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Microscopic protonation constants in tobramycin. An NMR and pH study with the aid of partially *N*-acetylated derivatives

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ABSTRACT

Three tetra-*N*-acetyl derivatives and one tri-*N*-acetyl derivative of tobramycin (**1**) have been prepared by partial *N*-acetylation. Comparison of the pK_a values, determined by NMR chemical shift titrations and pH titration of the derivatives, with those of unprotected **1** suggests that protonation equilibria at any particular amino group in **1** are not likely to be influenced by those at other sites. pH-Dependent conformational changes in **1** were assessed on the basis of ^1H and ^{13}C chemical shift changes in the derivatives.

INTRODUCTION

The exact molecular mechanisms responsible for the antibacterial activity of aminoglycoside antibiotics have not yet been fully elucidated[1]. There is no doubt, however, that the number and distribution pattern of amino groups in these molecules are significant with respect to biological function. It is furthermore anticipated that pH-dependent protonation should play a role in their binding and transport properties[2,3]. pK_a Values, estimated by ^{15}N and ^{13}C NMR chemical shift titration, have been published for apramycin[4–6], neomycin-B[7], and tobramycin[4,6]. Published values for apramycin and tobramycin, in particular, indicate relatively small differences in the basicities of the NH_2 groups in the same molecule. It is known, however, that the principle of stepwise dissociation breaks down with polyprotic acids or bases containing groups of similar basicities, and macroscopic pK_a values are therefore not related directly to the individual basicities of these groups[8]. In an attempt to obtain access to pK_a values of individual amino groups without interference from the others in tobramycin, we set out to synthesize

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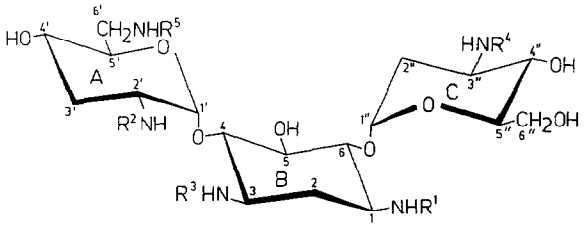
N-protected derivatives, each having a single amino group free. Partial *N*-acetylation led to the formation of two tetraacetyl derivatives, **3** and **4**, and one triacetyl derivative, **5**. A third tetra-*N*-acetyl derivative, **6**, was obtained via 6′-*N*-benzyloxycarbonyltobramycin[9] as an intermediate. The pK_a values of the free amino groups in these compounds have been determined by NMR and pH titration, and the results compared with data for unprotected tobramycin. Furthermore, pH-dependent conformational changes in tobramycin could be assessed, using ^1H and ^{13}C chemical shifts, with the aid of these partially protected derivatives.

RESULTS AND DISCUSSION

When tobramycin free base (**1**) is allowed to react with two equivalents of acetic anhydride in methanol at room temperature, TLC monitoring of the reaction indicates formation, within a few minutes, of four new compounds, **2**, **3**, **4**, and **5** (in the order of decreasing R_f values). Changing the tobramycin–acetic anhydride molar ratio or the reaction time did not influence appreciably the product distribution. Acetylation with acetic anhydride in aqueous solution at constant pH[10] (~ 9.0) led to a similar result, while use of triethylamine[11] in a water–methanol mixture yielded mainly **2** and **3** and only trace amounts of **4** and **5**. The products have been separated by column chromatography on silica gel.



	1	2	3	4	5	6
R^1	H	Ac	Ac	Ac	Ac	Ac
R^2	H	Ac	Ac	Ac	Ac	Ac
R^3	H	Ac	Ac	H	H	Ac
R^4	H	Ac	H	Ac	H	Ac
R^5	H	Ac	Ac	Ac	Ac	H

Compound **2** proved to be identical with the known[12,13] penta-*N*-acetyltobramycin. ^1H NMR data indicated the presence of four acetyl groups in both **3** and **4**, whereas three acetyl groups were detected in **5**. Another piece of evidence from the ^1H NMR spectra is that the chemical shifts of H-2′, H-3′e, and H-3′a display very little pH-dependence in **3**, **4**, and **5** (Table I); this shows that NH_2-2′ is

TABLE I

^{1}H NMR data [a]

