



## Systematic Chirality Investigations of Zn-TLM binding Sites by 2D-NOESY Back-calculations

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**Abstract :** The systematic chirality investigations were made on the basis of the fact that zinc-binding tallysomyacin (ZnTLMA) could have chiral centers (Zn, NC3, C6) at possible 4-, 5-, and 6-coordination models. Although our NMR data exhibit that the ligation sites are  $\beta$ -aminoalanine,  $\beta$ -hydroxyhistidine, and pyrimidine moiety, all possible coordination modes were tested out to see what kind of chiralities on NC3-C6 are favorable to each coordination mode. Tests were also made that take into account the specific configuration of functional groups, including  $\beta$ -aminoalanine, sugar ring, and  $\beta$ -hydroxyhistidine. Tests were finally extended to zinc-water binding and specific conformational studies by introducing various hydrogen bonding networks associated with the propionamide side chain and the carbamide group of mannose. Results of systematic chirality investigations exhibit that the S-S configuration of NC3-C6 is favorable to all of coordination models, but the R-S configuration, if exists at all, should have internal strain on C6 chiral center.

Antitumor antibiotics, including bleomycin and tallysomyacin, shown in Fig. 1, are clinically used against certain malignant lymphomas, squamous cell carcinomas, and testicular carcinoma. Metallo-bleomycins clearly demonstrated their ability to recognize and bind noncovalently to specific DNA base sequences.<sup>1</sup> In this view, the interest of metal ion activated DNA endonucleolytic cleavage reaction has been pursued in the past decade for the clinical purposes.<sup>1-2</sup> However, detailed structural feature and metal-binding mode of drugs were not clearly elucidated yet, and the nature of metal-mediated or metal-free drug mechanism of DNA cleavage is actively being studied. Difficulties in obtaining suitable crystal for drugs have hampered the X-ray crystallographic structural studies that may enhance the understanding of drug-metal-dependent chemical mechanisms. Therefore, structural studies have thus far relied on simple model complexes and on NMR studies for metallo-drug complexes. Interestingly, the stereospecific configuration of the  $\beta$ -carbon atom

