MUTATIONAL BIOSYNTHESIS BY IDIOTROPHS OF *MICROMONOSPORA PURPUREA*

II. CONVERSION OF NON-AMINO CONTAINING CYCLITOLS TO AMINOGLYCOSIDE ANTIBIOTICS

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A mutant of *Micromonospora purpurea*, which produces the gentamicin complex only when 2-deoxystreptamine is added to the fermentation medium, produces a new antibiotic complex, 2-hydroxygentamicin, when streptamine or 2,4,6/3,5-pentahydroxycyclohexanone is added to the fermentation medium. This mutant also produces the gentamicin complex when 2,4/3,5-tetrahydroxycyclohexanone is added to the fermentation medium. The C_1 and C_2 components of 2-hydroxygentamicin have broad spectrum *in vitro* antibacterial activity similar to the gentamicin C_1 and C_2 components, but with greater activity against some gentamicin-resistant strains.

SHEIR *et al.*[1] have devised a technique for producing semi-synthetic aminoglycoside antibiotics by supplementation of mutants, requiring exogenous 2-deoxystreptamine (**1**) with aminocyclitol analogs of 2-deoxystreptamine. Several groups[2~6] have successfully prepared new aminoglycoside antibiotics by this method of mutational biosynthesis.

In the accompanying publication[7] we described the preparation of new analogs of gentamicin C, a complex of antibiotics produced by *Micromonospora purpurea*, using this technique. We now report that the idiotroph that produced 2-hydroxygentamicin C (C_1 and C_2) upon supplementation with streptamine (**2**) affords the same antibiotic complex when supplemented with 2,4,6/3,5-pentahydroxycyclohexanone (**3**). Furthermore, when 2,4/3,5-tetrahydroxycyclohexanone (**9**) is fed to this idiotroph, the gentamicin C complex (C_1, C_2 and C_{1a}) is obtained.

We believe that this is the first report of a deoxystreptamine-requiring mutant that is capable of producing aminoglycoside antibiotics upon supplementation with a non-nitrogenous cyclitol. This mutant of *M. purpurea* appears to be blocked at an early stage in the biosynthesis of deoxystreptamine (**1**).

Materials and Methods

The mutant organism *M. purpurea* VIb-3P was maintained on agar slants of medium 1 consisting of (per liter, distilled H_2O) glucose, 10 g; soluble starch, 20 g; yeast extract, 5 g; N-Z-amine Type A (Difco), 5 g; $CaCO_3$, 1 g; and agar, 15 g. A first stage seed was prepared by inoculating a loopful from the slant to 50 ml of germination medium 2 consisting of (per liter, distilled H_2O) beef extract, 3 g; tryptone, 5 g; dextrose, 1 g; soluble starch, 24 g; yeast extract, 5 g; and $CaCO_3$, 4 g; and allowed to incubate for 4 days on a rotary shaker at 27~28°C. A

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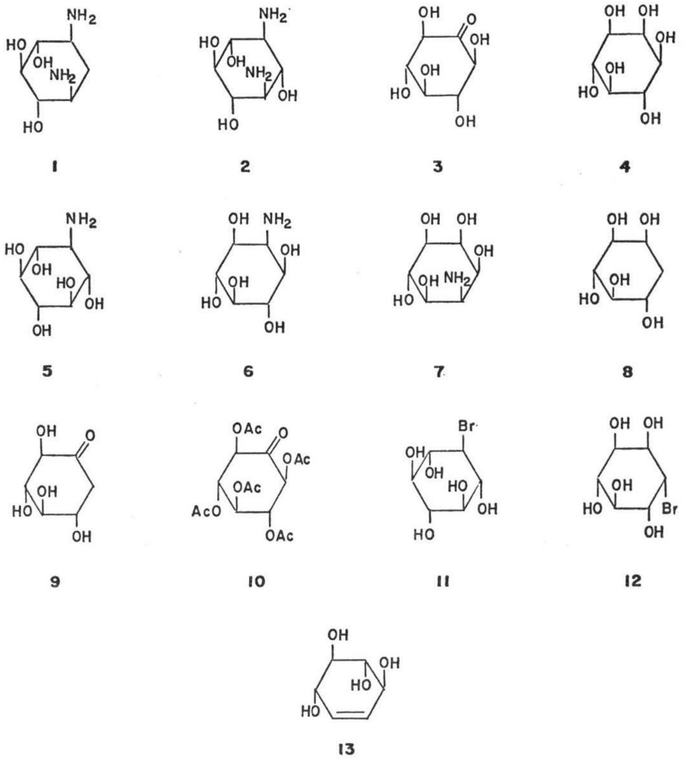
Fig. 1. Compounds tested for incorporation by *M. purpurea* VIb-3P.