Position	1 [b]	2	3	4	5	6
1′	5.76(0.65)	5.28(0.01)	5.29(0.02)	5.28(0.20)	5.22(0.24)	5.36(0.15)
2′	3.67(0.75)	3.95(~0)	3.95(−0.02)	4.08(0.10)	4.03(0.03)	4.02(0.10)
3′e	2.29(0.30)	1.95 [d](~0)	~2.0 [d](~0)	~2.0 [d](~0)	2.07(~0)	2.00(~0)
3′a	2.02(0.45)	1.63(0.02)	1.63(~0)	1.82(0.03)	1.78(0.06)	1.70(0.10)
2e	2.55(0.63)	1.95 [d](~0)	~1.9 [d](~0)	2.30(0.17)	2.23(0.23)	2.05(~0)
2a	1.99(0.80)	1.48(~0)	1.50(0.03)	1.71(0.36)	1.67(0.40)	1.52(0.10)
3	3.59(0.76)		> 3.3 [c]	~3.5(> 0.66)	> 3.4 [c](0.56)	
1″	5.12(0.11)	5.14(0.01)	5.14(0.07)	5.16(~0)	5.13(0.06)	5.13(0.02)
2″	3.95(049)	4.05(0.01)	3.74(0.26)	4.09(0.04)	3.75(0.27)	4.08(0.06)
3″	3.50(0.54)	3.53(−0.01)	3.40(0.43)	3.56(0.03)	3.75(0.40)	3.55(0.03)
Others						2.80(0.47) 2.86(0.50) [6′a,b]

[a] For solutions in D_2O at 23°C, pD ~ 2; reference: internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Protonation shifts, δ(pD ~ 2) − δ(pD ~ 11), are given in parenthesis. [b] Taken from ref 15. [c] Interchangeable with H-1. [d] Overlapped by acetyl methyl resonances.

acetylated in these derivatives. On the other hand, sizeable protonation shifts of the signals for H-2″ and and H-3″ and for H-2e and H-2a in **5** (Table I) indicate that NH_2-3″ and either NH_2-1 or NH_2-3 are free in this derivative. Similar considerations suggest that **3** has a free NH_2 in position 3″, whereas in **4** it is in position 1 or 3 (Table I). The positions of the free amino groups in these derivatives could then be unequivocally determined using amino group protonation shift measurements[4,14] in the ^{13}C NMR spectra. Only two signals show appreciable pH-dependence (Table II) for both **3** and **4**. This is in agreement with the presence of a single free amino group in both derivatives, as deduced from the ^{1}H NMR spectra. Comparison of the chemical shifts of the titratable carbon signals and the magnitudes of protonation shift increments measured for **3** (Table II) with literature data[4,15] allows these signals to be assigned to C-2″ and C-4″. It follows, therefore, that **3** has the free amino group in position 3″ (i.e., **3** is 1,3,2′,6′-tetra-*N*-acetyltobramycin). In the case of **4**, it is apparent that one of the β-upfield shifts is approximately twice that of the other one (Table II). In the free tobramycin molecule, such titration behavior has been observed[4,15] for C-4; the signal at 88.10/81.28 ppm (pH ~ 11/pH ~ 1) was therefore assigned to C-4 in **4** as well. The second resonance displays a regular protonation β-shift and, since this is a methylene carbon, it is assigned to C-2; **4** is therefore 1,2′,6′,3″-tetra-*N*-acetyltobramycin. The ^{13}C NMR spectra of **5** represent a superposition of those of **3** and **4** as far as the pH-titration behavior is concerned (Table II). Together with concurrent evidence, mentioned above, from ^{1}H NMR, **5** is identified thereby as 1,2′,6′-tri-*N*-acetyltobramycin. In addition to these partially *N*-protected derivatives, we have also synthesized 1,3,2′,3″-tetra-*N*-acetyltobramycin (**6**). 6′-*N*-Benzyloxycarbonyltobramycin[9] (**7**) was obtained using a recent procedure[16] elaborated for

TABLE II

^{13}C NMR data [a]

