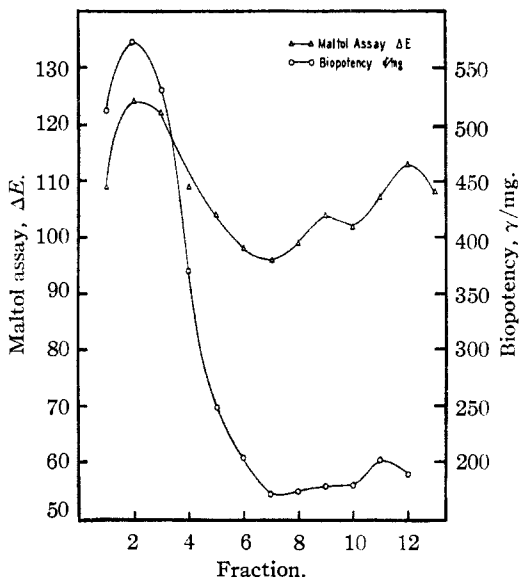

Fig. 2.—Chromatography of the streptomycin complex: O-O, biopotency; Δ-Δ, maltol assay.

When the bioactivities and ΔE-values for the above chromatographic fractions were plotted as ordinates against the fraction numbers in the order of their emergence from the column, the resulting curves (Fig. 2) showed the following characteristics: The biopotency curve reaches a maximum at the second fraction, drops to a low but rather constant value of about 175 γ/mg. and forms a second maximum of low intensity at Fraction 11. The triangle-marked curve, which illustrates the maltol-forming power of the same series of fractions, shows two distinct maxima of almost equal intensity at Fractions 2 and 12. An essentially identical curve resulted when the optical densities of the colored solutions obtained in the Sakaguchi reaction for guanido groups were plotted in the same fashion. It was clear from these results that the alumina column had effected separation of the concentrate into two entities each of which responded with about equal intensity to

(7) N. H. Coy, J. Fried and R. Donovick, unpublished data.

the characteristic chemical tests for streptomycin but whose bioactivities differed considerably. In order to demonstrate further that we were dealing with two separate chemical entities, Fractions 2 and 12 were subjected to a "Craig countercurrent distribution" procedure using 5% aqueous *p*-toluenesulfonic acid and *n*-butanol as the two liquid phases.[8] Examination of the 24 plate distribution curves showed that the major component of Fraction 2 was a substance having a distribution coefficient of one, whereas in Fraction 12 there predominated a more water-soluble substance having a distribution coefficient of 0.43. Since the fractions of high bioactivity and of a distribution coefficient of unity could readily be purified *via* the helianthate[9] or the reineckate sulfate[10] to give pure streptomycin, $C_{21}H_{39}O_{12}N_7$, the more water-soluble material present in Fractions 7 to 13 must constitute the new entity mannosidostreptomycin. For the further purification of mannosidostreptomycin, fractions of low biopotency (less than 250 γ/mg.) and a high maltol-forming capacity (ΔE greater than 90) were combined, freed from sulfate ion and precipitated with a solution of reinecke salt. Fractional crystallization of this precipitate yielded the trireineckate of mannosidostreptomycin entirely free from streptomycin[11] as large thin plates containing water of crystallization. Decomposition of this salt with silver sulfate and barium chloride yielded the trihydrochloride as a white amorphous powder. The latter was found to dissolve less readily in anhydrous methanol than the trihydrochloride of streptomycin and failed to form a crystalline or amorphous double salt with calcium chloride. In spite of the failure of pure mannosidostreptomycin to form a difficultly soluble calcium chloride double salt, the former was precipitated together with streptomycin, when a mixture of both streptomycins was treated with calcium chloride in methanol. This renders extremely difficult the preparation from such mixtures of pure streptomycin *via* the calcium chloride double salt. Conversely, it follows that the crystalline state of this salt *per se* is not a sufficient criterion of chemical purity.

Potentiometric titration of the trihydrochloride showed a midpoint at *p*H 7.6 from which an equivalent weight of 820 was computed. Analytical data on the trireineckate and the trihydrochloride were in agreement with a formula $C_{27}H_{49}O_{17}N_7$ for the free mannosidostreptomycin base. This formula also receives support from degradation studies, which have shown mannosidostreptomycin to be a D-mannoside of streptomycin.[12] Mannosidostreptomycin trihydrochloride has a ΔE of 126 in the maltol test indicating that on a molar basis the two streptomycins form approximately the same amount of maltol. Its antibiotic activity has been determined in yeast beef broth at *p*H 7.[13] Whereas 3.7 times more mannosidostreptomycin than streptomycin was required to inhibit the growth of *K. pneumoniae*, the two streptomycins were approximately equally active toward *E. typhosa*.

On catalytic hydrogenation mannosidostreptomycin trihydrochloride forms the dihydro derivative which possesses an antibiotic potency against *K. pneumoniae* equal to that of mannosidostreptomycin. As expected dihydromannosidostreptomycin fails to form maltol on heating with alkali and does not react with carbonyl reagents. In these and many other of its characteristics it closely resembles dihydrostreptomycin.

