NEBRAMYCIN: SEPARATION OF THE COMPLEX AND IDENTIFICATION OF FACTORS 4, 5, AND 5′

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By the use of improved isolation and separation methods, it has been shown that the major components of the nebramycin complex produced by *Streptomyces tenebrarius* are factor 2 (apramycin), factor 4 (6″-O-carbamoylkanamycin B), and factor 5′ (6″-O-carbamoyltobramycin). Factor 5, which is identical to kanamycin B, and tobramycin are not produced by *Streptomyces tenebrarius* but arise from the acid or base catalyzed hydrolysis of 6″-O-carbamoylkanamycin B and 6″-O-carbamoyltobramycin respectively.

The nebramycin complex of aminoglycoside antibiotics is produced by *Streptomyces tenebrarius*.[1,2] The complex has been separated into nebramycin factors 2, 4, 5 and 6 which have been characterized.[3] The structure of nebramycin factor 6, now named tobramycin, has been reported[4] and is shown in Fig. 2. During the course of the examination of production of the nebramycin complex by *Streptomyces tenebrarius*, STARK *et al.*[5] showed that the parent strain produces mainly nebramycin factors 2, 4 and 5′.

In this paper we describe an improved method for the isolation and separation of nebramycin factors 2, 4 and 5′. We have also proven that factor 5 is identical to kanamycin B. The kanamycin B and tobramycin present in the complex previously described[3] are formed by base catalyzed hydrolysis of factor 4 and factor 5′ during isolation.

Experimental

TLC detection of nebramycin factors.

Factors 2, 4 and 5′ were separated by thin-layer chromatography on Merck silica gel G plates using a solvent system composed of chloroform-methanol-28 % ammonium hydroxide (1:3:2). The compounds were detected by ninhydrin or a modification of the PAN-DUTCHER[6] reagent in which starch iodide was replaced by a stable reagent consisting of 100 mg benzidine, 100 mg KI and 2 ml glacial acetic acid in 100 ml of water.

Assay.

Antibiotic activity of samples was measured by a turbidimetric method with *Klebsiella pneumoniae* FDA KL4 as the test organism using a standard assigned an activity of 500 μg/mg.

Quantitative determination of nebramycin factors in broth.

An aqueous slurry of Amberlite CG-50 (NH_4^+), 100~200 mesh, was packed into a column having an inside diameter of 1.2 cm to a height of 10 cm. One hundred ml of filtered broth was applied to the column and the column was washed with water. The column was eluted by a convex gradient, which consisted of 75 ml of 0.05 N NH_4OH in a constant volume mixing chamber and 300 ml of 0.3 N NH_4OH in an open reservoir. One ml fractions were collected for a total of 60 fractions. One μl of each fraction was used for thin-layer chromatography to detect the separated components. Identical fractions were pooled, concentrated and dried. Weights of separated

components are presented in Table 1.

Recovery of nebramycin complex from whole broth.

To 10 liters of whole fermentation broth (1.1×10^7 μ) 1 liter of IRC 50 (H^+) was added. The mixture was stirred for 3 hours and then filtered through a wire-screen. The filtrate ($0.1\times10^7\mu$) was discarded and the resin was washed with five 1-liter portions of water. The resin was eluted with five 2-liter portions of 1 N NH_4OH. No activity was found in the first two eluates. Combined eluates 3, 4 and 5 contained 7.1×10^6 μ of activity (70 % of theory).

Separation of nebramycin factors 2, 4 and 5′ on a large scale.

A solution of the nebramycin complex in 3.75 liters of water (82×10^6 μ) was diluted to 20 liters and applied to a 10 cm diameter column which contained 20 liters of BioRex 70 (NH_4^+). The column was washed with 12 liters of water and then eluted with a gradient prepared by adding 0.2 N NH_4OH to a 50-liter constant volume reservoir which was charged with 0.05 N NH_4OH. The flow rate was 20 ml/min and fractions of 500 ml were collected.

The following fractions were combined, concentrated, and freeze-dried: fractions 39~100, 102 g of factor 2, 35×10^6 μ; fractions 115~154, 14g of factor 4, 8.1×10^6 μ; fractions 188~240, 24g of factor 5′, 30×10^6 μ. Eighty-nine per cent of the activity was recovered.

