MYOMYCIN, A NEW ANTIBIOTIC

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(Received for publication March 10, 1973)

The isolation and characterization of myomycin, a new broad spectrum antibiotic, are described. The isolation of *myo*-inositol, L-β-lysine and 3-amino-3-deoxy-D-mannose from hydrolyzates of myomycin established it to be an unusual member of the basic, water soluble family of cyclitol antibiotics.

From a soil-screening program in our laboratories an organism belonging to the *Nocardia* genus was discovered which produces a novel, broad-spectrum antibiotic. This compound, which we have named myomycin, is an unusual member of the large family of water soluble, basic (WSB) antibiotics that are derivatives of cyclohexane polyols. Many of these WSB antibiotics are glycosides of aminocyclitols such as streptamine and 2-deoxystreptamine. However, to date, only one WSB antibiotic (kasugamycin,[1] which contains *d*-inositol) has been reported to contain an inositol moiety, *i.e.*, a cyclitol in which nitrogen is not directly attached to the cyclohexane ring. Myomycin was found to be a derivative of *myo*-inositol and thus it represents a new example of a WSB cyclitol antibiotic.**

The isolation, characterization and structural study of myomycin constitute the subject of this paper.

Myomycin culture broths contain an antibiotic complex consisting of one major component (myomycin B) and two minor components (myomycins A and C) as shown by thin-layer chromatography of beer filtrates or of crude myomycin concentrates. The isolation of myomycin from beer filtrates involves the retention of the antibiotic complex on IRC-50 (NH_4^+) followed by elution with dilute acid. The antibiotic components in the neutralized eluate can be adsorbed on carbon and eluted with acidic 25 % aqueous acetone to yield a crude concentrate of myomycins A, B and C. Carbon chromatography of this material or of a concentrated IRC-50 eluate (neutralized with IR-45) leads to a main fraction of myomycin B containing a small amount of myomycin C followed by a fraction that contains only myomycin B. Crude concentrates containing all three of the myomycin components can also be purified by conversion to the Orange II salt. Regeneration of the antibiotic from this derivative produces material containing myomycins A, B and C with the same potency as material purified by carbon chromatography.

Myomycin C can be isolated by silica gel chromatography. This component represents 4~9 % of the complex and is 15 % as potent as myomycin B when assayed against *Bacillus subtilis* according to the procedure developed for the assay of the myomycin complex. This decrease in activity

* Deceased, August 17, 1972.

** *Myo*-inositol, although of common occurrence in plants and animals, is a rare constituent of antibiotics. The only antibiotics reported to contain *myo*-inositol are antiprotozoin[2] and the moenomycin complex.[3] These conpounds are acidic glycolipids that contain phosphorus.

Table 1. Elemental analysis of myomycin sulfate preparations

	Myomycin complex[a]	Myomycin B+C[b]	Myomycin B	Calcd. for $C_{27}H_{51}N_9O_{14}\cdot 2H_2SO_4\cdot 2H_2O$
% C	34.36	33.66	33.81	33.85
% H	6.18	6.01	6.26	6.21
% N	13.23	13.33	13.32	13.16
% S	7.07	6.94	6.49	6.69
% N (VAN SLYKE)	—	—	4.03	4.38 (for 3-NH_2)

[a] Prepared from the purified Orange II salt (see experimental) and contains myomycin A, B and C.
[b] From the main fraction of a carbon column.

Table 2. Summary of biological properties of myomycin complex sulfate[a]

Bacterial strain		M.I.C.[b] (μg/ml)	PD_{50}[c] (mg/kg)
Staphylococcus aureus	H-228	6.3	0.3
Staphylococcus aureus	UC-76	3.1	0.6
Aerobacter aerogenes	MGH-1	>100	>500
Escherichia coli (Vogel)		25	3.5
Klebsiella pneumoniae	AD	0.2	1.4
Klebsiella pneumoniae	MGH-2	6.3	1.0
Proteus mirabilis	MGH-1	50	14
Proteus vulgaris	UC-232	100	24
Pseudomonas aeruginosa	No. 733	25	38
Pseudomonas aeruginosa	No. 1396-1	25	54
Pseudomonas aeruginosa	Whittington	>100	>500
Salmonella typhimurium	V-31	12.5	< 20
Shigella sonnei	A-8	12.5	—
Shigella sonnei	C-10	6.3	2.4

Acute LD_{50} values in mice (mg/kg) as sulfate salt
IV=165; SC=*ca.* 1,100;
IP=800; PO>9,000

