LOCATION OF GUANIDINO AND UREIDO GROUPS IN BLUENSOMYCIN FROM ^{13}C NMR SPECTRA OF STREPTOMYCIN AND RELATED COMPOUNDS

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^{13}C NMR absorptions for streptomycin (**1**), dihydrostreptomycin (**2**) and bluensomycin allow the structure of bluensomycin to be assigned as **3A** rather than the alternative **3B**.

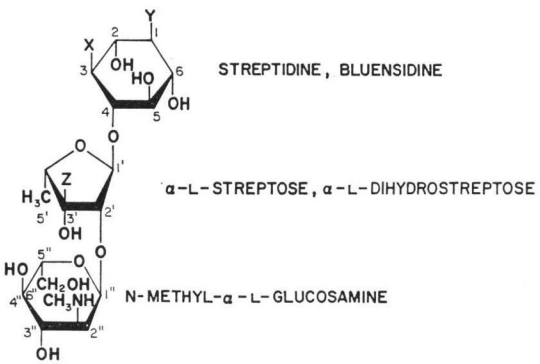
Nearly forty years after its discovery,[1] streptomycin (**1**) remains a clinically useful antibiotic, largely employed in the treatment of tuberculosis. In the course of our biosynthetic investigation of streptomycin it was necessary to assign the resonances in the ^{13}C NMR spectrum of streptomycin in order to locate label introduced from D-[6-^{13}C]glucose. The assignments were listed in a preliminary communication[2] dealing with the biosynthesis, but the arguments leading to them were not detailed and our ^{13}C NMR assignments differ considerably from those made earlier for streptomycin (**1**).[3] Similar arguments can be applied in assigning the ^{13}C NMR spectrum of the related antibiotic bluensomycin. Comparison of spectra for the two antibiotics allows the final stereochemical detail of the structure of bluensomycin to be assigned, giving **3A**.

Dihydrostreptomycin

Dihydrostreptomycin (**2**) occupies a central position structurally between streptomycin (**1**) and bluensomycin (**3A** or **3B**). To assign its ^{13}C NMR spectrum signals in the spectrum were first grouped by off-resonance decoupling into those due to methyl (two carbons), methylene (two), methine (fourteen), and quaternary (three) carbons, allowing assignment of the C-3′ quaternary carbon, the two guanidino quaternary carbons, and the C–CH_3 (C-5′) and N–CH_3 carbons.

Assignment of the remainder of the absorptions was by comparison of signals for dihydrostreptomycin (**2**) with those for sub-units of the molecule.

The signals from the sub-unit streptidine (**4**) were assigned (Table 1) from standard shift and molecular symmetry arguments. Assignment of C-5 at δ 75.6 and C-2 at δ 71.7 follows from two arguments. First, the position of C-5 should approximate that of the C-5 carbons in deoxystreptamine (**5**) and streptamine (**6**), which appear at δ

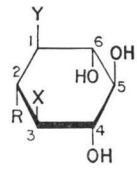


1. X=Y=–NHC(=NH)NH_2, Z=–CHO
2. X=Y=–NHC(=NH)NH_2, Z=–CH_2OH
3A. X=–NHC(=NH)NH_2, Y=–O–CO–NH_2, Z=–CH_2OH
3B. X=–O–CO–NH_2, Y=–NHC(=NH)NH_2, Z=–CH_2OH

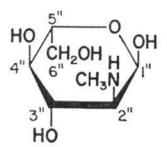
Table 1. ^{13}C NMR absorptions for bluensomycin,

Bluensamine (8)		Bluensidine (7)	Streptamine (6)		Streptidine (4)	*N*-Methyl-α-L-glucosamine (9)[a]	Benzyl α-L-dihydrostreptoside (11)[b]
pH 5.5	pH 8.0	pH 5.5	pH 5.5	pH 10.5	pH 5.5	pH 5.5	pH 5.0
							13.8, q[c]
						31.8	
56.6	56.6	59.0	57.3	57.2	59.8		
75.1	75.3	77.3	57.3	57.2	59.8		
						61.2	
						62.0	
							63.3, t
						70.4	
						70.4	
70.7	→ 72.7	71.2	67.1	→ 74.7	71.7		
73.9	74.3	72.2	70.2	→ 74.7	72.3		
						72.2	
75.1	75.3	74.6	75.5	76.2	75.6		
							78.6, d
70.7	→ 72.7	72.9	70.2	→ 74.7	72.3		
							80.4, s
							78.6, d
						88.4	
							107.2, d
		159.1			159.0		
		159.6			159.0		

