THE BINDING OF ZINC(II) TO BLEOMYCIN: AN INVESTIGATION USING ^{1}H NMR SPECTROSCOPY

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1. Introduction

The bleomycins (BLM) are a group of antibiotics isolated as the copper complexes from *Streptomyces verticillus* [1]. They are used in the treatment of squamous cell carcinomas. It has been suggested that their mode of action involves strand scission of DNA [2]. This strand scission is inhibited by metal ions such as Cu(II), Zn(II) and Co(II) [3] and a mechanism for the scission, involving the Fe(II) complex, has been suggested [4,5]. Knowledge of the structures of the metal ion complexes of BLM would appear to be a prerequisite to an understanding of its function.

In this study the binding of zinc(II) to BLM was investigated using ^{1}H nuclear magnetic resonance spectroscopy (NMR).

2. Experimental

BLM was a gift from Lundbeck Ltd, Luton, Bedfordshire. It was supplied in ampoules, as for injection (copper free and consisting of about 66% BLM A_2 and 33% BLM B_2, see fig.1).

Pulsed Fourier-transform ^{1}H NMR spectra were obtained at 270 MHz and 90 MHz on Bruker spectrometers at temp. 298°K. Quadrature detection was used on the 270 MHz spectrometer and 512 transients were accumulated. The residual water signal was suppressed by applying a pulse at the appropriate frequency either prior to application of the measure pulse or at all times except during data acquisition [6]. Single-phase detection was used on the 90 MHz spectrometer and 1000 transients were accumulated. Only the zinc titration was followed at both frequencies. ^2H_2O was used as solvent in all experiments and provided an internal field-frequency lock. Chemical shift values are quoted in parts per million (ppm) downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) as internal standard.

Solutions of BLM (1 mM) were made up in 20 mM cacodylate buffer, pH* 6.5 (pH* is a direct meter reading uncorrected for the ^{2}H isotope effect). The BLM concentration was checked by comparison of peak intensities with the peak intensity given by a standard concentration of DSS. Aliquots (10 μl) of a solution of spectroscopically pure $ZnSO_4 \cdot 7\ H_2O$ (10 mM) in ^2H_2O were added to 0.5 ml samples of the BLM solution. The pH* was re-adjusted after each addition to about 6.8 using 100 mM solutions of NaO^2H and/or ^2HCl in ^2H_2O. The pH was measured using a Pye-Ingold microelectrode and Radiometer pH meter 26.

3. Results and discussion

The various bleomycins, as shown in fig.1, differ only in their terminal amine residues. All the resonances in the ^{1}H NMR spectra (fig.2) of bleomycins A_2 and B_2 have recently been assigned [7], except for those from the carbohydrate and primary amide groups. The most prominent are those due to the imidazole C2(H) (A) and imidazole C4(H) (B), the pyrimidine methyl protons (C), the bithiazole protons (D) and the *S*-dimethyl protons (E) (the latter from BLM A_2 only).

On addition of the zinc(II) solution, at both 270 MHz and 90 MHz, the C2(H) (7.80 ppm), C4(H)

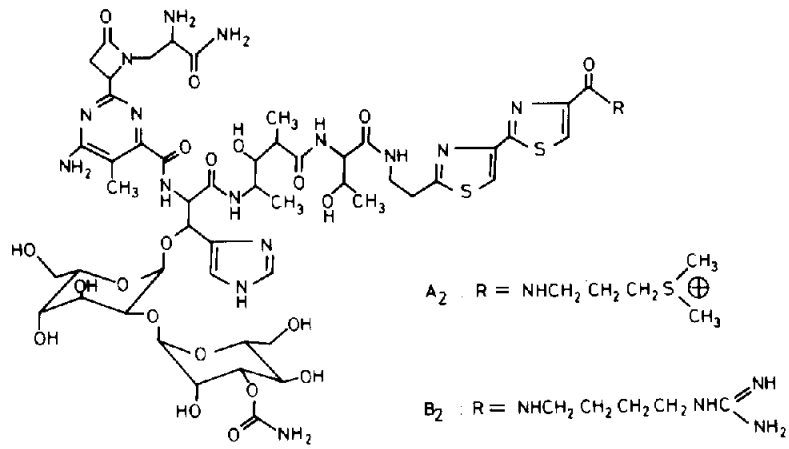

Fig.1. Structure of bleomycins (after [1]).

