Individual pKₐ Values of Tobramycin, Kanamycin B, Amikacin, Sisomicin, and Netilmicin Determined by Multinuclear NMR Spectroscopy

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ABSTRACT: NMR spectroscopy is a powerful technique for separating and measuring each distinct pKₐ value of the amino groups around aminoglycoside antibiotics. Unambiguous assignments were made for each individual amine substituent on 2-deoxystreptamine, tobramycin, kanamycin B, amikacin, sisomicin, and netilmicin using variations in the NMR spectroscopic chemical shift (δ) with ¹H, ¹³C, and ¹⁵N HMBC; the individual pKₐ values of netilmicin are reported for the first time.

1. INTRODUCTION

Aminoglycosides are clinically important microorganism-derived natural products, which consist of an aminocyclitol moiety 2-deoxystreptamine or a streptidine ring in streptomycin attached to amino sugars by glycosidic bonds.¹⁻² These polyamine-type alkaloids are primarily used for the treatment of infection by Gram-negative (aerobic) or Gram-positive bacteria.¹⁻⁵ The target at which these drugs act is found in the 16S fragment of ribosomal RNA (rRNA) located in the 30S subunit of the 70S bacterial ribosome, leading to cell death.⁶⁻⁸ The amino functional group substituents around the different rings of aminoglycoside antibiotics are key to the biological activities of these natural product alkaloids. The specific binding induced by the positively charged ammonium groups on aminoglycosides is to the negatively charged backbones of rRNA by electrostatic interactions.⁸

Ionization constants (pKₐ) provide key information about the physical and kinetic behavior of a chemical substance. The pKₐ values of a medication are significant physicochemical data and are therefore relevant to drug activity. This study is to determine individual pKₐ values by detailed nuclear magnetic resonance (NMR) spectroscopy of selected aminoglycoside alkaloids from Streptomyces and Micromonospora. To determine the individual pKₐ values, not available by potentiometric methods,⁹¹⁰ different NMR reporter nuclei have been employed. Studying the pKₐ values of the ionizable nitrogen atoms in these antibiotic alkaloids will afford a better understanding of their structure–activity relationships (SAR), especially the order in which these similar functional groups gain/lose protons. Such data will potentially help in understanding the order of target rRNA binding, in bacterial cells, of the key basic functional groups or their conjugate ammonium ions. The aim is to measure pKₐ values of individual amines on aminoglycosides by using new combinations of ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data.¹¹⁻¹³

2. RESULTS

2.1. pKₐ Values of the Individual Amino Groups of Tobramycin. Tobramycin is a 4,6-O-disubstituted 2-deoxystreptamine (see Figure 1). Tobramycin has five primary amine functional groups. Three of those amines are substituents on two amino sugar rings: 3-deoxynanosamine (nebrosamine) and 3-amino-3-deoxy-D-glucose and two are on a central cyclohexane ring (2-deoxystreptamine). The pKₐ determinations at every point on each curve were repeated twice using ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data. The average values of the chemical shifts of ¹H, ¹³C, and ¹⁵N HMBC of tobramycin at different pHs were plotted against the pH values of the solution. The pKₐ values of individual nitrogen atoms of tobramycin, shown in Figure 2 and Table 1, were extracted
from the inflection points of the nonlinear sigmoidal curves (Figure 2).

The average pKₐ values determined using ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data of each individual nitrogen atom on tobramycin are calculated to be: N-1 = 7.55, N-3 = 6.70, N-2′ = 7.75, N-6′ = 9.10, and N-3″ = 7.68. The assignment order of the average ionization constants within ±0.05 is N-6′ > N-2′ ≈ N-3″ > N-1 > N-3. These pKₐ values are consistent in the assignment order with those reported in the literature.¹¹,¹⁴

2.2. pKₐ Values of the Individual Amino Groups of Kanamycin B. Kanamycin B (Figure 3), like tobramycin (Figure 1), is a 4,6-O-disubstituted 2-deoxystreptamine. Kanamycin B has five primary amines. Three of those amines are substituents on two amino sugar rings: 3-amino-3-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose and two are on a central cyclohexane ring (2-deoxystreptamine). The chemical shifts of ¹H, ¹³C, and ¹⁵N HMBC of kanamycin B at different pH values were plotted against the pH values of the solution. The pKₐ values of individual nitrogen atoms of kanamycin B shown in Figure 4 and Table 2, were extracted from the inflection points of the nonlinear sigmoidal curves (Figure 4).

