Summary

6-Methyl-3-keto-2,3-dihydrothionaphthene has been prepared by several methods, and has been reduced to 6-methylthionaphthene, which has been oxidized in very low yield to 6-methyl-4,7-thionaphthenequinone. A by-product in the oxidation has been shown to be probably the 2,3-dihydro-6-methylthionaphthene sulfone. The action of chloroacetyl chloride and aluminum chloride on methyl *m*-tolyl sulfide leads to 3-keto-2,3-dihydro-6-methylthionaphthene and to a chloroacetyl compound, which has been proved to be 2-methyl-4-methylmercapto-ω-chloroacetophenone.

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Streptomyces Antibiotics. VIII. Isolation of Streptomycin

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Some chemical and biological properties of streptomycin helianthate, hydrochloride, sulfate and *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonate have been described.[1] The preliminary steps in the purification of the streptomycin concentrates and the preparation of these salts of streptomycin are described herein.

The first concentrates of streptomycin,[2] which were prepared for use in microbiological experiments, were obtained from the culture broths of *Streptomyces griseus* by the following steps: adsorption on Norite-A, elution with dilute acid, neutralization and concentration *in vacuo* to a residue.

The crystalline reineckate of streptomycin, which has been described,[3] was obtained by a sequence of steps similar to that used for streptothricin reineckate.[3] These steps were: charcoal adsorption, elution with mineral acid, precipitation with phosphotungstic acid, conversion of regenerated bases to crude picrate, chromatography of picrate and finally preparation of the reineckate.

Another method for the purification and isolation of streptomycin has been described.[4] The steps were as follows: clarification of broth with charcoal, adsorption on charcoal, elution with methanolic hydrogen chloride, and chromatography with aluminum oxide. A three-step charcoal adsorption process for the purification of streptomycin also has been used.[5]

Our sequence of steps for the isolation of streptomycin, which differs in several respects from those described above, is as follows: charcoal adsorption, elution with methanolic formic acid, precipitation with picric acid, conversion to the hydrochloride, chromatography, and finally conversion to the helianthate. The steps consisting of charcoal adsorption, elution with methanolic formic acid, precipitation with picric acid, and direct conversion to the hydrochloride were carried out essentially as described for streptothricin.[6] The concentrates of streptomycin hydrochloride showed an average activity of about 100–200 units/mg. The chromatographic step was carried out either with columns of aluminum oxide or Darco G-60. By these chromatographic procedures, fractions showing activities up to about 750 units/mg. were obtained.

Treatment of samples of streptomycin hydrochloride, which showed an activity of about 400 units/mg., or higher, with the sodium salt of helianthine (methyl orange) or of *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonic acid (orange II), yielded the corresponding crystalline salts of streptomycin. The helianthate was utilized for preparative purposes After recrystallization the helianthate was converted into the hydrochloride or other salts such as the hydrobromide or sulfate. The sulfate was obtained crystalline, but it was difficult to reproduce and it was frequently contaminated with streptidine sulfate.[7] The helianthate also was converted directly into the crystalline streptomycin trihydrochloride–calcium chloride double salt.[8] The desirable precautions in the conversion of the helianthate into other salts have already been noted.[7]

Potentiometric titrations were carried out on three salts of streptomycin and the data obtained were as follows: helianthate, eq. wt. 1600, $pK'a$ 7.50; calcd. for $C_{21}H_{37}N_7O_{12}\cdot 3C_{14}H_{15}N_3O_3S$, 1495.6; hydrochloride, eq. wt. 740, $pK'a''$ 7.66; calcd. for $C_{21}H_{37}N_7O_{12}\cdot 3$ HCl, 689; hydrobromide, eq. wt. 869; calcd. for $C_{21}H_{37}N_7O_{12}\cdot 3$ HBr, 823. As already stated,[8] a cryoscopic molecular weight determination on streptomycin hydrochloride in water gave a value of about 800 for the free base (calcd., 580). A colorimetric determination of the helianthine in streptomycin helianthate gave a value of 205 for the combining weight (calcd. combining weight, 193). The results of these determinations and the microanalytical

(1) Kuehl, Peck, Walti and Folkers, *Science*, **102**, 34 (1945).

(2) Schatz, Bugie and Waksman, *Proc. Soc. Exp. Biol. Med.*, **55**, 66 (1944).

