^{13}C-NMR STUDIES ON 1-N-[(*S*)-4-AMINO-2-HYDROXYBUTYL]KANAMYCIN A (BUTIKACIN) AND RELATED AMINOGLYCOSIDES

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The ^{13}C-NMR spectra of 1-N-[(*S*)-4-amino-2-hydroxybutyl]kanamycin A (butikacin) (Fig. 1, **1b**) and some of its related compounds have been recorded and are tabulated, assigned and discussed. The chemical shifts of many of the carbon nuclei are shown to be reasonably invariant amongst this series of compounds. A procedure is described for the determination of the (*R*,*S*)-epimer ratio of kanamycin A derivatives which have N-substituted groups containing a chiral centre.

^{13}C-NMR spectroscopy has become a powerful technique for the structural elucidation and characterisation of aminocyclitol-aminoglycoside antibiotics[1~7]. In this present paper, the fully assigned ^{13}C-NMR spectra are described for the aminoglycoside antibiotic 1-N-[(*S*)-4-amino-2-hydroxybutyl]kanamycin A (butikacin) and its related compounds including the *R*-epimer (**1c**), and the 3- and 3″-N-substituted positional isomers (Fig. 1, **1d** and **1e**). The synthesis and antibacterial activities of butikacin and some of its 1-N-[(*S*)-ω-amino-2-hydroxyalkyl] analogues have been reported[8].

The large number of publications on the application of ^{13}C-NMR spectroscopy for the structural elucidation of aminoglycosides in recent years has afforded a wealth of ^{13}C-NMR spectral data on common aminoglycosides and their constituent moieties for workers in this field. Much of these data have been utilised in this present work. In the ^{13}C-NMR spectra of a structurally similar series of aminoglycosides obtained under identical conditions, the chemical shifts of nuclei in a particular environment are relatively invariant, provided there is no molecular modification within three bonds of the carbon nucleus under examination. It is therefore possible to make unambiguous assignments by comparison with the chemical shifts of comparable ^{13}C nuclei in structurally similar model compounds whose spectra have been assigned.

Well-resolved ^{13}C-NMR spectra were obtained for all the compounds examined and spectral assignment was facilitated by ^{13}C chemical shift changes produced by changes of the pD of their solutions in deuterium oxide; the "β-shift" effect. Deuteronation of amino groups causes large upfield shifts of the resonance signals of ^{13}C nuclei which are β- to the amino groups[3,5].

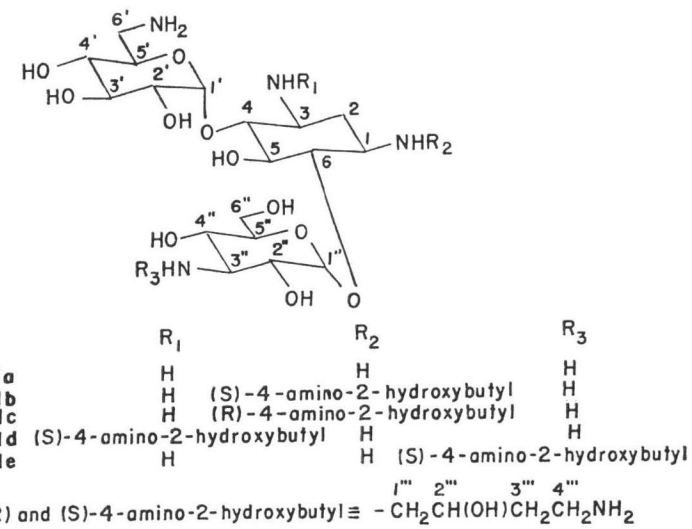
These β-carbons can be readily assigned and this coupled with substituent effects and glycosidation shifts, which are documented[9], provides unambiguous assignment of the ^{13}C-NMR spectra of aminoglycosides. Further confirmation of spectral assignments was achieved from single frequency off-resonance decoupling (SFORD) experiments.

Finally, the potential racemisation of the (*S*)-4-amino-2-hydroxybutyl moiety during the synthesis of butikacin has been monitored and ratios of the (*S*)-**1b** to (*R*)-**1c** epimers determined by quantitative ^{13}C-NMR spectroscopy.

Results and Discussion

The ^{13}C chemical shifts of butikacin (**1b**) and its related compounds (Fig. 1) are tabulated in Table 1.

