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CARBON-13 N.M.R. STUDIES OF THE INITIAL BINDING OF Cu(II) TO AMINOGLYCOSIDE ANTIBIOTICS - A USEFUL STRUCTURAL AND FUNCTIONAL PROBE.

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Spectroscopic techniques have played an increasingly important role in the structure elucidation of the aminoglycoside antibiotics [1] during the past decade. Initially using mass spectrometry and more recently in combination with ^{13}C n.m.r spectroscopy and chiroptical methods, much has been learned in terms of the gross structures of these medicinally important substances. In this paper, we wish to report on a physico-chemical technique that probes into the functional features of these molecules as exemplified by studies on two representative aminoglycosides, kanamycin A and ribostamycin. Because of the presence of vicinal amino alcohol functions in these and related antibiotics, it was anticipated that an effective interaction with paramagnetic divalent cations would result in solution, a process that can be conveniently studied by ^{13}C n.m.r spectroscopy particularly at very low metal/ligand ratios [2] due to the specific broadening of some resonances [3]. Addition of small increments of copper sulfate pentahydrate or copper acetate hydrate to a solution of kanamycin A (free base) in D_2O, caused the selective and discernible broadening of some resonances in its ^{13}C n.m.r spectrum [4] (Fig. 1). Thus, at a Cu^{++}/ligand ratio of $9x10^{-4}$, signals corresponding to three ^{13}C nuclei were visibly affected. The addition was continued until a ratio of $30x10^{-4}$ of Cu^{++}/ligand was reached, at which time, the signals due to two other ^{13}C nuclei were sequentially broadened. The progression of signal broadening and their eventual levelling off is shown in Fig. 1, where it is clear that the signals associated with the ^{13}C nuclei of the 3-amino-3-deoxy-α-D-glucopyranosyl (kanosamine) portion are selectively affected in the order C-3", C-4" > C-1" ≫ C-6". Next to be affected was C-6' of the 6-amino-6-deoxy-α-D-glucopyranosyl portion, but at

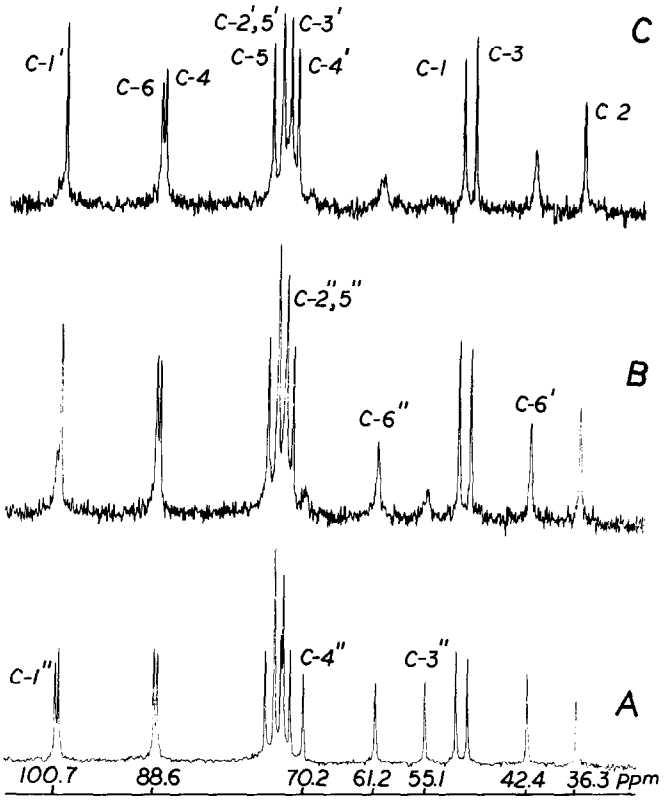
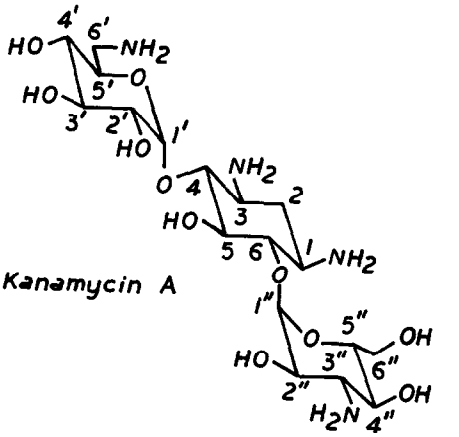


Fig. 1. 22.6 MHz Fourier transform ^{13}C n.m.r spectra of kanamycin A (free base) in D_2O: A. pD 10.9 no $CuSO_4$; B. $9x10^{-4}$ Cu^{++}/ligand; C. $30x10^{-4}$ Cu^{++}/ligand. Chemical shifts in ppm downfield from TMS (dioxane at 67.4 ppm, standard).



nearly an order of magnitude higher of Cu^{++}/ligand ratio. With increasing amounts of Cu^{++}, the paramagnetic effect was eventually felt through the entire carbon skeleton of the molecule resulting in the leveling off of the remaining resonances. The signals due to C-2" and C-5" are also broadened selectively, but the process cannot be followed because of interfering signals. Cu^{++} titration of kanamycin A monosulfate, in which all the signals of the kanosamine portion are well resolved due to β-shifts (except for C-5"), clearly showed selective broadening and eventual levelling off of these signals at a Cu^{++}/ligand ratio of $22x10^{-4}$. Interestingly, C-6' was somewhat slower in being affected compared to kanamycin free base, presumably due to the higher proportion of protonated species at that position. The results were subjected to statistical analysis [5] and a graphical representation in the case of kanamycin A is shown in Fig. 2, where the slope of the lines are a function of the line broadening. Those lines with the greatest slopes