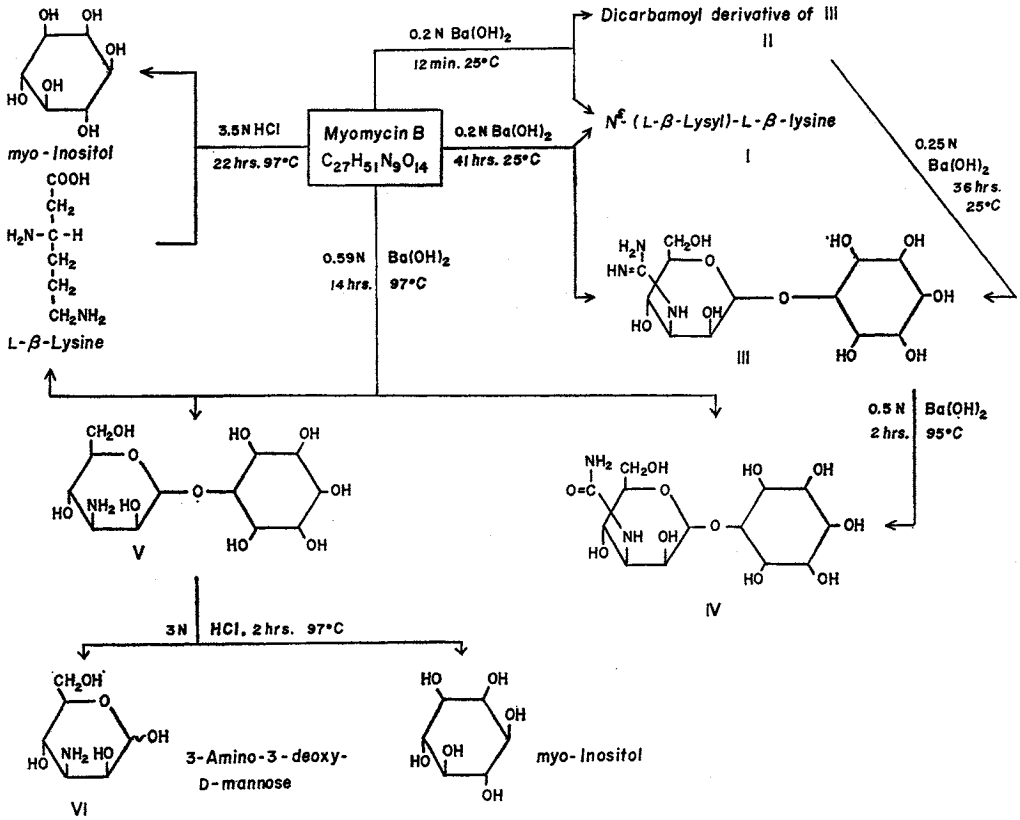
[a] This complex contained myomycin A, B and C. The weights given correspond to the sulfate salt.
[b] Microtitration in tryptic soy broth.
[c] Single SC-50 % protective dose in mice.

may be due to partial decomposition of myomycin C during its isolation. Myomycin A was isolated as a single component by thin-layer chromatography on silica gel.

The physical, chemical and biological properties of the purified myomycin fractions normally isolated (pure B, B+C and A+B+C) are essentially identical. Although the same products were obtained using myomycin complexes, the chemical degradation results reported herein are those derived from the pure major component, myomycin B.

Myomycin is a strongly basic antibiotic which is conveniently isolated as the amorphous sulfate salt. The salt is very soluble in water but nearly insoluble in methanol and organic solvents such as ethyl acetate, chloroform, and acetone. All of the various preparations of the sulfate salt display a rotation of $[\alpha]_D^{25}=-4.5\pm1°$ in 1.5~2 % aqueous solutions and exhibit pK'a values in water of 8.0 and above 11. The antibiotic has no ultraviolet absorption maximum above 220 nm and possesses no definite melting point but gradually darkens above 200°C. Myomycin gives positive reactions in the ninhydrin and SAKAGUCHI tests and negative reactions toward the maltol and ELSON-MORGAN tests. Analytical data are listed in Table 1 and some biological properties of myomycin are given in Table 2. Myomycin hydrochloride and acetate salts, prepared by passing myomycin sulfate over Dowex-1×2 in the appropriate salt form, are also amorphous solids and are much more methanol-soluble than myomycin sulfate. Whereas myomycin is quite stable in acid, it is extremely unstable in basic media. Therefore, it is not practical to isolate the antibiotic as the free base.

Fig. 1.



Hydrolysis of myomycin in 3.5 N HCl at 97°C for 22 hours (Fig. 1) yields NH_4Cl, CO_2, an unidentified basic compound (SAKAGUCHI-negative), *myo*-inositol, and L-β-lysine (characterized as its crystalline H_2SO_4 salt). In several hydrolysis experiments, the last two compounds were repeatedly isolated in the approximate molar ratio of 1:2, respectively, suggesting that either two separate β-lysine fragments are located in the molecule or that the dipeptide, β-lysyl-β-lysine, is present as in the case of streptolin.[4]

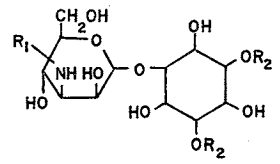
A greater insight into the structure of myomycin was gained by alkaline hydrolysis (see Fig. 1). All antimicrobial activity is lost when a solution of myomycin in 0.2N $Ba(OH)_2$ is allowed to stand at room temperature for 5 minutes. If the reaction is stopped at this point by the addition of H_2SO_4, no CO_2 is produced and from the hydrolyzate an amino acid (**I**) and a basic, SAKAGUCHI-positive compound (**II**) can be isolated. The latter could not be obtained crystalline but the analyses of the purified amorphous hydrochloride and sulfate salts indicate that $C_{15}H_{27}N_5O_{12}$ is the empirical formula for **II**. This compound when hydrolyzed in 0.25N $Ba(OH)_2$ for 36 hours at 25°C gave rise to two equivalents of CO_2 and a new SAKAGUCHI-positive basic compound (**III**), $C_{13}H_{25}N_3O_{10}$, which was characterized as its crystalline hydrochloride. **III**·HCl, in addition to the amino acid (**I**), could also be obtained from 0.2N $Ba(OH)_2$ hydrolysis of myomycin for 41 hours at 25°C. The infrared spectra of myomycin and **II**·HCl both exhibited a strong band at 1730 cm^{-1} characteristic of $R\text{-}O\text{-}\overset{O}{\overset{\|}{C}}\text{-}NH_2$ whereas this absorption was absent in the infrared spectrum of **III**·

HCl. Also, the change of empirical formula of **II**→**III**, a loss of 2(CHNO), and the isolation of two equivalents of CO_2 suggest that **II** is a dicarbamoyl derivative of **III**.