(3) Fried and Wintersteiner, *Science*, **101**, 613 (1945).

(4) Carter, Clark, Dickman, Loo, Skell and Strong, *J. Biol. Chem.*, **160**, 337 (1945).

(5) Le Page and Campbell, *ibid.*, **162**, 163 (1946).

(6) Peck, Walti, Graber, Flynn, Hoffhine, Allfrey and Folkers, THIS JOURNAL, **68**, 772 (1946).

(7) Peck, Graber, Walti, Peel, Hoffhine and Folkers, *ibid.*, **68**, 29 (1946).

(8) Peck, Brink, Kuehl, Flynn, Walti and Folkers, *ibid.*, **67**, 1866 (1945).

data are in agreement with the molecular formula $C_{21}H_{37-39}N_7O_{12}\cdot 3HX$ for these streptomycin salts. Only one of the three salt groups of streptomycin can be titrated with sodium hydroxide solution.

Experimental

We are indebted to Dr. H. B. Woodruff and Mr. D. Hendlin of the Microbiological Department for the assays. *B. subtilis* was employed as a test organism in a cup assay method which they developed and which was similar to the paper-disc plate method.[9]

Preparation of Crude Concentrates of Streptomycin Hydrochloride from Culture Broths.—The streptomycin was adsorbed on charcoal from the culture broths of *Streptomyces griseus* and eluted from the adsorbate with methyl alcohol–water solutions containing formic acid. The eluate was concentrated into a crude amorphous streptomycin formate. The streptomycin was purified further by precipitation with picric acid followed by conversion to the hydrochloride. The details of the manipulations of these steps are essentially the same as those described for streptothricin.[6] The preparations of streptomycin hydrochloride obtained by these procedures showed 100–200 units/mg.

TABLE I

CHROMATOGRAPHIC PURIFICATION OF STREPTOMYCIN HYDROCHLORIDE WITH ALUMINUM OXIDE

Adsorbate		Adsorbent			Products		
g.	Activity units/mg.	g.	Type	Eluates ml.	g.	Activity units/mg.	% Activity[a] recovered
45	72	800	Alumina	200	2.42	372	28
				600	9.67	154	46
				2000	8.67	113	30
							104
30.9	110	600	Alumina	500	4.122	380	46
				500	2.687	150	12
				1500	3.145	160	15
							73
20	150	400	Alumina	100	1.040	370	12
				500	4.795	330	53
				500	1.851	220	13
				1000	1.651	240	13
							91
11.4	170	245	4:1	50	0.434	690	16
			Alumina	100	1.275	400	26
			supercel	100	0.847	210	9
				200	0.987	180	10
				630	1.333	200	14
							74
16.6	200	333	4:1	50	0.237	0	...
			Alumina	100	3.678	410	45
			supercel	100	1.711	190	10
				1000	2.786	160	13
							88
6.0	225	160	Alumina	250	0.698	500	28
				150	1.031	490	34
				250	0.729	290	14
				350	0.4132	160	4
							75
40	354	400	Alumina	100	10.6	720	54
				100	7.2	538	27
				200	6.0	433	18
							99
1.0	500	30	Alumina	15	0.147	640	19
				15	0.147	700	21
				50	0.146	620	19
				100	0.141	540	15
				35[b]	0.317	420	27
							101

[a] The accuracy of the assay method was estimated to be ±15%. [b] Water.

(9) Loo, Shell, Thornberry, Ehrlich, McGuire, Savage and Sylvester, *J. Bact.*, **50**, 701–714 (1945).

Chromatographic Purification of Streptomycin with Aluminum Oxide.—Columns of acid-washed aluminum oxide were prepared and used for the chromatographic purification of streptomycin in the same manner as has been described for streptothricin.[6] The data obtained on representative concentrates of streptomycin hydrochloride are summarized in Table I.

Aluminum oxide was also suspended in methanol solutions of crude streptomycin hydrochloride for the removal of gross impurities. From a given sample of concentrate, the fractions obtained by chromatography were of higher activity than those obtained by treatment with a suspension of aluminum oxide.

Chromatographic Purification of Streptomycin with Darco G-60.—The columns of Darco G-60 and filter paper mixture were used for the purification of streptomycin according to the procedure which has been described for streptothricin.[6] A summary of typical results is given in Table II.