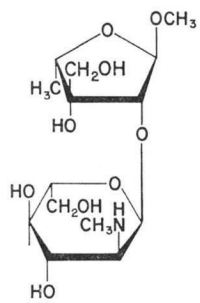
[a] Measured as a mutarotated mixture. [b] Benzyl carbons: α-C, 71.4 ppm, t[c]; C-1, 137.8, s; *o*-C's 129.2, s=singlet, d=doublet, t=triplet, q=quartet. [d] Methoxyl C: 56.4 ppm, q.[e] [e] $CONH_2$ in bluenso-



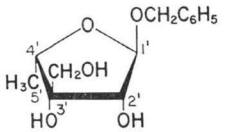
4. X=Y=–NHC(=NH)NH_2, R=–OH
5. X=Y=–NH_2, R=–H
6. X=Y=–NH_2, R=–OH
7. X=–$OCONH_2$, Y=–NHC(=NH)NH_2, R=–OH
8. X=–OH, Y=–NH_2, R=–OH



9. *N*-Methyl-α-L-glucosamine



10. Methyl-α-L-dihydrostreptobiosaminide



11. Benzyl-α-L-dihydrostreptoside

dihydrostreptomycin, streptomycin and model compounds.

Methyl α-L-dihydrostrepto-biosaminide (**10**)[d] pH 5.5	Assignment	Bluensomycin (**3A**)	Dihydrostreptomycin (**2**)			Streptomycin (**1**)
		pH 5.5	pH 5.5	pH 9.5	pH 12.0	pH 5.5
13.3, q[c]	C-5′	13.4, q[c]	13.5, q	13.6	13.7	13.4
32.7, q	N-CH_3	32.8, q	32.8, q	→34.3	34.4	33.1
	C-3	58.2, d	59.1, d	59.0	→ 59.5	59.0
	C-1	*77.0*, d	59.8, d	59.8	→ 60.2	59.7
60.8, t	C-6″	61.3, t	61.3, t	61.8	61.8	61.2
62.1, d	C-2″	62.1, d	62.1, d	→63.9	64.0	62.3
63.8, t	-CH_2OH	64.1, t	64.2, t	63.9	64.0	*90.5*
70.1, d	C-4″	70.3, d	70.3, d	71.0	71.1	70.1
70.3, d	C-3″	70.2, d	70.3, d	→72.7	72.8	70.4
	C-2	*71.0*, d	71.5, d	71.5	→ 72.1	71.5
	C-6	*72.2*, d	72.4, d	72.4	→ 72.8	72.4
73.6, d	C-5″	73.6, d	73.6, d	73.5	73.5	73.7
	C-5	*73.6*, d	74.2, d	74.3	→ 74.5	74.2
78.1, d	C-4′	78.6, d	78.5, d	78.4	78.4	78.3
	C-4	*78.9*, d	78.7, d	79.2	→ 79.5	78.9
81.3, s	C-3′	81.7, s	81.7, s	81.4	81.4	*83.1*
85.8, d	C-2′	84.9, d	84.9, d	85.8	85.7	85.3
95.5, d	C-1″	94.6, d	94.4, d	→98.3	98.4	95.2
107.3, d	C-1′	106.8, d	106.7, d	106.9	106.9	106.7
	C-3-NH-C[=NH]NH_2	158.6, s	158.6, s	158.7	158.8	158.6
	C-1-NH-C[=NH]NH_2	159.7, s[e]	159.2, s	159.2	159.3	159.1