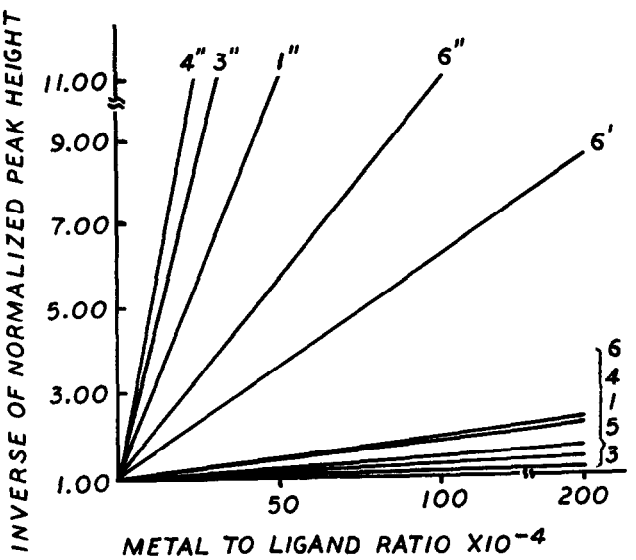
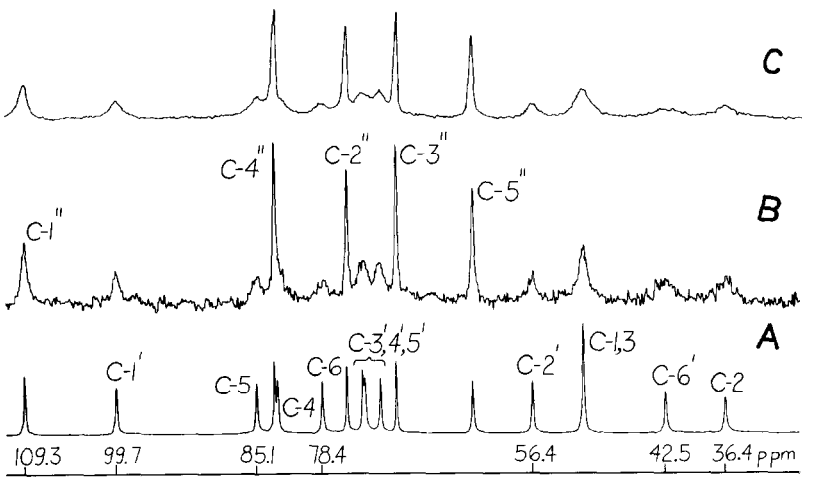


Fig. 2. Peak broadening index for kanamycin A and $CuSO_4$. Procedure: i. peak heights are normalized to the height of the 100.3 ppm peak (in mm), ii. peak heights are normalized to 100 for solutions with no Cu^{++}, iii. reciprocal peak heights are calculated and plotted versus Cu++/ligand.

correspond to ^{13}C nuclei at or very near the primary site of metal binding. In spite of the large number of functional groups and the possibility of complicated equilibria involving several species, the selectivity for C-3"/C-4" (and possibly C-2") at a specific Cu^{++}/ligand ratio is remarkable. Interestingly, other amino groups, diols, and non-vicinal amino alcohols in kanamycin A are, in general, not primary sites for chelation, as evidenced by the fact that with the exception of C-6', the ring carbon atoms of the 2-deoxystreptamine and 6-amino-6-deoxy-α-D-glucopyranosyl portions remain largely unaffected at very low Cu^{++}/ligand ratios (Fig. 1c).

The Cu^{++} titration technique could be applied to other aminoglycosides containing *cis* and *trans* (gauche) amino alcohols, N-methylamino alcohols, as well as diamino functions with essentially the same results, although the magnitudes of signal broadening, hence the relative strengths of the chelates differed. Thus, at a Cu^{++}/ligand ratio of 47×10^{-4}, the ^{13}C n.m.r spectrum of ribostamycin[6], which unlike kanamycin, is a 4,5-di-O-glycoside of 2-deoxystreptamine, was reduced to five peaks corresponding to the resonances of the D-ribofuranosyl portion of the molecule. The ^{13}C nuclei of the pseudodisaccharide portion were thus selectively affected as a result of chelation of the amino alcohol functions with the paramagnetic cation (Fig. 3).



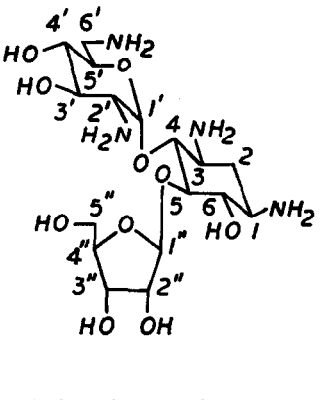


Fig. 3. 22.6 MHz Fourier transform ^{13}C n.m.r spectra of ribostamycin in D_2O: A. no $CuSO_4$; B. $30x10^{-4}$ Cu^{++}/ligand; C. $47x10^{-4}$ Cu^{++}/ligand. Chemical shifts from TMS (dioxane at 67.4 ppm, standard).

In conclusion it is evident that the Cu^{++} titration technique as described here can be a useful tool in studying the structural and stereochemical features of aminoglycoside antibiotics, particularly in conjunction with other methods. It provides a method for simplification of ^{13}C n.m.r spectra of these substances [7], and in this respect it could complement the study of β-shifts induced at acid pH.

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