UDC 615.779.931:576.852.1

62. Sueo Tatsuoka, Tsunaharu Kusaka, Akira Miyake, Michitaka Inoue, Hiromu Hitomi, Yutaka Shiraishi, Hidesuke Iwasaki, and Masahiko Imanishi: Studies on Antibiotics. XVI. Isolation and Identification of Dihydrostreptomycin produced by a New Streptomyces, *Streptomyces humidus nov. sp.*

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.**)

The liquid culture of *Streptomyces humidus*, which was isolated by Nakazawa and Shibata,** produced a basic antibiotic substance having growth inhibitory activity against gram-positive, gram-negative, and acid-fast bacteria. This substance was provisionally named Antibiotic No. 23572.

Antibiotic No. 23572, in crude state, is positive to Sakaguchi reaction but negative to Maltol reaction. It shows an antibacterial spectrum similar to those of streptomycin and dihydrostreptomycin, and moreover has no activity against streptomycin-resistant bacteria.

These data suggest that Antibiotic No. 23572 belongs to the so-called streptomycin group but differs from streptomycin, mannosidostreptomycin, and hydroxystreptomycin which are produced directly by streptomycetes.

When purification of Antibiotic No. 23572 was continued, it was obtained in pure state in the form of its sulfate. Comparison of physicochemical and microbiological properties of this sulfate with those of the sulfates of streptomycin-group antibiotics revealed that Antibiotic No. 23572 is identical with dihydrostreptomycin. This fact was also confirmed by the examination of its degradation products.

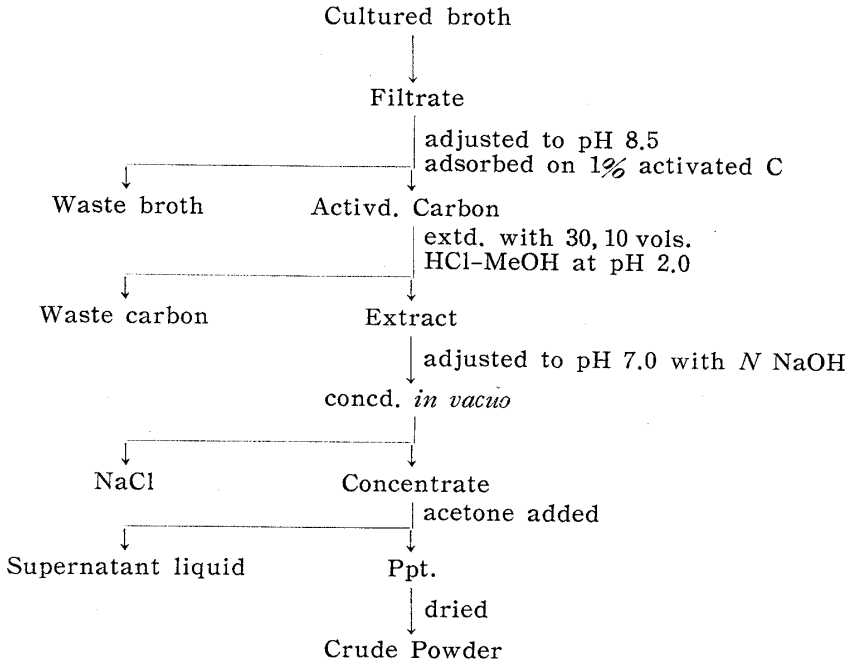

Chart 1. Purification Process with Activated Carbon

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** Isolation and taxonomical consideration of this new streptomyces and the preliminary studies on the production of Antibiotic No. 23572 by the microörganism on various media will be reported shortly in J. Agr. Chem. Soc. Japan.