Hydrolysis of **III** in 0.5 N $Ba(OH)_2$ at 95°C for 2 hours produced a crystalline, neutral compound **IV** ($C_{13}H_{24}N_2O_{11}$) whereas prolonged basic hydrolysis gave rise to a crystalline basic compound (**V**) ($C_{12}H_{23}NO_{10}$). The guanidino absorption bands at 1680, 1670 and 1620 cm^{-1} in the SAKAGUCHI-positive **III**·HCl are replaced by a single band at 1660 cm^{-1} in the neutral **IV** and no carbonyl absorption is present in the infrared spectrum of the base **V**. Both **IV** and **V** were SAKAGUCHI-negative. The results attending the conversion **III**→**IV**→**V** are characteristic of the transformation $R\text{-}NH\text{-}\overset{\overset{NH}{\|}}{C}\text{-}NH_2 \rightarrow R\text{-}NH\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH_2 \rightarrow R\text{-}NH_2$ and thus **III** and **IV** are the guanidino and uredio derivatives, respectively, of the parent amino compound **V**. Compounds **IV** and **V** were first isolated from vigorous alkaline hydrolysis of myomycin. As expected, **IV** can be converted to **V** by prolonged alkaline hydrolysis.

Compound **V** proved to be a valuable degradation product since upon hydrolysis (3 N HCl for 2 hours at 97°C) it was found to yield *myo*-inositol and 3-amino-3-deoxy-D-mannose (**VI**).[5] The latter compound was characterized as its crystalline hydrochloride which proved to be identical to an authentic sample.* Thus, myomycin contains a guanidino sugar, a structural unit not yet found in other antibiotics. To our knowledge this is the first time that a derivative of 3-amino-3-deoxy-D-mannose has been found in an antibiotic although its 2-epimer, kanosamine, is present in the kanamycins[6] and its 6-deoxy analog, mycosamine, occurs in polyenic macrolides such as nystatin, pimaricin, and tetrins A and B.[7]

The above degradative information permits the following structural assignments.



V	$R_1 = R_2 = H$
IV	$R_1 = CO\cdot NH_2$ $R_2 = H$
III	$R_1 = C(=NH)NH_2$ $R_2 = H$
II (?)	$R_1 = C(=NH)NH_2$ $R_2 = CO\cdot NH_2$

The uncertainty in **II** was attempted to be removed by periodate oxidation studies, the results of which are summarized in Table 3. The positioning of the two carbamoyl groups as shown in **II** is indicated since any other arrangement would contain a vicinal dihydroxy system which should be oxidized rapidly (as found for **III**) under the conditions employed. However, the slow, general oxidation of myomycin and **II** preclude a definite assignment of a symmetrically trisubstituted inositol and therefore this structure is tentative. The fact that no formaldehyde was formed in the periodate oxidation of **III** supports the pyranose form of the sugar component and rules out a furanose ring in which the C_5 and C_6 hydroxyl groups are unsubstituted. The anomeric configuration of the sugar and the position of glycosidic attachment to *myo*-inositol have not been determined.

* We are pleased to acknowledge the helpful cooperation of Dr. H. H. BAER who supplied us with this sample.

Table 3. Periodate oxidation results

	Moles I 0_4^-/Mole substrate				Molar yield of:			
					HCO_2H		CH_2O	
Time (hrs.)	1.0	4	24	52	28	54	3	22
Myomycin	0.13	0.15	0.23	0.23	—	0.21	—	—
Compd. II	0.24	0.37	0.77	0.92	—	0.28	—	0.10
Compd. III	3.8	4.5	5.2	—	2.95	3.25	0.05	0.07

In the nmr spectra (D_2O), the C-1 hydrogen in the mannose moiety of compounds **III**→**V** appears at approximately δ 5.4 ($J<1$ Hz) relative to TMS as an external standard.

Two structural units of myomycin not yet considered are L-β-lysine and compound **I** which were isolated from vigorous acid and mild alkaline hydrolysis, respectively. The relation of **I** to β-lysine was soon inferred when it was found that vigorous acid hydrolysis of **I** yielded L-β-lysine in good yield as the only ninhydrin-positive product. The consideration that **I** was a dipeptide of L-β-lysine was strengthened when analysis of the picrate and PHABS salts of **I** gave results consistent with **I**=$C_{12}H_{26}N_4O_3$ (MW 274) and was confirmed when the mass spectrum of **I**-free base exhibited an M+1 peak at 275. Finally, crystalline N, N′, N″-triacetyl compound **I** was prepared; analytical results of this compound agreed with $C_{12}H_{26}N_4O_3(CH_2CO)_3$ and although its mass spectrum did not give a molecular ion (the highest mass recorded was M-60=340), the corresponding methyl ester gave the expected M=414. Thus, the two equivalents of L-β-lysine derived from myomycin B are present as a (β-lysyl)$_2$ dipeptide, the structure of which was shown to be N^{ε}-(L-β-lysyl)-L-β-lysine as described in the experimental section. This dipeptide, as recently established by other means, also occurs in racemomycin C[8] and streptothricin D (streptolin).[9]

The condensation of compounds **I** and **II** with a loss of H_2O leads to an empirical formula of $C_{27}H_{51}N_9O_{14}$ as proposed in Table 1. To account for myomycin's functionality (4 basic groups), compound **II** is joined to the carboxyl group of the dipeptide **I**. Because of the extreme base lability of the function that links the dipeptide to **II**, we initially ruled out an ester linkage and therefore considered that the guanidino group of **II** is acylated by the β-lysyl dipeptide. However the rapid hydrolysis of α-amino acid esters of glucose[10] at pH 8 indicates that the placement of the dipeptide moiety in myomycin remains to be rigorously proven. The results of our structural studies of myomycin B lead to the partial structure **II** wherein one of the hydrogens on N or O is

replaced by L, L-$CH_2CH_2CH_2\underset{\underset{NH_2}{|}}{C}HCH_2CO\cdot NHCH_2CH_2CH_2\underset{\underset{NH_2}{|}}{C}HCH_2\overset{\overset{O}{\|}}{C}$—.