Experimental

Chromatographic Separation of Mannosidostreptomycin from Streptomycin.—Ten grams of streptomycin complex (400 γ/mg.) was dissolved in 100 ml. of 80% methanol and percolated at a flow rate of between 80 and 100 drops per minute through a column of 3.2 cm. diameter containing 450 g. of alumina, which had previously been washed with dilute sulfuric acid to a *p*H of 4.5 and reactivated at 150°. The column was washed with 80% methanol and the effluent solution was tested by means of the Sakaguchi reaction. When 490 ml. of solvent had passed through the column the Sakaguchi test became positive and the effluent solution was collected in five fractions totalling 1080 ml. Water was then substituted for 80% methanol as the eluting agent and two more fractions were collected. The streptomycin was recovered from the eluates by distilling off the methanol *in vacuo*, filtering the resulting aqueous solutions and lyophilizing them. Table I summarizes the data obtained in a typical experiment.

TABLE I

SEPARATION OF MANNOSIDOSTREPTOMYCIN FROM STREPTOMYCIN BY CHROMATOGRAPHY ON ALUMINA

Eluates[a] ml.	g.	Biopotency, γ/mg.	Maltol assay ΔE 1% 1 cm.	Weight ratio[b] Mannosidostreptomycin to Streptomycin
240	4.88	640	131	0.27
160	1.23	354	119	2.1
80	0.37	309	107	3.5
80	.26	246	108	5.3
520	.83	334	108	7.3
330	.59	286	116	6.2
130	.54	305	121	4.3

[a] The first five eluates are 80% methanol; the remaining two are water. [b] The figures in this column are based on countercurrent distribution data using the system mentioned in Ref. 11.

The maltol assays[14] were carried out in the following manner: Between 2 and 3 mg. of the sample was weighed into

(8) E. Titus and J. Fried, *J. Biol. Chem.*, **168**, 393 (1947); **174**, 57 (1948).

(9) F. A. Kuehl, Jr., R. L. Peck, C. E. Hoffhine, Jr., R. P. Graber and K. Folkers, THIS JOURNAL, **68**, 1460 (1946).

(10) J. Fried and O. Wintersteiner, *Science*, **104**, 273 (1946).

(11) The quantitative estimation of the two streptomycins in a mixture has been carried out by countercurrent distribution using a system (Plaut and McCormack, to be published) in which the distribution coefficient of streptomycin is seven times greater than that of mannosidostreptomycin. With this system it has been possible to detect the presence of as little as 1–2% of streptomycin in preparations of mannosidostreptomycin.

(12) J. Fried and H. E. Stavely, THIS JOURNAL, **69**, 1549 (1947).

(13) G. Rake, C. M. McKee, F. E. Pansy and R. Donovick, *Proc. Soc. Exp. Biol. Med.*, **65**, 107 (1947).

(14) The details of this procedure were worked out by Dr. N. H. Coy of the Squibb Biological Laboratories and by Mr. F. Russo-Alesi of the Division of Organic Chemistry.

a 6″ test-tube and 5 ml. of 0.1 *N* sodium hydroxide was added. The solution was immediately transferred to a 1 cm. Corex cell and its optical density at 325 mμ measured against a water blank in a Beckman quartz spectrophotometer. The solution was then heated in a boiling water bath for ten minutes. To prevent losses of water by evaporation a small glass funnel was placed into the test-tube and air was gently blown across the top half of the tube. At the end of the heating period the tube was placed into an ice-bath and the cooled solution was diluted with water to one-tenth the original concentration. The optical density was read again at 325 mμ and the $\Delta E^{1\%}_{1\,cm.}$ computed according to the formula

$$\Delta E^{1\%}_{1\,cm.} = \frac{D_{\text{after heating}}}{\text{concn.}} - \frac{D_{\text{before heating.}}}{\text{concn.}}$$

Mannosidostreptomycin Trireineckate.—Nineteen and three-tenths grams of combined chromatographic fractions containing 80% of mannosidostreptomycin or more was dissolved in 200 ml. of water and the sulfate ion which had been eluted from the sulfuric acid washed alumina was replaced by chloride ion by means of a 2% barium chloride solution. The barium sulfate precipitate was removed by filtration over a bed of Darco G-60, and the filtrate was diluted with water to a total volume of 380 ml. To this solution was added at 40° a solution of 25.1 g. of reinecke salt in 600 ml. of water and the mixture was allowed to cool very slowly. Upon standing at room temperature for fifteen hours, a copious crystalline precipitate formed, which was redissolved by warming to 40° leaving a small residue of amorphous and sandy crystalline material. This was removed by filtration, and the filtrate was allowed to crystallize slowly at room temperature. Recrystallization of the resulting material from 770 ml. of warm water yielded 6.9 g. of pure mannosidostreptomycin trireineckate, which contained less than 1.5% of streptomycin. Allowing the original and the second mother liquors to cool in the refrigerator afforded two crops of crystals which were combined and recrystallized from water yielding 6.5 g. of material containing about 8% of streptomycin. This was recrystallized from 535 ml. of water together with a fraction (2.9 g.) obtained by concentrating and chilling in the refrigerator the mother liquors from the above 6.9 g. of pure trireineckate. The resulting crop of crystals amounted to 3.4 g., which raises the total yield of pure product to 10.3 g.