5% inoculum was then transferred to flasks of medium 2 with and without a potential precursor (500 μg/ml) and these were incubated as above. Preliminary detection of antibiotic activity was determined by disc-agar diffusion testing of broths against *Bacillus subtilis*. In addition paper and silica gel chromatography were used. The chromatography systems were (1) Whatman No. 1 paper saturated with 0.95 M sulfate-bisulfate and developed in descending fashion in 80% aqueous ethanol +1.5% NaCl followed by bioautography using *B. subtilis* and (2) silica gel 60F254 thin-layer plates (TLC) developed in the lower phase of $CHCl_3$ - MeOH - Conc. NH_3 (28%) (1:1:1) followed by bioautography using *B. subtilis*.

Isolation of Antibiotics

A 5% inoculum of *M. purpurea* VIb-3P growing in medium 2 was transferred to 500 ml of medium 2 and this was incubated for 3 days. One liter of this second stage seed was used to inoculate 9 liters of production medium (medium 4) consisting of (per liter, distilled H_2O) beef extract, 3 g; yeast extract, 5 g; soybean meal, 5 g; maltose, 1 g; starch, 24 g; casamino acid, 1 g; $CaCO_3$ 4 g; $CoCl_2 \cdot 6H_2O$, 1 mg in tanks agitated at 400 rpm and sparged with filtered air at 5 liters/min at 28~29°C for 48 hours. The growth from this third stage seed was used to inoculate the tanks of production medium containing the cyclitol or aminocyclitol. The purified antibiotic mixture and the individual gentamicin C or 2-hydroxy gentamicin C components were obtained as described in the accompanying publication.[7]

Preparation of Aminocyclitols and Characterization of Antibiotics

All melting points are uncorrected. The mass spectra reported were measured with a Joelco JMS-1-OCS mass spectrograph. Infrared spectra were determined on a Model-21 Perkin Elmer infrared spectrophotometer. ^{1}H-NMR spectral measurements were made on a Varian HA-100 spectrophotometer using TMS as an internal standard. ^{13}C-NMR spectral measurements were made on a Varian XL 100 spectrophotometer. Shifts were referenced to internal dioxane and are reported relative to TMS. D_2O used as the solvent was obtained from fresh ampules. Cell manipulations were done in a dry box under N_2.

Aminocyclitols and Cyclitols

2-Deoxystreptamine ·2HBr (**1**) was obtained from neomycin sulfate by the procedure of KUEHL *et al.*[8]

Streptamine ·H_2SO_4 (**2**) was obtained from streptomycin by the procedure of PECK *et al.*[9]

2, 4, 6/3, 5-Pentahydroxycyclohexanone (**3**) was prepared from *myo*inositol (**4**), obtained from R. W. Greef & Co. Inc., by microbiological oxidation using *Acetobacter suboxydans*, a method described by CHARGOFF *et al.*[10]

*Scyllo*inosamine-2 (**5**) and *myo*inosamine-2 (**6**) were prepared from inosose, **3** by the method of CARTER *et al.*[11] *Myo*inosamine-4 (**7**) was prepared by the method of MAY and MOSETTIG.[12]

Microbiological oxidation of **8** using *Acetobacter suboxydans* afforded the *dl* 2, 4/3, 5-tetrahydroxycyclohexanone (**9**), a procedure used by POSTERNAK[13] for the oxidation of the individual enantiomers of viboquercitol (**8**).