Crystallization of nebramycin factor 4.

Crude nebramycin factor 4, 10 g, was heated to reflux in 400 ml of 2B ethanol. The hot mixture was filtered. The residue was heated overnight under reflux in an additional 400 ml of 2B ethanol. The extracts were cooled to room temperature and factor 4 was recovered by filtration. The first extract yielded 5.46 g of factor 4, $[\alpha]_D+122.8°$ (c 7.8, H_2O).

Anal. Calc. for $C_{19}H_{38}N_6O_{11}\cdot H_2O$: C 41.90, H 7.40, N 15.43
Found: C 41.91, H 7.53, N 15.16

The second extract gave an additional 1.39 g of factor 4.

Penta-N-acetyl nebramycin factor 4.

A 1.0 g sample of factor 4 was suspended in 100 ml of absolute methanol. When 10 ml of acetic anhydride was added, the solid material dissolved. The solution was stirred at room temperature for 7 days. The methanol was removed *in vacuo* with a rotary evaporator and the oily residue was crystallized from aqueous methanol. Filtration of the mixture gave 0.67 g of penta-N-acetyl nebramycin factor 4. $[\alpha]_D+112°$ (c 1.5, H_2O).

Anal. Calc. for $C_{29}H_{48}N_6O_{16}$: C 47.28, H 6.57, N 11.41
Found: C 47.35, H 6.71, N 11.21

Crystallization of nebramycin factor 5′.

Crude nebramycin factor 5′, 23 g, was heated under reflux with decolorizing carbon in 1.2 liter of 2B ethanol. The mixture was filtered while hot and the filtrate was allowed to cool to room temperature and evaporate until crystallization occurred. Factor 5′, 6.9 g, was recovered by filtration. $[\alpha]_D+120°$ (c 1, H_2O).

Anal. Calc. for $C_{19}H_{38}N_6O_{10}\cdot 2H_2O$: C 41.75, H 7.75, N 15.38
Found: C 41.80, H 7.37, N 15.07

Upon further evaporation, an additional 1.9 g of factor 5′ was recovered from the filtrate.

Penta-N-acetyl nebramycin factor 5′.

A 2 g sample of factor 5′ was suspended in 200 ml absolute methanol. When 20 ml of acetic anhydride was added, the solids went into solution. After 30 minutes, a solid reprecipitated. The mixture was stirred at room temperature for 16 hours and the product, 2.0 g of penta-N-acetyl nebramycin factor 5′ was isolated by filtration. $[\alpha]_D+105°$ (c 10, H_2O).

Anal. Calc. for $C_{29}H_{48}N_6O_{15}\cdot H_2O$: C 47.15, H 6.82, N 11.38
Found: C 47.37, H 6.60, N 11.27

Conversion of factor 4 to factor 5.

A solution of 10 g of nebramycin factor 4 in 1 liter of 0.1 N NaOH was heated on a steam bath, 85~90°C, for 22 hours. The solution was allowed to cool to room temperature and its pH was adjusted to 9.4 with 60 ml of 1N HCl. BioRex 70 (NH_4^+), 400 ml, was added to this solution and

the mixture was poured into a 2.5 mm I.D. column. The effluent, 1.1 liter, was inactive. The column was washed with 1.4 liter of distilled water and finally with 1 liter of 1N NH_4OH. The water wash was inactive. The eluate from the NH_4OH was collected in 20 ml fractions. Fractions 18~25 were combined and concentrated to an oil. The oil was dissolved in 100 ml of boiling absolute methanol. The gelatinous solid, 5.1 g of nebramycin factor 5, which formed when the solution was cooled to room temperature was recovered by filtration. $[\alpha]_D+139°$ (c 4.6, H_2O).

Anal. Calc. for $C_{18}H_{37}N_5O_{10}\cdot H_2O$: C 43.11, H 7.84, N 13.96
Found: C 43.21, H 7.86, N 13.69

Penta-N-acetyl nebramycin factor 5.

A 1.0 g sample of factor 5 was suspended in 100 ml of absolute methanol. Ten ml of acetic anhydride was added and the mixture was stirred at room temperature for 16 hours. Penta-N-acetyl nebramycin factor 5, 0.8 g, was recovered by filtration of the mixture. $[\alpha]_D+115°$ (c 10, H_2O).