Upon drying *in vacuo* at 80° the crystals lost 8.10% of water, which was rapidly regained when the material was exposed to laboratory air. The dried trireineckate melted at 178–179° (dec. cor.).

Anal. Calcd. for $C_{27}H_{49}O_{17}N_7 \cdot 3HCl\ [(NH_3)_2(SCN)_4] \cdot 2H_2O$: C, 26.97; H, 4.29; N, 20.15; S, 22.11; Cl, 8.98. Found: C, 26.89; H, 4.24; N, 20.1; S, 22.2; Cl, 8.70.

Conversion of Mannosidostreptomycin Trireineckate into the Trihydrochloride.—To a warm solution of 3.4 g. of the trireineckate in 190 ml. of water was added 154 ml. of a 0.6% aqueous solution of silver sulfate. The precipitated silver reineckate was removed by filtration and to the filtrate was added a 2% aqueous solution of barium chloride until a sample failed to give a precipitate with either barium chloride or dilute sulfuric acid. The mixture was filtered over a bed of Darco G-60 and the filtrate was lyophilized. One and five-tenths grams of a white amorphous powder was obtained, which after drying *in vacuo* at 100° melted with decomposition at 190–200°. The melting point of this salt is of little significance since it varied somewhat for different preparations: $[\alpha]^{25}D$ −47 (c = 1.35 in water). The substance was analyzed after drying at 140° for two hours.

Anal. Calcd. for $C_{27}H_{49}O_{17}N_7 \cdot 3HCl \cdot H_2O$: C, 37.26; H, 6.24; N, 11.25; Cl, 12.23. Found: C, 36.85; H, 6.11; N, 11.3; Cl, 12.83; ash, 0.5.

A solution of 58.8 mg. of the trihydrochloride in 2 ml. of water was titrated with 0.1 *N* sodium hydroxide. The slope of the titration curve showed a minimum at 0.36 ml. of sodium hydroxide and *p*H 7.60 (equiv. wt., 820. Calcd. for $C_{27}H_{49}O_{17}N_7 \cdot 3HCl \cdot H_2O$: 871).

Isolation of Maltol from Mannosidostreptomycin.—A solution of 320 mg. of mannosidostreptomycin trihydrochloride in 50 ml. of 0.1 *N* sodium hydroxide was heated on the steam-bath for fifteen minutes. The cooled solution was acidified with 1 *N* hydrochloric acid and extracted three times with 50 ml. of chloroform. The chloroform solutions were dried over sodium sulfate and evaporated to dryness. The residue after two sublimations yielded 17 mg. of crystals which melted at 160–161° (cor.) and gave no depression in melting point with an authentic sample of maltol.

Anal. Calcd. for $C_6H_6O_3$: C, 57.14; H, 4.80. Found: C, 57.14; H, 4.91.

Hydrogenation of Mannosidostreptomycin.—A solution of 1.038 g. of mannosidostreptomycin trihydrochloride in 20 ml. of water was shaken in an atmosphere of hydrogen in the presence of 150 mg. of platinum oxide. At the end of eighteen hours 37 ml. of hydrogen had been taken up; calculated for 1 mole, 30 ml. The catalyst was removed by filtration and the filtrate was lyophilized. 940 mg. of an amorphous powder was obtained which gave $\Delta E^{1\%}_{1\,cm.}$ = 0.73 in the maltol test. After drying at 100° for two hours the substance melted with decomposition at 194–199° (cor.) and had $[\alpha]^{25}D$ −55° (c = 0.90 in water). For analysis a sample was dried to constant weight at 140°.

Anal. Calcd. for $C_{27}H_{51}O_{17}N_7 \cdot 3HCl \cdot H_2O$: C, 37.14; H, 6.47; N, 11.23; Cl, 12.19. Found: C, 37.16; H, 6.36; N, 10.9; Cl, 12.9; ash, negligible.

Acknowledgment.—The authors wish to express their appreciation to Dr. James A. Shannon and to Dr. Oskar Wintersteiner for their stimulating interest in this work. They also wish to thank Mr. Edward A. Paredes, Mr. Joseph Feltzin and Miss Doris E. Walz for their able technical assistance, Mr. Frank Russo-Alesi for the maltol determinations and Mr. Joseph F. Alicino and his associates for the microanalytcial determinations.

Summary

Streptomycin concentrates have been shown to contain in addition to streptomycin a second antibiotic, which has been termed streptomycin B or mannosidostreptomycin. This new entity has been separated from streptomycin by chromatography on alumina and isolated in pure form as the crystalline reineckate. Like streptomycin it forms maltol on treatment with alkali, gives positive qualitative tests for guanido groups and forms a dihydro-derivative on catalytic hydrogenation. Analytical data are in agreement with the formula $C_{27}H_{49}O_{17}N_7$ for the mannosidostreptomycin base.

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