Carbon	1 [b]	2	3	4	5	6
1′	99.99(5.96)	98.06(0.48)	98.77(0.59)	98.60(−0.22)	98.81(0.28)	98.27(−0.06)
2′	49.38(1.45)	47.83(0.22)	47.83(0.05)	48.48(0.92)	48.61(1.04)	49.04(−0.03)
3′	35.25(5.90)	31.58(−1.10)	31.39(−1.41)	31.67(0.62)	31.84(0.80)	32.87(0.03)
4′	66.48(1.82)	64.94(0.17)	64.87(0.04)	65.76(0.75)	65.80(0.82)	65.03(0.07)
5′	74.16(3.80)	72.76(0.38)	72.26(0.24)	72.43(0.15)	72.50(−0.20)	71.70(2.70)
6′	41.94(0.87)	39.70(0.06)	39.63(0.02)	40.18(0.63)	40.27(0.70)	40.70(0.73)
1	50.46(0.72)	48.78(−0.24)	49.00(0.10)	48.64(−1.14)	49.69(−0.13)	48.08(−0.06)
2	35.94(8.12)	32.91(1.68)	32.84(1.51)	34.28(3.94)	34.31(3.94)	31.93(0.15)
3	49.67(1.09)	48.29(0.15)	48.18(0.02)	49.13(0.68)	49.14(0.80)	47.76(0.12)
4	86.95(9.75)	80.37(0.72)	79.87(0.27)	88.10(6.82)	88.40(7.09)	81.31(0.10)
5	74.70(0.48)	75.14(−0.30)	75.56(−0.02)	74.39(0.31)	74.42(0.31)	75.35(0.13)
6	88.40(4.79)	81.41(−0.02)	81.80(0.01)	81.79(0.98)	82.21(0.93)	79.08(~0.17)
1″	100.04(−0.51)	96.55(−0.33)	96.23(0.11)	98.35(0.83)	98.51(0.99)	95.40(0.09)
2″	71.17(4.07)	70.19(0.48)	71.47(3.46)	69.86(0.06)	71.74(3.76)	69.84(0.06)
3″	54.57(−0.44)	54.53(0.44)	54.07(−1.15)	54.09(0.05)	54.21(−1.03)	54.06(−0.02)
4″	69.82(4.29)	67.86(0.49)	69.32(3.78)	67.41(0.05)	69.34(3.88)	67.48(−0.0)
5″	72.53(−0.40)	71.66(0.32)	71.65(0.24)	72.35(−0.12)	72.30(0.26)	72.44(0.00)
6″	60.79(0.75)	60.43(0.31)	60.33(0.74)	60.10(0.01)	60.34(0.82)	60.19(−0.05)

[a] For solutions in D_2O at 23°, pD ~ 11; reference: internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Protonation shifts, δ(pD ~ 11) − δ(pD ~ 1), are given in parentheses. [b] Taken from ref 15.

regioselective acylation of carbohydrates. *N*-Acetylation with acetic anhydride in methanol followed by hydrogenolytic deprotection then provided **6** in good yield. The structure of **6** is corroborated by the pH-dependence of ^{13}C chemical shifts, with only one carbon resonance revealing a protonation β-upfield shift (Table II); this can only be for C-5′. ^{1}H NMR data in Table I also support this assignment.

pK_a *Studies.*—Compounds **3**, **4**, and **6** offer the possibility to determine pK_a values of individual amino groups and compare them with those obtained for the partially protected derivative **5** and the unprotected parent compound, **1**. We have used both ^{13}C NMR chemical shift- and volumetric pH-titration methods for determining pK_a values. The pH-titration curves (not shown) display, as expected, well-defined, narrow steps for the tetra-*N*-acetyl derivatives **3**, **4**, and **6**; the transition is wider for the tri-*N*-acetyl derivative **5**, and very broad for tobramycin (**1**) itself. A theoretical fit, with the aid of the PSEQUAD program[17], of the protonation equilibria, assuming six (**1**, $\mathbf{1}^+$, $\mathbf{1}^{2+}$, ... $\mathbf{1}^{5+}$) and three (**5**, $\mathbf{5}^+$, $\mathbf{5}^{2+}$) species for **1** and **5**, and two for each of **3**, **4**, and **6**, respectively, yielded the protonation constants collected in Table III. From a concentration distribution calculated[17] for **1** (Fig. 1), it is evident that at least two protonated species are present at any pH in this protonation equilibrium. Another set of pK_a values can be obtained by NMR chemical shift titrations. For NH_2 groups, pH-dependence of ^{15}N chemical shifts appears to provide the most obvious direct access to pK_a values. An early ^{15}N NMR study[6] of tobramycin provided the values displayed in

TABLE III

Comparison of pK_a values [a]

Compound	Position					Method [b]
	1	2′	3	3″	6′	
1	7.6	8.0; 7.9; 8.2	7.4	7.6; 7.8; 7.7	8.7 8.9	A
	7.4	8.2; 8.0	7.2	8.1; 8.0	8.9	B
	6.2 [c]	7.6	7.4 [c]	7.4	8.6	C
	5.7 [c]	7.8 [c]	7.1 [c]	8.3 [c]	8.9 [c]	D
3				8.0; 8.1		B
				8.1		D
4			7.2			B
			7.1			D
5			7.0	8.2; 8.1		B
			6.9 [c]	8.1 [c]		D
6					9.1	B
					8.6	D

[a] Determined for D_2O solutions at 23°C for methods A and B; pK_a values are corrected[19] for the deuterium isotope effect: $pK_a(H) = pK_a(D) - 0.4$. [b] A, ^1H NMR, B, ^{13}C NMR; C, ^{15}N NMR; determined[6] for solutions in 9:1 H_2O–D_2O; D, pH-titration. [c] Interchangeable assignments.