After calculating, using ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data, the average pKₐ values of each individual nitrogen atom on kanamycin B are N-1 = 8.10, N-3 = 6.78, N-2′ = 7.36, N-6′ = 8.97, and N-3″ = 7.65. The assignment order of the average ionization constants is N-6′ > N-1 > N-3″ > N-2′ > N-3. In the absence of any kanamycin B published pKₐ data determined using NMR spectroscopy, these are therefore reported for the first time.

2.3. pKₐ Values of the Individual Amino Groups of Amikacin. Amikacin has four primary amines, which are substituents on two amino sugar rings: 3-amino-3-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose, a central cyclohexane ring (2-deoxystreptamine), and 1-amino-α-hydroxybutanolic acid (see Figure 5). The chemical shifts of ¹H, ¹³C, and ¹⁵N HMBC of amikacin at different pH values were plotted against the pH values of the solution. The pKₐ values of the individual amino groups of amikacin, shown in Figure 6 and Table 3, were extracted from the inflection points of the nonlinear sigmoidal curves (Figure 6). After calculating, the average pKₐ values, using ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data, of each amino group on amikacin are N-3 = 7.64, N-6′ = 8.81, N-3″ = 8.05, and N-4″ = 9.89. The assignment order of the average ionization constants is N-4″ > N-6′ > N-3″ > N-3. These pKₐ values are consistent in magnitude and in assignment order with those reported in the literature.¹⁶

2.4. pKₐ Values of the Individual Amino Groups of Sisomicin. Sisomicin has four primary amines and a secondary N-methylamine. Those amines are substituents on two amino sugar rings: dehydro-purpurosamine and garosamine and a central cyclohexane ring (2-deoxystreptamine) (see Figure 7). The chemical structure of sisomicin is similar to that of netilmicin, with a primary amine as N-1 for the N-ethyl of netilmicin the only difference. The chemical shifts of ¹H, ¹³C, and ¹⁵N HMBC of sisomicin at different pHs were plotted against the pH values of the solution. The pKₐ values of the individual nitrogen atoms of sisomicin, shown in Figure 8 and Table 4, were extracted from the inflection points of the nonlinear sigmoidal curves. After calculating, the average pKₐ values, using ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data, of each individual nitrogen atom on sisomicin are N-1 = 7.42, N-3 = 6.22, N-2′ = 8.00, N-6′ = 9.30, and N-3″ = 8.50.
The assignment order of the average ionization constants is $\text{N-6}'> \text{N-3}'' > \text{N-2}'> \text{N-1} > \text{N-3}$. These $pK_a$ values are consistent in magnitude and in assignment order with those reported in the literature.

Some NMR spectra obtained for sisomicin, typical of these experiments, are shown below (Figure 9A). These illustrate the ease of applying our technique to determine the chemical shifts. While peak heights do reduce on dilution during titration with an aqueous base, there was no significant increase in line width. The $^1\text{H}$ spectra for the various aminoglycosides at the extremes of pH are well-resolved, as in these conditions the amines are either fully protonated or fully deprotonated. At

![3-amino-3-deoxy-D-glucose](image)

**Figure 3. Kanamycin B.**

The assignment order of the average ionization constants is $\text{N-6}'> \text{N-3}'' > \text{N-2}'> \text{N-1} > \text{N-3}$. These $pK_a$ values are consistent in magnitude and in assignment order with those reported in the literature.\(^{17}\)

Some NMR spectra obtained for sisomicin, typical of these experiments, are shown below (Figure 9A). These illustrate the ease of applying our technique to determine the chemical shifts. While peak heights do reduce on dilution during titration with an aqueous base, there was no significant increase in line width. The $^1\text{H}$ spectra for the various aminoglycosides at the extremes of pH are well-resolved, as in these conditions the amines are either fully protonated or fully deprotonated. At

![NMR titration curves](image)