The elemental analysis was obtained on the osazone derivative of **9**: mp 208~209°C (from EtOH).

Anal. Calcd. for $C_{18}H_{20}N_4O_3$: C, 63.52; H, 5.92; N, 16.43.
Found: C, 63.5; H, 6.0; N, 16.5.

2, 4, 6/3, 5-Pentahydroxycyclohexanone pentaacetate (**10**) was prepared by the method of KLUYVER and BOEZAARDT.[14]

dl-Viboquercitol (**8**), bromoscylloquercitol-A (**11**), bromo*vibo*quercitol-A (**12**) and conduritol B (**13**) were prepared from *myo*inositol (**4**) by the method of McCASLAND and HORSWILL.[15]

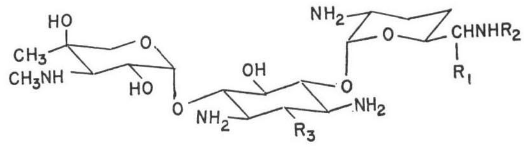
Structures are shown in Fig. 1.

Characterization of Antibiotics

The 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**) were compared by tlc, ms, nmr and elemental analysis.[7] The gentamicins C_1 (**16**), C_2 (**17**) and C_{1a} (**18**) were compared by tlc and ms. Structures are shown in Fig. 2.

In addition to the 2-hyroxygentamicins C_1 (**14**) and C_2 (**15**) obtained from the experiments in which inosose **3** was used for supplementation of mutant VIb-3P, streptamine (**2**) was also isolated as its H_2SO_4 salt. It was identical to authentic **2** by tlc (silicagel, Brinkman 60F254, $CHCl_3$-MeOH-conc. NH_4OH, 3:4:1, Rf 0.2 and 1:1:1 system, Rf 0.07, no separations on co-chromatography) and ir comparisons. The elemental analysis was also in agreement.

Fig. 2. Antibiotics produced by *M. purpurea* VIb-3P.



(**14**) 2-Hydroxygentamicin C_1: $R_1=R_2=CH_3$; $R_3=OH$
(**15**) 2-Hydroxygentamicin C_2: $R_1=CH_3$; $R_2=H$; $R_3=OH$
(**16**) Gentamicin C_1: $R_1=R_2=CH_3$; $R_3=H$
(**17**) Gentamicin C_2: $R_1=CH_3$; $R_2=R_3=H$
(**18**) Gentamicin C_{1a}: $R_1=R_2=R_3=H$

Anal. Calcd. for $C_6H_{14}N_2O_4 \cdot H_2SO_4$: C, 26.09; H, 5.84; N, 10.14; S, 11.61.
Found: C, 26.1; H, 5.4; N, 10.1; S, 12.0.

Degradation of 2-Hydroxygentamicins C_1 (**14**) and C_2 (**15**) Obtained from Inosose **3** Supplementation

A 10~20 mg sample of the mixture of 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**) in 0.3 ml of 6 N HCl in a 0.5-mm capillary tube was heated in refluxing 6 N HCl for 6 hours. The mixture was allowed to stand at room temperature for 2 days and was then diluted with 1.5 ml of EtOH. The resulting clear supernatant liquid was decanted from the solid residue. The solid residue was identical to streptamine (**2**) when compared by tlc analysis (silica gel, Brinkman 60F254, $CHCl_3$-MeOH-conc.NH_3 (28%); 3:4:2. Its Rf was 0.1 as compared to 0.2 for deoxystreptamine (**1**); spots were visualized with ninhydrin spray or acid charring.

The hexaacetate was prepared and had a mp comparable to authentic hexaacetate prepared from streptamine.[9] Vapor phase chromatographic comparisons of the two hexaacetates indicated that they were identical (R.T. 10.6 minutes for streptamine hexaacetate *versus* R.T. 9.1 minutes for deoxystreptamine pentaacetate, 3-ft glass OV-25 column).