(7.27 ppm) and pyrimidine–Me (2.03 ppm) resonances of free BLM decreased in intensity. New resonances appeared (at 8.07 ppm, 7.36 ppm and 2.44 ppm), increasing in intensity with increasing zinc concentration. These are labelled L,M,N, respectively, in fig.2. There were other slight changes throughout the spectrum, but, significantly, the bithiazole and *S*-dimethyl proton resonances are unaffected in position and intensity by the addition of zinc. The pH* at which the experiment was performed is well above the pK_a of the imidazole residue (4.7 [8]) and avoids the complication of pH shifts of peaks. The changes observed are complete at a 1:1 mole ratio of Zn:BLM.

It seems reasonable to assign the new peaks in the spectrum of the zinc–BLM complex as follows: pyrimidine–Me 2.44 ppm, imidazole C2(H) 8.07 ppm and imidazole C4(H) 7.36 ppm, on the assumption that the zinc will not affect the chemical shift sufficiently to invert the positions of the C2(H) imidazole and C4(H) imidazole resonances. The pyrimidine–Me resonance can be assigned more confidently because of its high intensity and good separation from neighbouring peaks.

The zinc(II) is therefore in slow exchange [9] at both 270 MHz and 90 MHz since the intensity changes described above are observed, rather than continuous shifts in peak position. The upper limit on the exchange rate can be derived since $\tau_m \Delta\omega_m >> 1$ for slow exchange, where $\Delta\omega_m$ is the chemical shift difference of the proton in the bound and free environments, in radians, and τ_m is the lifetime of the bound form [9]. This condition refers to individual resonances and if it is applied to the resonance with the smallest $\Delta\omega_m$ value, it will also apply for larger $\Delta\omega_m$ values. The upper limit for τ_m can then be obtained. Thus, for the C4(H) resonance, $\Delta\omega_m$ is 49.6 radians or 7.9 Hz at 90 MHz. Therefore $\tau_m >> \frac{1}{49.6}$ s and the dissociation rate $(1/\tau_m) << 49.6\ s^{-1}$.

The large effect of zinc on the pyrimidine–Me and imidazole resonances suggests that these residues of BLM are ligands. Furthermore the imidazole resonances in the spectrum of the zinc complex are not sensitive to pH* at values greater than 4.7. At pH* values below 4.7 the zinc(II) begins to be released from the complex and peaks due to free BLM reappear at positions depending on the pH* [7]. The zinc is completely released by pH* 3.7. The zinc is at all times in slow exchange.

These results are consistent with conclusions reached on the basis of chemical evidence [10]. The other two ligands suggested [10], the α-amino group of the terminal diaminopropionic acid residue and the nitrogen of the carbamoyl group, cannot be observed under conditions employed in this work due to complete exchange of their protons with deuterium from the solvent.

The insensitivity of the resonances associated with the *S*-dimethyl group and the bithiazole moiety to zinc(II)-binding suggests that they are not included in the first coordination sphere. A model constructed on