**Figure 4. NMR titration curves for the (A) $^1\text{H}$, (B) $^{13}\text{C}$, and (C) $^{15}\text{N}$ HMBC chemical shifts of 1.315–0.822 M kanamycin in 99.97% D$_2$O at 25 °C and (D) $pK_a$ values of individual nitrogen atoms of kanamycin B determined using $^1\text{H}$ (red), $^{13}\text{C}$ (blue), and $^{15}\text{N}$ HMBC (green) NMR spectroscopy.**

| Table 1. $pK_a$ Values of Individual Nitrogen Atoms of Tobramycin Determined Using $^1\text{H}$, $^{13}\text{C}$, and $^{15}\text{N}$ HMBC NMR Spectroscopy in This Work and Then Compared With the Published Data, as Indicated |
|---------------------------------------------------------------|
| **individual nitrogen atoms $pK_a$** | method |
| | N-1 | N-3 | N-2' | N-6' | N-3' |
| $^{15}\text{N}$ NMR\(^a\) | 7.40 | 6.20 | 7.60 | 8.60 | 7.40 |
| $^1\text{H}$ NMR\(^b\) | 7.30 | 6.60 | 7.50 | 8.40 | 7.30 |
| $^{15}\text{N}$ NMR\(^b\) | 7.40 | 6.60 | 7.70 | 8.50 | 7.40 |
| $^1\text{H}$ NMR\(^c\) | 7.51 ± 0.03 | 6.60 ± 0.05 | 7.80 ± 0.05 | 9.07 ± 0.10\(^d\) | 7.62 ± 0.08 |
| $^{13}\text{C}$ NMR\(^c\) | 7.61 ± 0.07 | 6.80 ± 0.15 | 7.71 ± 0.07 | 9.15 ± 0.05 | 7.71 ± 0.03 |
| $^{15}\text{N}$ HMBC NMR\(^c\) | 7.55 ± 0.05 | 6.70 ± 0.05 | 7.75 ± 0.05 | 9.10 ± 0.05 | 7.70 ± 0.05 |

$^a pK_a$ values of individual nitrogen atoms of tobramycin determined using $^{15}\text{N}$ NMR spectroscopy in H$_2$O/D$_2$O (90:10 v/v) relative to $^{15}\text{NH}_4\text{Cl}$ at 25 °C.\(^{11}\) $^b pK_a$ values of individual nitrogen atoms of tobramycin determined using $^1\text{H}$ NMR spectroscopy and $^{15}\text{N}$ NMR spectroscopy in D$_2$O relative to TMS at 25 °C.\(^{14}\) $^c$This work. $^d$The $pK_a$ value of N-6' of tobramycin determined using $^1\text{H}$ NMR spectroscopy (in this work) is the average $pK_a$ of the values of N-6' obtained using $^1\text{H}$ NMR spectroscopic data for 6'a (9.05) and 6'b (9.10).

| Table 2. $pK_a$ Values of Individual Nitrogen Atoms of Kanamycin B Determined Using $^1\text{H}$, $^{13}\text{C}$, and $^{15}\text{N}$ HMBC NMR Spectroscopy and Then Compared With the Published Data, as Indicated |
|---------------------------------------------------------------|
| **individual nitrogen atoms $pK_a$** | method |
| | N-1 | N-3 | N-2' | N-6' | N-3' |
| $^1\text{H}$ NMR\(^a\) | 8.12 | 6.04 | 9.03 | 7.46 |
| $^1\text{H}$ NMR\(^b\) | 8.16 | 6.71 | 7.35 | 8.93\(^c\) | 7.60 |
| $^{13}\text{C}$ NMR\(^c\) | 8.10 | 6.80 | 7.40 | 9.10 | 7.70 |
| $^{15}\text{N}$ HMBC NMR\(^c\) | 8.05 | 6.85 | 7.35 | 8.90 | 7.65 |

$^a pK_a$ values of individual nitrogen atoms of kanamycin A (note, which lacks an N-2' amine) determined using $^1\text{H}$ NMR spectroscopy in D$_2$O relative to TSP at 25 °C.\(^{15}\) Note also that there are no literature data for the $pK_a$ values of kanamycin B.\(^b\)This work. $^c$The $pK_a$ value of N-6' of kanamycin B determined using $^1\text{H}$ NMR spectroscopy (in this work) is the average $pK_a$ of the values of N-6' obtained using $^1\text{H}$ NMR spectroscopic data for 6'a (8.95) and 6'b (8.90).
more intermediate values of pH, the situation is more complicated, as at any time the bases will tend to be in an equilibrium state between protonation/deprotonation, and this may be expected to impact on the spectral appearance, as potentially the rates of exchange approach the NMR time scale. However, as seen in the stack plot of the $^1$H spectra for sisomicin at all pD points (Figure 9A), while there is a little line broadening observed, the line shapes remain distinct and accurate chemical shift information can easily be gleaned.