Fig. 1.



	R_1	R_2	R_3
1a	H	H	H
1b	H	(S)-4-amino-2-hydroxybutyl	H
1c	H	(R)-4-amino-2-hydroxybutyl	H
1d	(S)-4-amino-2-hydroxybutyl	H	H
1e	H	H	(S)-4-amino-2-hydroxybutyl

(R) and (S)-4-amino-2-hydroxybutyl ≡ $-\overset{1'''}{CH_2}\overset{2'''}{CH(OH)}\overset{3'''}{CH_2}\overset{4'''}{CH_2}NH_2$

The ^{13}C-NMR spectra of butikacin (**1b**) obtained using proton noise decoupling showed 22 resonances at pD 3, and 19 resonances, incorporating three degenerate pairs, at pD 11. A single resonance of double intensity appeared in the spectrum of butikacin at 101.1 ppm at pD 11 but this separated into two resonances at 97.1 and 101.8 ppm on deuteronation at pD 3. These resonances were assigned to C-1′ and C-1″ respectively by comparison with the ^{13}C-NMR spectra of kanamycin A (**1a**). The 4.0 ppm upfield shift of the C-1′ resonance on deuteronation is typical for this anomeric carbon in the ^{13}C-NMR spectra of other similar aminoglycosides[6]. The shift is anomalous since C-1′ is not β- to an amino group and arises due to steric interaction between the 6-amino-6-deoxy glucose and 2-deoxystreptamine rings[10]. The small downfield shift of 0.7 ppm by the C-1″ carbon resonance is characteristic of this particular carbon nucleus[6]. The anomeric carbons C-1′ and C-1″ in compounds **1c**, **1d** and **1e** were assigned using parallel arguments to those proposed for butikacin. The increased shielding of C-1′ by 2.1 ppm in **1d** at pD 3, compared to butikacin, is indicative of an increase in the steric shift due to the presence of the N-(*S*)-4-amino-2-hydroxybutyl moiety on the 3-amino group.

The aglycon carbon resonances in butikacin were readily identified since these appear in the region 85~90 ppm[2,5]. The resonances at 88.7 and 86.9 ppm at pD 11 indicated that there were two glycosidic links in the molecule. Since both resonances exhibited deuteronation shifts, their positions were recognised as being β- to amino groups. Therefore, the resonances were associated with carbons 4 and 6 of the 2-deoxystreptamine ring which defined the sites of glycosidation. The anomalous deuteronation shift of 10.3 ppm of one resonance defines this as the C-4 carbon by analogy with the ^{13}C-NMR spectrum of kanamycin A[10]. By analogy with butikacin, comparable evidence defined the C-4 and C-6 resonances of compounds **1c**, **1d** and **1e**.

The C-6 resonances of both **1b** and **1c** at pD 11 are shielded by 2.3 and 2.4 ppm respectively relative to the C-6 resonance in **1a** at the same pD. However, the C-4 resonances have similar chemical shifts in the spectra of **1b**, **1c** and **1a**. The upfield shifts of 2.3 and 2.4 ppm are typically caused by the N-alkylation of an amino group in the β-position[1]. Similarly, the C-4 resonance of **1d** at pD 11 is shielded by 2.4 ppm relative to the C-4 resonance of kanamycin A and pD 11 whereas the C-6

Table 1. ^{13}C-Chemical shifts of compounds **1a**~**1e** at pD 3 together with deuteronation shifts.