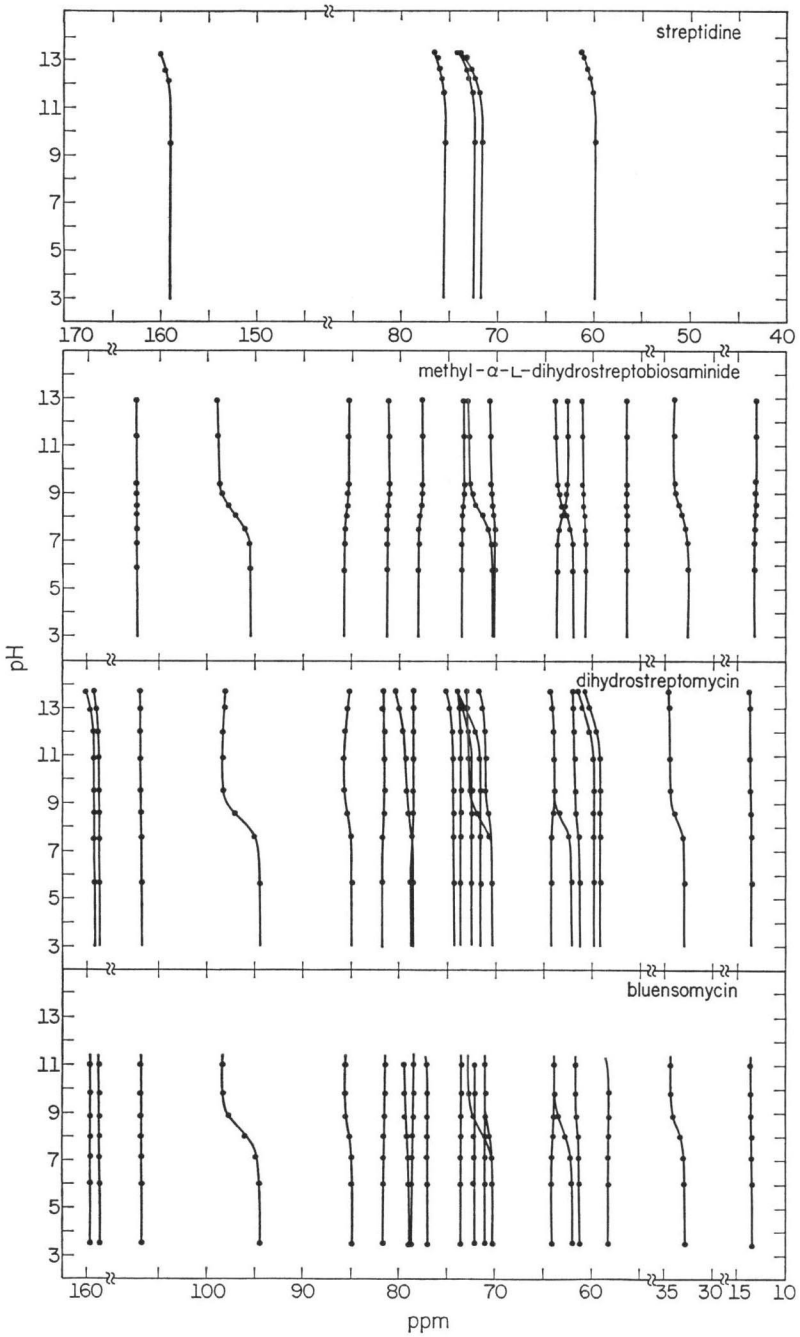
d; *m*-C's 129.6, d; *p*-*C* 129.2, d. [c] Multiplicity in off-resonance decoupled spectrum: mycin.

75.8 and 75.5, respectively. The distinction between C-2 and C-5 in streptamine (**6**) is clearcut, being based on the large downfield shift in the resonance for C-2 (β to two nitrogens) on raising the pH (Table 1).[4,5,6] The assignment of C-5 in deoxystreptamine (**5**) is based on symmetry considerations.[6,7] Second, the resonance at δ 71.7 shifts downfield at a greater rate at high pH than that at δ 75.6 (Fig. 1), since C-2 is β to the guanidino groupings.

The resonances of *N*-methyl-α-L-glucosamine (**9**), shown in Table 1, can be assigned by comparison with the very similar chemical shifts of α-D-glucosamine.[8,9] In the spectrum of methyl α-L-dihydrostreptobiosaminide (**10**) the resonances due to the *N*-methyl-α-L-glucosamine unit are found at the same chemical shifts (Table 1) except that C-1″ and, to a lesser extent, C-5″ are shifted downfield by glycoside formation. The remaining resonances in methyl α-L-dihydrostreptobiosaminide (**10**) belong to the dihydrostreptose unit. These are approximated (Table 1) in the model compound benzyl α-L-dihydrostreptoside (**11**),[10] in which C-5′ is the only methyl carbon, C-3′ the only quaternary carbon, the 3′-hydroxymethyl carbon the only methylene carbon, C-1′ the only anomeric carbon, and both C-2′ and C-4′ are at δ 78.6. In methyl α-L-dihydrostreptobiosaminide (**10**) these dihydrostreptose carbons are found at similar chemical shifts except that the glycosidically attached C-2′ is found downfield, at δ 85.8.

