REDUCTION PRODUCTS OF SPECTINOMYCIN

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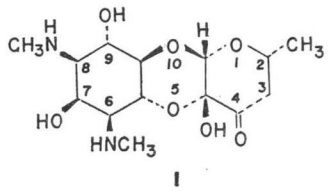
The two epimers of dihydrospectinomycin have been separated and identified structurally. Four tetrahydrospectinomycins have also been prepared.

Spectinomycin* (Fig. 1, **1**) has a structure[1,2] unique among the aminocyclitol antibiotics in which actinamine forms both acetal and hemiacetal linkages with actinospectose, a sugar also found in uscharidin.[3] Reaction with sodium borohydride or catalytic hydrogenation reduces spectinomycin to dihydrospectinomycin as reported previously.[1] We now report the separation and characterization of the two epimeric dihydrospectinomycins (Fig. 2, **2a**, **b**) expected from these reductions and four new reduction products, the epimeric tetrahydrospectinomycins (Fig. 5; **6a**, **b**, **7a**, **b**).

Fig. 1. Spectinomycin



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Dihydrospectinomycins

The NMR spectra of the products of either catalytic hydrogenation or sodium borohydride reduction of **1** were indicative of mixtures of two very similar compounds. While most absorptions were identical there were two distinct singlets which could only be ascribed to the C-10a (anomeric) hydrogen one at 4.55 ppm and the other at 4.70 ppm (Table 1). There were also two very similar doublets due to the C-2α methyl group.

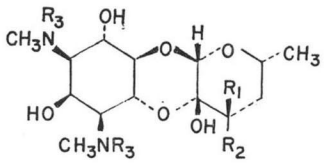
Table 1. Physical properties of the epimeric dihydrospectinomycin dihydrochlorides

	Isomer **2a**	Isomer **2b**
$[\alpha]_D$ (*c* 1, H_2O, pH 7)	+31°	+26°
Melting point	200~202°C	206~214°C
NMR Chemical shifts (DMSO-d^6)		
C-10a (anomeric H)	4.55 ppm	4.70 ppm
C-2α (methyl doublet)	1.11 ppm	1.06 ppm
	1.22 ppm	1.17 ppm
IR, Carbonyl absorption	1600 cm^{-1}	1600 cm^{-1}
	1575 cm^{-1}	1585 cm^{-1} (sh)

The two compounds were separated from each other on an analytical or preparative basis by ion exclusion chromatography over Dowex 1×2 or AG-1×2 resins. These materials were

* Formerly known as actinospectacin. Trobicin® is the registered U.S. trademark of The Upjohn Company for spectinomycin hydrochloride. Additional trademarks include Togamycin and Stanilo.

Fig. 2. The Dihydrospectinomycins



2a R_1 = H ; R_2 = OH ; R_3 = H
2b R_1 = OH ; R_2 = H ; R_3 = H
4a R_1 = H ; R_2 = OH ; R_3 = CBZ
4b R_1 = OH ; R_2 = H ; R_3 = CBZ

Fig. 3. Dihydro-γ-pyrone from dihydrospectinomycin

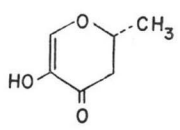


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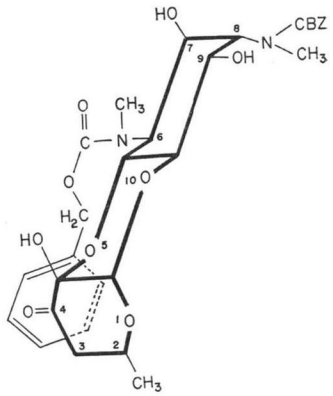
Fig. 4. N, N′-*bis*(Carbobenzyloxy) spectinomycin (**5**)



then characterized as the crystalline dihydrochlorides. Additional differentiating physical characteristics for these materials are shown in Table 1.

The mixture resulting from catalytic reduction of spectinomycin has been shown to have the structure assigned to dihydrospectinomycin on the basis of its periodate oxidation products,[1] a method which would not distinguish the epimers. Mass spectra of the epimers further substantiate the identity of their gross structures. These mass spectra are virtually identical, with an M^+-H_2O peak at 316. A major peak for each at *m/e* 128 is due to the γ-pyrone[4] **3** (Fig. 3) which arises from thermal cleavage. The latter was also isolated from both compounds as a crystalline solid by heating *in vacuo* at 200°C. This same mass ion occurs in mass spectra of *Calotropis* glycosides such as tetrahydrouscharidine[3] which contain a dihydroactinospectose moiety.