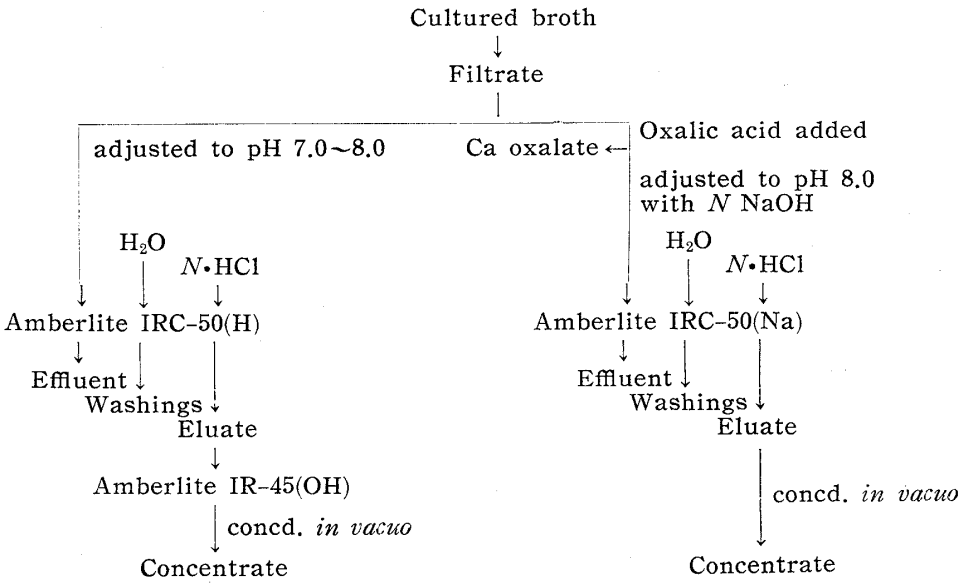

Chart 2. Purification Process with Ion Exchanger

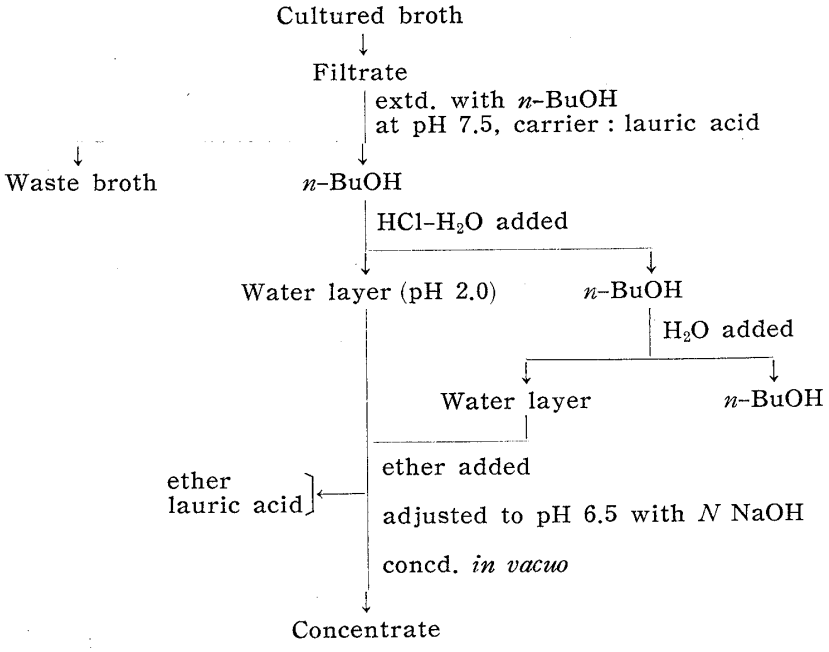

Chart 3. Purification Process with Organic Solvent

Extraction of Antibiotic No. 23572 from Culture Broth—One of the compositions of the culture medium was as follows : Glucose 2.0%, meat extract 0.6%, peptone 1.0%, NaCl 0.6%, and $CaCO_3$ 0.6%. *St. humidus nov. sp.* was used as the antibiotic-producing microörganism and the cultivation was conducted under aerobic submerged conditions. The cultivation was preferably conducted at a temperature of about 24~30° over a period of 3~8 days. The separation of the active principle from the filtered broth could be effected by any of the three methods, namely, adsorption by suitable adsorbents and elution, adsorption by suitable ion exchangers and elution, and extraction by suitable water-immiscible organic solvents. Some examples are shown in Charts 1, 2, and 3.