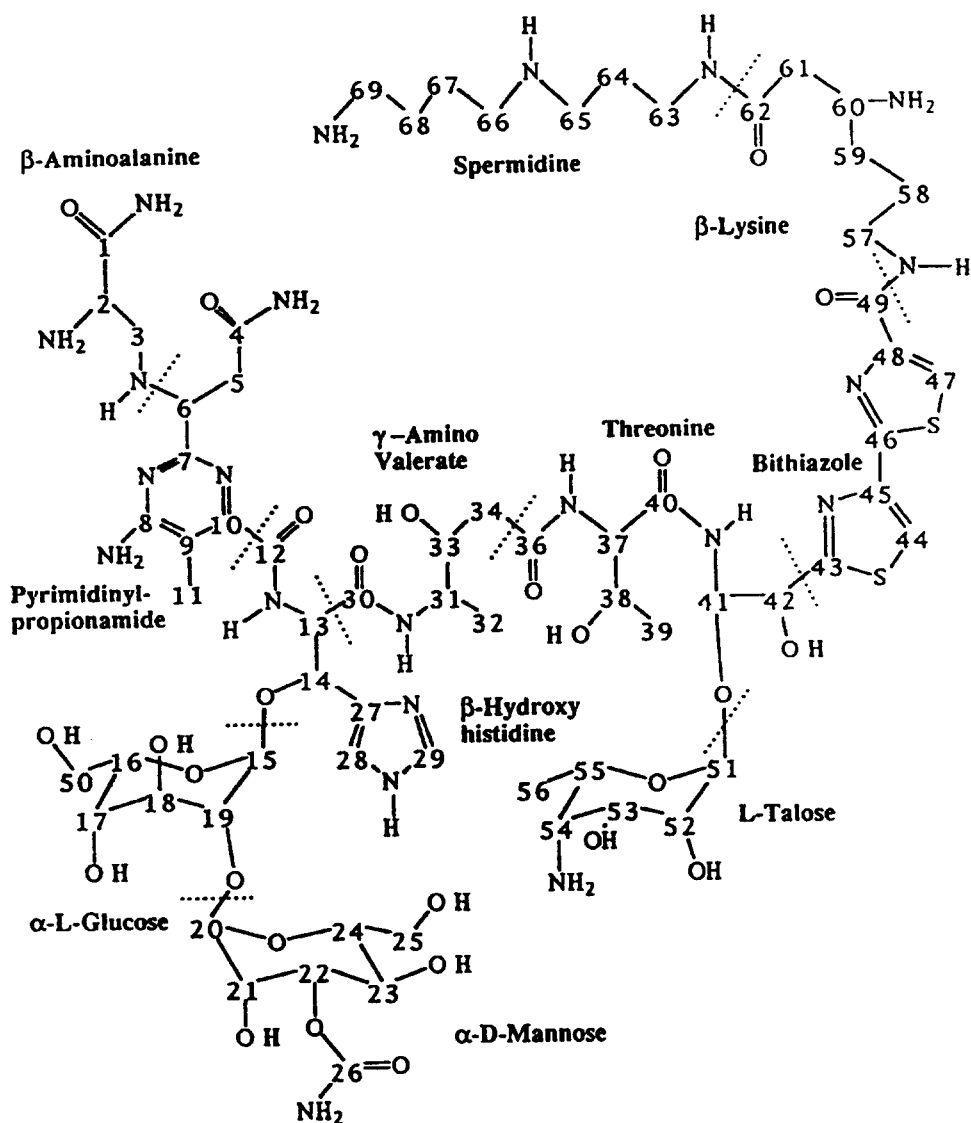


Fig. 1. Molecular structure of tallysomycin-A with functional groups and numbering scheme

of pyrimidinyl propionamide (C6) previously made on the basis of model studies recently came into question. Recent model synthesis of bleomycin (a pseudodipeptide containing pyrimidinyl propionamide of bleomycin) exhibits that pseudodipeptide can have both S- and R-configuration at C6 chiral center. The chirality of C6 was originally determined to be S-configuration based on the X-ray crystal structures of a Cu-P3A model complex and an acid hydrolysis degradation product of bleomycin.<sup>3</sup> In order to understand the conformational geometry of zinc in addition to tallysomyacin, modern NMR techniques were applied and subsequently systematic chirality-dependent conformation were studied with NMR-based distance geometry and 2D-NOE back-calculation methods.

## Experimental Section

**Preparation of ZnTLMA :** A standard ZnCl<sub>2</sub> solution was prepared by dissolving highly pure ZnCl<sub>2</sub> hexahydrate (99.98%) in deionized water and by determining the concentration of ZnCl<sub>2</sub> solution with titration of the standard EDTA solution (0.1M). The standard ZnCl<sub>2</sub> solution was subsequently lyophilized three times to minimize the amount of H<sub>2</sub>O. A zinc titration of tallysomyacin-A(TLMA, purchased at Bristol Myer Co.) was performed in order to monitor the formation of ZnTLMA. The degree of complexation both in H<sub>2</sub>O and D<sub>2</sub>O was checked by REDFIELD NMR pulse sequence. Finally, the sample of ZnTLMA (6.7 mM) was dissolved in 500 ml of D<sub>2</sub>O and placed in an NMR tube.

**NMR Data :** <sup>1</sup>H-NMR spectroscopy was performed on a Nicolet 360-MHz and GN-500 MHz, GEQ-600 MHz spectrometers. Spectra were typically recorded for samples dissolved in 99.98% D<sub>2</sub>O with the following parameters: 90° pulses presaturation of HOD; 16K data points; 0.5 Hz line broadening; 32 scans. 1-1 echo experiments were carried out with a sample dissolved in the mixture of H<sub>2</sub>O(90%)/ D<sub>2</sub>O(10%) in order to obtain the optimal temperature and pH condition of ZnTLMA. Raw NMR data were transferred via ethernet to SGI 4D/25 Silicon Graphics Personal Iris computers before being converted into readable format and then processed with programs FELIX and FTNMR (Hare Research Inc.). Phase-Sensitive NOE Spectroscopy (PSNOE) was performed with a 512 × 2048 (GN-500) or 1024 × 2048 (GEQ-600) data matrix size with 16 scans per t<sub>1</sub> and spectra were zero-filled to 2048 × 2048 real points. The delay time between scans was 1.0 s, and the mixing time was 300 ms. Gaussian line broadening of 2 Hz was used in the t<sub>2</sub> dimension before Fourier transformation. A 90° shifted sinebell squared filtering was used in the t<sub>1</sub> dimension before Fourier transformation. For NMR-based DG computations and 2D-NOESY back-calculation methods, NOESY data were collected at five different mixing times of 5, 50, 100, 250, 500 ms.

