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CONFORMATIONAL FLEXIBILITY OF BLEOMYCIN-A_2: A CARBON-13 SPIN-LATTICE RELAXATION TIME STUDY

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Summary: The extent of segmental motion of the backbone and sidechain carbons of the glycopeptide antibiotic, bleomycin-A_2 in aqueous solution, has been determined from the natural abundance carbon-13 spin-lattice relaxation times (T_1) measured at 67.89 MHz. The backbone carbons of the central residues have an average T_1 of 163 msec, corresponding to an isotropic rotational correlation time of 3.9×10^{-10} sec. The backbone and sidechain carbons exhibit varying degrees of motional freedom. The solution conformation of bleomycin-A_2 is discussed in terms of the conformational flexibility available to these carbons.

The bleomycins represent a family of glycopeptide antibiotics that exhibit antibacterial and antitumor activities. They are clinically employed in cancer chemotherapy (1,2). Complexes of bleomycin with certain radioactive materials are also employed in the detection of a broad range of solid tumors and malignant lymphomas (2-4). The antineoplastic activity of bleomycin has been ascribed to its ability to induce single strand scission and fragmentation of DNA (2,5,6). The proton (7a) and carbon NMR assignments (8) of this antibiotic have already been reported. Several experimental (2,6, 9-11) and theoretical (12,13) studies have dealt with the interaction of bleomycin with metal ions and DNA. Characterization of the free solution conformation and dynamics of bleomycin serves as a logical starting point in delineating the interaction of this antibiotic with metal ions and nucleic acids. Towards this end, we have analyzed the ^{13}C spin-lattice relaxation times of specific carbon atoms of bleomycin-A_2 (Bleo-A_2), the major natural congener of bleomycin. These parameters are sensitive indicators of

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molecular conformational flexibility (14). This investigation provides a basis for identifying regions of secondary or tertiary structure in the Bleo-A_2 molecule, since such interactions are expected to restrict the conformational mobility of the participating carbon atoms.

Materials and Methods: The various bleomycins differ only with respect to the structure of the C-terminal amine. The commercial drug, Blenoxane (supplied by Bristol Laboratories, Syracuse, N.Y.) consists predominantly of a 3:1 mixture of two of the congeners, Bleo-A_2 and bleomycin-B_2 (Bleo-B_2) with trace quantities of other congeners. Most of the proton and carbon-13 resonances are identical for Bleo-A_2 and Bleo-B_2 and evidence from chemical shift studies (7) suggests similar solution conformational characteristics for these two congeners. For this reason, in the present investigation, the carbon-13 spin-lattice relaxation times of Blenoxane have been analyzed in terms of the conformational flexibility of the major congener, Bleo-A_2. The commercial drug was used in the experiments without further purification. A concentrated solution (250 mM in D_2O) of Blenoxane was prepared (pH_m = 5, where pH_m is the pH meter reading uncorrected for deuterium isotope effects) and all the carbon-13 studies were performed in 10 mm or 15 mm o.d. NMR sample tubes. The carbon-13 experiments, under broad band proton noise decoupling conditions, were performed at 67.89 MHz (on Bruker WH-270 and HX-270 spectrometers). Additional carbon-13 T_1s were measured at 37.7 MHz. Spin-lattice relaxation times (T_1) of the individual carbons of Blenoxane, were measured (accuracy $\pm$20%) through spin inversion-recovery experiments. The assignment of the various resonance peaks to the respective carbons of Bleo-A_2 has been given earlier by Naganawa et al (8).