TABLE II

CHROMATOGRAPHIC PURIFICATION OF STREPTOMYCIN HYDROCHLORIDE WITH DARCO G-60

Adsorbate		Adsorbent			Products		
G.	Units/mg.	Darco G-60 g.	Filter paper pulp, g.	Eluates ml.	g.	Activity units/mg.	Activity recovered, %
1.90	250	55	15	25	0.232	350	17
				25	.419	320	28
				150	.730	220	34
2.04	250	144	16	25	.100	700	14
				27	.148	540	15
				25	.111	360	8
				50	.130	270	7
				75	.115	250	6
2.75	350	145	15	25	.202	780	16
				25	.355	600	21
				50	.820	400	33
				50	.367	220	8
				250	.208	210	4
3.20	350	136	14	40	.041	880	3
				25	.332	870	26
				25	.503	530	24
				25	.502	410	18
				500	1.150	375	38
2.7	300	120	20	25	0.046	650	4
				25	.092	540	6
				50	.369	540	25
				50	.584	500	36
				500	1.090	300	40

Streptomycin Helianthate.—A 26.3-g. sample of streptomycin hydrochloride (500 units/mg.) was dissolved in 390 ml. of methanol and the solution was heated to approximately 55°. A solution containing 20.8 g. of methyl orange in 1456 ml. of water was heated to 75° and added to the methanol solution. After standing overnight at 10°, the mixture was centrifuged and the crystalline product

was washed with approximately 200 ml. of water. Recrystallization of the helianthate was accomplished by dissolving it in 3 liters of hot 33% aqueous methanol and allowing the solution to stand overnight at 10°. The helianthate was then removed by centrifuging and washed with 200 ml. of water and with 200 ml. each of isopropanol, acetone and ether. The weight of the dried product was 32 g. (87% yield).

Anal. Calcd. for $C_{21}H_{37}N_7O_{12}(C_{14}H_{16}N_3O_3S)_3$: C, 50.59; H, 5.53; N, 14.99; S, 6.43. Calcd. for $C_{21}H_{39}N_7O_{12}$-$(C_{14}H_{16}N_3O_3S)_3$: C, 50.52; H, 5.65; N, 14.97; S, 6.42. Found: C, 50.38; H, 5.86; N, 15.02; S, 5.76.

In the melting point determination on the micro-block, the helianthate decomposed at 220–226°. The crystals were dark red in color, but under the microscope they appeared as flat yellow plates. Upon drying, the crystals lost solvent of crystallization and changed into an amorphous copper-like powder.

Anal. Found: C, 50.53; H, 5.83; N, 14.34.

After one more recrystallization, the following data were obtained.

Anal. Found: C, 50.45; H, 5.83; N, 14.81.

After three more recrystallizations, the following data were obtained.

Anal. Found: C, 50.64; H, 5.90; N, 14.13.

Conversion of Streptomycin Helianthate to Streptomycin Hydrochloride.—A 29-g. sample of streptomycin helianthate was added to 465 ml. of concentrated hydrochloric acid–methanol solution (ratio 1:26 by volume). After the mixture was thoroughly stirred, the suspension was filtered through a thin layer of Darco G-60 and the filter cake was washed with 85 ml. of methanol. The filtrate and washings were poured into 4500 ml. of acetone and the mixture was centrifuged. The precipitated streptomycin hydrochloride was washed with acetone and dried; yield 13 g. (97%), activity about 785 units/mg., $[\alpha]_D$ −86.7° (*c*, 1% in water). A sample was dried at 100° *in vacuo* to constant weight before analytical determinations were made.

Anal. Calcd. for $C_{21}H_{37}N_7O_{12}\cdot 3HCl$: C, 36.61; H, 5.85; N, 14.23; Cl, 15.44. Calcd. for $C_{21}H_{39}N_7O_{12}\cdot 3HCl$: C, 36.50; H, 6.13; N, 14.19; Cl, 15.39. Found: C, 36.80; H, 6.09; N, 14.39; Cl, 15.59.

Conversion of Streptomycin Helianthate to Streptomycin Hydrobromide.—When a 219-mg. sample of streptomycin helianthate was treated with methanol–hydrobromic acid in the manner described for the hydrochloride, streptomycin hydrobromide was obtained as an amorphous white powder, yield 98 mg., activity about 680 units/mg. The product was difficult to purify.