Typically, the $^{13}$C data points were determined by HSQC (overlay plot Figure 9B) in 20 min, as direct detection via a simple 1D $^{13}$C experiment may have been significantly longer in duration (depending upon the concentration). The use of the HSQC experiment increases the sensitivity as signal detection is through the proton channel and thus is intrinsically far more sensitive. In addition, the use of the second dimension gives advantages in assignment by reducing the incidence of the overlapping peaks. For the $^{15}$N data obtained via $^{15}$N−$^1$H HMBC, typically ~45 min was required.
HMBC of netilmicin at different pH values of the solution. The pK_a values of the individual nitrogen atoms of sisomicin determined using 1H NMR spectroscopy in D_2O relative to TSP at 25 °C and (D) pK_a values of the individual nitrogen atoms of sisomicin determined using 1H (red), 13C (blue), and 15N HMBC (green) NMR spectroscopy.

Table 4. pK_a Values of Individual Nitrogen Atoms of Sisomicin Determined Using 1H, 13C, and 15N HMBC NMR Spectroscopy in This Work and Then Compared With the Published Data, as Indicated

| method           | individual nitrogen atoms | pK_a |
|------------------|---------------------------|------|
| 1H NMR           | N-1 | 7.34 | 6.11 | 7.93 | 9.45 | 8.63 |
| 13C NMR          | N-2 '' | 7.42 | 6.18 | 7.98 | 9.35 | 8.51 |
| 15N HMBC NMR     | N-3 | 7.45 | 6.25 | 7.99 | 9.28 | 8.45 |
| 1H NMR           | N-6' | 7.41 | 6.24 | 8.05 | 9.29 | 8.55 |

*pK_a values of individual nitrogen atoms of sisomicin determined using 1H NMR spectroscopy in D_2O relative to TSP at 25 °C.*

**2.6. pK_a Measurement Studies on 2-Deoxystreptamine.** The reproducibility of the data obtained from these 1H, 13C, and 15N HMBC NMR spectroscopic experiments was determined by three repetitions using a simple diamine as a model compound, 2-deoxystreptamine (Figure 12). It was observed that the majority of the error bars for the pH values and for the chemical shifts were of a similar size as the (small, typical) symbols used to plot the points on the nonlinear sigmoidal curves. Typically, pK_a values were accurate to ±0.1 and sometimes even down to ±0.3.

1H and 13C NMR spectroscopic data were measured at pD 1.44, 8.30, and 11.68 for the model compound 2-deoxystreptamine at three fixed temperatures 25, 35, and 50 °C. The results showed that the chemical shifts corresponding to the H-1/3 and C-1/3 of 2-deoxystreptamine did not shift with the temperature increasing from 25 to 50 °C at low or high pD. One possible explanation of this observation is that the chemical shifts of these protons and carbons of 2-deoxystreptamine were not temperature dependent. Therefore, we concluded that the pK_a values of the amino groups on aminoglycosides will not be affected by increasing the temperature in this typical NMR experiment range. 1H NMR spectroscopic data were measured at two concentrations of 0.631 and 0.157 M at low pD (~2) for 2-deoxystreptamine. The obtained results showed that the chemical shifts corresponding to the H-1/3 of 2-deoxystreptamine did not shift with the changing concentration levels. Thus, the pK_a values of N-1/3 on 2-deoxystreptamine were not affected by changing their concentrations, at least in this typical NMR concentration range.
The $^{13}$C NMR (125.77 MHz) spectra of 0.631−0.369 M 2-deoxystreptamine in 99.97% D$_2$O at 25 °C recorded between pD 2.28 and 12.00 showed the effects of dilution on titration, but no significant line broadening in the stack plot (Figure 13).