Carbon	Chemical shifts (δ) ppm									
	1a		**1b**		**1c**		**1d**		**1e**	
	pD 3	pD 11 −pD 3	pD 3	pD 11 −pD 3	pD 3	pD 11 −pD 3	pD 3	pD 11 −pD 3	pD 3	pD 11 −pD 3
1	51.0	0.3	58.1d	−0.2	56.9d	0.2	51.0d	0.4	50.9d	0.3
2	28.6	8.0	26.7t	6.8	26.4t	6.9	26.2t	6.9	28.7t	8.0
3	48.9	1.2	49.0d	1.0	48.7d	1.4	54.4d	0.1	48.7d	1.4
4	78.9	9.6	78.4d	10.3	79.1d	9.2	78.1d	8.0	79.3d	8.9
5	73.8*	1.1	74.2†d	1.2	74.0d	1.6	73.9†d	1.5	74.0d	0.9
6	84.9	4.3	84.3d	2.6	84.5d	2.3	85.0d	3.6	85.0d	4.7
1′	96.9	4.6	97.1d	4.0	96.7d	4.0	94.8d	5.9	96.7d	5.3
2′	71.9	1.2	72.2d	0.8	71.9*d	1.2	71.8*d	1.2	71.8*d	1.8
3′	73.2	1.4	73.1d	1.3	73.4d	0.8	71.8*d	1.2	73.3*d	1.6
4′	72.1	0.2	71.9d	0.3	71.9*d	0.3	71.8*d	0.1	71.8*d	0.3
5′	69.8	4.5	69.8d	4.6	69.8d	4.4	69.9d	4.1	69.8d	4.5
6′	41.6	1.2	41.7t	1.1	41.5t	1.3	41.5t	1.1	41.5t	1.4
1″	101.5	−1.0	101.8d	−0.7	101.8d	−0.5	101.4d	−0.5	101.6d	−1.3
2″	69.3	4.1	69.2d	4.1	69.3d	3.8	69.2d	4.0	67.8d	4.9
3″	56.1	−0.5	56.2d	−0.7	56.1d	−0.6	56.1d	−0.7	61.5d	0.4
4″	66.6	4.0	66.5d	4.0	66.6d	3.9	66.8d	3.7	65.9d	3.7
5″	73.8*	−0.4	74.0†d	−0.5	74.0d	−0.5	73.7†d	0.3	73.3*d	0.3
6″	61.0	0.6	61.1t	0.5	61.1t	0.5	61.3t	0.1	61.1t	0.6
1‴	—	—	51.4t	1.6	50.7t	1.4	49.9t	1.2	50.3t	4.4
2‴	—	—	66.1d	3.8	65.4d	3.9	66.0d	1.8	65.1d	4.9
3‴	—	—	32.7t	5.7	32.6t	5.9	32.6t	5.7	32.6t	5.8
4‴	—	—	37.5t	0.9	37.6t	0.9	37.6t	1.0	37.6t	1.1

In Table 1, all upfield deuteronation shifts are designated positive.
Assignments d and t show doublet or triplet multiplicities from single frequency off-resonance decoupling (SFORD) experiments.
δ: ppm from TMS (δ=0.0 ppm) as an external standard.
* Assignments within any vertical column are degenerate.
† Assignments within any vertical column may be interchanged.

resonance has essentially the same chemical shift in the spectra of both compounds.

In the spectrum of **1e** at pD 11, the C-4 and C-6 resonances have similar chemical shifts to those observed in kanamycin A at pD 11. However, the C-2″ and C-4″ resonances are both shielded by 1.0 ppm compared to their equivalents in kanamycin A. The shielding of C-2″ and C-4″ in **1e** is indicative of N-alkylation at their common β-carbon, C-3″. This reduced shielding may be caused by the equatorial hydroxyl groups at C-2″ and C-4″ whose combined negative inductive effect clearly counteracts some of the shielding provided by the N-(*S*)-4-amino-2-hydroxybutyl group at C-3″. These observations provide evidence that **1b** and **1c** are N-alkylated at C-1; **1d** is N-alkylated at C-3 and **1e** is N-alkylated at C-3″.

The magnitudes of the alkylation shifts at the N-alkylated carbons are fairly consistent at pD 3 and pD 11. These are downfield shifts which range from 4.4~7.1 ppm. The magnitude and direction of these alkylation shifts are typical for N-alkylated aminoglycosides. In the spectrum of N-methyl-2-deoxystreptamine[1], the C-1 carbon resonance moves downfield 7.6 ppm while C-3 is not shifted com-

pared to their respective positions in the spectra of 2-deoxystreptamine.

The C-2 methylene carbon in butikacin is identified by the high field resonance at 33.5 ppm at pD 11. This resonance, for which SFORD reveals a triplet, is observed at the same position as the C-2 methylene resonance of N-methyl-2-deoxystreptamine[1] and the upfield shift of 6.8 ppm on deuteronation indicates that the C-2 carbon is β- to two amino groups. The resonance at 32.7 ppm (pD 3) is attributed to the C-3‴ carbon of the N-(*S*)-4-amino-2-hydroxybutyl group by comparison to analogous carbons, designated S3, in the spectrum of butirosin A sulphate[4] and Cβ in the spectrum of amikacin[7] at the same pD. This resonance, for which SFORD shows an expected triplet, shifts upfield by 5.7 ppm on deuteronation and verifies that C-3‴ in butikacin is β- to an amino group.