Experimental*

Fermentation: The organism producing myomycin was isolated from a soil sample collected

* Melting points were taken in capillary tubes in a Thomas-Hoover Unimelt Apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed using 95% EtOH-H_2O-HOAc-2M NH_3 (79:21:5:10) on ChromAR-500 silica gel sheets and bioautography on *B. subtilis*. Approximate R_f values for myomycins A, B and C in this system are 0.21, 0.41 and 0.63, respectively. Whatman 3 MM paper and *t*-butanol-HOAc-H_2O (2:2:1) were used for paper chromatography. Myomycin, **V**, **I**, β-lysine, and 3-amino-mannose (R_f 0.05, 0.15, 0.20, 0.30, 0.31) were detected with ninhydrin; compounds **II** and **III** (R_f~0.18) were detected by SAKAGUCHI test or an $NaIO_4$-$KMnO_4$ spray.

near Crosby, North Dakota, and was characterized as a member of the *Nocardia* genus. This culture was named *Nocardia* sp. J327 (NRRL 5338, 5339). Stirred-jar or tank fermentations were conducted in a nutrient medium containing 2% glucose, 1% glycerol, 1% soy bean oil, 44% protein (solvent extracted), 0.5% animal peptone, 0.5% distiller's solubles, 0.2% NH_4Cl, 0.5% NaCl, and 0.25% $CaCO_3$ at an initial pH of 7.5 for 96~120 hours at 28~32°C.

Isolation:

Method 1.

The harvested beer (1,130 liters) was treated with 4.5 kg of oxalic acid; the mixture was stirred for 1/2 hour and the pH adjusted from 2.1 to 6.0 with 7 liters of concentrated ammonium hydroxide. After standing overnight at 5°C, the oxalated beer was stirred with 45 kg of Celite 545 and the mixture filtered. The filtrate was stirred with 34 liters of IRC-50 (H^+) for 2 hours keeping the pH between 6 and 6.5 by periodic additions of ammonium hydroxide. The resin was separated from the supernatant, washed with 190 liters of deionized water, and charged into a 15 cm glass column. The packed column was washed with deionized water until the effluent was clear, and the antibiotic was eluted with 0.6 N H_2SO_4. That portion of the eluate, containing the majority of the antibiotic (57 liters) according to microbial assay, was adjusted to pH 6 with 10 N sodium hydroxide and treated with 5.95 kg of Darco G-60 and 4.5 kg of Celite 545. The mixture was stirred for 1 hour and then filtered in a plate and frame unit. The carbon cake was washed with 30 liters of water and blown dry. The partially dried cake was resuspended in 57 liters of 25% aqueous acetone at pH 2.5 and the mixture agitated for 1/2 hour keeping the mixture at pH 2.5 with dilute sulfuric acid. The mixture was filtered and the carbon reextracted with 42 liters of 25% aqueous acetone at pH 2.5. Both 25% aqueous acetone extracts were combined, the pH adjusted to pH 5 with IR-45 (OH^-) resin, and the aqueous solution was concentrated to 800 ml *in vacuo*. The concentrate was filtered and the filtrate was treated with 8 liters of methyl alcohol and 10 ml of a 50% solution of triethylamine sulfate in aqueous methanol. The resulting white precipitate was digested overnight at 5°C, filtered, and dried. The weight of this product was 98 g and corresponded to a myomycin complex of approximately 52% purity as determined by disc/plate agar diffusion assays using *B. subtilis* as a test organism.

A solution of 50 g of the above material in 1 liter of water was treated with 54.4 g of Orange II [*p*-(2-hydroxy-1-naphthylazo) benzenesulfonic acid sodium salt] in 1.47 liters of water with stirring. The resulting mixture was stored for 3 days at 5°C and then filtered. The precipitate was washed with 1.5 liters of water and dissolved in 22.5 liters of hot 50% aqueous methanol. The hot solution was filtered from a small amount of insoluble material and then stored at 5°C for 2 days. The precipitate that formed was filtered off and washed with 1.5 liters of water. This material was dissolved in 28 liters of hot 90% methanol. The hot solution was decanted from a small amount of red-orange oil, cooled to about 40°C and then treated, with stirring, with an excess (62 ml) of a 36% solution of triethylamine sulfate. After storing this mixture at 5°C overnight the precipitated myomycin sulfate was filtered off and washed with 1 liter of methanol. The precipitate was dissolved in 0.45 liter of water and the light orange solution (pH 4.8) was passed over 230 ml of Dowex 1×2 (SO_4^{--}). The effluent plus 0.65 liter water wash of the resin were combined and stirred with 4.3 g of Darco G-60. The carbon was filtered off and the filtrate was freeze-dried to afford 25.1 g of myomycin complex sulfate (assay, 975 μg/mg) as a white solid. $[\alpha]_D^{25}$ −3.7° (*c* 2.0, H_2O); pK'a (H_2O), 7.9~8.0 (approx. equivalent weight, 440); no ultraviolet maximum above 220 nm; no definite melting point, but decomposes over a wide range above 200°C. Thin-layer chromatography of this material showed it was an antibiotic complex containing myomycins A, B and C. Analytical results are recorded in Table 1.

A solution of 850 mg of this product in 60 ml of water was slowly passed over a 1.5×12.5 cm column of Dowex 1×2 (Cl^-). The effluent plus a 40 ml water were combined and freeze-dried to yield 770 mg of myomycin complex hydrochloride. $[\alpha]_D^{25}$ −2.7° (*c* 1.1, H_2O); pK'a (H_2O)=8 (equiv. wt=435); no definite melting point, with gradual decomposition beginning at about 170°C.

Anal. Calcd. for $C_{27}H_{51}N_9O_{14}\cdot 4HCl\cdot H_2O$:

C, 36.45; H, 6.46; N, 14.17 (VAN SLYKE for 3-NH_2, 4.72); O, 26.98; Cl, 15.94.
Found:
C, 36.56; H, 6.44; N, 13.87 (VAN SLYKE=4.30); O, 27.06; Cl, 16.13; S and ash, 0.0.

Method 2.