Note the inversion of the chemical shift order for carbons labeled 5 and 4,6 and the stepped transition arising from double deprotonation of the two amine functional groups, most evidently reported for carbon 2. The $^{15}$N HMBC chemical shift (ppm) in 99.97% D$_2$O (50.67 MHz) of N-1/3 of 2-deoxystreptamine (0.631−0.369 M) at pH 1.53 is 48.17 ppm and at pH 11.84 is 40.45 ppm measured relative to external nitromethane (CH$_3$NO$_2$/CDCl$_3$ 1:1, v/v) at −511.72 ppm at 25 °C. Not unexpectedly, this symmetrical diamine molecule only provides one signal in $^{15}$N HMBC spectroscopy.
3. DISCUSSION

The pKₐ values of the individual amino groups of tobramycin, kanamycin B, amikacin, sisomicin, and netilmicin were determined using chemical shift (δ) variation with ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopy. The chemical shifts of ¹H, ¹³C, and ¹⁵N of these natural products depend on their chemical environment. Consequently, the gradual change in acidity or basicity leads to subtle alterations in their chemical shifts. The ionization constants were measured for every amine on each polyamine-type aminoglycoside alkaloid. Unambiguous assignments were made for each individual proton, carbon, and amine substituent on these clinically important aminoglycoside antibiotics using combinations of ¹H, ¹³C, HSQC, HMBC, NOESY, and ¹⁵N HMBC NMR spectroscopy. Where the proton and carbon signals overlap, ¹H−¹³C HSQC was used to determine the chemical shifts of each of the protons and carbons (see Figure 9B). These chemical shifts

Table 5. pKₐ Values of the Individual Nitrogen Atoms of Netilmicin Determined Using ¹H, ¹³C, and ¹⁵N HMBC NMR Spectroscopy in This Work and Then Compared With the Published Data, as Indicated

| method       | individual nitrogen atoms pKₐ | N-1 | N-3 | N-2′ | N-6′ | N-3″ |
|--------------|-------------------------------|-----|-----|------|------|------|
| ¹H NMR       |                               | 8.15| 6.55| 8.10 | 9.27 | 8.48 |
| ¹³C NMR      |                               | 8.11| 6.50| 8.11 | 9.32 | 8.50 |
| ¹⁵N HMBC NMR |                               | 8.20| 6.51| 8.23 | 9.37 | 8.45 |

"This work. As far as can be determined, there are no literature data for the pKₐ values of netilmicin. bThe pKₐ value of N-6′ of netilmicin determined using ¹H NMR spectroscopy (in this work) is the average pKₐ of the values of N-6′ obtained using ¹H NMR spectroscopic data for 6′a (9.29) and 6′b (9.25)."
were then plotted against the pH; the pKₐ values were extracted from the inflection points of these sigmoidal curves.

The reason for using NMR spectroscopy rather than potentiometry or UV spectrophotometry is that NMR spectroscopy is a powerful technique for separating and measuring the distinct pKₐ values of the similar amino groups located around the aminoglycosides. The NMR signals measured at low pH are diagnostic of the (>99%) protonated forms of these amino substituents, ammonium ions. Likewise, the signals obtained at high pH indicate the (>99%) free-base amines on these alkaloids. The key ¹H and ¹³N NMR peaks (from protons located on the carbon atom adjacent to the amine of interest) shift downfield (to higher ppm) with decreasing pH. Correspondingly, the ¹H and ¹³N NMR spectroscopic data associated with each amine free-base functional group resonated at lower chemical shifts (ppm) than for its conjugate protonated amine salt. However, the reverse is true for the ¹³C chemical shifts. This phenomenon is due to the change in the electron transition type at the nitrogen from n → π⁎ to σ → π⁎, therefore the ΔE will increase. However, the σ will decrease and, as was indeed observed, shielding resulted.

The use of D₂O instead of H₂O as a solvent is a routine procedure for NMR spectroscopy, including for in situ NMR titration. However, the use of D₂O raises the problem of the relationship between the pKₐ values measured in D₂O and in H₂O. The comparisons of pH and pD determined data are not straightforward because the binding affinities of protonating groups are, in general, different for H⁺ and D⁺. For this reason, the apparent pKₐ values measured in D₂O and expressed using pD, a measure of D⁺ concentration, are not the same as the corresponding values, measured in H₂O and expressed in pH, the well-known measure of H⁺ concentration. A proposed and widely accepted equation pH = pD − 0.4 is derived for ionic strength I = 0.001 mol dm⁻³ and 25 °C. However, another paper used pH = pD − 0.44 for ionic strength I = 0.01 mol dm⁻³ and 22 °C and another paper used pH = pD − 0.5 for ionic strength I = 0.1 mol dm⁻³ and 25 °C. Although the differences between the subtracted values, 0.4, 0.44, and 0.5, are not large, it has a significant participation in the systematic error, particularly for high ionic strengths and temperatures as the diversity of relationships between pKₐ (D₂O) and pKₐ (H₂O) is both ionic strength and temperature dependent. In these studies, the guidelines for NMR measurements for the determination of high and low pKₐ values, given in the IUPAC Technical Report, have been followed. Therefore, the measured pD values were converted into pH values by the subtraction of 0.5.