Anal. Calcd. for $C_{21}H_{37}N_7O_{12}\cdot 3HBr$: C, 30.67; H, 4.91; N, 11.92; Br, 29.1; eq. wt., 822. Calcd. for $C_{21}H_{39}$-$N_7O_{12}\cdot 3HBr$: C, 30.60; H, 5.13; N, 11.88; Br, 29.1. Found: C, 31.25; H, 5.58; N, 11.61; Br, 28.0; eq. wt., 869 (potentiometric titration).

Streptomycin *p*-(2-Hydroxy-1-naphthylazo)-benzenesulfonate.—Five grams of streptomycin hydrochloride, activity about 700 units/mg., $[\alpha]_D$ −86.5° (*c*, 0.9% in water), was dissolved in 75 ml. of methanol and a solution of 5 g. of Orange II dissolved in 280 ml. of hot water was added. On cooling, the bulky crystalline precipitate was collected by centrifugation and recrystallized twice from hot aqueous methanol. The crystals were then collected by filtration and washed with water, isopropanol, acetone and ether, and dried at 25° *in vacuo* over phosphorus pentoxide. The yield was 6.3 g., activity about 300 units/mg.

Anal. Calcd. for $C_{21}H_{37}N_7O_{12}(C_{16}H_{12}N_2O_4S)_3\cdot 5H_2O$: C, 50.09; H, 5.06; N, 11.01; H_2O, 5.4. Calcd. for $C_{21}H_{39}$-$N_7O_{12}(C_{16}H_{12}N_2O_4S)_3\cdot 5H_2O$: C, 50.02; H, 5.17; N, 10.99; H_2O, 5.4. Found: C, 50.01; H, 5.34; N, 11.03; weight loss on drying to constant weight at 100° *in vacuo*, 5.3.

Conversion of Streptomycin Helianthate to Streptomycin Sulfate.—A sample of streptomycin helianthate weighing 32.6 g. was suspended in 150 ml. of water by stirring mechanically. Thirty milliliters of 2 *N* sulfuric acid was added slowly and the stirring was continued for forty-five minutes. At the end of this period, the helianthine was in crystalline form and was removed by filtration through a layer of Darco G-60. The colorless filtrate was lyophilized, yielding a voluminous fluffy white powder. After drying for two hours at 100° *in vacuo*, the product weighed 12.2 g. It showed $[\alpha]_D$ −79.5° (*c*, 1% in water) and had an activity of about 700 units/mg. This preparation contained 0.99% of ash and the values for carbon, hydrogen and nitrogen given below are corrected to an ash-free basis.

Anal. Calcd. for $(C_{21}H_{37}N_7O_{12})_2\cdot 3H_2SO_4$: C, 34.75; H, 5.54; N, 13.47; SO_4, 19.80. Calcd. for $(C_{21}H_{39}N_7O_{12})_2\cdot$ $3H_2SO_4$: C, 34.61; H, 5.80; N, 13.45; SO_4, 19.76. Found: C, 34.45; H, 6.16; N, 13.45; SO_4, 20.2.

Conversion of Streptomycin Sulfate to the Free Base.—A sample of amorphous streptomycin sulfate weighing 126 mg. was dissolved in about 5 ml. of water and the solution was treated with the calculated amount of barium hydroxide solution. After removal of barium sulfate, the filtrate was concentrated to a residue. After washing with acetone and drying, streptomycin base was obtained as an amorphous light yellow powder; 520 units/mg.

Anal. Calcd. for $C_{21}H_{37}N_7O_{12}$: C, 43.49; H, 6.45; N, 16.91; calcd. for $C_{21}H_{39}N_7O_{12}$: C, 43.37; H, 6.76; N, 16.85. Found: C, 43.03; H, 6.57; N, 15.87.

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Summary

Streptomycin has been isolated from the culture broths of *Streptomyces griseus* by the following sequence of steps: charcoal adsorption, elution with methanolic formic acid, precipitation with picric acid, conversion to the hydrochloride, chromatography with aluminum oxide or Darco G-60, and finally conversion to the crystalline helianthate.

The results of various determinations and analyses are in agreement with the formula $C_{21}H_{37-39}N_7O_{12}$ for streptomycin and show that it has three basic functional groups.

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