Unfiltered beer (9,550 liters) was processed in the same manner as described above except that the IRC-50 resin was stirred for 15 hours and the active acidic eluates were adjusted to pH 5 with IR-45 (OH^-) resin. The eluate was concentrated to 18.7 liters *in vacuo* and then charged onto a column containing 47 kg of Darco G-60 and 40 kg of Celite 545 (the column was washed with 95 liters of pH 5 deionized water prior to loading). The carbon column was developed with approximately 2,300 liters of water. Initially, cuts of approximately 18 liters were taken and as the water development progressed, the volumes of the fractions were increased to as high as 256 liters. An aliquot of each fraction was chromatographed on silica gel as described above. After the column hold-up (189 liters), the initial water eluates from the carbon column contained myomycins B and C according to TLC and the same constituents were found in the subsequent fractions until a total of 685 liters of eluate was collected. The ensuing water eluates (a total of 1,425 liters) contained only myomycin B according to TLC. The isolation of myomycin B from the first 256 liters of the above eluate of 1,425 liters will be described here as an example of the group.

The 685 liters combined eluate containing myomycins B and C was concentrated to 9 liters. On standing at 5°C, the concentrate deposited inert material which was filtered off. The clear filtrate was applied to a column of 250 ml of Dowex 50×16 (H^+ form) resin. The column was developed with water and those cuts containing the antibiotic were combined, and adjusted batchwise to pH 5 with IR-45 (OH^- form). The resin was filtered off and the filtrate was polished by filtering through an 8μ Millipore membrane. The filtrate was freeze-dried, to afford 878 g of myomycin sulfate (assay, 993 μg/mg) which was shown by TLC to contain only myomycins B and C; $[\alpha]_D^{25}$—3.2° (*c* 1.0, H_2O). Analytical data are presented in Table 1.

The 256 liters containing myomycin B were concentrated to 1 liter and passed over a column of 75 ml of Dowex 50×16 (H^+). Myomycin B sulfate (104 g; assay—940 μg/mg was isolated in the same manner as described above. $[\alpha]_D^{25}$—4.9° (*c* 1.0, H_2O); indefinite decomposition beginning above 200° C; no ultraviolet absorption maximum above 220 nm; pK'a (H_2O) 8.0 and about 11; ir absorption maxima (KBr disk) at 3430 (broad), 2940, 1735, 1645, 1500, 1460, 1405, 1350, 1075, 700 and 620 cm^{-1}. Analytical results are given in Table 1.

Isolation of *myo*-inositol and L-β-lysine: A solution of 1.008 g (1.05 mmoles) of myomycin B sulfate in 120 ml of 3.5N HCl was heated at 97°C for 22 hours. The hydrolyzate was concentrated to near dryness *in vacuo* and the residual HCl removed by repeated co-distillations with water *in vacuo*. An aqueous solution of the residue was adjusted to pH 4.3 with IR-45(OH^-), treated with 0.3 g of Darco G-60, and then filtered. The colorless filtrate was freeze-dried to yield 1.047 g of residue. This material was dissolved in 10 ml of water and applied to a 1.54×14 cm column of Dowex 50W×2 (H^+) (50~100 mesh). The resin was eluted as follows:

Fraction No.	Eluant	Weight of residue (mg)
1	65 ml water	166
2	35 ml water	nil
3	40 ml 0.33 N HCl	5
4	40 ml 〃	211
5	40 ml 〃	80
6	50 ml 〃	15
7	45 ml 〃	1
8	90 ml 1.2 N HCl	406
9	85 ml 〃	80
10	90 ml 〃	11

Fraction 1 was adjusted to pH 4.2 with IR-45 (OH^-) before it was evaporated to dryness. The

white residue (166 mg) was crystallized from aqueous ethanol to yield 142 mg (0.79 mmoles) of *myo*-inositol, mp 221.5~223.5°; $[\alpha]_D^{25}$ 0, as shown by the comparison of its infrared spectrum with the spectrum of an authentic sample. This material was recrystallized (95 % recovery) to afford a product, mp 222.5~223.5°C, that did not depress the melting point of a known sample of *myo*-inositol.

Fractions 4 through 6 contained ammonium chloride (isolated and identified by paper chromatography and its infrared spectrum) and an organic base of unknown structure. According to paper chromatography, the material in Fractions 8 and 9 was slightly inhomogeneous. These residues were combined in 35 ml of water and this solution was passed over 14 ml of Dowex 1×2 (OH^-). The resin was washed with water and the main fraction eluted with 90 ml of 0.4 M acetic acid. This eluate was concentrated to dryness *in vacuo*. A solution of the residue in a small volume of water was adjusted to pH 1.5 with sulfuric acid. The slow addition of methanol to this solution (*ca.* 3.2 ml) caused the precipitation of 429 mg (1.76 mmoles) of crystalline L-β-lysine·H_2SO_4, mp 224~226°C d, $[\alpha]_D^{25}$+15.3° (*c* 1.63, H_2O), as shown by the comparison of its infrared spectrum with that of an authentic sample and an undepressed mixed melting point.

Anal. Calcd. for $C_6H_{14}N_2O_2 \cdot H_2SO_4$: C, 29.50; H, 6.60; N, 11.47; S, 13.13.
Found: C, 29.55; H, 6.52; N, 11.67; S, 13.13.