The twelve carbon resonances of the α-L-dihydrostreptobiosaminide (**10**) unit can all be identified easily in the spectrum of dihydrostreptomycin (**2**), since most are shifted downfield by 0.5 ppm or less, while those shifted upfield — C-2′ (−0.9 ppm), C-1″ (−1.1 ppm), and C-1′ (−0.6 ppm) — all appear at

Fig. 1. pH profiles of streptidine, methyl α-L-dihydrostreptobiosaminide, dihydrostreptomycin, and bluensomycin.



unequivocal positions. The carbons of streptidine (**4**) can also be identified in the spectrum of dihydrostreptomycin (**2**) if one allows for the shifts in the vicinity of C-4 of streptidine [the α-carbon (C-4) moving downfield (by 6.4 ppm); the β-carbons (C-3 and C-5) moving upfield (by 0.7 and 1.4 ppm, respectively)]. These shifts approximate those observed for the deoxystreptamine (**5**) moiety of neamine.[7,9]

Additional evidence for the correctness of the assignments in dihydrostreptomycin (**2**) is found in the changes in chemical shift with increasing pH.[4,5,6] The pH profiles of streptidine (**4**), methyl α-L-dihydrostreptobiosaminide (**10**), and dihydrostreptomycin (**2**) are shown in Fig. 1. It is clear from Fig. 1 and Table 1 that the carbons α (C-2'' and N-CH_3) and β (C-1'', C-3'') to the nitrogen of the less basic *N*-methyl-L-glucosamine unit move downfield by 1.5 to 3.9 ppm between pH 5.5 and 9.5 (indicated by arrows in Table 1), while other carbons undergo much smaller shifts in this pH range. Further, all streptidine carbons shift downfield by 0.3~0.6 ppm between pH 9.5 and 13.5 (Fig. 1 and Table 1), while no other carbons in dihydrostreptomycin (**2**) shift.

Streptomycin

Resonances for the carbon atoms of streptomycin (**1**) are assigned directly from those of dihydrostreptomycin. The 3'-formyl carbon and C-3' undergo the only significant shifts (+26.3 and +1.4 ppm, respectively), as italicized in Table 1.

BOCK *et al.*, assigned the carbon resonances of streptomycin (**1**) and dihydrostreptomycin (**2**) in a study of α- and β-streptomycins.[3] Their assignments for dihydrostreptomycin (**2**) disagree with ours for the signals at δ 61.3, 62.1, 71.5, 72.4, 73.6 and 74.2 (all of which were only tentatively assigned by them) and for those at δ 78.7 and 84.9. Similarly, their assignments for streptomycin disagree with ours for the same signals. Since BOCK *et al.*, observed no shifts in the spectra with pH,[3] and the spectrum of streptidine (**4**) was not reported, we believe our assignments are more satisfying. Calculations indicate that their acidic and basic solutions would in any case have had virtually identical pH values because of the large buffering capacities of the concentrated streptomycin solutions used.

Bluensomycin

Bluensomycin[11,12] has lower antimicrobial activity but also lower toxicity than dihydrostreptomycin (**2**),[11] and differs from it in the replacement of one guanidino group of **2** by a ureido group in bluensomycin; it thus has either structure **3A** or **3B**.[11]* BARLOW and ANDERSON[13] studied bluensomycin with the tetramminecopper reagent (TACu) and, on balance, proposed that the $[M]_{TACu}$ value favored formula **3A**. In the present study we have carried out an analysis of the ^{13}C NMR spectrum of bluensomycin, which definitively establishes the structure of bluensomycin as **3A**, in agreement with the previous tentative assignment.

Comparing the ^{13}C NMR spectra (Table 1) of dihydrostreptomycin (**2**) and bluensomycin (**3A** or **3B**) shows that the absorptions differ by more than 0.1 ppm for only eight carbons — the six ring carbons and one of the guanidino carbons of streptidine (the latter a ureido carbon in bluensomycin), and C-1'' of *N*-methyl-L-glucosamine (0.2 ppm). To complete the structural assignment of bluensomycin it is required only that one assign these signals in bluensomycin.

Bluensidine (**7**), the aminocyclitol in bluensomycin, lacks the plane of symmetry of streptidine and, therefore, has eight carbon resonances (Table 1). Of the eight, three can be rapidly and unequivocally assigned by comparison with streptidine (**4**). These are the guanidino carbon (δ 159.1), the ureido carbon (δ 159.6) and the ring carbon attached to nitrogen (δ 59.0). By comparing the shift in the resonance of this ring carbon on conversion of bluensidine (**7**) to bluensomycin (**3A** or **3B**) with those observed in

* Streptobiosamine was located adjacent to the guanidino or ureido group by periodate data,[11] and on C-4 rather than on C-6 of **3A**~**3B** (using the numbering shown) by the nearly identical rotations of bluensomycin and dihydrostreptomycin[11] and their nearly identical behavior[13] in Cupra B solution, which distinguishes the absolute stereochemical relationship of cyclohexanediols, including aminocyclitols.[14]

the conversion of streptidine (**4**) to dihydrostreptomycin (**2**) it is possible to distinguish between structures **3A** and **3B**.