Results, Analysis and Discussion: The recently revised (15) structure of Bleo-A_2 together with the NT_1 values (where N is the number of directly bonded hydrogens) obtained in the present investigation for the protonated carbons is shown in Fig. 1. The expressions for the analysis of T_1 data in terms of ^{13}C-^{1}H dipolar interactions and rotational correlation times are available in the literature (16-18). A plot of T_1 as a function of τ_{eff}, the isotropic rotational correlation time, gives a curve exhibiting a minimum, and thus for large molecules each experimental T_1 value can yield, two values for the τ_{eff}. We have confirmed, by performing measurements at a lower field (38 MHz), that the ^{13}C relaxation times of Bleo-A_2 correspond to the shorter correlation time side of the T_1 vs τ_{eff} plot. In this paper, we will confine ourselves to a discussion of the relative mobilities in terms of net effective motion, i.e. the greater NT_1, the greater is the motional freedom available for the protonated carbon. A detailed analysis of the T_1 data invoking multiple internal rotations, nonexponential autocorrelation functions (19-21) and nonspherical models (22) for the molecular shape, is beyond the scope of this short communication. These aspects will be examined elsewhere.

Bleomycin-A_2 is a linear peptide consisting of the amino acids I-VI and a terminal amine residue (VII) (Fig. 1). Carbamoyl mannopyranosyl

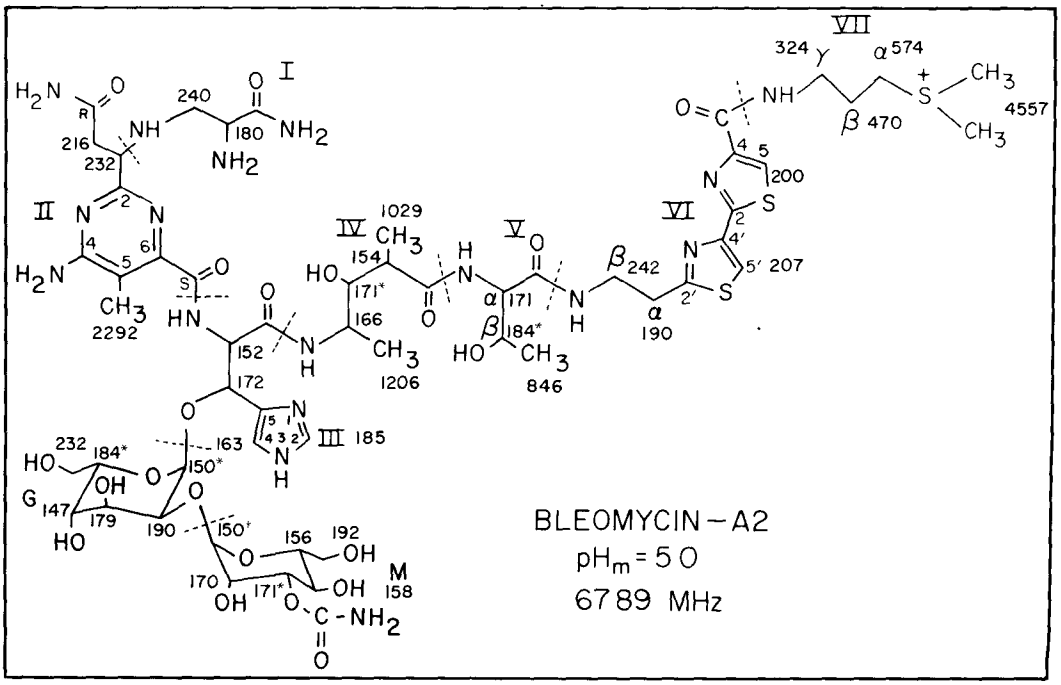

Figure 1: Primary sequence of bleomycin-A_2 showing NT_1 values (in msec) obtained at 67.89 MHz for protonated carbons. Numbers with superscripts indicate resonance overlap. Boundaries between the labeled residues are indicated with broken lines.

gulose is attached to the peptide through an oxygen at the β-carbon of the hydroxyhistidine residue (III). The NT_1 values along the backbone (Fig. 1) suggest regions with varying degrees of conformational flexibility. In the following, we will discuss the conformational freedom of the different moieties of Bleo-A_2.