Table III. The low sensitivity and the difficulties of obtaining unequivocal assignments (for NH_2 groups with rapidly exchanging protons, assignments are based on low-sensitivity 1D or 2D methods based on long-range couplings) make this

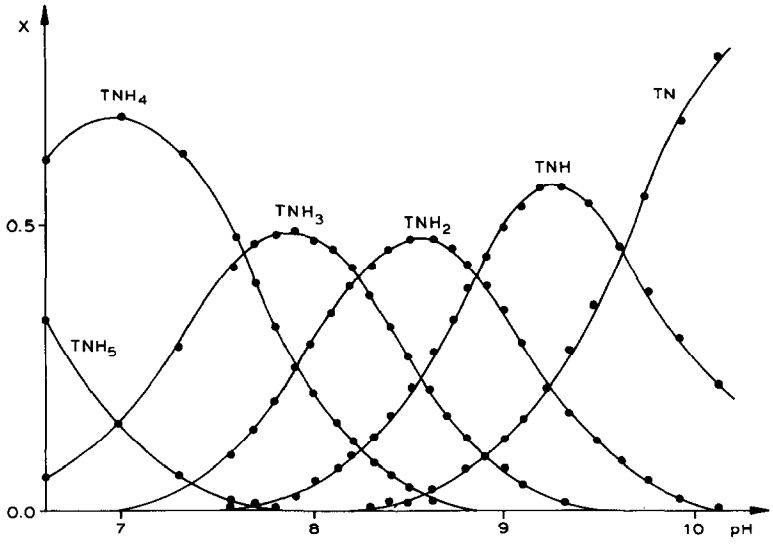


Fig. 1. Concentration distribution of different species in the protonation equilibria of 1 as a function of the pH calculated by the PSEQUAD[17] routine: X denotes the molar ratio.

method unattractive for routine applications. The well-known upfield shifts of β-carbon resonances[4,14] upon NH_2-protonation offer a much better alternative. Very often, the presence of more than one β-carbon provides access to multiple data for a given protonation process. ^{13}C Chemical shift titration curves have been published[4] for tobramycin without calculation of the protonation constants. We have repeated this experiment (data not shown) and extracted a set of pK_a values as displayed in Table III. It is seen that when titration of a given NH_2-group affects two β-carbon resonances (sites 2′ and 3″, for instance), the pK_a values calculated therefrom are in good agreement. For sites 1 and 3, on the other hand, only C-6 and C-4, respectively, have been considered for calculation of pK_a values because C-2 is affected by protonation processes at both of the amino groups in question.

It is well known that amino group protonation influences the chemical shifts of protons attached to carbon atoms in α, β, and γ positions. Therefore, ^1H NMR titration curves can, in addition, be exploited for the determination of protonation constants. We have determined the pH-dependence of ^1H chemical shifts for nonexchangeable hydrogens of tobramycin (Fig. 2), based on our previous complete ^1H-assignments[15]. For the reasons mentioned above, protonation constants for amino groups in positions 1 and 3 have been calculated from titration curves for H-6 and H-4, respectively (Fig. 2); δ-effects (e.g., shift of H-4 due to protona-