L-β-Lysine was also identified by means of the *p*-(*p*-hydroxyphenylazo) benzenesulfonate salt, mp 240~241°C d, and the picrate, mp 203~205°C d. The melting points of these derivatives agree with the reported melting points of the corresponding salts of L-β-lysine.[11] The infrared spectrum of the picrate was identical to the infrared spectrum of the picrate of L-β-lysine isolated from viomycin.[11]

Isolation of x-(3-deoxy-3-guanidino-D-mannopyranosyl)-*myo*-inositol hydrochloride (**III**·HCl): A solution of 1.54 g (1.61 mmoles) of myomycin B sulfate in 77 ml of water was treated with 51 ml of 0.51 N $Ba(OH)_2$. The mixture was allowed to stand for 41 hours at 25°C and then adjusted to pH 4.2 with H_2SO_4. The $BaSO_4$ was filtered off and the filtrate was passed over a 2×13 cm column of Dowex 1×2 (OH^-). The effluent and 75 ml of water wash were combined and freeze-dried to yield 658 mg of a white solid. A solution of this material in 2 ml of water was acidified with 3 N HCl and diluted to 2.8 ml. The slow addition of ethanol caused the precipitation of a crystalline solid. This mixture was further diluted with ethanol and chilled overnight at 3°C. The crystalline product (569 mg; 84 %) melted at 253~256°C with prior softening at 248°C. The analytical sample of **III**·HCl was obtained (85 % recovery) by an additional recrystallization from aqueous ethanol, mp 255~256°C d; $[\alpha]_D^{25}$−22° (*c* 1.05, H_2O); this compound is ninhydrin-negative but gives a positive SAKAGUCHI test.

Anal. Calcd. for $C_{13}H_{25}N_3O_{10} \cdot HCl$: C, 37.19; H, 6.24; N, 10.01; Cl, 8.45.
Found: C, 37.12; H, 6.36; N, 9.97; Cl, 8.14.

The above Dowex 1×2 (OH^-) column yielded no eluate solids upon further washing with water. However, 770 mg of a yellow oil was eluted with 170 ml of 0.4 M acetic acid. Paper chromatography indicated that this material was mainly N-(L-β-lysyl)-L-β-lysine. This product was converted to the same N, N′, N″-triacetyl derivative, mp 231~233°C, as described below.

Alkaline hydrolysis of myomycin to a dicarbamoyl derivative (**II**) of x-(3-deoxy-3-guanidino-D-mannopyranosyl)-*myo*-inositol and N^{ε}-(L-β-lysyl)-L-β-lysine (**I**).

Method 1: A solution of 2.39 g (2.50 mmoles) of myomycin B sulfate in 33 ml of water was treated with 137 ml of 0.24 N $Ba(OH)_2$. After 12 minutes the resulting mixture was quickly adjusted to pH 4.3 with 2 N H_2SO_4 and then stored at 3°C overnight. The $BaSO_4$ was filtered off and washed with water. The filtrate and wash were combined (280 ml) and passed through a 2×15 cm column of freshly prepared Dowex 1×2 (OH^-). The effluent plus 100 ml of a water wash of the resin were collected in a beaker containing 1.2 ml of 2 N H_2SO_4 so that the pH was always 6 or lower. This combined fraction was adjusted to pH 4 with H_2SO_4 and freeze-dried to yield 1.35 g of a white solid which was ninhydrin-negative but gave a positive SAKAGUCHI test. A

portion of this product (1.31 g) readily dissolved in 6 ml of water. A trace of insoluble material was filtered off and the filtrate diluted and freeze-dried to afford 1.30 g (100 % yield) of **II**-sulfate as an amorphous, granular solid that could not be crystallized, mp 224~225°C d with previous contraction beginning at 215°C; $[\alpha]_D^{25}$ −18.4° (*c* 1.0, H_2O).

Anal. Calcd. for $C_{15}H_{27}N_5O_{12}\cdot 0.5H_2SO_4\cdot H_2O$: C, 33.58; H, 5.64; N, 13.06; S, 2.99.
Found: C, 33.28; H, 5.59; N, 12.88; S, 3.30.

By allowing a solution of **II** in 0.25N $Ba(OH)_2$ to stand for 36 hours at 25°C, 1.8 equivalents of CO_2 was detected and an 82 % yield of crystalline **III**·HCl, mp 256~257°C d, was isolated as described above.

A second water wash (80 ml) of the above Dowex 1×2 resin afforded no additional material, but elution with 160 ml of 0.4 M acetic acid yielded 1.04 g (92 %) of the acetate salt of **I** as a nearly colorless, ninhydrin-positive, viscous oil; $[\alpha]_D^{25}$ +20.6° (*c* 1.54, H_2O); the mass spectrum showed a weak M+1 ion at 275.

Paper chromatography of this product showed it to be slightly inhomogeneous (major zone at R_f 0.20). This material (455 mg) could be purified by chromatography over a Darco G-60: Celite 545 (9 g: 9 g) column. Elution with water gave nearly homogeneous **I**-acetate (343 mg) as a colorless glass; the sulfate or hydrobromide salts of **I** could not be obtained crystalline. The hydrochloride was isolated as a colorless glass, $[\alpha]_D^{20}$ +23.2° (*c* 1.37, H_2O); pK'a (H_2O) = ~2.8, 8.2, ~10 (estimated mol. wt, 330). A pulsed nmr spectrum (D_2O) recorded on a 100 MHz spectrometer with FOURIER transform* revealed five well-separated multiplets which are assigned as follows: δ from external TMS 2.35 (8H, 2-CH_2CH_2-); 3.25 (4H, 2-CH_2CO); 3.68 (2H, -CH_2ND_2); 3.88 (2H, -CH_2ND-CO); and 4.25 (2H, 2-$CHND_2$). The fact that the last three signals are in a 2:2:2 ratio clearly indicates that this dipeptide is N^{ε}-(β-lysyl)-β-lysine. The other possibility, N^{β}-(β-lysyl)-β-lysine would require a proton ratio of 4:1:1, respectively, for 2(CH_2-ND_2), -CH-ND_2, and -CH-ND-CO.

The picrate of compound **I** was obtained as a yellow solid, mp 200~202°C d with prior contraction.

Anal. Calcd. for $C_{12}H_{23}N_4O_3\cdot 3(C_3H_3N_3O_7)$: C, 37.47; H, 3.67; N, 18.94.
Found: C, 37.25; H, 3.71; N, 19.24.

The *p*-(*p*-hydroxyphenylazo) benzensulfonate salt was isolated as an orange solid, mp 205~213°C d with prior darkening; $[\alpha]_D^{25}$ +11.5° (*c* 0.24, 95 % EtOH).