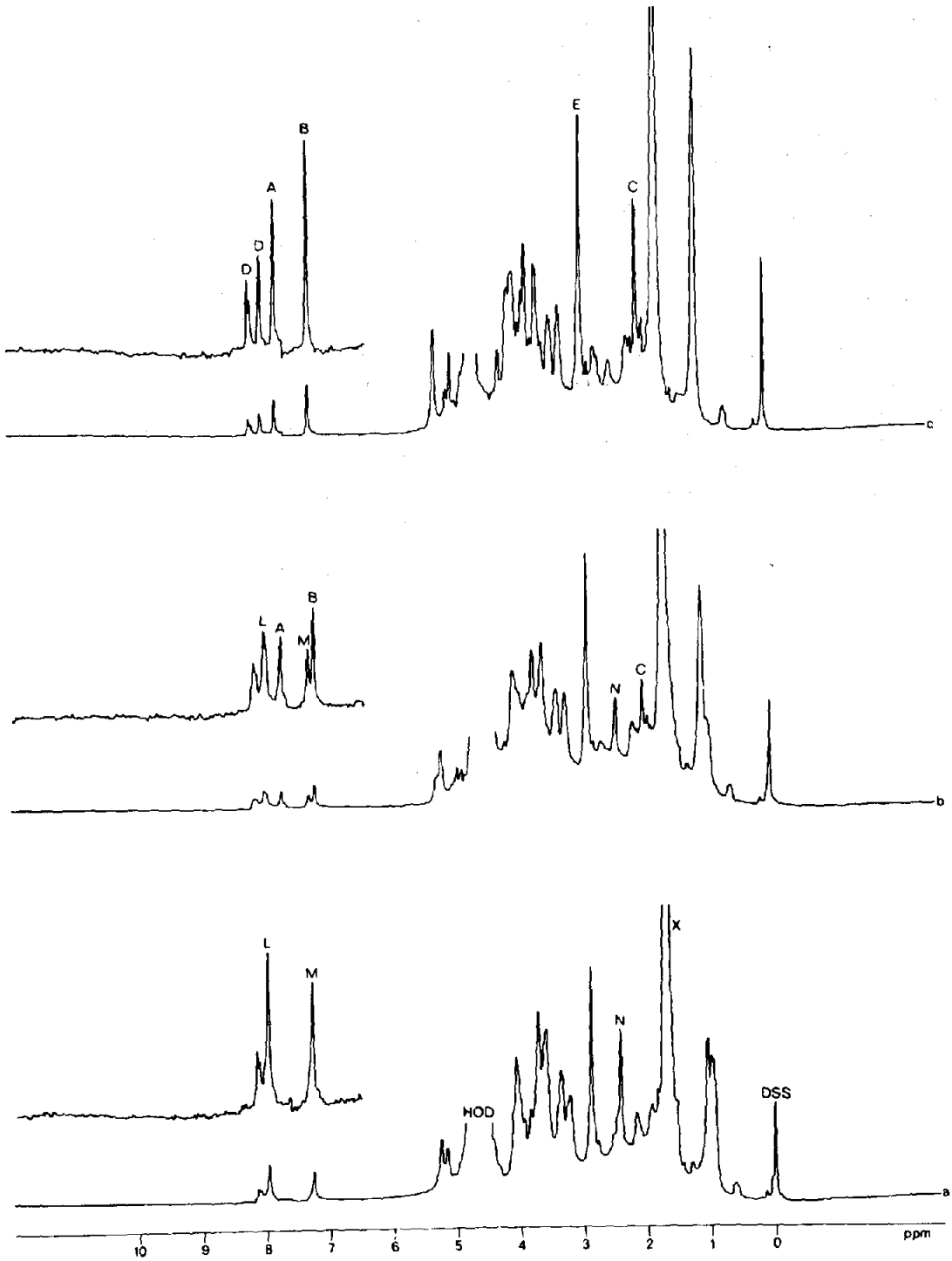

Fig.2. ^{1}H NMR spectra at 270 MHz of BLM with (a) 1 mol, (b) 0.4 mol, (c) 0 mol Zn(II)/mol BLM. The vertical scale of the aromatic region is expanded 4 times in each case. X is a resonance associated with the buffer. For other labels, see text.

the assumption that the imidazole, pyrimidine, α-amino group of the terminal diaminopropionic acid residue and the nitrogen of the carbamoyl group act as ligands leaves the bithiazole 'tail' free. It has been suggested [11] that it is the latter which interacts with the DNA. The bleomycin may therefore owe its activity to this bifunctionality, one part acting as a ligand for metal ions, the other interacting with DNA.

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