Depending on both, the chemical structures and the acid–base properties of tobramycin, kanamycin B, amikacin, sisomicin, and netilmicin, the amino substituents can be classified into three groups: primary amines attached directly to the amino sugar ring (R-NH₃⁺), primary aminomethylene groups (R-CH₂NH₂), and in sisomicin and netilmicin secondary amines (N-methyl and an additional N-ethyl on N-1 in netilmicin) are found. ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data indicated that the lowest pKₐ values were the primary amines (R-NH₃⁺) attached directly to the sugar ring. The average pKₐ value for the primary amines attached directly to the amino sugar ring (R-NH₃⁺) using ¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopic data is 7.41 and for the primary aminomethylene groups (R-CH₂NH₂) is 9.09 (see Table 6). This value may well result from the primary aminomethylene groups being less sterically hindered than the primary amines attached directly to the amino sugar rings, respectively.

### 4. CONCLUSIONS

¹H, ¹³C, and ¹⁵N HMBC NMR spectroscopy is a powerful technique for the measurement of the individual pKₐ values. Unambiguous assignments have been made for each amine substituent on tobramycin, kanamycin B, amikacin, sisomicin, and netilmicin using variations in the chemical shift. The individual pKₐ values of netilmicin are reported for the first time. At the concentrations (~0.9 M) used in these studies, ¹H NMR spectroscopy was shown to be less time consuming (2 min per data point) than ¹³C (20 min per data point) and ¹⁵N HMBC (~45 min per data point) NMR spectroscopy. The NMR equipment used in these studies is now standard for many NMR facilities, where 500 MHz is ubiquitous for small-molecule research. The data were also obtained with a standard BBFO probe (tunable X-channel). In the last decade, cryoprobes have become far more common in NMR laboratories, increasing the sensitivity of the probe by chilling the receivers with either liquid helium or nitrogen. Typically, a nitrogen-cooled cryoprobe can increase sensitivity by four-fold. The availability of a cryoprobe would therefore increase the S/N. However, the ¹⁵N experiments would still be best achieved by 2D methods, although the availability of such a probe may impact on making 1D ¹⁵C NMR measurements quicker.

¹H NMR spectroscopy is the most preferable method for measuring individual pKₐ values. These results demonstrate the analysis by NMR techniques of individual amine basicity, which is impossible by other analytical techniques. Therefore, knowing each amine’s basicity in a polyamine can lead to selective functionalization and a more precise knowledge of the molecule at physiological pH can lead to a deeper understanding of SAR.

### 5. EXPERIMENTAL SECTION

#### 5.1. Materials and General Methods

Deuterium oxide (D₂O), DCl, and NaOD were purchased from Goss Scientific. The purchased DCl was a 20% concentration solution in D₂O. NaOD was a 30% concentration solution in D₂O. 2-Deoxystreptamine dihydrobromide, tobramycin free base, kanamycin B sulfate, amikacin sulfate, sisomicin sulfate, netilmicin sulfate, potassium hydrogen phthalate, disodium tetra-borate, trimethylsilylpropanoic acid (TMSP), and nitromethane (CH₃NO₂) were purchased from Sigma-Aldrich (U.K.).

#### 5.2. Instrumentation

The NMR spectra including ¹H, ¹³C, HSQC, HMBC, NOESY, and ¹⁵N HMBC were recorded on Bruker Avance III (operating at 500.13 MHz for ¹H, 125.77 MHz for ¹³C, 300.13 MHz for ¹⁵N).