Anal. Calcd. for $C_{12}H_{23}N_4O_3\cdot 3(C_{12}H_{10}N_2O_4S)$: C, 51.97; H, 5.09; N, 12.63; S, 8.67.
Found: C, 51.72; H, 5.31; N, 12.61; S, 8.53.

Compound **I**-acetate was converted to the free base by retention on IRC-50 (NH_4^+) followed by elution with 0.2 M NH_3. The mass spectrum of the resulting colorless, viscous oil, showed an M+1 peak at 275. Later it was found that crude **I**-acetate need not be purified by carbon chromatography but could be converted directly to the crystalline N, N', N''-triacetyl derivative by stirring 520 mg of **I**-acetate salt with 14 ml of pyridine and 7 ml of acetic anhydride for 7 hours. The reaction mixture was allowed to stand overnight and then concentrated to dryness *in vacuo*. The residue (477 mg) was dissolved in 12 ml of water. The resulting solution was warmed for 10 minutes on the steam bath, cooled, and then passed over 5 ml of Dowex 50×2 (H^+). The effluent and a 90 ml water wash of the resin were combined and freeze-dried to afford 438 mg of a white solid, mp 225~227°C. Repeated crystallization of this material from 95 % EtOH gave the analytical sample, mp 232~234°C; $[\alpha]_D^{25}$ −4.6° (*c* 1.0, H_2O). The largest ion detected in the mass spectrum was at *m/e* 340.

Anal. Calcd. for $C_{18}H_{32}N_4O_6$: C, 53.98; H, 8.06; N, 13.99.
Found: C, 53.84; H, 8.03; N, 13.85.

The methyl ester was prepared by treating an MeOH solution of the above compound with a slight excess of ethereal diazomethane. The product was crystallized from 95 % EtOH or MeOH-

* We are grateful to Digilab, Inc., Cambridge, Mass. 02139, for recording this spectrum.

EtOAc, mp 225~227°C; $[\alpha]_D^{25}$—5.2° (*c* 0.50, MeOH). The mass spectrum showed the expected molecular ion at 414 in addition to a prominent peak at 270 which has been attributed[9] to [Ac$_2$-β-lys-NHCH$_2$CH$_2$CH$_2$]$^+$.

Anal. Calcd. for $C_{19}H_{34}N_4O_6$: C, 55.05; H, 8.27; N, 13.52.
Found: C, 55.02; H, 8.00; N, 13.68.

Method 2: A solution of 1.21 g (1.26 mmoles) of myomycin B in 49 ml of water was treated with 33 ml of 0.5 N Ba(OH)$_2$. After standing at 25°C for 12 minutes with occasional stirring, the reaction mixture was quickly neutralized with H_2SO_4 and then adjusted to pH 4.3 with dilute H_2SO_4. The resulting mixture was chilled at 3°C overnight and filtered. The filtrate (plus the water wash) was freeze-dried to yield 1.22 g of a white solid. This material was dissolved in 12 ml of water and a small amount of insoluble residue was filtered off. The filtrate contained 1.19 g of solid which was dissolved in 5 ml of water and applied to a 1.3×23 cm column of Dowex 50W×2 (H^+). The resin was washed with water and then eluted with 0.33 N HCl in 85 ml fractions. The major amount of SAKAGUCHI-positive material appeared in the first two fractions which were concentrated to dryness *in vacuo*. Each of the two residues was dissolved in a small amount of MeOH and these solutions were treated with excess ethyl acetate to precipitate white, amorphous solids; total yield, 596 mg (90 %). A portion of the product from the second fraction was dissolved in water; freeze-drying afforded **II**·HCl as an amorphous solid, mp 205~210°C d with prior contraction beginning at *ca.* 185°; $[\alpha]_D^{25}$—17.6° (*c* 1.33, H_2O).

Anal. Calcd. for $C_{15}H_{27}N_5O_{12}\cdot HCl\cdot H_2O$: C, 34.39; H, 5.77; N, 13.37; Cl, 6.77.
Found: C, 34.58; H, 5.60; N, 13.10; Cl, 7.17.

Further elution of the Dowex 50 with 0.33 N HCl beyond Fraction 3 yielded no significant material. Elution was then conducted with 1.6 N HCl. Most of the material was eluted between 80~400 ml and was isolated as a nearly colorless, ninhydrin-positive, viscous oil; (510 mg); $[\alpha]_D^{25}$ +19.0° (*c* 1.5, H_2O), pK'a (H_2O)=2.6, 8.2, 9.9 (mol wt=336). This material was the hydrochloride of **I** as determined by paper chromatography and conversion to the *p*-(*p*-hydroxyphenylazo) benzenesulfonate salt.

Periodate Experiments: Solutions containing 35~90 mg of sample in 10 ml of 0.05~0.07M $NaIO_4$ were stored at 2°C. The extent of IO_4^- consumption was determined by the addition of excess arsenite followed by titration with I_2 as described by DRYHURST.[12] Formic acid was determined by titration with 0.01 N NaOH and formaldehyde was determined using chromotropic acid.[13]

Isolation of x-(3-deoxy-3-uredio-D-mannopyranosyl)-*myo*-inositol (**IV**) and x-(3-amino-3-deoxy-D-mannopyranosyl)-*myo*-inositol (**V**).

1. From myomycin: A mixture of 1.05 g of myomycin complex, 7.5 g of Ba(OH)$_2\cdot 8H_2O$, and 80 ml of water was heated at 97°C for 14 hours. The excess Ba(OH)$_2$ was then converted to $BaCO_3$ with CO_2 and the mixture filtered. The filtrate was concentrated to 16 ml and adjusted to pH 6.5 with H_2SO_4. The insoluble material was filtered off and the filtrate passed over 30 ml of Dowex 1×2 (OH^-). After the first hold-up volume, elution of the resin with 230 ml of water gave 365 mg of a mixture which was separated into two components by chromatography over 25 ml of Amberlite CG-50 (NH_4^+). The first compound was readily eluted with water and weighed 110 mg. It was crystallized from aqueous ethanol to afford 86 mg of neutral, ninhydrin-negative **IV**, mp 185~186°C d; $[\alpha]_D^{25}$—43.6° (*c* 1.04, H_2O).