Isolation of crystalline Sulfate of Antibiotic No. 23572—For further purification of Antibiotic No. 23572, chromatography on activated carbon is recommendable. The concentrate of the crude hydrochloride of the antibiotic, obtained by the above methods, was suitable as the starting material. The size of the activated carbon was specially made uniform within the range of 150~180 mesh. Distilled water was suitable as eluant. The details of this procedure are shown in Chart 4.

Using this purification procedure, calcium and ferric ions as well as colored impurities which coexisted originally or intermixed in the course of the previous procedures were removed.

All fractions of the eluate containing the active principle were combined and concentrated

almost to dryness. Dehydration by acetone gave the hydrochloride in amorphous form. This amorphous product was crystallized by the ordinary method used in the crystallization of dihydrostreptomycin.

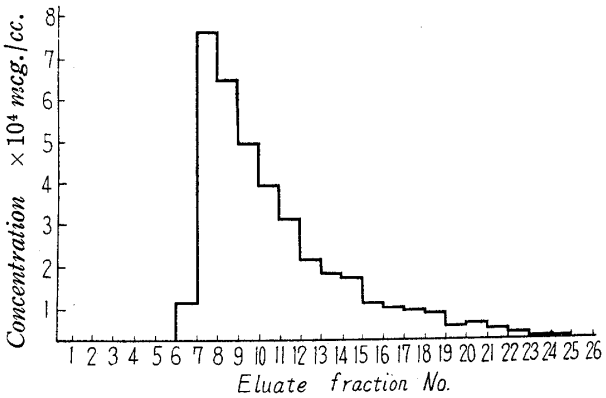

Chart 4. Fractional Distribution in Chromatography

Activd. Carbon	: 150~200 mesh 40 g. (150 cc.)
Column	: Diameter, 4 cm.
Concentrate applied	: 25 cc., 273,000 mcg./cc.
Velocity	: 1~2 cc./min.
Eluant	: Distilled water
Fractionation	: 20 cc. each
Yield	: 7.5 g. (potency)

TABLE I. Comparison of Antibiotic No. 23572 with Dihydrostreptomycin

	Antibiotic No. 23572	Dihydrostreptomycin
Sulfate, m.p.(°C)	250~255(decomp.)	255~265[a] 250[b]
Analysis	C 34.45, 34.11 H 6.42, 6.54 N 12.98 S 6.43	$C_{21}H_{41}N_7O_{12}\cdot{}^3/_2\,H_2SO_4$[c]
Specific rotation	$[\alpha]_D^{18}$ −87.5°(c=1.0, H_2O)	$[\alpha]_D^{25}$ −88°, −88.5°[a,b]
Color reactions	Maltol — Sakaguchi +	Maltol —[d] Sakaguchi +[e]
Acute toxicity (mg./kg.)	250 (i.v.) 2,000 (s.c.) 1,900 (i.p.)	250 (i.v.) 2,000 (s.c.) 1,900 (i.p.)
Indices of refraction	α 1.545±0.002 β 1.556±0.005 γ 1.564±0.002	α 1.552±0.002[b] β 1.558±0.004 γ 1.566±0.002
Alkaline stability	Indistinguishable[c]	
Stability in the presence of carbonyl reagents and cysteine	// [c]	
Paper chromatographic Rf	// [f]	
Helianthate		
m.p.(°C)	222~225(decomp.)	
Analyses	C 50.19, H 5.93 N 14.93, S 6.80	Calcd. C 50.46, H 5.79[g] N 14.94, S 6.40
Reineckate		
m.p.(°C)	203~205(decomp.)[h]	
Analyses	C 25.42, H 4.40 S 24.64	Calcd. C 25.72, H 4.06 S 24.96

