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^{1}H NMR Study of Heteroassociation of Daunomycin and Propidium Iodide in Aqueous Solution*

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Abstract—Heteroassociation of an anthracycline antibiotic Daunomycin (DAU) and phenanthridine dye Propidium iodide (PI) in aqueous solution was studied by ^{1}H NMR spectroscopy. The complex PI–DAU is stabilized mainly by dispersion van der Waals interactions and hydrogen bond between the 3(8)-amino group of the dye and 9-acetyl group of DAU. This conclusion follows from comparison of parameters of DAU–PI heteroassociation and complex formation of DAU with aromatic dyes, Proflavine and Ethidium bromide, under the same conditions.

Studies of heteroassociation of biologically active aromatic molecules are important from both theoretical and practical viewpoints. First, they provide information on the nature of physicochemical interactions which affect the affinity of aromatic molecules in solution and depend on structural features of the chromophore and side chains of the aromatic ligand. Second, from the medicobiological viewpoint, heterocomplexes formed by aromatic compounds and their concurrent binding to receptors could influence the solubility and efficiency of antibiotics. For example, caffeine and nicotinamide (vitamin PP) increase the solubility of a series of antibiotics in aqueous solution [1, 2]. Aromatic compounds isolated from food raw materials, such as polyphenols and methylxanthines, can act as regulators of pharmacological activity of antibiotics and DNA protectors from complex formation with aromatic mutagens [3–6]. This problem also includes some aspects of using antibiotics in combination with other drugs in pharmacotherapy [7, 8]. Thus studies of heteroassociation of aromatic molecules are related both to the solubility and efficiency of antibiotics and to the diet in chemotherapy [9, 10].

Various heteroassociation models have been proposed in the recent years for aromatic molecules [4, 5, 11–15]. However, most of these are characterized by fairly severe limitations which restrict their practical application (for details, see [16, 17]). As a rule, the proposed models [4, 5, 11–15] do not consider the possibility for formation of multidimensional aggregates of any size via both self-association and heteroassociation. Also, they provide no analytical expressions which could be convenient for experimental data processing. In order to reliably determine NMR parameters (at a sufficiently high signal-to-noise ratio) it is necessary to perform measurements at relatively large concentrations of aromatic compounds (about several mmol/l). Therefore, models for analysis of NMR data should take into account formation of not only dimeric but also associates of higher order via self- and heteroassociation of molecules [18].

We recently developed a statistical–thermodynamic heteroassociation model for analyzing NMR data of aromatic molecules in a mixed solution [16, 17]. According to this model, molecules form infinite-dimensional aggregates via both self-association and heteroassociation, and there are no limitations for the equilibrium self-association constants. In the present study we applied the developed model [16, 17] to the analysis of heteroassociation of an anthracycline antibiotic Daunomycin (DAU), which exhibits antitumor activity, and phenanthridine dye Propidium iodide (PI), which possesses pronounced mutagenic properties, using one- and two-dimensional ^{1}H NMR spectroscopy (500 MHz). The structures of DAU and

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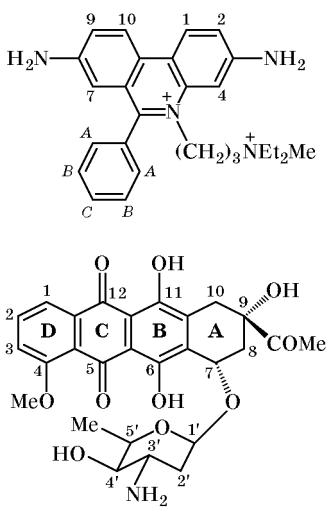

Fig. 1. Structural formulas of (a) Propidium iodide and (b) Daunomycin; unexchangeable protons are denoted.

PI are shown in Fig. 1. Previously [19], we examined heteroassociation of DAU with another phenanthridine dye, Ethidium bromide (EB), under similar conditions. Structural and thermodynamic analysis of the system EB–DAU led us to conclude [19] that the complex of EB with DAU is stabilized by both dispersion van der Waals interactions and hydrogen bond between the 3-amino group of the dye and the 9-acetyl group of DAU. Unlike Ethidium bromide, the molecule of Propidium iodide contains a longer aminoalkyl side chain and has an additional positive charge, which strongly influence its self-association parameters. The self-association constant of PI (K_{PI} = 63 ± 6 l/mol [16]) is by a factor of ~5 smaller than that of EB (K_{EB} = 305 ± 14) l/mol [20]) at 298 K. Comparison of the heteroassociation parameters of the phenanthridine dyes with those of Daunomycin led us to draw some conclusions on the nature of physicochemical interactions and the role of side chains in the aromatic molecules in the formation of heterocomplexes in solution.