Anal. Calc. for $C_{28}H_{47}N_5O_{15}\cdot H_2O$: C 47.25, H 6.94, N 9.84
Found: C 47.27, H 6.59, N 10.05

Conversion of factor 5′ to tobramycin.

A solution of factor 5′ (1.0 g) in 100 ml of 0.1 N NaOH was heated at 95~100°C for 8 hours. The solution was adjusted to pH 10.0 with sulfuric acid and applied to a 2.5×50 cm BioRex 70 (NH_4^+) column. The column was eluted with a gradient 2 liters of 0.01 N NH_4OH in the mixing chamber and 0.5 N NH_4OH in the reservoir. Fractions containing 20~25 ml were collected. Fractions 57 and 58 gave 0.02 g of recovered factor 5′ and fractions 119~131 gave 0.80 g of amorphous tobramycin. Recrystallization of this material from ethanol gave 0.72 g of crystalline tobramycin, X-ray powder pattern identical to that of tobramycin standard.

Isolation of $BaCO_3$ from the hydrolysis of nebramycin factor 5′ with $Ba(OH)_2$.

A solution of 5 g (10 mmoles) of nebramycin factor 5′ and 6.3 g of $Ba(OH)_2\cdot 8H_2O$ in 200 ml of water was heated under reflux for 1 hour. The cooled reaction mixture was filtered. The X-ray powder pattern of the solid, 1.77 g, 9 mmoles $BaCO_3$, was identical to that of authentic $BaCO_3$. The filtrate contained tobramycin.

In a similar manner $BaCO_3$ was isolated from the conversion of factor 4 to factor 5.

Determination of ammonia in nebramycin factors 4, 5 and 5′ and tobramycin.

Small samples of nebramycin factors 4, 5, and 5′ and tobramycin were hydrolyzed with 6 N HCl for 1 hour at 120°C. Quantitative ammonia determinations were performed on these hydrolysates by the alkaline-phenol method.[7] The results are shown in Table 3.

Identity of kanamycin B and nebramycin factor 5.

A solution of 200 mg of kanamycin B sulfate (Kanendomycin, Meiji Seika Kaisha, Ltd.) in 0.5 ml of H_2O was applied to a 0.7×11.0 cm column of AG 1×4 (OH^-). The column was eluted with water and 3 ml fractions were collected. Fractions 1~4 were combined and freeze-dried. The solides (140 mg) were dissolved in a mixture of 15 ml methanol and 1.5 ml acetic anhydride. After the mixture was stirred at room temperature for 6 hours the solids which had formed were removed by filtration, washed with methanol and dried. The crystals, $[\alpha]_D+115°$ (c 0.29, H_2O) had the correct analysis for penta-N-acetyl kanamycin B. The X-ray powder pattern of this sample of penta-N-kanamycin B was identical to that of penta-N-acetyl nebramycin factor 5.

Results and Discussion

In support of a study of the biosynthesis of the nebramycin complex by strains of *Streptomyces tenebrarius*,[5] we developed a method for the quantitative determination of the factors being produced. Filtered broth is passed through a column of Amberlite CG-50 (NH_4^+) resin. The column is washed with water and the nebramycin factors are eluted with a convex gradient[8] prepared by mixing 0.2 ~0.3 N NH_4OH with 0.05 N NH_4OH in a closed vessel. Nebramycin factors 2 and 4 and a new compound, factor 5′, are eluted in that order. Under these conditions of isolation and separation,

Table 1. Quantitative determination of nebramycin factors produced by *Streptomyces tenebrarius**.

Culture	Factor 2		Factor 4		Factor 5′	
	mg	%	mg	%	mg	%
Parent	85.7	77	11.7	10	14	13
Strain 12	109.6	54	46.5	23	46.7	23
Strain 23	72.8	57	4.3	3	50	39

* Reported as mg isolated from 100 ml of filtered fermentation broth and percent factor in complex.

Table 2. Physical data on nebramycin factors.