a) I. A. Solomons, P. P. Regna : Science, **109**, 515(1949).
b) F. J. Wolf, E. T. Elmendorf, R. G. Denkewalter, M. Tishler : *Ibid.*, **109**, 515(1949).
c) Q. R. Bartz, J. Controulis, H. M. Crooks, Jr., M. C. Rebstock : J. Am. Chem. Soc., **68**, 2163(1946).
d) D. C. Grove, W. A. Randall : "Assay Methods of Antibiotics," Medical Encyclopedia, Inc., New York, 40(1955).
e) H. E. Carter, R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell, W. A. Strong: J. Biol. Chem., **160**, 337(1945).
f) F. H. Stodola, O. L. Shotwell, A. M. Borud, R. G. Benedict, A. C. Riley : J. Am. Chem. Soc., **73**, 2290(1951).
g) R. L. Peck, C. E. Hoffhine, Jr., K. Folkers : *Ibid.*, **68**, 1390(1946).
h) J. Fried, O. Wintersteiner : *Ibid.*, **69**, 79(1947).

Properties of the Sulfate of Antibiotic No. 23572—In Table I, physicochemical properties, biological stability, and toxicity of the sulfate are shown for comparison with those of dihydrostreptomycin sulfate. As seen in the Table they are in good agreement. The infrared absorption spectra of Antibiotic No. 23572 sulfate and dihydrostreptomycin sulfate are shown in Fig. 1. There is found no difference between them. The antibacterial spectra of Antibiotic No. 23572 sulfate and dihydrostreptomycin sulfate are shown in Table II. They are also in complete agreement.

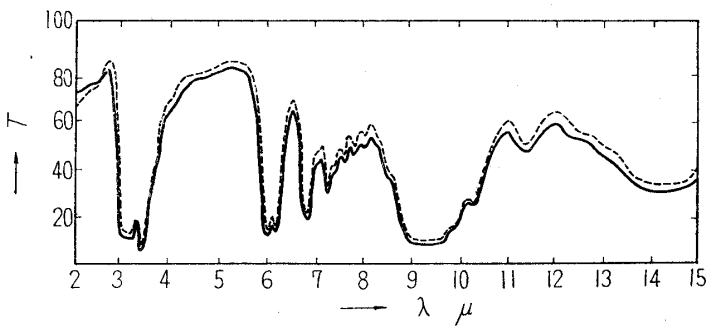

Fig. 1.
Infrared Spectra of Antibiotic No. 23572 Sulfate and Dihydrostreptomycin Sulfate

------ : Dihydrostreptomycin sulfate
——— : Antibiotic No. 23572 sulfate

TABLE II. Comparison of Antibacterial Spectra of Crystalline Sulfates of Antibiotic No. 23572 and Dihydrostreptomycin

No.	Test organisms	Min. inhibitory concn. (mcg./cc.)	
		Antibiotic No. 23572	Dihydrostreptomycin
1	*Staphylococcus aureus* Terajima	1	1
2	*Bacillus subtilis* PCI-219	0.5～0.2	0.5～0.2
3	*Salmonella typhosa*	50	50
4	*Shigella dysenteriae*	1	1
5	*Vibrio cholerae*	8	8
6	*Proteus vulgaris*	5	5
7	*Escherichia coli*	5	5
8	*Pseudomonas aeruginosa*	10	10
9	*Aspergillus niger*	>100	—
10	*Penicillium notatum*	>100	—
11	*Saccharomyces cerevisiae*	>100	—
12	*Candida albicans*	>100	—
13	*Mycobacterium* 607	1	—
14	*Mycobacterium avium*	1	—

Medium : Nos. 1～ 8 Bouillon-agar
Nos. 9～12 Glucose-bouillon-agar
Nos. 13～14 Glycerine-bouillon-agar

According to the method of Bartz, *et al.*,[3] Antibiotic No. 23572 sulfate, dihydrostreptomycin sulfate, and acetone were respectively added to the aqueous solution of thiosemicarbazide. The ultraviolet absorption spectra of these reaction mixtures are shown in Fig. 2. It is apparent from Fig. 2 that Antibiotic No. 23572 shows the same absorption curve as that of dihydrostreptomycin and that the two substances have no aldehyde radical.