The ^{13}C-NMR spectra of butikacin show six resonances in the region 35 ~ 60 ppm whose positions are characteristic of carbons directly bonded to nitrogen. The resonances at 42.8 and 55.5 ppm (pD 11) are assigned to the C-6′ and C-3″ carbons by comparison with the spectra of kanamycin A. A resonance at 38.4 ppm which shifts to 37.5 ppm on deuteronation is assigned to C-4‴ by analogy with the spectrum of butirosin A[4] and amikacin[7]. The assignments of C-6′ and C-4‴ were confirmed by SFORD experiments which revealed two triplets. In the spectrum of 1-N-methyl-2-deoxystreptamine[1], the C-1 resonance moves downfield 7.6 ppm and the position of the C-3 resonance remains unchanged compared to their respective positions in the spectrum of 2-deoxystreptamine[2]. On this evidence the resonance at 57.9 ppm, which is 6.5 ppm downfield of the C-1 carbon resonance of kanamycin A, was assigned to the C-1 carbon of butikacin and the resonance at 50.0 ppm was assigned to the C-3 carbon since this appeared at the same position as the C-3 carbon resonance of kanamycin A. The remaining resonance at 53.0 ppm was assigned to the C-1‴ carbon for which SFORD showed a triplet.

The ^{13}C-NMR spectra of butikacin exhibit ten resonances in the range 60 ~ 76 ppm. These resonances are characteristic of carbons bonded directly to oxygen. The assignments of these resonances were aided by comparing the spectrum of butikacin (pD 11) to the spectra, recorded under identical conditions, of the following model compounds: methyl 3-amino-3-deoxy-α-D-glucopyranoside, methyl 6-amino-6-deoxy-α-D-glucopyranoside, the aminocyclitol 2-deoxystreptamine and kanamycin A[5].

The resonance at 61.6 ppm was assigned to the hydroxymethyl carbon, C-6″ by comparison with the analogous spectrum of methyl 3-amino-3-deoxy-α-D-glucopyranoside[5] and this assignment was verified from SFORD experiments. The resonances at 73.3 and 70.5 ppm were assigned to the hydroxymethine carbon, C-2″ and C-4″ and both these carbons show the expected deuteronation shifts. The C-5 resonance at 75.4 ppm was identified by comparison with the spectra of kanamycin A. The resonances at 73.0, 74.4 and 72.2 ppm were assigned to the hydroxymethine carbons C-2′, C-3′ and C-4′ respectively by comparison with the spectra of methyl 6-amino-6-deoxy-α-D-glucopyranoside[5].

The two resonances at 74.4 and 73.5 ppm were assigned to the C-5′ and C-5″ carbons which are directly bonded to ring oxygen atoms. Since only the C-5′ resonance undergoes a deuteronation shift, these carbons were readily distinguished. The remaining resonance at 69.9 ppm was assigned to C-2‴. This undergoes a 3.8 ppm upfield shift on deuteronation.

The ^{13}C-NMR evidence presented so far has established the structure of butikacin. This indicates that the molecular structure consists of a 2-deoxystreptamine ring linked at the C-4 and C-6 positions

to 6-amino-6-deoxy-α-D-glucose and 3-amino-3-deoxy-α-D-glucose respectively. An (*S*)-4-amino-2-hydroxybutyl group is attached to the C-1 amino group. Similar evidence has been presented and discussed to confirm the structures of compounds **1c** to **1e** inclusive.

Examination of the deuteronation shifts of the carbons in the N-(*S*)-4-amino-2-hydroxybutyl side chain of compounds **1d** and **1c** has revealed some interesting results. In compound **1d**, C-2‴ is shielded by *ca.* 2.0 ppm compared to the C-2‴ carbons in compounds **1b** and **1e** at pD 11. This results in a deuteronation shift of only 1.8 ppm upfield in the case of **1d** as opposed to 3.8 ppm for butikacin and 4.9 ppm for **1e**. These latter values are those normally expected for carbons which are β- to amino groups. Similarly C-1‴ is shielded by 1.9 ppm compared to C-1‴ in butikacin. It is suggested that in **1d**, the protons bonded to C-1‴ and C-2‴ experience intramolecular steric perturbations which shield the C-1‴ and C-2‴ nuclei. This occurs as a direct consequence of the close proximity of the 6-amino-6-deoxy-α-D-glucose ring. KOCH *et al.*[5] have showed that a preferred rotamer conformation exists in many aminoglycosides in which the 6-amino-6-deoxy-α-D-glucose ring is inclined towards the C-3 carbon of the 2-deoxystreptamine ring. This not only results in shielding of the C-3 carbon nucleus but also shields the carbon nuclei present in substituent groups attached to the C-3 amino group.