Compound	Molecular formula	PMR*		Assignment
		ppm**	Integral	
Factor 2, apramycin	$C_{21}H_{41}N_5O_{11}$***			
Factor 4 6″–O–carbamoyl-kanamycin B	$C_{19}H_{38}N_6O_{11}\cdot H_2O$	6.63 (d)	1 H	anomeric H
		5.75 (d)	1 H	anomeric H
		5.0~4.8 (m)	2 H	CH_2OCOND_2
		4.8~3.8 (m)	15 H	CHOD
				$CHND_3+$,
				CH_2ND_3+
		3.4~2.2 (m)	2 H	CH_2
Factor 5 kanamycin B	$C_{18}H_{37}N_5O_{10}\cdot H_2O$	6.58 (d)	1 H	anomeric H
		5.75 (d)	1 H	anomeric H
		4.9~3.8 (m)	17 H	CHOD, CH_2OD
				$CHND_3+$
				CH_2ND_3+
		3.5~2.2 (m)	2 H	CH_2
Factor 5′ 6″–O–carbamoyl-tobramycin	$C_{19}H_{38}N_6O_{10}\cdot 2H_2O$	6.29 (d)	1 H	anomeric H
		5.60 (d)	1 H	anomeric H
		5.0~4.7 (m)	2 H	CH_2OCOND_2
		4.7~3.8 (m)	14 H	CHOD
				CH_2ND_3+,
				$CHND_3+$
		3.5~2.2 (m)	4 H	CH_2
Tobramycin	$C_{18}H_{37}N_5O_9\cdot H_2O$	6.38 (d)	1 H	anomeric H
		5.73 (d)	1 H	anomeric H
		4.8~3.8 (m)	16 H	CHOD, CH_2OD
				$CHND_3+$,
				CH_2ND_3+
		3.5~2.2 (m)	4 H	CH_2
Ethyl carbamate	$C_3H_7NO_2$	4.55 (q)	2	CH_2OCOND_2
		1.67 (t)	3	CH_3
Diethyl urea	$C_5H_{12}N_2O$	3.67 (q)	2	CH_2NDCO
		1.60 (t)	3	CH_3

* Varian HA 60, D_2O–DCl.
** Relative to tetramethylsilane, external standard in coaxial capillary.
*** Dr. S. O'Connor, 165th ACS National Meeting.

factor 5 (kanamycin B) and tobramycin are not detected in the fermentation broth. The ratios of factors 2, 4, and 5′ produced[5] by several *Streptomyces tenebrarius* strains are shown in Table 1.

Modification of the isolation and separation scheme described above has enabled us to purify and characterize nebramycin factors 2, 4, and 5′. Thus, treatment of whole broth with IRC 50 (H^+) leads to quantitative removal of the complex from the broth. The loaded resin, which is recovered by filtration of the mixture through a wire-screen, is washed with water, the complex is eluted from the resin with 1 N NH_4OH and the active eluate is concentrated under vacuum to remove excess ammonia. The ammonia-free concentrate is then applied to a Bio-Rex 70 (NH_4^+) column and separated by elution with the convex gradient described above. In this manner large amounts of factors 2, 4 and 5′ can be isolated. These factors as well as factor 5 have been crystallized from methanol or ethanol. Revised molecular formulas of these compounds are given in Table 2.

Crystalline nebramycin factor 5 has a molecular formula that is identical to the molecular formula of kanamycin B.[9] In addition, the optical rotations of these compounds are similar. When nebramycin factor 5 and kanamycin B were compared directly to one another by paper chromatography,[8] slight variations in their movement were observed. We have prepared the penta-N-acetyl derivative of each compound and find that the X-ray powder patterns the two penta-N-acetyl compounds are identical. Thus, nebramycin factor 5 is identical to kanamycin B.

In the initial work on the isolation and separation of the nebramycin complex,[3] factors 2, 4, kanamycin B and tobramycin were isolated and characterized. The current report shows that the

Fig. 1. Procedures for the isolation of the nebramycin complex.