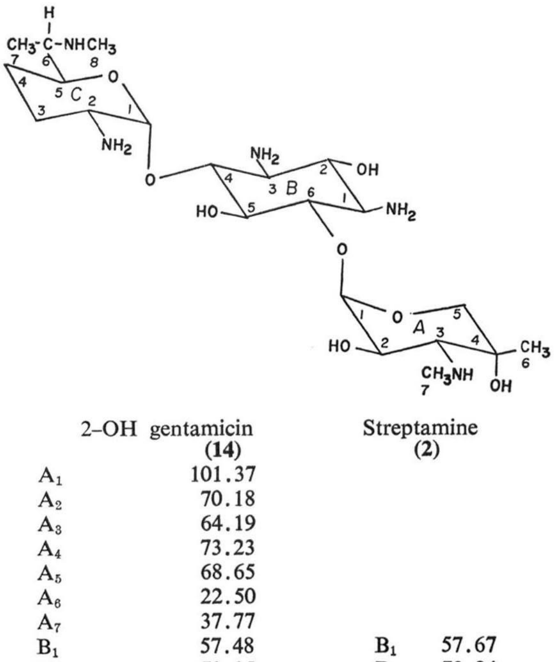
In Vitro Testing

The antibiotics prepared were tested by tube dilution tests in MUELLER-HINTON broth by standard techniques.[16]

Results and Discussion

In the accompanying publication,[7] we described the isolation of an idiotroph of *Micromonospora purpurea* which we coded VIb-3P. This idiotroph afforded the usual mixture of gentamicins [C_1 (**16**), C_2 (**17**) and C_{1a} (**18**)] when a growing culture was supplemented with 2-deoxystreptamine (**1**). When streptamine (**2**) was supplemented to the growing culture, a mixture of 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**) was obtained. When 2, 4, 6/3, 5-pentahydroxycyclohexanone (**3**), a non-nitrogenous cyclitol obtained by microbiological oxidation of *myo*inositol (**4**), was fed to a growing culture of *M. purpurea* VIb-3P, the same mixture of 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**) could be isolated from the broth. In addition, streptamine (**2**) could be isolated from the broth indicating that the inosose **3** was converted to the aminocyclitol streptamine (**2**) by *M. purpurea* VIb-3P.

Fig. 3. ^{13}C Chemical shifts of 2-hydroxygentamicin C_1 (**14**) and streptamine (**2**)



	2-OH gentamicin (**14**)		Streptamine (**2**)
A_1	101.37		
A_2	70.18		
A_3	64.19		
A_4	73.23		
A_5	68.65		
A_6	22.50		
A_7	37.77		
B_1	57.48	B_1	57.67
B_2	73.95	B_2	73.24
B_3	56.33	B_3	57.67
B_4	83.92	B_4	73.91
B_5	75.01	B_5	76.32
B_6	83.30	B_6	73.91
C_1	101.97		
C_2	50.66		
C_3	26.75		
C_4	25.51		
C_5	72.02		
C_6	58.27		
C_7	13.69		
C_8	33.02		