Examination of the chemical shift of C-1‴ in **1e** at pD 11 shows that this carbon is deshielded by 1.7 ppm compared to C-1‴ in butikacin. The amino group at C-3‴ is unique in that it is β- to two carbons bearing equatorial hydroxyl groups whose combined negative inductive effect may account for this difference.

Since **1b** and **1c** are epimers it was postulated that directly comparable carbon nuclei (*e.g.* C-1, C-1‴ and C-2‴) within the vicinity of the chiral centre C-2‴ would have different chemical shifts in the two epimers. This phenomenon was observed; the largest difference in chemical shift occurring for carbon nuclei C-1 (*ca.* 1 ppm) at pD 3 and pD 11. Measurements of the intensities of the C-2‴ carbon lines in the spectra, using the technique of quantitative ^{13}C-NMR spectroscopy[11], indicated levels of **1c** present in bulk lots of butikacin.

The method postulates that (a) the spin-lattice relaxation times (T_1) of comparable carbons in both epimers are equal and (b) that the amount of Nuclear OVERHAUSER Effect signal enhancement from bonded protons on the carbon nuclei of interest is identical for comparable carbon nuclei in each epimer. Results in our laboratory have confirmed the validity of these two fundamental assumptions.

Four samples of butikacin, spiked with known levels of **1c** were prepared and their ^{13}C-NMR spectra recorded under identical conditions. The intensities of the C-2‴ peaks arising from **1b** and **1c** were acquired from the data outputs associated with the FOURIER-transformed spectra.

Table 2. Data relating to the quantitative determination of compound **1c** in samples of butikacin.

Synthetic mixtures of epimers		^{13}C-Peak intensities* of C-2‴ carbons		Calculated % of **1c** present in butikacin
% **1c**	% Butikacin	**1c**	Butikacin	
4.5	95.5	13	204	6.0
9.2	90.8	29	261	10.0
12.7	87.3	17	118	12.6
17.3	82.7	30	138	17.9

* Arbitrary units.

A graph of % **1c** in butikacin (synthetic mixtures) against % **1c** in butikacin (determined by ^{13}C-NMR peak intensities of C-2‴ carbons) was plotted. A linear correlation graph was obtained and the results were processed by computer using linear regression analysis. A correlation coefficient of 0.995 was obtained. The data are presented in Table 2.

The results in Table 2 were used to assess the value of quantitative C-^{13}NMR spectroscopy as a technique for determining the levels of **1c** in bulk lots of butikacin. For this purpose, the method was considered satisfactory for determining levels of **1c** in butikacin down to 4% relative. Although used as a quality control and in-process control monitoring technique initially, it was later considered unsuitable for routine use and was superseded by an h.p.l.c. method.

Experimental

^{13}C-NMR Spectra

Proton noise decoupled ^{13}C-NMR spectra were recorded at a frequency of 25.2 MHz on a Varian XL-100-15 spectrometer operating in the FOURIER Transform mode. The instrument used deuterium oxide, the solvent for all this work, as a source of deuterium for the lock signal.

A Varian 620L computer was used to accumulate 10~30,000 transients of 5,000 Hz width and the proton noise decoupler used a 1,500 Hz bandwidth to ensure complete decoupling. Square wave modulation of the proton decoupling frequencies using a Level R.C. Oscillator assured consistency in decoupling power over the total bandwidth. SFORD experiments were accomplished by off-setting the proton noise decoupler by 400 Hz.

In general, 300~400 mg of the sample under examination was dissolved in 1.5 ml of deuterium oxide and the pD of the solution was adjusted with 0.1 M deuteriochloric acid or sodium deuteroxide as appropriate. Chemical shifts were measured relative to the ^{13}C signal of tetramethylsilane (δ=0.00 ppm) as an external standard enclosed in a 1-mm glass capillary.

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