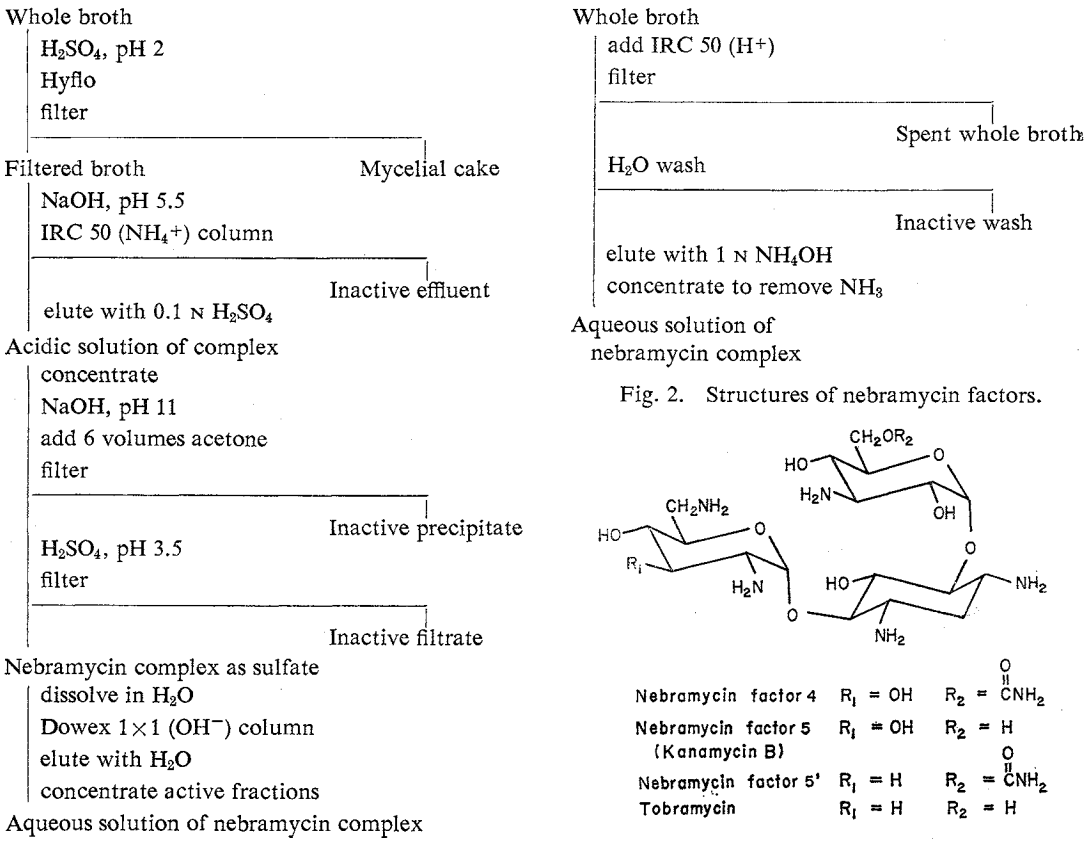

Fig. 2. Structures of nebramycin factors.

Nebramycin factor 4	R_1 = OH	R_2 = CONH$_2$
Nebramycin factor 5 (Kanamycin B)	R_1 = OH	R_2 = H
Nebramycin factor 5′	R_1 = H	R_2 = CONH$_2$
Tobramycin	R_1 = H	R_2 = H

complex produced by *Streptomyces tenebrarius* consists of factors 2, 4 and 5′. Kanamycin B and tobramycin are not present in the nebramycin complex when it is isolated by the method described above. A clue to the cause of this apparent change in the composition of the nebramycin complex was obtained by comparing this simplified procedure for isolating the nebramycin complex from whole broth to the original procedure (see Fig. 1). At two steps of the original procedure,[3] the complex was exposed to strongly basic conditions. After the complex was eluted from an IRC 50 (NH_4^+) column with dilute sulfuric acid, the pH was adjusted to eleven and inactive solids were precipitated with acetone. The precipitation at this pH stood overnight. Later the sulfated form of the complex was converted to the free base by passage through a Dowex 1×1 (OH^-) resin. Since either of these steps might cause hydrolysis of unstable compounds, we examined the effect of aqueous base on factors 4 and 5′. We have now shown that factor 4 is converted to kanamycin B when heated in 0.1 N NaOH at 99°C. Under similar conditions, factor 5′ is converted to tobramycin.