Table 1. Parameters of heteroassociation of Daunomycin and Propidium iodide in 0.1 M phosphate buffer, pD 7.1

Temperature, K	Protons of DAU	δ_c, ppm	Protons of PI	δ_c, ppm	K, l/mol
303	2-H	7.61	1-H	7.81	
	1-H	7.31	10-H	7.74	
K_{DAU} = 580 ± 110	3-H	7.32	9-H	7.08	560 ± 60
K_{PI} = 46 ± 5	OCH_3	3.93	4-H	6.92	
	10-H_{eq}	2.96	2-H	6.89	
	10-H_{ax}	2.75	7-H	6.13	
313	2-H	7.66	1-H	7.74	
	1-H	7.21	10-H	7.68	
K_{DAU} = 370 ± 90	3-H	7.33	9-H	7.05	340 ± 90
K_{PI} = 35 ± 4	OCH_3	3.93	4-H	6.99	
	10-H_{eq}	2.92	2-H	6.81	
	10-H_{ax}	2.78	7-H	6.19	

The structural and thermodynamic parameters for complex formation of Daunomycin with Propidium iodide were determined, as it was done previously for the system Ethidium bromide–Daunomycin [19], by analysis of proton chemical shifts of the dye and antibiotic in solution at various concentrations and temperature. Figure 2 shows the plots of proton chemical shifts of PI and DAU versus concentration at 303 K and versus temperature. While varying the concentration of DAU, the concentration of PI was maintained constant (P_0 = 0.79 mM). The self-association equilibrium constant of DAU is more than an order of magnitude greater than the self-association constant of PI (K_{PI} = 46 [16], K_{DAU} = 580 l/mol [21] at 303 K); therefore, the concentration of DAU has a stronger effect on the equilibrium distribution of aggregates than does the concentration of the dye.

As previously [17, 19], the experimental data were analyzed following the general molecular heteroassociation model which assumes the existence of a dynamic equilibrium in solution. Here, the equilibrium involves infinitely dimensional self-association and heteroassociation with formation of different complexes (Scheme 1):

Scheme 1.

$$A_1 + A_i \overset{K_A}{\rightleftharpoons} A_{i+1} \quad (a)$$

$$P_1 + P_j \overset{K_P}{\rightleftharpoons} P_{j+1} \quad (b)$$

$$A_i + P_j \overset{K}{\rightleftharpoons} A_i P_j \quad (c)$$

$$P_j A_i + P_i \overset{K}{\rightleftharpoons} P_j A_i P_l \quad (d)$$

$$A_k + P_j A_i \overset{K}{\rightleftharpoons} A_i P_j A_k \quad (e)$$

Here, A_1 and P_1 are, respectively, monomeric DAU and PI; A_i, A_k, P_j, and P_l are aggregates containing

i or *k* molecules of DAU and *j* or *l* monomers pf PI. The equilibrium self-association constants of DAU (K_A) and PI (K_P) and DAU–PI heteroassociation constant K were assumed not to depend on the number of molecules in the aggregates and complexes. In terms of the above model, the dependence of the observed proton chemical shifts of DAU (A) and PI (P) may be represented as follows [17, 19]:

For protons of Daunomycin:

$$\delta_A = \frac{a_1}{a_0}\left[\delta_{mA}\left(2(1 + K_A a_1) - \frac{1}{(1 - K_A a_1)^2}\right) + 2\delta_{dA}\left(\frac{1}{(1 - K_A a_1)^2} - 1 - K_A a_1\right) + \delta_{cA}\frac{K p_1}{(1 - K_A a_1)^2 (1 - K_P p_1)} \times \left(1 + \frac{K p_1}{2(1 - K_P p_1)} + \frac{K a_1}{1 - K_A a_1}\right)\right];$$

For protons of Propidium iodide:

$$\delta_A = \frac{p_1}{p_0}\left[\delta_{mP}\left(2(1 + K_P p_1) - \frac{1}{(1 - K_P p_1)^2}\right) + 2\delta_{dP}\left(\frac{1}{(1 - K_P p_1)^2} - 1 - K_P p_1\right) + \delta_{cP}\frac{K a_1}{(1 - K_P p_1)^2 (1 - K_A a_1)} \times \left(1 + \frac{K a_1}{2(1 - K_A a_1)} + \frac{K p_1}{1 - K_P p_1}\right)\right].$$