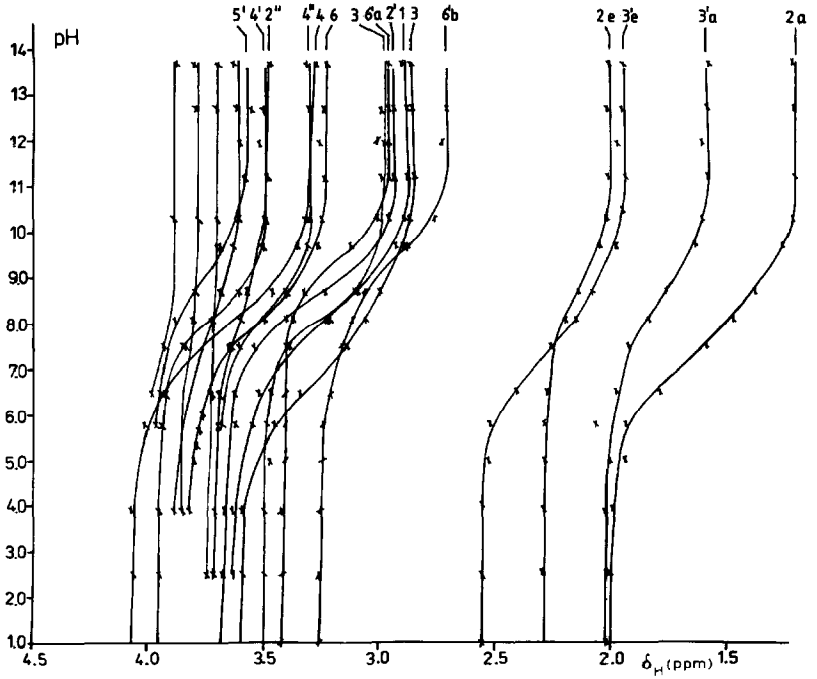


Fig. 2. pH-Dependence of ^1H chemical shifts for non-anomeric CHs in **1** based on the assignments in ref 15. Labels identify curves of titratable ^1H signals only.

tion of NH_2-1) were neglected ($\Delta\delta$ at CH_2-6″ is ~ 0.04–0.09 ppm, see ref 15). pK_a Values from this source are in good agreement with data obtained from ^{13}C NMR (Table III).

The macroscopic protonation constants calculated from volumetric pH-titrations for **1** and **5** cannot be assigned clearly in terms of individual protonation processes at any particular site, since more than one NH_2-group is affected, due to the similar basicities, at any pH (see above, Fig. 1); in other words, the principle of stepwise protonation breaks down here, as mentioned above. On the other hand, pK_a values derived from NMR data are certainly assignable to individual sites in **1** or **5** but, at the same time, they may still be composite values in the sense that they represent contributions from all components in the protonation equilibrium. These values can therefore be termed as *pseudo*-microscopic pK_a values in contradistinction to true microscopic constants characteristic for single, well-defined protonation events. Obviously, pK_a values, determined either by pH titration or NMR, for the *N*-protected derivatives **3**, **4**, and **6**, each having a single amino group free, represent true microscopic values. Then, if we assume that blocking any four of the five amino groups in **1** does not affect significantly the basicity of the one remaining free, the above pK_a values can be considered as reasonable estimates of the respective microscopic dissociation constants in **1**. It is remarkable that, in this particular case, these values are close to the respective *pseudo*-microscopic pK_a values of **1** obtained by ^{13}C or ^{1}H NMR chemical shift titrations (Table III). Consequently, protonation of any one of the five NH_2 groups in **1** appears not to be appreciably influenced by protonation at other sites. Since interactions between groups capable of protonation can originate from either through-bond (inductive) or through-space (electrostatic) effects, this observation indicates the absence of both at least for positions 3, 6′, and 3″. The vanishing of through-bond interactions can be rationalized on the grounds that any two amino groups in **1** are separated by at least four bonds. On the other hand, electrostatic repulsion between protonated amino groups in positions 3 and 6′ has been ascertained by ^{13}C NMR (see below). The pertinent microscopic pK_a values for **4** and **6**, respectively, are, however, identical with apparent (or *pseudo*-microscopic) values for **1**, within experimental error (Table III). It appears, therefore, that these two groups move to positions, by virtue of the conformational change manifested in ^{13}C chemical shifts (see below), such that their distance becomes large enough to allow practically independent protonation at each site. In other words, the lack of a through-space (electrostatic) effect on pK_a values in this particular case is a consequence of conformational mobility. The present study provides no direct data for the microscopic pK_a values of NH_2-1 and NH_2-2′ in **1**. Following the lines of the foregoing reasoning, it seems likely, however, that these values are close to the respective *pseudo*-microscopic pK_a values obtained from ^{1}H or ^{13}C chemical shift titration experiments (Table III). While the agreement between data from these two sources is very good, those from ^{15}N NMR[6] deviate significantly, especially for NH_2-1 (or NH_2-3). The cause of this discrepancy is not clear to us at present, but

we notice that published[6] ^{15}N titration curves for sites 1, 3, and 2′ show four crossover points in the pH range of interest and, as the number of experimental points is only five in this range, it is somewhat difficult to trace and identify the resonance signals at each point along the titration curve.