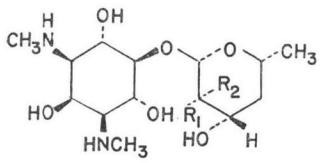
The assignment of configuration was made on the basis of a study of N, N′-*bis*(carbobenzyloxy) derivatives. Dihydrospectinomycin epimeric mixture obtained by catalytic reduction of **1** was converted into N, N′-*bis*(carbobenzyloxy) dihydrospectinomycin **4a, b** (Fig. 2) which was then separated into epimers by silica gel chromatography. Hydrogenolysis of these products then afforded crystalline **2a** and **2b**. An examination of a COREY-PAULING-KOLTUN model of N, N′-*bis*(CBZ)spectinomycin (**5**, Fig. 4) shows the *endo* surface of the actinospectose moiety to be severely hindered at the carbonyl function by the CBZ group on the nitrogen of C-6. When **5** was reduced with a hindered reagent, lithium *tri*(*t*-butoxy) aluminum hydride,[5] exclusive yields of a single epimer of the CBZ-dihydrospectinomycin were obtained. For this epimer, formula **4a** could be assigned based on mechanistic considerations for hydride reductions of bicyclic ketones.[6,7] This of course permitted assignments for the hydrogenolysis products **2a** and **2b** as well.

While it was necessary to use the reaction with the hindered hydride for assignments of structure, it is of interest to note that the CBZ group hinders the carbonyl sufficiently to direct catalytic hydrogenation as well. The total product from Pt-catalyzed hydrogenation of **5** also consisted of a single epimer (**4a**) under the same conditions which afforded equal quantities of **2a** and **2b** when spectinomycin itself was reduced.

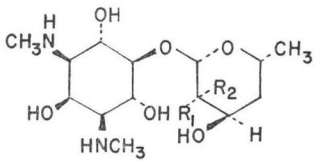
Tetrahydrospectinomycins

Sodium borohydride reduction in absolute methanol converts **1** to **2a** and **2b**. This reduction proceeds further however in 50 % aqueous methanol to afford a mixture of tetrahydrospectinomycins. These compounds each had five free hydroxyls as shown by a molecular ion of 696 for each trimethylsilyl derivative. Considerable additional molecular disruption was evidenced by the large change in optical rotation (now negative) and the total loss of antibacterial activity. This was consistent with assignment of the second reduction site to C-4a at the hemiacetal function, producing compounds which no longer contain the tricyclic ring system. The occurence of this additional reduction suggests that in aqueous solution spectinomycin or dihydrospectinomycin is in equilibrium with open ring forms such as **8** and **9**.

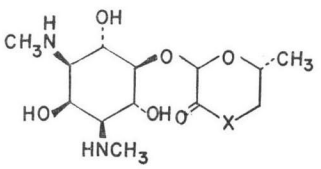
Fig. 5. Tetrahydrospectinomycins and related intermediates



6a,b: R_1 = H or OH, R_2 = OH or H



7a,b: R_1 = H or OH, R_2 = OH or H



8: X = C=O
9: X = CHOH

While the stereochemical assignments at the new epimeric site have not been made, each pair has been individually prepared from the separate dihydrospectinomycin epimers, **6a** and **6b** from **2a**, and **7a** and **7b** from **2b**. Each had a different retention time when chromatographed on AG 1×2 resin.

Biological Activities

The dihydrospectinomycins showed a fraction of the antibacterial activity of spectinomycin *in vitro* and *in vivo*. Epimer **2a** was about 30 % as active as spectinomycin and **2b** showed about 40 % of the activity of **1** *vs. E. coli* UC 527 (Table 2) *in vitro*. *In vivo*, mice infected with *Klebsiella pneumoniae* were protected by subcutaneous administration of **2a** at a CD_{50} of 226 mg/kg, and by **2b** at 75 mg/kg representing *ca.* 10 % and 25 %, respectively, of the corresponding activity of spectinomycin dihydrochloride.