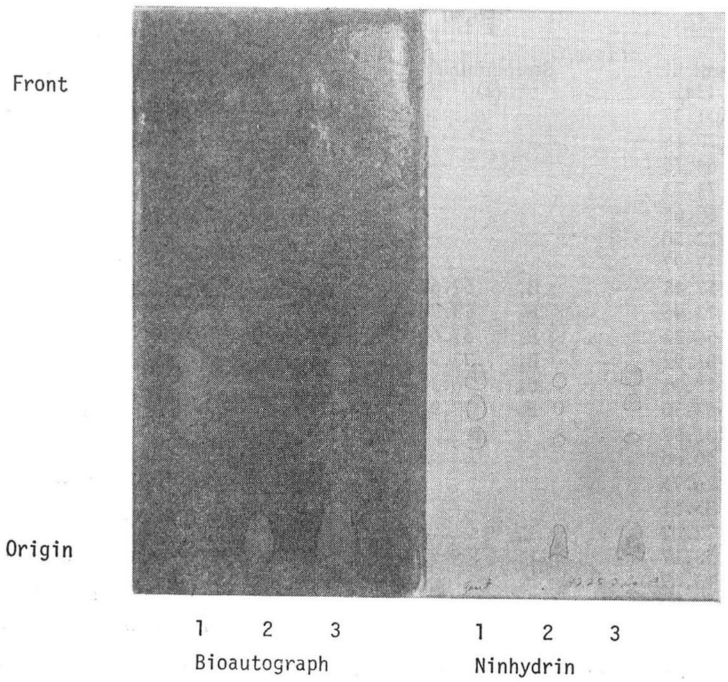
The 2-hydroxygentamicin components C_1 (**14**) and C_2 (**15**) from the inosose **3** supplementation were isolated as described earlier[7] and were found to be identical to the 2-hydroxygentamicin C_1 (**14**) and C_2 (**15**) components isolated from the streptamine (**2**) supplementation by tlc, pmr and ms comparisons as well as by elemental analysis of their H_2SO_4 salts.[7] In addition, acid degradation of the 2-hydroxygentamicin mixture, obtained from the inosose **3** supplementation, afforded streptamine which was also characterized by tlc and vpc comparison to authentic streptamine (**2**) and streptamine hexaacetate.

The C_1 (**14**) components of the 2-hydroxygentamicin obtained from the inosose **3** and streptamine (**2**) supplementations were also compared by ^{13}C-nmr analysis. The lines of the spectra of the two samples (in D_2O, N_2 atmosphere) formed two sets with a one to one correspondence. However, the values of the chemical shifts of corresponding lines differed in many instances far beyond the limits of reproducibility. These differences could arise from either different isomers or from small differences in pH of the solution. The two samples were combined in a 1:2 ratio and the resulting spectrum showed only one set of lines clearly indicating that the two samples were identical. The ^{13}C chemical shifts are shown in Fig. 3. The values shown for the purpurosamine ($C_1 \sim C_8$) and the garosamine ($A_1 \sim A_7$) carbons correspond very well with the values reported by MORTON *et al.*[17] for gentamicin antibiotics. ^{13}C-nmr comparison of the 2-hydroxygentamicin C_2 (**15**) component from the inosose **3** and streptamine (**2**) sources could not be accomplished to our satisfaction because of different impurities present in the two samples.

The pentaacetate of 2, 4, 6/3, 5-pentahydroxycyclohexanone (**10**) also afforded the complex of 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**). *Myo*inositol (**4**) did not produce antibiotic when supplemented to a growing broth of *M. purpurea* VIb-3P. *Scyllo*inosamine-2 (**5**) when supplemented to *M. purpurea* VIb-3P afforded a mixture of the 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**). The C_1 component was identified by ms and tlc analyses. *Myo*inosamine-2 (**6**) and *myo*inosamine-4 (**7**) as well as the bromocyclitols (**11**) and (**12**) were not incorporated to form antibiotic.

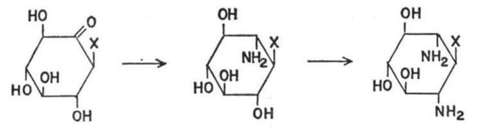
RINEHART *et al.*[18] have suggested a deoxyinosose (Scheme I, X=H) as an intermediate in the biosynthetic pathway to deoxystreptamine (**1**) in the deoxystreptamine aminoglycoside anti-

Fig. 4. Tlc chromatography of the gentamicin complex compared to the product obtained by supplementation of *dl*-deoxyinosose (**9**) to the broth of growing *M. purpurea* VIb-3P.
Solvent system; $CHCl_3$-MeOH-conc. NH_4OH (1 : 1 : 1, lower phase).



1=gentamicin. 2 and 3=different concentrations of product obtained from supplementation with **9**.