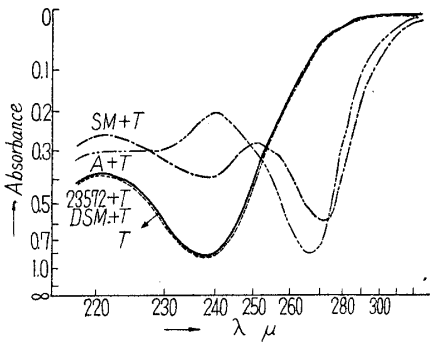

Fig. 2. Effect of Thiosemicarbazide on Ultraviolet Spectra of Antibiotic No. 23572 and Dihydrostreptomycin

23572 : Antibiotic No. 23572
DSM : Dihydrostreptomycin sulfate
SM : Streptomycin sulfate
A : Acetone
T : Thiosemicarbazide

Helianthate and Reineckate of Antibiotic No. 23572—The physicochemical constants of the helianthate and reineckate of Antibiotic No. 23572 were in complete agreement with those of the corresponding salts of dihydrostreptomycin as shown in Table I.

Degradation Products of Antibiotic No. 23572—As mentioned above, the physicochemical, microbiological, and biological properties of the sulfate and two other salts of Antibiotic No. 23572 showed good agreement with those of the corresponding salts of dihydrostreptomycin. Then, the degradation products of Antibiotic No. 23572 were compared with those of dihydrostreptomycin.

According to the method of Stodola, *et al.*,[6] Antibiotic No. 23572 hydrochloride (I) was subjected to methanolysis and out of the degradation products, streptidine (II), and α- (III) and β-methylpentaacetyldihydrostreptobiosaminide (IV) were isolated. (III) was further degraded and out of the products, N-methyl-L-glucosamine was isolated as its pentaacetyl derivative (V). The physicochemical properties of (II), (III), (IV), and (V) are shown in Table III. The infrared absorption spectra of (II), (III), and (V) are shown in Figs. 3, 4, and 5 for comparison with those of the corresponding degradation products of dihydrostreptomycin.

Table III. Degradation Products of Antibiotic No. 23572

Product	m.p.(°C)	$[\alpha]_D$ (c, solvent)	Mol. formula	Analyses (%) Calcd. C	H	N	S	Found C	H	N	S
Streptidine picrate[a]	271~273 (decomp.)		$C_8H_{18}O_4N_6 \cdot 2C_6H_3O_7N_3$	33.34	3.36	23.33		33.46	3.44	23.49	
Streptidine sulfate[a]	290~310 (decomp.)		$C_8H_{18}O_4N_6 \cdot H_2SO_4 \cdot H_2O$	25.40	5.86	22.20	8.47	25.61	6.06	22.02	8.63
α-Methylpentaacetyldihydrostreptobiosaminide[b,c]	194.5	$[\alpha]^{17}$ −120.0° (1.0, $CHCl_3$)	$C_{13}H_{19}O_8N(CH_3CO)_5(OCH_3)$	51.15	6.62	2.49		51.09	6.73	2.40	
β-Methylpentaacetyldihydrostreptobiosaminide[b]	155~156	$[\alpha]^{16}$ −36° (1.0, $CHCl_3$)						50.71	6.62	2.65	
Pentaacetyl-N-methyl-L-glucosamine[b,c,d]	158~159	$[\alpha]^{15}$ −102° (0.7, $CHCl_3$)	$C_7H_{10}O_5N(CH_3CO)_5$	50.62	6.25	3.47		50.71	6.14	3.24	

a) H.E. Carter, *et al.*: Science, **103**, 53(1946).
b) N.G. Brink, F.A. Kuehl, Jr., E.H. Flynn, K. Folkers: J. Am. Chem. Soc., **68**, 2557(1946).
c) J. Fried, O. Wintersteiner: *Ibid.*, **69**, 79(1947).
d) F.H. Stodola, *et al.*: *Ibid.* **73**, 2290(1951).