Backbone carbon T_1s of the central residues III, IV and V are relatively constant and vary from 154 msec to 171 msec with an average of 163 msec. Using the model of an isotropically reorienting rigid molecule (17) we find that this relaxation time corresponds to an isotropic rotational correlation time $\tau_{eff} = 3.9 \times 10^{-10}$ sec. This value for Bleo-A_2 may be compared with the correlation times estimated for the peptide hormones oxytocin (23), lysine vasopressin (23), angiotensin (24) and the luteinizing hormone-releasing hormone (LH-RH) (17). Since the latter peptides were studied under experimental conditions (e.g., pH, concentration, and temperature) somewhat different from that of bleomycin, the comparison is not rigorous. The hormones oxytocin, lysine vasopressin and (Ile^5) angiotensin have similar molecular weights ($\sim$1000) and similar correlation times, $\tau_{eff} \sim 5 \times 10^{-10}$ sec. From the average

NT_1 value of 170 msec (at 68 MHz) for the central residues (17) of the linear peptide hormone LH-RH (M.W. $\sim$1200), we estimate a somewhat shorter effective correlation time of 3.6×10^{-10} sec. This was attributed to increased segmental motion along the backbone of LH-RH (17). The effective correlation time of 3.9×10^{-10} sec for the central residues of Bleo-A_2 (M.W.$\sim$1500), thus, appears to be reasonable. Assuming a hard sphere diameter of 14.6 Å (estimated from CPK models of Bleo-A_2) and using a viscosity of 10.4 mP for D_2O, we obtain from the Stokes-Einstein relation (25) a value $\tau_{mol} \sim 4 \times 10^{-10}$ sec at 308°K. Such good agreement between τ_{mol} and τ_{eff} is, to some extent, fortuitous in view of the approximations involved in the expression for τ_{mol}, and the large error associated with measuring hard sphere diameters from molecular models. Since the most restricted α carbons of oxytocin and lysine vasopressin (M.W. $\sim$1000) have correlation times of the order of 5×10^{-10} sec, we would expect a slightly longer effective correlation time for Bleo-A_2 in view of its larger molecular weight. The significantly shorter correlation time of 3.9×10^{-10} sec may be indicative of segmental motion along the backbone of Bleo-A_2, which is somewhat less significant in the cyclic octapeptides oxytocin and lysine vasopressin.

The α- and β-carbons of L-threonine have similar T_1 values. The β-carbon of threonine in the model pentapeptide Gly-Gly-Thr-Gly-Gly shows (26) greater motional freedom than the corresponding C^{α}, the ratio of their T_1s being $\sim$1.4. The substantially lower T_1 ratio (1.08) of Bleo-A_2 (though measured at different experimental conditions viz, resonance frequency, pH, concentration, temperature) suggests that in the case of Bleo-A_2 the sidechain of threonine experiences restricted mobility about the C^{α}-C^{β} bond, either because of steric hindrance from other parts of the molecule or due to an internal hydrogen bond involving the hydroxyl proton. The methyl carbon of threonine has a long relaxation time because of fast internal rotation around its symmetry axis. Using Woessner's model for isotropic rotational diffusion with one degree of internal rotation (16,27), we obtain the correlation time for methyl group internal rotation, $\tau_G \sim 1.2 \times 10^{-11}$ sec assuming that the isotropic rotational correlation time corresponds to that of the β-carbon

The α- and γ-methyl carbons of the valeric acid residue (IV) also have long NT_1 values, as a result of fast internal rotation. Using Woessner's model (16,27) we compute internal rotation correlation times of 4.7×10^{-12} sec and 2.9×10^{-12} sec, respectively, for the α- and γ-methyl carbons. The T_1s of the protonated carbons of the histidine ring in L-erythro-β-hydroxyhistidine are similar to that of the β-carbon, suggesting restricted rotation of the ring about the C^{γ}-C^{β} bond. Using the appropriate angles in Woessner's model (16,27) for orientation of the C-H vectors of the histidine ring with

respect to the axis of internal rotation, we obtain $\tau_G \gtrsim 1 \times 10^{-10}$ sec. In making this estimate we have assumed an isotropic correlation time corresponding to the β-carbon T_1. The methine carbon T_1 values of the gulose and mannose moieties are similar to those of the central residue carbons, and thus the motional freedom of these moieties is similar to that of the central residues. In these moieties, the NT_1 values for the C(6) carbons are slightly longer, as a result of increased segmental motion available for these carbons.