## NMR Observations and 2D-NOE Back-Calculations

**NMR Observations :** Through-space connectivities associated with zinc-binding site were obtained by performing the NMR signal assignments and volume integration as shown in Fig. 2. The conformation that C6 and NC3(next metal binding site in the macrocyclic ring) have S- and R-configuration, respectively, has been used in most of metal-bleomycin (or derivatives) studies. Interestingly, our recent observations in NMR-based structural studies of zinc-binding tallysomyacin A (ZnTLMA) exhibit that the chirality of C6 may have a R-configuration. A medium intensity NOE from proton of C6 (H6) to mannose (H22) exhibits that the chirality of C6 may be reversed to be R-configuration. Several weak NOEs from  $\beta$ -aminoalanine (H3,3') and  $\alpha$ -carbon atom of pyrimidinyl propionamide (H5,5') toward the mannose sugar ring (H21, H22) which is geometrically close to pyrimidine moiety were observed. In addition, experimental strong NOE observations from H3,3' to H5,5' reveal that two side chains  $\beta$ -aminoalanine and propionamide are geometrically close to each other (cis-configuration). The zinc-complexation to TLMA give rise to an chiral center on NC3 which is important in determining the C6 configuration. Previous chiralities of NC3-C6 determined to be R-S are not consistent with experimentally observed strong NOEs associated with H3,3 and H5'5'. The weak NOE observation from H6 to H3,3' at NOE spectra (250 ms) exhibit that the  $\beta$ -aminoalanine side chain is in trans-configuration with H6. Modern NMR-based distance geometry (DG) methods and NOE back-calculations were utilized in order to access the chirality of C6 in this study. NOE experiments were performed both in D<sub>2</sub>O (5, 50, 100, 250, 300, 500 ms) and H<sub>2</sub>O (190 ms) to see NOE behaviors and to obtain NOE-derived distance information for exchangeable and non- exchangeable protons.

The chirality test was based on the fact that ZnTLMA has chiral centers (Zn, NC3, C6) in 4-, 5-, and 6-coordination models. Although our NMR data exhibit that the ligation sites are  $\beta$ -aminoalanine,  $\beta$ -hydroxyhistidine, and pyrimidine moiety, all possible coordination modes were examined to see what kind of chiralities on NC3-C6 is preferred in each coordination mode.<sup>4</sup> Tests were also made that take into account the specific configuration of functional groups, including  $\beta$ -aminoalanine, sugar ring, and  $\beta$ -hydroxyhistidine. Tests were finally extended to zinc-water binding and specific conformational studies by introducing various hydrogen bonding networks associated with the propionamide side chain and the carbamide group of mannose and 4-coordination(4C) modes (NC2-NC3-NC10-NC29, NC2-NC3-NC10-NC12), 5-coordination(5C) modes (NC2-NC3-NC10-NC12 -NC29, NC2-NC3-NC10-NC12-OC26), and 6-coordination(6C) modes (NC2-NC3-NC10-NC12-NC29-OC26, NC2-NC3 -NC10-NC12-OC26-H<sub>2</sub>O~NC29 (hydrogen bond on NC29-H<sub>2</sub>O), NC2-NC3-NC10-NC12-OC26 -H<sub>2</sub>O) associated with Zn-NC3-C6 chiral center were tested on the

basis of NMR-based DG computations and 2D NOE back-calculations. DG structures have been generated by utilizing the routine 2D-NOE back-calculations and DG computation procedures. In order to access the conformations of specific functional groups (imidazole, sugar, and aminoalanine) participate in the active metal binding sites, a molecular plane [pyrimidine ring(right) plus a ring(left) associated with C6, NC3, Zn, NC10, C7] was defined. By this definition, the positions of the functional groups (imidazole ring,  $\beta$ -aminoalanine, and sugar ring) were redefined as up and down. For example, R-configuration of Zn can give rise to the up and down positions of imidazole ring in 4-coordination modes.