Conformational considerations.—Differences in the chemical shift of H-1′ in the protonated and free base forms of kanamycin A and in certain synthetic analogs have been interpreted[2] in terms of conformational changes around the O–C-4 glycosidic bond brought about by H-bonding of NH_2-3 with the sugar-ring O-5′ in the protonated state. In derivatives bearing an amino group at position 2′, like kanamycin B or tobramycin, the chemical shift of H-1′, is, however, largely influenced by the direct (β) effect of the protonation at NH_2-2′ and indirect conformational contributions, if any, cannot be easily separated. Our partially protected derivatives **4** and **5** have NH_2-2′ blocked and NH_2-3 free, and are therefore suitable substances to test the validity of Lemieux's hypothesis[2] in such cases as well. It is seen from Table I that, on moving from high to low pH, the signals for H-1′ suffer appreciable low-field shifts whenever NH_2-3 is free (**4** and **5**), whereas no chemical shift change is observed when this NH_2 group (together with NH_2-2′, of course) is blocked (**2** and **3**). This is further evidence to support the conjecture that protonation at NH_2-3 is the principal cause for the pH-dependence of the chemical shift of H-1′ possibly by the mechanism suggested[2]. On the other hand, other factors, such as a repulsive interaction between protonated NH_2-3 and NH_2-6′, have also been proposed[18], on the basis of ^{13}C NMR studies, as a source for pH-dependent conformational change around the O–C-4 glycosidic bond in aminoglycoside antibiotics. The present data seem to provide support for such a mechanism. Reduced ^{13}C β-protonation shifts are observed for C-4 in **4** and **5** relative to that in free tobramycin (**1**, Table II). Assuming that the conformational contribution (rotation around the O–C-4 bond) of the protonation shift at C-4 has two main sources, as mentioned, the decreased values observed for **4** and **5** can be rationalized as being the result of diminished interaction between the protonated NH_2-3 and acetylated NH_2-6′ in these molecules. Derivative **6** represents the inverse situation, in that interaction between protonated NH_2-6′ and acetylated NH_2-3 may be envisaged at low pH. This may explain the protonation shift observed for the signal of H-1′ in **6** (Table I), which is comparable in magnitude to those seen for **4** and **5**. In agreement with this reasoning, no pH-dependence is observed for the chemical shift of H-1′ when both NH_2-3 and NH_2-6′ are acetylated as in **2** and **3** (Table I).

EXPERIMENTAL

General methods.—Optical rotations were determined in a 10-cm micro-cell with a Perkin–Elmer model 141 MC polarimeter. TLC was performed on DC-Alurolle Kieselgel 60 F_{254} (E. Merck), using a mixture of 9:1 MeOH–aq NH_3 (1%) for developing; visualization was done by spraying the sheets with 5% H_2SO_4 in

EtOH and heating. A Radiometer model PHM 85 pH-meter equipped with glass and calomel electrodes was used for the volumetric pH-titrations. Tetramethylammonium hydroxide was used as a base and measurements were performed with a Radiometer model ABU 80 automatic burette in an N_2 atmosphere with continuous stirring at 25°C, the ionic strength being adjusted to 0.1 M with tetramethylammonium chloride. Calculation of pK_a values was done with the aid of the PSEQUAD least-squares iterative fitting routine[17]. ^1H NMR and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, with a Bruker model WP 200 SY spectrometer for solutions in D_2O. The pH of the samples was measured directly in 5-mm NMR tubes, using a combination glass micro-electrode with calomel reference (Cole Parmer). Assignments of the NMR spectra were achieved through combined use of the 2D methods DQ-COSY (phase sensitive), single and double relayed COSY, and $^1H,^{13}C$ COSY in magnitude representation as described previously[15]. All chemical shifts are referenced to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Thermospray mass spectra were obtained with a VG TRIO-2 (VG Biotech, UK) instrument connected to a Waters 600 HPLC pump in an isocratic mode. Samples were dissolved in 0.1 M NH_4OAc or NaOAc buffer–MeOH mixtures (1:1). The PSP interface tip temperature was 210°C. Samples were introduced without column in the bypass mode.

Partial N-*acetylation of tobramycin* (**1**).–(*A*) Tobramycin free base (**1**; 2.0 g, 4.28 mmol) was dissolved in MeOH (80 mL), and Ac_2O (0.85 mL, 8.33 mmol) was added at room temperature. After 10 min, the mixture was evaporated to dryness under diminished pressure at 50°C. The residue was applied in two portions to a column (3 × 30 cm) of Silica Gel 60 (E. Merck) and eluted with 95:5 MeOH–aq NH_3 (1%). Mixed fractions were rechromatographed on smaller columns. The following compounds were isolated.

1,3,2′,6′,3″-Penta-*N*-acetyltobramycin (**2**): white powder (0.86 g, 26.9%); mp 272–274°C (dec), $[\alpha]_D^{23}$ + 97.8° (*c* 1.08, H_2O); TLC: R_f 0.63; lit.[13] mp 248–260°C (dec); $[\alpha]_D^{20}$ + 106.1° (H_2O).