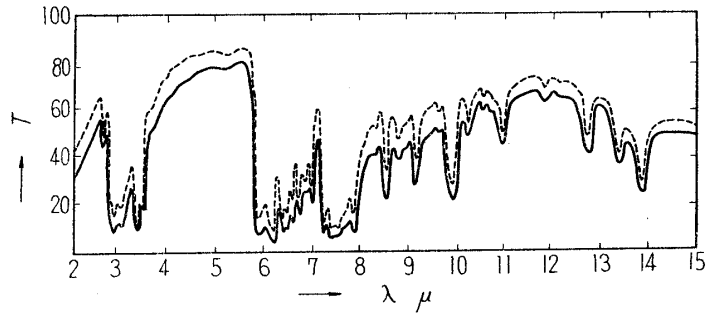

Fig. 3. Infrared Spectra of Streptidine Picrate

------ : From dihydrostreptomycin
—— : From Antibiotic No. 23572

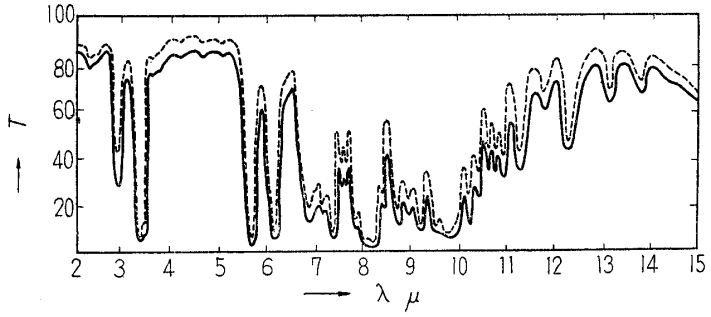

Fig. 4. Infrared Spectra of α-Methylpentaacetyldihydrobiosaminide

------ : From dihydrostreptomycin
—— : From Antibiotic No. 23572

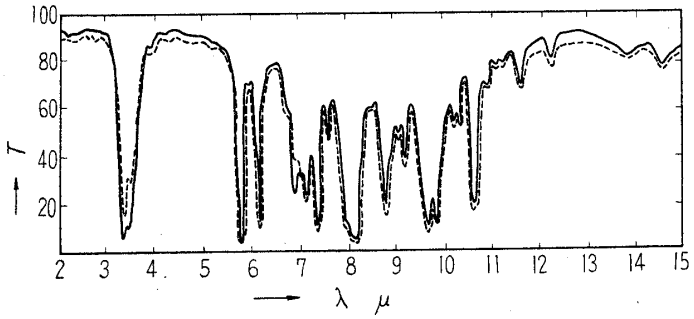

Fig. 5.
Infrared Spectra of Pentaacetyl N-Methyl-L-glucosamine
------ : From dihydrostreptomycin
—— : From Antibiotic No. 23572

From these data it was recognized that Antibiotic No. 23572 is identical with dihydrostreptomycin, and that dihydrostreptomycin, which has hitherto been prepared only by chemical hydrogenation of streptomycin, can be produced directly by the cultivation of *St. humidus nov. sp.*

The authors wish to express their gratitude to Dr. S. Kuwada, Director of the Takeda Research Laboratories, and to Dr. K. Sato, Director of the Institute for Fermentation, Osaka, for their kind advice throughout the course of this work, to Messrs. K. Nakazawa and M. Shibata for their supply of the culture broth and their helpful suggestions and assistances, to Dr. T. Kanzawa and his staff members, and to the members, who took part in the microbiological assay and tank fermentation.