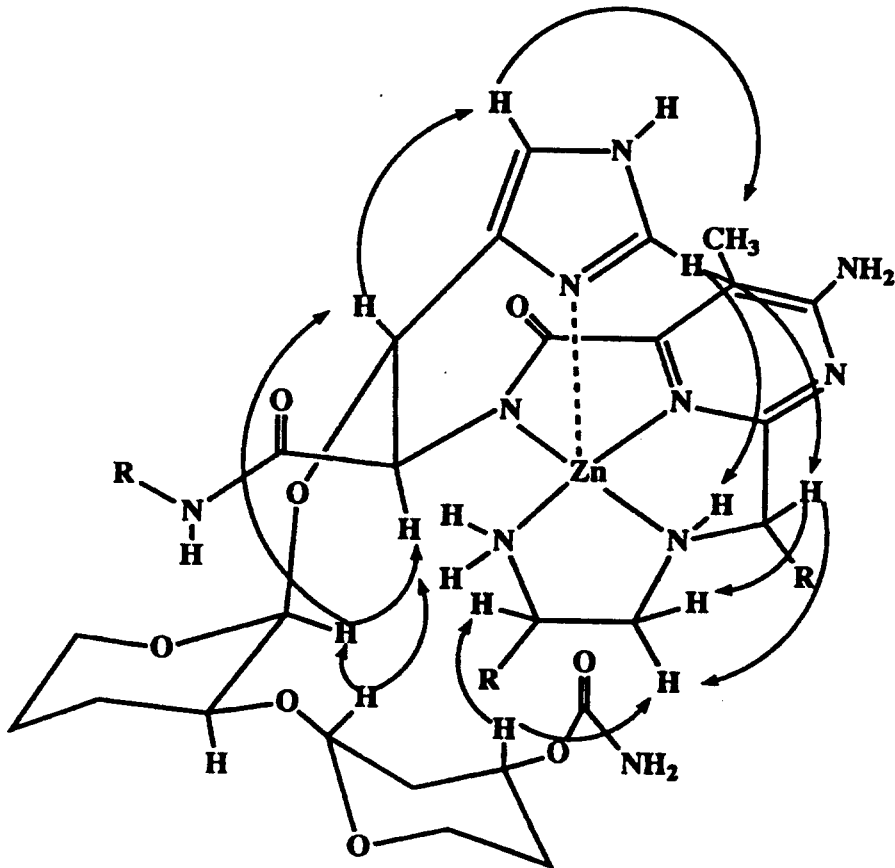


Fig. 2. Some important NOE connectivities near metal-binding site.

**Systematic Chirality Investigations :** The NOE restraints were obtained via qualitative assessment of the NOE cross peak volumes in the NOESY spectra. However, no restraints were included to attempt to fit the spin diffusion cross peaks. Instead, loose NOE restraints were used for cross-peaks classified as strong, medium, and weak. The ranges of initial NOE restraints were assigned with 2.0-2.5 Å (strong cross peak intensities), 2.0-3.5 Å (medium cross peak intensities), and 2.0-4.5 Å (weak cross peak intensities). The reasons for the NOE assessments for structure generation and refinement are described.