1,3,2′,6′-Tetra-*N*-acetyltobramycin (**3**): white amorphous powder (0.38 g, 12.8%); TLC: R_f 0.34; mp 269–272°C (dec); $[\alpha]_D^{23}$ + 101.3° (*c* 1.5, H_2O). MS, *m/z* (%): 636 (100) $[M + H]^+$. Anal. Calcd for $C_{26}H_{45}N_5O_{13} \cdot H_2CO_3$: C, 46.48; H, 6.79; N, 10.04. Found: C, 45.73, H, 6.90; N, 10.34.

1,2′,6′,3″-Tetra-N-acetyltobramycin (**4**): white amorphous powder (0.22 g, 7.4%); TLC: R_f 0.45; mp 205–213°C (dec); $[\alpha]_D^{23}$ + 91.8° (*c* 1.3, H_2O). MS, *m/z* (%): 636 (100) $[M + H]^+$, 658 (50) $[M + Na]^+$. Anal. Calcd for $C_{26}H_{45}N_5O_{13} \cdot 1.5\ H_2O$: C, 47.13; H, 7.25; N, 10.57. Found: C, 47.00; H, 7.07; N, 10.24.

1,2′,6′-Tri-*N*-acetyltobramycin (**5**): white amorphous powder (0.22 g, 7.9%); TLC; R_f 0.25; mp 288–292°C (dec); $[\alpha]_D^{23}$ + 79.8° (*c* 3.4, H_2O). MS, *m/z* (%): 594 (100) $[M + H]^+$. Anal. Calcd for $C_{24}H_{43}N_5O_{12} \cdot H_2CO_3 \cdot 0.5\ H_2O$: C, 45.18; H, 6.92; N, 10.54. Found: C, 44.87; H, 6.70; N, 10.55.

(*B*) Tobramycin free base (**1**; 1.0 g, 22 mmol) was dissolved in 3:2 water–MeOH (50 mL) followed by addition of Et_3N (0.2 mL, 0.6 equiv) and Ac_2O (1.3 mL, 6

equiv.). After standing for 15 min at room temperature, evaporation and column chromatography (see above) furnished **2** and **3** in ~40 and ~30% yields, respectively; minute amounts of **4** and **5** were detected by TLC.

(*C*) Tobramycin free base (**1**; 1.3 g, 2.9 mmol) was dissolved in water (20 mL) and the pH of the solution was adjusted to 9.0 using 1 M NaOH. Acetic anhydride (1.12 M) in MeCN solution (2.5 mL, 2 equiv.) was added in small portions and the pH adjusted to 9.0 ± 0.5 after each addition. After a few minutes at room temperature, evaporation to dryness followed by column chromatography (see above) yielded **2**, **3**, **4**, and **5** in ~30, ~20, ~15, and ~10 yields, respectively.

1,3,2',3''-Tetra-N-*acetyltobramycin* (**6**).—To a solution of **7** (0.327 g, 0.54 mmol) in dry MeOH (30 mL) was added Ac_2O (0.22 mL, 2.30 mmol), and the mixture was stirred at room temperature. The reaction was monitored by TLC (10:10:1 CH_2Cl_2–MeOH–aq NH_3) and was complete after 30 min. Removal of the volatiles in vacuo gave a solid residue which was dissolved in 7:3 MeOH–water (20 mL), and treated with Pd–C (60 mg) under H_2. After 1 h, the reaction, monitored by TLC (10:10:1 CH_2Cl_2–MeOH–aq NH_3), was complete. The catalyst was removed by filtration through a Celite pad. After thorough washing with MeOH and treatment with a strongly basic anion-exchange resin (Dowex 1, OH^- form), the filtrate was evaporated and the residue recrystallized from EtOH–ether, to yield **6** (0.310 g, 85.7%), mp 275–300°C (dec); $[\alpha]_D^{20} + 52.5°$ (*c* 1.0, H_2O). Anal. Calcd for $C_{26}H_{45}N_5O_{13} \cdot 2\ H_2O$: C, 47.20; H, 7.51; N, 10.58. Found: C, 47.73; H, 7.75; N, 10.29.