Experimental

Picrate of Streptidine (II)—Streptidine picrate was obtained from Antibiotic No. 23572 by the method of Stodola, *et al.*[1] as follows: Two grams of the hydrochloride of Antibiotic No. 23572 was dissolved in 100 cc. of dehyd. MeOH containing 1% of HCl. After standing for 48 hrs. at room temperature, 200 cc. of ether was added to the solution with stirring and the resulting white precipitate (streptidine hydrochloride) was separated by centrifugation. The precipitate was dissolved in 20 cc. of water and a saturated picric acid solution was added. After standing for several hrs. the precipitated crystals were separated and recrystallized from water to give 400 mg. of streptidine picrate as yellowish needles, m.p. 271~273° (decomp.). *Anal.* Calcd. for $C_8H_{18}O_4N_6 \cdot 2\,C_6H_3O_7N_3$: C, 33.34; H, 3.36; N, 23.33. Found: C, 33.46; H, 3.44; N, 23.49.

Infrared absorption spectrum of the picrate was in accord with that of the corresponding picrate prepared from commercial dihydrostreptomycin as shown in Fig. 3.

Sulfate of Streptidine (II)—Streptidine sulfate was obtained from Antibiotic No. 23572 by the method of Wintersteiner, *et al.*[2] as follows: The sulfate of Antibiotic No. 23572 was dissolved in 4 volumes of *N* H_2SO_4. After standing for 48 hrs. at 45°, the resulting crude streptidine sulfate was collected by filtration. Recrystallization from water gave pure streptidine sulfate as colorless plates, m.p. 290~310°(decomp.). *Anal.* Calcd. for $C_8H_{18}O_4N_6 \cdot H_2SO_4 \cdot H_2O$: C, 25.40; H, 5.86; N, 22.20; S, 8.47. Found: C, 25.61; H, 6.06; N, 22.02; S, 8.63.

α-Methylpentaacetyldihydrostreptobiosaminide (III)—The supernatant of the above streptidine hydrochloride was neutralized with MeOH solution of NaOH, the resulting NaCl was separated by centrifugation, and the solvent was distilled off under reduced pressure, leaving a white powder. The residue was dissolved in 20 cc. of pyridine and 7 cc. of Ac_2O was added. After standing overnight, water was added to the reaction mixture and the solvent was distilled off. The residue was recrystallized from MeOH to obtain α-methylpentaacetyldihydrostreptobiosaminide, colorless needles, m.p. 194.5°. The yield was 700 mg. *Anal.* Calcd. for $C_{13}H_{19}O_8N(CH_3CO)_5(OCH_3)$: C, 51.15; H, 6.62; N, 2.49. Found: C, 51.09; H, 6.73; N, 2.40. $[\alpha]_D^{17}$ −120.0°(c=1.0, $CHCl_3$).

Infrared absorption spectrum of the resultant (III) was in complete agreement with that of α-methylpentaacetyldihydrostreptobiosaminide obtained from commercial dihydrostreptmycin as shown in Fig. 4. No depression of the melting point was observed when mixed with α-methylpentaacetyldihydrostreptobiosaminide obtained from commercial dihydrostreptomycin.

β-Methylpentaacetyldihydrostreptobiosaminide (IV)—β-Methylpentaacetyldihydrostreptobiosaminide was obtained from Antibiotic No. 23572 by the method of Brink *et al.*[3] as follows: Ten grams of the hydrochloride of Antibiotic No. 23572 was dissolved in 500 cc. of MeOH containing 1% of HCl. After standing overnight at room temperature, the solvent was distilled off under reduced pressure. The residue was dissolved in 750 cc. of MeOH and 450 cc. of ether was added. The solution

1) F. H. Stodola, *et al.*: J. Am. Chem. Soc., **73**, 2290(1951).
2) J. Fried, O. Wintersteiner: *Ibid.*, **69**, 79(1947).
3) N. G. Brink, F. A. Kuehl, Jr., E. H. Elynn, K. Folkers: *Ibid.*, **68**, 2557(1946).