### Table 6. Average pKₐ Values of the Individual Nitrogen Atoms of the Indicated Aminoglycosides Determined Using ¹H, ¹³C, and ¹⁵N HMBC NMR Spectroscopy

| aminglycoside       | individual nitrogen atoms pKₐ | N-1   | N-3  | N-2  | N-6  | N-3' | N-4' |
|---------------------|--------------------------------|-------|------|------|------|------|------|
| tobramycin          |                                | 7.55  | 6.70 | 7.75 | 9.10 | 7.68 |
| kanamycin B         |                                | 8.10  | 6.78 | 7.36 | 8.97 | 7.65 |
| amikacin            |                                | 7.64  |      | 8.81 | 8.05 | 9.89 |
| sisomicin           |                                | 7.42  | 6.22 | 8.00 | 9.30 | 8.50 |
| netilmicin          |                                | 8.15  | 6.52 | 8.14 | 9.29 | 8.47 |
MHz for $^{13}$C and 50.67 MHz for $^{15}$N HMBC) spectrometers at 25 °C. MestReNova and Bruker Topspin have been used for processing the spectra. $^1$H and $^{13}$C chemical shifts (δ) were observed and are reported in parts per million (ppm) relative to trimethylsilylpropanoic acid (TMSP) at 0.00 ppm as an internal reference and $^{15}$N HMBC chemical shifts were measured relative to external nitromethane (CH$_3$NO$_2$ in CDCl$_3$ (1:1, v/v) at −511.72 ppm). The total recording time differs for each isotope as follows: 2, 20, and ~45 min per data point for $^1$H, $^{13}$C, and $^{15}$N HMBC NMR spectroscopy, respectively.

5.3. Calibration of a 5 mm NMR Tube-pH Electrode. A 5 mm NMR tube-pH electrode purchased from Sigma-Aldrich (U.K.) was used for measuring the pH values. The electrode easily fitted into the 5 mm NMR tubes. Standard buffers of 0.40 M potassium hydroxide phthalate in H$_2$O, pH 4.00, and 0.01 M disodium tetra-borate in H$_2$O, pH 9.18, were used for calibrating the 5 mm NMR tube-pH electrode. All of the measurements were carried out at 25 °C.

5.4. Reproducibility, Errors, and Consistency. The data obtained from these $^1$H, $^{13}$C, and $^{15}$N HMBC NMR spectroscopic experiments were determined reproducibly by repetitions (n = 3) using the simple symmetrical diamine 2-deoxystreptamine as a model compound. Similarly, each of the different NMR experiments was then repeated for tobramycin (n = 2) to calculate the errors in the measurement of: pH values, chemical shifts (δ), and pK$_a$ values. The majority of the error bars for the pH values and for the chemical shifts were of a similar size as the (small, typical) symbols used to plot the points on the nonlinear sigmoidal curves. Typically, the pK$_a$ values were accurate to ±0.1 and sometimes even down to ±0.03. Therefore, having determined by the experiment that the typical size of the errors is small, it was judged that n = 1 was sufficient for obtaining further NMR spectroscopic data for each of kanamycin B, amikacin, sisomicin, and netilmicin. As the chemical shifts of the protons and carbons of 2-deoxystreptamine were not temperature dependent, the pK$_a$ values of the amino groups on aminoglycosides will not be affected by increasing the temperature in this typical NMR experiment range. Likewise, the $^1$H NMR spectroscopic data for 2-deoxystreptamine, measured at 0.630 and 0.157 M, at low pH (~2), showed that the chemical shifts corresponding to the H-1/3 of 2-deoxystreptamine did not shift with the changing concentration levels. Thus, the pK$_a$ values of N-1/3 on 2-deoxystreptamine were not affected by changing their concentrations, at least in this typical NMR concentration range.

5.5. pK$_a$ Determination Using $^1$H, $^{13}$C, and $^{15}$N HMBC NMR Spectroscopy. Aminoglycoside analyte solutions were typically prepared at ~635 to 525 mg/mL, ~1.3 to 0.9 M analyte, beginning from an acidic pH and adjusting with 0.5 M NaOD (~9 × 20 μL) to pH = 14, when the final concentration will have been diluted by ~33% to ~0.8 to 0.6 M. The pH values were adjusted using 0.5 M NaOD and 0.5 M DCl. MestReNova and Bruker Topspin were used for the analysis of the recorded spectra. Chemical shifts of $^1$H, $^{13}$C, and $^{15}$N HMBC of aminoglycosides at varying pH values were plotted against the pH values. The nonlinear sigmoidal curve and the inflection point of the sigmoidal curve were determined using GraphPad Prism 7 (version 2017), after subtraction of 0.5 to convert the measured pD values into pH values. The pK$_a$ values of the individual nitrogen atoms of each aminoglycoside are extracted from the inflection points of the sigmoidal curves.

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