6'-N-*Benzyloxycarbonyltobramycin* (**7**).—To a stirred solution of anhyd 1-hydroxy-1*H*-benzotriazole (0.405 g, 3.00 mmol) and Et_3N (0.418 mL, 3.00 mmol) in dry dioxane (15 mL) was added benzyl chloroformate (0.43 mL, 3.00 mmol) slowly at room temperature over a period of 20 min. The mixture was stirred for a further 30 min, then a solution of tobramycin (0.935 g, 2.00 mmol) in water (10 mL) was added in one portion, and stirring was continued for 20 h. After evaporation and co-evaporation with toluene, the residue was purified by column chromatography on silica gel (12:12:1 → 5:5:1 CH_2Cl_2–MeOH–aq NH_3) to give **7** (418 mg, 34.8%). ^{13}C NMR (D_2O, pD ~ 11): δ (protonation shift), 100.00 (5.15) (C-1'), 50.28 (1.30) (C-2'), 35.88 (5.68) (C-3'), 67.30 (0.83) (C-4'), 75.31 (−0.18) (C-5'), 42.40 (1.25) (C-6'), 51.30 (0.57) (C-1), 36.60 (7.87) (C-2), 50.66 (1.00) (C-3), 89.06 (9.02) (C-4), 73.49 (−0.99) (C-5), 89.00 (4.57) (C-6), 101.55 (~0) (C-1''), 72.89 (3.77) (C-2''), 55.30 (~0.72) (C-3''), 70.43 (5.60) (C-4''), 73.23 (−1.29) (C-5''), 61.47 (0.53) (C-6''), 128.51, 129.16, 129.53, 137.06 (phenyl), 167.20 (C=O). These chemical shift data are identical with those given in ref 9 for 6'-*N*-Cbz-tobramycin ("CbzA"). This product was used without further purification for the preparation of **6**.

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REFERENCES

1 H. Umezawa and I.R. Hooper (Eds.), *Aminoglycoside Antibiotics*, Springer, Berlin, 1982.
2 R.U. Lemieux, T.L. Nagabhushan, K.L. Clemetson, and L.C.N. Tucker, *Can. J. Chem.*, 51 (1973) 53–66.
3 A. Van Schepdael, J. Delcourt, M. Mulier, R. Busson, L. Verbist, H.J. Vanderhaeghe, M.P. Mingeot-Leclercq, P.M. Tulkens, and P.J. Claes, *J. Med. Chem.*, 34 (1991) 1468–1475.
4 K.F. Koch, J.A. Rhoades, E.W. Hagaman, and E. Wenkert, *J. Am. Chem. Soc.*, 96 (1974) 3300–3305.
5 J.W. Paschal and D.E. Dorman, *Org. Magn. Reson.*, 11 (1978) 632–634.
6 D.E. Dorman, J.W. Paschal, and K.E. Merkel, *J. Am. Chem. Soc.*, 98 (1976) 6885–6888.
7 R.E. Botto and B. Coxon, *J. Am. Chem. Soc.*, 105 (1983) 1021–1028.
8 G.M. Bodner, *J. Chem. Educ.*, 63 (1986) 246–247.
9 K.F. Koch, K.E. Merkel, S.C. O'Connor, J.L. Occolowitz, J.W. Paschal, and D.E. Dorman, *J. Org. Chem.*, 43 (1978) 1430–1434.
10 N.H. Andersen, H.L. Eaton, K.T. Nguyen, C. Hartzell, R.J. Nelson, and J.H. Priest, *Biochemistry*, 27 (1988) 2782–2790.
11 J.J. Wright, A. Cooper, P.J.L. Daniels, T.L. Nagabhushan, D. Rane, W.N. Turner, and J. Weinstein, *J. Antibiot.*, 29 (1976) 714–719.
12 K.F. Koch and J.A. Rhoades, *Antimicrob. Agents Chemother.*, (1970) 309–313.
13 G.I. Eneva, S.L. Spassov, M.A. Haimova, and J. Sandström, *Magn. Reson. Chem.*, 30 (1992) 841–846.
14 J.G. Batchelor, J. Feeney, and G.C.K. Roberts, *J. Magn. Reson.*, 20 (1975) 19–38.
15 L. Szilágyi, *Carbohydr. Res.*, 170 (1987) 1–17.
16 I.F. Pelyvás, T.K. Lindhorst, H. Streicher, and J. Thiem, *Synthesis*, (1991) 1015–1018.
17 L. Zékány and I. Nagypál, in D. Leggett (Ed.), *Computational Methods for the Determination of Stability Constants*, Plenum, New York, 1985, pp 291–352.
18 P.J.L. Daniels, A.K. Mallams, S.W. McCombie, J.B. Morton, T.L. Nagabhushan, D.F. Rane, P. Reichert, and J.J. Wright, *J. Chem. Soc., Perkin Trans. 1*, (1981) 2209–2227.
19 R.G. Bates, *Determination of pH. Theory and Practice*, Wiley, New York, 1964, pp. 218–220.