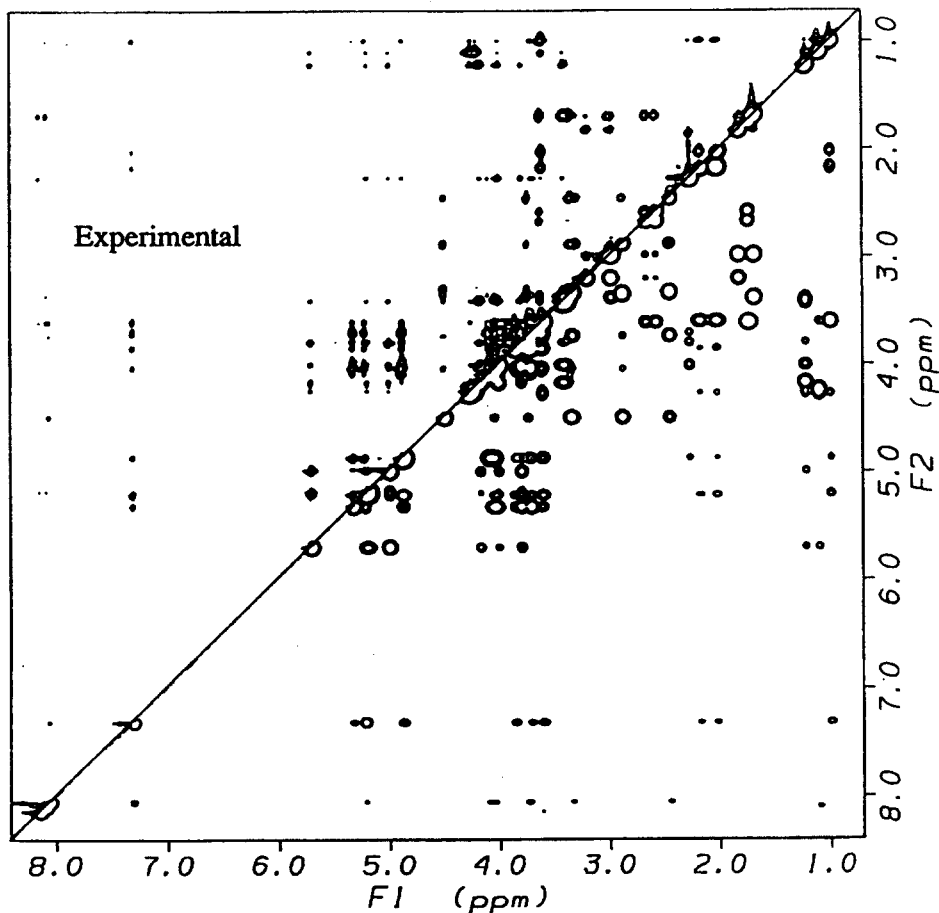


Fig. 3. Comparison of experimental and back-calculated 2D-NOESY spectra at 250 ms mixing time. The Back-calculated NOE spectrum was obtained from a distance geometry structure with coordination geometry Zn-NC2,NC3,NC10,NC12,NC29 mode.

Distance geometry(DG)<sup>5</sup> structures were generated and refined by using primary restraints and loose NOE-derived distance restraints. Trial distances generated by selecting random distances between the upper and lower bounds of each element were embedded in 3-dimensional space by the metric matrix method and subjected to simulated annealing (SA) and conjugated gradient minimization (CGM). The embedded initial coordinates containing violations of the upper and lower boundary restraints were then refined. After minimization to a moderate target penalty (ca.  $0.3 \text{ \AA}^2$ ), the initial DG structure was saved, and new DG structures were generated by performing two  $10 \text{ \AA}$  randomization of atom positions, followed by SA and CGM refinement. Once a low-penalty (penalty = squared sum of the covalent and experimental bounds violation) structure was stored, new starting coordinates were then obtained by performing different embedding, SA, CGM algorithms. Further refinement was achieved by application of variable velocity simulated annealing (to maximum penalty values of  $10\text{-}20 \text{ \AA}^2$ ), SHAKE (to penalties of ca.  $10 \text{ \AA}^2$ ) and CGM algorithms.