Table 2. Comparative MIC's (mcg/ml) for spectinomycin and the epimeric dihydrospectinomycins

Organism	Spectinomycin	Epimer **2a**	Epimer **2b**
Staphylococcus aureus 284 (UC 76)	31.2	125	62.5
Staphylococcus aureus UC 6570	31.2	>1,000	250
Staphylococcus aureus UC 746	15.6	125	62.5
Escherichia coli UC 45	7.8	15.6	31.2
Klebsiella pneumoniae UC 57	7.8	15.6	7.8
Salmonella schottmuelleri UC 126	15.6	>1,000	>1,000
Pseudomonas aeruginosa UC 95	62.5	1,000	500
Streptococcus faecalis UC 694	62.5	>1,000	>1,000

Experimental

Spectroscopic Methods

Mass spectra were obtained with an LKB 9000 spectrometer and a Varian MAT CH_7 equipped with an F & M Model 402 gas chromatograph.

NMR spectra were obtained with a Varian T-60 spectrometer on solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in d_6-dimethylsulfoxide or d-chloroform.

Chromatographic Procedures

Thin-layer chromatograms were run on Uniplate® silica gel GF plates (250 micron thickness) by Analtech, Inc., using chloroform-methanol (97:3, v/v) as the solvent system.

Ion-exclusion chromatograms were run in columns by Chromatronix, served by a Milton-Roy Minipump, Model 196-89 and monitored by an R_4 Differential Refractometer (Waters Associates) and a Bendix 143A Photoelectric Polarimeter. Packing and conditions are described in the following procedures.

Antibacterial testing procedures have been described by C. LEWIS, *et al.*[8]

Separation of Dihydrospectinomycins 2a and 2b by Chromatography on AG 1×2 Anion Exchange Resin (OH^- form)

A solution containing both dihydrospectinomycins (prepared by catalytic hydrogenation) was chromatographed on a 1″×11″ column of AG 1×2 resin (200~400 mesh, OH^- form, Bio-Rad), and eluted with deionized water at a flow rate of 120 ml/hour and a pressure of 10 psi. The column effluent was monitored with a differential refractometer, and the individual peaks collected.

The dihydro epimers appeared at elution times of approximately 9 hours and 16 hours, respectively. Aliquots of each peak were evaporated to dryness and examined by mass spectrometry. Each showed an M^+-H_2O peak at *m/e* 316 but neither gave a molecular ion. The remainder of each fraction was evaporated to a small volume, acidified to pH 3.0 with dilute HCl; the dihydrochlorides crystallized.

Isomer **2a** (eluted first): m.p. 200~202°, $[\alpha]_D$+31° (*c* 1.0, water at pH 7.0).

Anal: Calcd. for $C_{14}H_{26}N_2O_7 \cdot 2HCl \cdot 2H_2O$: C, 37.92; H, 7.22; N, 6.32; Cl, 16.03.
Found: C, 38.31; H, 7.23; N, 6.32; Cl, 17.16.

Isomer **2b**: m.p. 206~214°, $[\alpha]_D$+26° (*c* 1.0, water at pH 7.0).

Anal. Found: C, 38.62; H, 6.99; N, 6.76; Cl, 18.09.

Borohydride Reduction of Spectinomycin to Tetrahydrospectinomycin Epimers **6a** and **6b**

Spectinomycin sulfate tetrahydrate (5 g; 10 mmol) was slurried with 50 ml Dowex 2×8 resin (OH^- form) in 50 ml water for 15 minutes. This was filtered and the resin washed with 20 ml water. Methanol (70 ml) was added to the combined filtrate and washings, followed by 1.16 g (26 mmole) sodium borohydride over a period of 15 minutes. The solution was stirred 15 minutes longer, then neutralized to pH 7 with dilute sulfuric acid and concentrated *in vacuo* to about 10 ml. The concentrated solution was applied to a column (1″×16″) of AG 1×2 resin (OH^- form) monitored with a polarimeter and a differential refractometer. Two passes separated the products into two fractions of negative optical rotation. The individual peaks were collected separately and evaporated to dryness. The major fraction (eluting first) was obtained as an amorphous solid, but the second peak material crystallized from methanol as fine needles m.p. 227~231°C, $[\alpha]_D$−22° (*c* 0.9612, water).