Anal. Calcd. for $C_{13}H_{24}N_2O_{11}\cdot H_2O$: C, 38.80; H, 6.51; N, 6.96.
Calcd. for $C_{13}H_{24}N_2O_{11}$: C, 40.62; H. 6.29; N, 7.29.
Found (dried at 50°): C, 38.60; H, 6.36; N, 6.86.
Found (dried at 95°): C, 40.30; H, 6.12; N, 7.14.

The second component was slowly eluted from the Amberlite CG-50 resin with water. This material (pale yellow; 195 mg) crystallized from aqueous ethanol to give 166 mg of ninhydrin-positive **V**, mp 253~256°C d with prior darkening starting at about 240°C. The analytical sample

(151 mg) was obtained by recrystallization from aqueous methanol and exhibited the same mp; pK'a (water)=7.6; equiv. wt=335; $[\alpha]_D^{25}$—24.1° (*c* 0.99, H_2O).

Anal. Calcd. for $C_{12}H_{23}NO_{10}$: C, 42.23; H, 6.79; N, 4.10.
Found: C, 42.34; H, 6.89; N, 3.97.

Compound **V**·HCl crystallized from aqueous ethanol, mp 205~207°C d.

Anal. Calcd. for $C_{12}H_{23}NO_{10}\cdot HCl\cdot 0.5H_2O$: C, 37.26; H, 6.52; N, 3.62; Cl, 9.17.
Found: C, 37.42; H, 6.26; N, 3.58; Cl, 9.15.

Prolonged hydrolysis in hot 0.5 N $Ba(OH)_2$ converted **IV** to **V**. Further elution of the above Dowex 1×2 resin with 0.3 M acetic acid (190 ml) yielded 440 mg of an oil which, by paper chromatography, was shown to be a mixture of β-lysine and N-(β-lysyl)-β-lysine. Vigorous hydrolysis (6 N HCl, 97°C for 24 hours) converted this mixture to material which yielded only a single zone, corresponding to β-lysine, upon paper chromatography. From the 6 N HCl hydrolyzate, 224 mg of crystalline L-β-lysine·H_2SO_4 was isolated.

2. From **III**: A solution of 210 mg of **III**·HCl in 18 ml of 0.5 N $Ba(OH)_2$ was heated at 95°C for 2 hours. The reaction mixture was adjusted to pH 5 with H_2SO_4 and then filtered. The filtrate was passed over a 1.0×9 cm column of Dowex 50×2 (H^+). The effluent and water wash of the resin contained 128 mg of a solid. Crystallization of this material from aqueous ethanol gave 116 mg of product, mp 185~187°C, with an infrared spectrum identical to that of **IV** isolated from myomycin. Further elution of the resin with 0.2 M NH_4OH yielded 62 mg of a residue which was crystallized from aqueous ethanol to afford 51 mg of product, mp 248~250°C d, with an infrared spectrum identical to that of **V** isolated from myomycin.

When the $Ba(OH)_2$ hydrolysis of **II** was extended to 48 hours, essentially no **IV** was present. The only compound isolated was **V**.

Hydrolysis of x-(3-amino-3-deoxy-D-mannopyranosyl)-*myo*-inositol (**V**) to 3-amino-3-deoxy-D-mannose·HCl and *myo*-inositol.

A solution of 98 mg of **V** in 10 ml of 3 N HCl was heated at 97°C for 2 hours. The mixture was then concentrated to dryness *in vacuo* and the remaining HCl removed by repeated evaporations of aqueous solutions of the residue *in vacuo*. An aqueous solution (7 ml) of the final residue was slowly passed over 2 ml of Dowex 50×4 (H^+). The effluent plus water wash (20 ml) were combined and afforded 50 mg of a crystalline solid. This material was recrystallized from aqueous ethanol to yield 45 mg of *myo*-inositol, mp 222.5~224.5°C, as shown by its infrared spectrum.

Elution of the Dowex 50×4 resin with 45 ml of 0.32 N HCl followed by 25 ml of 1.6 N HCl gave a total of 66 mg of a solid residue. A solution of this material in 1.3 ml of water was applied to a 1.8-cm column containing a mixture of 4 g of Darco G-60 + 4 g of Celite 545. The major fraction was eluted with water to give 45 mg of material which was dissolved in 2 ml of methanol. This solution was filtered from a small amount of insoluble material and the filtrate concentrated to dryness. The residue was recrystallized twice from moist acetic acid to afford 21 mg of crystalline 3-amino-3-deoxy-D-mannose·HCl, mp 163~164°C d, $[\alpha]_D^{25}$+14.6°→+5.1° in 4 hours (*c* 0.96, H_2O), the infrared spectrum of which was identical with that of an authentic sample of 3-amino-3-deoxy-D-mannose hydrochloride.[5]

Anal. Calcd. for $C_6H_{13}NO_5\cdot HCl$: C, 33.42; H, 6.54; N, 6.50; Cl, 16.44.
Found: C, 33.70; H, 6.49; N, 6.14; Cl, 16.66.

Acknowledgments

The authors wish to express their appreciation to Dr. J. D. HOWELLS and associates for supplying myomycin fermentation broths; to Mr. NEIL WILLMER and associates for the isolation of pilot plant quantities of myomycin; to Dr. C. L. HEIFETZ and associates for chemotherapeutic data; to Dr. J. M. VANDENBELT and associates for physical constants and spectral data; and to Mr. C. E. CHILDS and associates for microanalyses.

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