was passed through a column packed with 210 g. of acid-washed alumina impregnated with a 2 : 1 MeOH-ether mixture. The column was then washed with 1000 cc. of a 3 : 2 MeOH-ether mixture. The effluent was evaporated to dryness under reduced pressure. The residue was acetylated by being allowed to stand overnight at room temperature with 10 cc. of Ac_2O and 10 cc. of pyridine. Water was then added and the solution was evaporated to dryness *in vacuo*. The residue was dissolved in $CHCl_3$ and the $CHCl_3$ solution was washed successively with water, dil. H_2SO_4, and water. After $CHCl_3$ was distilled off, the brownish solid residue was boiled for about 2 mins. with 100 cc. of ether, and the ether solution was separated by decantation. The same process was repeated again. The ether-insoluble fraction was crystallized from MeOH, yielding α-methylpentaacetyldihydrostreptobiosaminide, m.p. 194.5°. The ether solution was concentrated to about 50 cc. and petr. ether was added to precipitate white crystals. Recrystallization from MeOH gave about 100 mg. of β-methylpentaacetyldihydrostreptobiosaminide, m.p. 155~156°. No depression of the melting point was observed, when mixed with the β-isomer prepared from dihydrostreptomycin in the same way as described above. *Anal.* Found : C, 50.71; H, 6.62; N, 2.65. $[\alpha]_D^{16}$ −36°(c=1.0, $CHCl_3$).

Infrared absorption spectrum of the product was in complete agreement with that of β-methylpentaacetyldihydrostreptobiosaminide obtained from commercial dihydrostreptomycin.

Pentaacetyl-N-methyl-L-glucosamine (V)—A solution of 260 mg. of α-methylpentaacetyldihydrostreptobiosaminide obtained from Antibiotic No. 23572 in 20 cc. of 10% HCl was refluxed for 3 hrs. After cooling, the brownish solution was decolorized wth charcoal and evaporated to dryness *in vacuo*. The residue was acetylated with 1 cc. of Ac_2O and 3 cc. of pyridine at room temperature. After addition of water, the solution was evaporated to dryness. The residue was dissolved in 50 cc. of a 7 : 3 benzene-petr. ether mixture. The solution was passed through a column packed with 4 g. of acid-washed alumina impregnated with petr. ether, whereupon the acetylation product was adsorbed by the acid-washed alumina. The column was treated with 100 cc. of a 7 : 3 benzene-$CHCl_3$ mixture to elute the objective substance. The effluent was concentrated to about 10 cc. and 30 cc. of ether was added to precipitate crystals. Recrystallization from $CHCl_3$-ether gave 25 mg. of needles, pentaacetyl-N-methyl-L-glucosamine, m.p. 158~159°. *Anal.* Calcd. for $C_{17}H_{25}O_{10}N$: C, 50.62; H, 6.25; N, 3.47. Found : C, 50.71; H, 6.14; N, 3.24. $[\alpha]_D^{15}$ −102°(c=0.7, $CHCl_3$).

No depression of the melting point was observed when mixed with pentaacetyl-N-methyl-L-glucosamine obtained from commercial dihydrostreptomycin.

Summary

Isolation and chemical studies were carried out on an antibiotic which was produced by a new streptomyces, *Streptomyces humidus nov. sp.* From the physical, chemical, and microbiological properties of this antibiotic in crude state, it was found that this antibiotic belonged to the so-called streptomycin-group antibiotics, but differed from streptomycin, mannosidostreptomycin, and hydroxystreptomycin, which are produced directly by streptomycetes. This antibiotic was finally isolated as a pure crystalline sulfate. The physicochemical and microbiological properties of this sulfate and physicochemical properties of its degradation products showed that this sulfate was nothing but dihydrostreptomycin sulfate.

(Received April 23, 1957)