Anal: Calcd. for $C_{14}H_{28}N_2O_7$: C, 49.99; H, 8.39; N, 8.33
Found: C, 50.26; H, 8.62; N, 8.12

Each of the two products was converted into the dihydrochloride with 2N HCl (to pH 3) followed by lyophilization, and finally by crystallization from methanol-acetone.

First peak material: $[\alpha]_D$−25° (*c* 1.0, water)

Anal: Calcd. for $C_{14}H_{28}N_2O_7 \cdot 2HCl \cdot H_2O$: C, 39.34; H, 7.49; N, 6.56; Cl, 16.63
Found: C, 38.75; H, 7.30; N, 6.23; Cl, 16.53

The penta-TMS derivative gave M+ at *m/e* 696.

Second peak material: $[\alpha]_D$ −19° (*c* 1.0, water).

Anal: Calcd. as above. Found: C, 39.22; H, 7.24; N, 5.12; Cl, 16.61

Borohydride Reduction of Dihydrospectinomycin Epimer **2a**, to Tetrahydro-Epimers **6a** and **6b**

The dihydrochloride of epimer **2a** (0.5 g) was slurried in water with small increments of Dowex 2×8 resin (OH^- form) until the pH reached 9.0, then it was filtered and the resin washed with a little water. The filtrate and washings (20 ml combined) were treated with 0.5 g $NaBH_4$ added in small amounts over 30 minutes. The solution was then cooled in ice and excess borohydride decomposed by the addition of 10 N sulfuric acid. The solution was then reslurried with Dowex 2×8 to a pH of 9.5, and concentrated to about 15 ml at 40°C *in vacuo*. This was then applied to a column of AG 1×2 as previously described and chromatographed in water at 92 ml/hour while monitoring with a polarimeter and differential refractometer. Two peaks with negative optical rotations were seen, the first very sharp (RT 1.75 hours) and the second much smaller and rather broader (T 2.75 hours). Each peak was collected separately and taken to dryness at 45°C under reduced pressure. The first peak gave a viscous gum (243 mg), but the second gave a crystalline solid (61 mg) that was recrystallized from methanol as needles m.p. 227~231, $[\alpha]_D$ −22° (*c* 1.0, water). The ir and mass spectra were identical to those of the "second peak material" from reduction of spectinomycin itself (isomer **6a** or **6b**).

Each of the epimers was silylated in hexamethyldisilazane plus trimethylchlorosilane (MDS/TMCS) in dimethylformamide at room temperature overnight, then run on a 6′ column of 3 % OV-1 at 210°C. Retention time (RT) for the crystalline epimer was 16.3 minutes and for the amorphous epimer 17.4 minutes. At 200°C they were well separated. GC/mass spectrography in each case gave a molecular ion at *m/e* 696.

Borohydride Reduction of Dihydrospectinomycin Isomer **2b**, to Tetrahydro-Epimers **7a** and **7b**

The hydrochloride of epimer **2b** (0.5 g) was reduced in exactly the same manner as described for epimer **2b** above, and chromatographed under the same conditions. Two peaks were again obtained, each of negative optical rotation. The first was very sharp (retention time 2 hours), and the second, low and very broad (eluting between 3.5 to 6.5 hours). Concentration of each peak fraction to dryness under reduced pressure gave 254 mg of amorphous gum and 127 mg solid, respectively.

The solid from the second peak was recrystallized three times from methanol - 2-propanol to give needles m.p. 189~194°C, $[\alpha]_D$ −58° (*c* 0.75, water). The analysis fitted a methanol solvate.

Anal: Calcd. for $C_{14}H_{28}N_2O_7 \cdot CH_3OH$: C, 48.91; H, 8.70; N, 7.61
Found: C, 49.09; H, 8.49; N, 7.85

As in the case of tetrahydro-epimers **6a** and **6b**, both compounds were silylated with HMDS/TMCS and examined by GC/mass spec. Retention times on the 3 % OV-1 column were 14.2 minutes for the amorphous epimer and 15.7 minutes for the crystalline one at 210°C. Molecular ions at *m/e* were obtained in each case.