The complete time course for nuclear relaxation was then determined for each refined structure via numerical integration of the Bloch equations. As described previously, this approach accurately accounts for spin diffusion. Generic Z-leakage rate constant  $K_{z1}$ , accounting for the loss of Z-magnetization within mixing period, of  $1 \text{ sec}^{-1}$  ( $3 \text{ sec}^{-1}$  for Me11, Me32, Me39, Me56) was used. A cross relaxation rate constant  $K_{cr}$  of  $60 \text{ sec}^{-1}$  which governs the cross relaxation rate was used in this NOE back-calculation.  $K_{cr}$  was determined by using NOE build-up curve comparisons in advance with well resolved germinal protons which have structurally known internuclear distances with  $1.8 \text{ \AA}$ . The cross relaxation rate term includes intramolecular dipolar relaxation, effect of chemical exchange, quadrupolar relaxation, the possible contributions from spin rotation, scalar relaxation, and multiple quantum effects. 2D NOE back-calculation gives a list of normalized auto- and cross peak intensities for selected increasing mixing times. Profiles of these outputs provide the theoretical NOE build-up curves. The results of back-calculation are then assigned to GNOE in order to generate the theoretical 2D NOEs. A consecutive serial files, obtained from GNOE calculation for different mixing times, are incorporated into FELIX to generate 2D NOE back-calculated spectra which can be directly compared with experimental spectra. The back-calculated 2D NOE spectra of a DG-NOE structure (lowest penalty value,  $0.08 \text{ \AA}^2$ ) are consistent with experimental NOE spectra(see Fig. 3).

## Results and Discussion

A relatively new NMR-based DG approaches and 2D NOE back-calculations have enabled the systematic chirality investigation of ZnTLMA. Not all the results of the systematic chirality investigations for the ZnTLMA are consistent with the original R-configuration of C6. Rather, the S-configuration of C6 is in good agreement with

experimental 2D NOESY spectra. Consistencies between experimental and back-calculated NOESY spectra are the major criteria for evaluating the suitability of the DG structures. As identified in structure determination for small molecule, 2D NOE back-calculation plays an important role in identifying specific orientation and prochiral assignments.

The 4-coordination models were better consistent with experimental NOE data than any other models. Although two binding-modes (NC2-NC3-NC10-NC29, NC2-NC3-NC10-NC12) were resulting in good fitting, the former was better than the latter in terms of penalty and 2D-NOEs. In the former mode, the *S-S* chirality (NC3-C6) gives best results (in the experimental and back-calculated spectra). Two good structures (R-R, S-S on NC3-C6) were obtained in the latter mode, but it turned out that the S-S configuration yielded a bad 2D-NOE back-calculation results.

In the case of 5-coordination model test, there are two different binding-modes (NC2-NC3-NC10-NC12-NC29, NC2-NC3-NC10-NC12-OC26). The former was better than the latter in terms of penalty and 2D back-calculations. The *S-S* chiralities of NC3-C6 resulted in better fitting than any other different chirality of models. In the latter mode, the S-S of NC3-C6 was better than any other different chirality. However, this mode gave higher internal strains on C14, NC3, C6 chiral centers and bad 2D back-calculations.

In the case of 6-coordination model test, there are three different binding-modes (NC2-NC3-NC10-NC12-NC29-OC26, NC2-NC3-NC10-NC12-OC26-H<sub>2</sub>O, NC2-NC3-NC10-NC12-OC26-H<sub>2</sub>O~NC29 hydrogen bonding) that have been tested out. Compared with 4C, 5C models, some of 6C models gave higher penalty values and resulted in bad 2D back-calculations. In general, the tight distance or the long distance from Zn to NC29 gave internal strains on NC3 and C6 atoms. In the first binding-mode, both S-S and R-R of NC3-C6 were observed to result in better fitting than other combinations in 2D NOEs. However, higher internal strains on C14, NC3, C6 were observed. In the second binding-mode, a relatively low penalty value (0.07 Å<sup>2</sup>) for the R-R configuration of NC3-C6 chiral centers was obtained but, the 2D back-calculation showed that this is not a good model. The third binding-mode gave relatively higher penalty and bad results in 2D-NOE back-calculations. Results of systematic chirality investigations exhibit that the S-S configuration of NC3-C6 is preferred in all of coordination models, but the R-S configuration, if exist at all, should have internal strains on C6 chiral center.

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