Scheme 1



biotics. In light of our results with inosose **3** it became apparent that *M. purpurea* VIb-3P may be a mutant blocked somewhere before the suggested deoxyinosose intermediate in the biosynthetic pathway to deoxystreptamine (**1**). We prepared *dl*-viboquercitol (**8**) from the bromocyclitol (**12**)[15] and by means of a microbiological oxidation using *Acetobacter suboxydans*[10,13] we obtained *dl* 2, 4/3, 5-tetrahydroxycyclohexanone (**9**).

According to the suggested biosynthetic pathway, one enantiomer of **9** should form deoxystreptamine (**1**). In fact, when **9** was supplemented to a growing broth of *M. purpurea* VIb-3P, the gentamicin complex [C_1 (**16**), C_2 (**17**) and C_{1a} (**18**)] of antibiotics was produced as shown by tlc (Fig. 4) and by comparison of the mass peaks and fragments of the isolated C_1, C_2 and C_{1a} components with authentic gentamicin C_1, C_2 and C_{1a} components.

The scheme suggested by RINEHART *et al.*[18] seems to be borne out by the results that we have obtained with our deoxystreptamine blocked mutant by conversion of **9** to the gentamicin complex and by the conversion of **3** and **5** to the 2-hydroxygentamicin complex.

We have also made the interesting observation that whereas *myo*inositol (**4**) is not converted by this organism to **3** and then to antibiotic as in the biosynthetic pathway to streptomycin,[19] *dl*-viboquercitol (**8**) did afford gentamicin as shown by tlc comparison of the product obtained after supplementation of a growing broth of *M. purpurea* VIb-3P. The unsaturated cyclitol

Table 1. *In vitro* antibacterial activity of 2-hydroxygentamicin C_1 and C_2 compared to gentamicin C_1 and C_2.

Test organism	Minimum inhibitory concentration mcg/ml			
	2–OH Gentamicin		Gentamicin	
	C_1 (**14**)	C_2 (**15**)	C_1 (**16**)	C_2 (**17**)
Staphylococcus aureus Smith	0.78	0.39	0.39	0.2
Escherichia coli Vogel	3.13	3.13	3.13	1.56
Escherichia coli W677/HJR66	50	100	>100	>100
Escherichia coli JR 35	3.13	1.56	3.13	1.56
Escherichia coli JR 76.2	6.25	12.5	50	50
Escherichia coli JR 89	100	50	100	50
Escherichia coli K12ML1629	3.13	1.56	1.56	1.56
Enterobacter cloacae A-20960	3.13	6.25	25	25
Klebsiella pneumoniae A-2063	6.25	6.25	50	50
Klebsiella pneumoniae 39645	3.13	1.56	1.56	0.78
Proteus mirabilis MGH-1	6.25	1.56	1.56	1.56
Providencia stuartii A-20894	>100	50	>100	100
Providencia 164	>100	25	>100	50
Pseudomonas aeruginosa A-20897	50	>100	>100	>100
Pseudomonas aeruginosa A-20741	>100	>100	>100	>100
Pseudomonas aeruginosa MGH-2	6.25	3.13	3.13	1.56
Pseudomonas aeruginosa C	6.25	3.13	3.13	1.56
Pseudomonas aeruginosa #2	6.25	1.56	1.56	0.78

(**13**) also afforded the gentamicin complex as identified by tlc comparison as well as mass spectral comparisons of the isolated C_1, C_2 and C_{1a} components with the authentic gentamicins. The conversion of **13** to **9** is not inconceivable.

The 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**) were screened against a series of gram-negative and gram-positive organisms by the tube dilution technique (Table 1).

The new 2-hydroxygentamicins C_1 (**14**) and C_2 (**15**) have the same spectrum of activity as gentamicin C_1 (**16**) and C_2 (**17**). Comparison of the C_1 and C_2 components against some gentamicin-sensitive strains of *P. aeruginosa* suggests that the new antibiotics may be less potent against these organisms. The new antibiotics have greater activity against the gentamicin-resistant strains, *E. coli* JR76.2, *E. cloacae* A-20960 and *K. pneumoniae* A-20636. These organisms are known to contain the 2″-OH-adenylating enzyme (GAS).

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