N, N′-*bis*-(Carbobenzyloxy)-dihydrospectinomycins, Isomers **4a** and **4b**

To a solution of 20 g (47 mmol) of dihydrospectinomycin dihydrochloride (a mixture of isomers prepared by catalytic reduction of spectinomycin) in 100 ml of cold water was added 20 ml of 6 N NaOH. This solution was then added to 200 ml of water containing 15 g each of $NaHCO_3$ and Na_2CO_3 (pH 9.4). It was chilled to 0~10°C and 50 g (290 mmol) of benzyl chloroformate was added with vigorous stirring. After two hours the mixture was extracted

with 300 ml methylene chloride. The extract was washed exhaustively with eight water washes, then dried over $MgSO_4$ and concentrated to a syrup. A white powder (14 g) was next precipitated from a solution of this syrup in ether with Skellysolve B. A 10-g aliquot of this material was chromatographed over 1 kg of silica in a 3″ (i.d.) column developed first with 3 liters of 5% methanol in chloroform, then with 3 liters of 6% methanol in chloroform. Fractions of 20 ml were collected. From fractions 230~270, 1.96 g of a compound (**4a**) was collected. The NMR spectrum was consistent for a N, N′-*bis*-carbobenzylated dihydrospectinomycin. The absorption attributed to the anomeric hydrogen was located at 4.65 ppm. From fractions 289~323, 2.74 g of a second substance (**4b**) was obtained. Its NMR was virtually identical with that of **4a** save for the location of the singlet ascribed to the anomeric hydrogen which now appeared at 4.82 ppm.

Anal: Calcd. for $C_{30}H_{38}N_2O_{11}\cdot 1/4\ H_2O$: C, 59.38; H, 6.34; N, 4.62.
Found; (**4a**) C, 59.21; H, 6.42; N, 4.58.
(**4b**) C, 59.11: H, 6.33; N, 4.62.

N, N′-*bis*-(Carbobenzyloxy)dihydrospectinomycin, Isomer **4a** by Hydride Reduction

To 1 g (1.6 mmol) of N, N′-*bis*-(carbobenzyloxy)spectinomycin[1] in 40 ml of dry tetrahydrofuran, was added, with stirring, 1.5 g (6.0 mmol) of Li-*tri*(*t*-butoxy)AlH. Following 16 hours at room temperature a second 1.5 g portion of hydride was added. An hour later the mixture was concentrated to 10 ml, diluted to 60 ml with water, and acidified with 1 N HCl to pH 1.5. This solution was extracted sequentially with methylene chloride and ethyl acetate and the combined extracts were water-washed and evaporated to afford 640 mg of white solid. Chromatography over silica, developed by chloroform-methanol (97:3) followed by precipitation from ethyl acetate with Skellysolve B gave 350 mg of amorphous solid which appeared as a single spot on TLC(SiO_2:$CHCl_3$-MeOH, 97:3). The NMR spectrum was identical with that for **4a** above with the C-10a (anomeric) hydrogen singlet appearing at 4.65 ppm.

N, N,′-*bis*-(Carbobenzyloxy)dihydrospectinomycin (**4a**) by Catalytic Hydrogenation

A 4-g (6.6 mmol) quantity of N, N′-*bis*-(carbobenzyloxy)spectinomycin was hydrogenated in 100 ml of 97% ethanol over 500 mg of platinum oxide catalyst at 40 psig for one hour in a Parr shaker. The product, 3.0 g, was isolated by evaporation and purified further by precipitation from ethyl acetate with Skellysolve B. Its NMR spectrum was again identical with that of **4a**. No **4b** was present either by TLC or NMR.

Dihydrospectinomycin Dihydrochloride **2b** by Hydrogenolysis of **4b**

A solution of 300 mg (0.5 mmol) of **4b** in 100 ml of methanol containing 25 mg of 30% palladium/charcoal catalyst was shaken for two hours at a hydrogen pressure of 40 psig. Following filtration and evaporation, the residue was converted into dihydrochloride with 0.5 ml of 1 N HCl and then crystallized from acetone and water, yielding 80 mg, mp 203~210°C dec. Identification was made by a comparison of its NMR spectrum with that of **2b** obtained by ion-exclusion chromatography.

Dihydrospectinomycin Dihydrochloride **2a** by Hydrogenolysis of **4a**

The procedure used for **4b** was also applied to 9.1 g of **4a** to yield 5.6 g of **2a**. Identification was made by a comparison of its NMR spectrum with that of **2a** obtained by ion exclusion chromatography.

Acknowledgement

We wish to thank Messers. C. LEWIS and G. E. ZURENKO for biological data on some of these compounds and Mr. J. A. SHILEY for able technical assistance.

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