The C-terminal dipeptide moiety (residues VI and VII) appears to be relatively more flexible than the central residues. This is especially true of the terminal residue (VII), whose methylene carbons show a monotonic increase in the NT_1 values from 324 msec to 574 msec towards the C-terminus, as a result of a corresponding increase in segmental motion. The methyl carbons of this residue have an NT_1 value longer than 4 sec as a result of fast internal rotation superimposed on the fast segmental motion of the terminal methylene carbons. The relative motional freedom of the terminal residue may facilitate ionic binding of the terminal cationic portion to DNA phosphates, a step which is believed to be the first stage in the reaction of Bleo-A_2 with DNA (6,9). The protonated carbons of the bithiazole moiety have similar T_1 values suggesting similar degrees of motional freedom for these carbons, i.e. there is no large gradation in the segmental motion across the C(2)-C(4') bond connecting the two rings. Crystallographic studies indicate (28) that the two rings are almost coplanar. Preservation of this coplanarity in solution also might provide one possible explanation for the similarity of T_1 values, since the two rings would move, in this case, as a single unit. A small degree of motional freedom about the C(2)-C(4') bond cannot, however, be excluded. The magnitude of the T_1 values indicates that the conformational flexibility of the bithiazole moiety and that of adjacent α-and β-methylene carbons in residue VI is intermediate to that of the central residues and the terminal residue (VII). The larger NT_1 values of the methylene carbons of residue VI could be explained by the somewhat increased motional freedom available to these carbons due to the absence of bulky side chains. This behavior is entirely similar to that of the α-carbon of the central glycine residues in LH-RH (17), and its des-glycinamide[10] N-ethylamide analog (29).

The N-terminal dipeptide (residues I and II) shows an interesting variation in its segmental motion. The methine and methylene carbons of residue II have NT_1 values substantially longer than the central residue carbon T_1 value of 163 msec. In addition, the methyl carbon of the pyrimidine has NT_1 = 2292 msec, which is longer than the NT_1 values of methyl

groups associated with the central residues. These observations suggest that residue II experiences greater freedom in its segmental motion than the central residues III, IV and V and the sugar moieties. The methylene carbon of residue I shows an NT_1 value similar to the methine and methylene carbon NT_1 values of the penultimate residue II, and hence it has a similar degree of motional freedom. Interestingly enough, this freedom does not appear to be transmitted to the α-carbon of the terminal residue, which has a T_1 value of 180 msec, fairly close to the central residue average T_1 of 163 msec. This terminal α-carbon experiences restricted mobility perhaps because of a hydrogen bond involving one of the amide hydrogens or due to steric forces from other parts of the molecule. That this carbon may be involved in some type of secondary or tertiary structure is also indicated from the ^{13}C chemical shift studies (7b).

In conclusion, the carbon-13 spin-lattice relaxation times proved to be particularly sensitive in delineating the conformational flexibility of bleomycin-A_2 in aqueous solution. There is evidence of segmental motion along the backbone of Bleo-A_2. An effective rotational correlation time of 3.9×10^{-10} sec has been estimated for the backbone carbons of the central residues (III, IV, V). The conformational freedom of the disaccharide moiety is similar to that of the central residues. The sidechain of threonine (residue V) and the histidine ring of residue III show restricted segmental motion. The C-terminal cationic group appears to be relatively more mobile in its segmental motion. Carbon-13 chemical shift studies (7b) of Bleo-A_2 give evidence for very limited interaction between the C-terminal tripeptide (residues V-VII) and the N-terminal tetrapeptide (residues I-IV). It is probable that the C-terminal dipeptide or at least the terminal residue extend(s) into solution. The α-carbon of the N-terminal residue appears to be somewhat restricted in its motion whereas the adjacent, penultimate residue appears to be more mobile than the central residues.

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