From the molecular formulas of purified nebramycin factors, which are shown in Table 2, the conversion of factor 4 to factor 5 and the conversion of factor 5′ to tobramycin can be explained by the loss of carbon dioxide and ammonia as shown below.

$$\underset{\text{Factor 4}}{C_{19}H_{38}N_6N_{11}} + H_2O \xrightarrow{OH^-} \underset{\text{Kanamycin B}}{C_{18}H_{37}N_5O_{10}} + CO_2 + NH_3$$

$$\underset{\text{Factor 5}}{C_{19}H_{38}N_6O_{10}} + H_2O \xrightarrow{OH^-} \underset{\text{Tobramycin}}{C_{18}H_{37}N_5O_9} + CO_2 + NH_3$$

In fact $BaCO_5$ has been isolated from the hydrolysis of both factor 4 and factor 5′ with 0.1 N $Ba(OH)_2$. Furthermore, hydrolysis of the factors with 6 N hydrochloric acid liberates 1 equivalent of ammonia per mole of factors 4 and 5′ (see Table 3). Only traces of ammonia are found in the hydrolysates of kanamycin B and tobramycin. On the basis of these data, it appears that factors 4 and 5′ are carbamoyl or ureido derivatives of kanamycin B and tobramycin respectively.

The exact nature of the $-CONH_2$ group in factors 4 and 5′ was elucidated by preparing their penta-N-acetyl derivatives and by studying the infrared and nuclear magnetic resonance spectra of factors 4 and 5′.

The infrared spectra of the nebramycin factors are characteristic of aminoglycodides. However, in addition of strong bands for OH and NH_2 vibrations, the spectrum of factor 4 has a band at 5.8 μ and the spectrum of 5′ has a band at 5.9 μ. The position of this band is characteristic of the carbamoyl group rather than ureido group.[10]

When treated with acetic anhydride in methanol, aminoglycosides are converted to their N-acetylated derivatives. We have prepared the N-acetyl derivatives of nebramycin factors 4, 5 and 5′ by this method. The derivatives were shown by microanalysis to be penta-N-

Table 3. Determination of ammonia in hydrolysates of nebramycin factors[a].

	mgN/mg Sample	
	Theory	Found
Factor 4	0.0266[b]	0.0261
Factor 5	0.0000[c]	0.0059
Factor 5′	0.0274[b]	0.0220
Tobramycin	0.0000[c]	0.0064
Methyl 3-amino-3-deoxy-β[D]-glycopyranoside[d]	0.0000[c]	0.0022
Nebramin[d]	0.0000[c]	0.0063

[a] See ref. 7.
[b] Release of 1 mole NH_3/mole compound.
[c] Release of no NH_3.
[d] See ref. 4.

acetyl nebramycin factor 4, penta-N-acetyl factor 5, and penta-N-acetyl factor 5′. Under these mild conditions the $-CONH_2$ would not be acetylated; hence there are five free amino groups in factors 4 and 5′ and the $-CONH_2$ group must be present as a carbamate.

The NMR spectra of the nebramycin factors is summarized in Table 2. The only difference between the spectrum of factor 4 and that of kanamycin B is in the region of 5.0~3.8 ppm. Thus, the NMR spectrum of kanamycin B shows a complex pattern for 17 protons between 4.9 and 3.8 ppm, while that of factor 4 shows a 2-proton multiplet at 5.0~4.8 ppm and a complex pattern for the remaining 15 protons between 4.8~3.8 ppm. A similar difference is seen between the spectrum of tobramycin and that of factor 5′. The 2-proton multiplet is at the same chemical shift as the CH_2 group in ethyl carbamate and much further down field that the CH_2 group in diethyl urea; once again, we place the $-CONH_2$ moiety on an OH rather than an NH_2. Since the multiplet integrates for approximately 2 protons, we conclude that the carbamoyl group is attached to the 6-hydroxyl of the 3-amino glucose unit of these compounds.

The carbon magnetic resonance spectra of these compounds provides further evidence for the attachment of the carbamoyl group to this hydroxyl group.[11]

The structures of nebramycin factor 4 (6″-O-carbamoyl-kanamycin B), nebramycin factor 5 (kanamycin B), nebramycin factor 5′ (6″-O-carbamoyltobramycin) and tobramycin are given in Fig. 2.

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