On glycoside formation the glycosidically bound carbon, C-4, of streptidine (**4**) shifts downfield by 6.3 ppm (α carbon), while the β carbons, C-3 and C-5, move upfield by 0.8 and 1.4 ppm, respectively. The other three carbons (γ, δ) shift by less than 0.2 ppm.

In bluensomycin (**3A** or **3B**) the guanidine-bearing ring carbon is shifted upfield relative to bluensidine by 0.8 ppm, to δ 58.2. In structure **3A** the N-substituted carbon is β to the site of glycoside formation, while in the alternative structure **3B** it is δ. This shift of −0.8 ppm is consistent with the carbon's being β, but not with its being δ to the site of glycoside formation. The observed change is, in fact, identical to that observed for C-3 of streptidine (**4**) relative to dihydrostreptomycin (**2**) and is also consistent with those changes observed for C-3 of deoxystreptamine (**5**) on conversion to neamine[6,7] and nebramine.[6] Structure **3A** is, therefore, assigned to bluensomycin.

The remaining five ring carbons of the bluensidine portion of bluensomycin are assigned on the basis of changes in chemical shifts for bluensamine (**8**) relative to bluensidine and in those of bluensidine relative to bluensomycin. Due to a plane of symmetry only four resonances are observed for bluensamine (**8**). The resonances at δ 56.6 and 73.9 can be assigned unequivocally to C-3 (the amino-bearing carbon) and C-6 based on shift and intensity arguments. The signal for the pair of carbons at δ 70.7 is assigned to C-2 and C-4, β to the amino substituent, as this resonance shifts downfield at higher pH. The remaining signal, for the pair of carbons at δ 75.1, is assigned by difference to C-1 and C-5.

Using streptidine (**4**) and streptamine (**6**) as model compounds chemical shift information on guanidine formation indicates $\Delta\delta_g^\alpha = 2.5$, $\Delta\delta_g^\beta = 2.3$ ppm. Similarly, shifts on carbamate formation ($\Delta\delta_C$) were obtained from the published data on kanamycin B, tobramycin and their respective carbamates[8] [$\Delta\delta_C^\alpha = +3.3$, $\Delta\delta_C^\beta = -1.8$ ppm].

Application of these shift data for guanidine and carbamate formation to bluensamine (**8**) allows calculation of the chemical shifts of bluensidine (**7**), which agree with experimental values within 1.1 ppm (Calcd. C-1 75.1, C-2 73.0, C-3 59.1, C-4 71.2, C-5 78.4, C-6 72.1 ppm).

By applying the known changes in the streptidine (**4**) resonances on conversion to dihydrostreptomycin (**2**) [$\Delta\delta^\alpha$ +6.3; $\Delta\delta^\beta$ −0.8, −1.4; $\Delta\delta^\gamma$ −0.2, +0.1; $\Delta\delta^\delta$ −0.0 ppm] to the bluensidine situation, tentative assignment can be made of the remaining bluensidine resonances in the bluensomycin spectrum (as italicized in Table 1). Using these assignments the observed changes for bluensomycin [$\Delta\delta^\alpha$ +6.0; $\Delta\delta^\beta$ −1.0, −0.8; $\Delta\delta^\gamma$ −0.2, 0.0; $\Delta\delta^\delta$ −0.3] parallel those for dihydrostreptomycin.

The pH profiles for bluensomycin (**3A**) and bluensidine (**7**) up to pH 11.5 are shown in Fig. 1. However, as both bluensomycin (**3A**) and bluensidine (**7**) are unstable at pH > 11 it was not possible to confirm the assignments by observing the induced shifts in the carbons of the bluensidine moiety on deprotonation of the guanidine grouping, as was possible for dihydrostreptomycin (**2**).

Experimental

All spectra were determined on aqueous 1 M solutions at 305K using a Varian CFT-20 spectrometer with D_2O providing an internal lock signal. Chemical shifts were measured relative to dioxane (δ 67.4) as an internal standard. The spectral window used was 4000 Hz with a flip angle of 60° and an acquisition time of 0.5 second. The data were collected over 4K data points and, following a small exponential weighting, transformed over 8K data points.

Each compound was either made up in water and the pH adjusted with 2 M potassium hydroxide,

or dissolved in 2 M potassium hydroxide and the pH adjusted with 2 M hydrochloric acid. Nitrogen was bubbled through each sample for 10 minutes prior to determination of its spectrum.

All pH measurements were carried out with a "Radiometer" pH meter, Model 23, fitted with a "Metrohm" AG 9100 combined electrode assembly. Even at high pH it was not necessary to apply a correction for alkali error as all alkaline solutions were based on potassium hydroxide.

Acknowledgments

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