Here, a_0, p_0 and a_1, p_1 are the molar concentrations of DAU and PI; the subscript "0" refers to initial concentration, and "1", to concentration of the monomer. The quantities δ_{mA}, δ_{dA}, δ_{mP}, and δ_{dP} and the equilibrium constants K_A and K_R (Table 1) were determined from the data of independent experiments which were carried out under identical conditions [16, 21]. It follows that the observed concentration dependences of proton chemical shifts of PI and DAU in mixed solvents (Fig. 2a) are functions of two unknown parameters δ_c and K. The latter can be determined by the calculation routine described in [16]. Table 1 gives the values of δ_c and K calculated for 303 and 313 K.

The thermodynamic parameters, enthalpy ΔH and entropy ΔS, of heteroassociation of DAU with PI

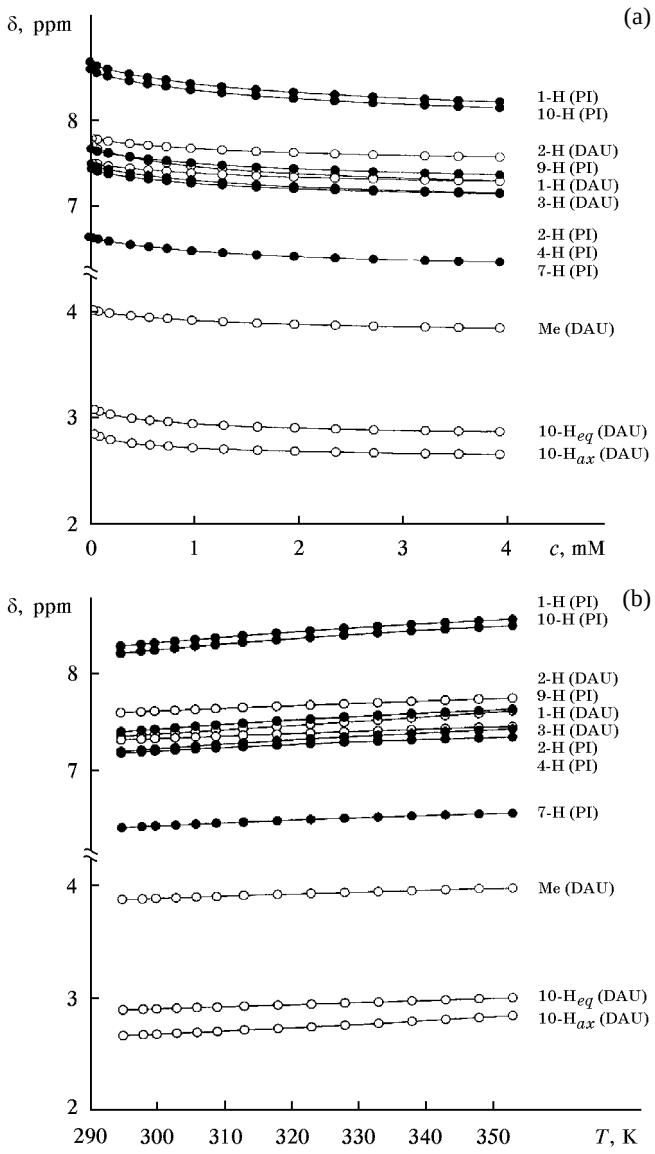

Fig. 2 Experimental dependences of proton chemical shifts of Daunomycin (DAU) and Propidium iodide (PI) in a mixed solution: (a) versus concentration of DAU at 303 K, p_0 = 0.79 mM and (b) versus temperature at p_0 = 0.79 and a_0 = 1.61 mM.

were determined from the temperature dependences of proton chemical shifts in a mixed solvent (Fig. 2b) using the additivity model [16, 20]. Table 2 contains the calculated enthalpies and entropies of complex formation of DAU with PI in aqueous solution.

The data in Table 1 show that the PI–DAU heteroassociation constant coincides with the self-association equilibrium constant of DAU within the error of determination but considerably exceeds the self-association of PI. According to the results of studies of heteroassociation of aromatic ligands possessing

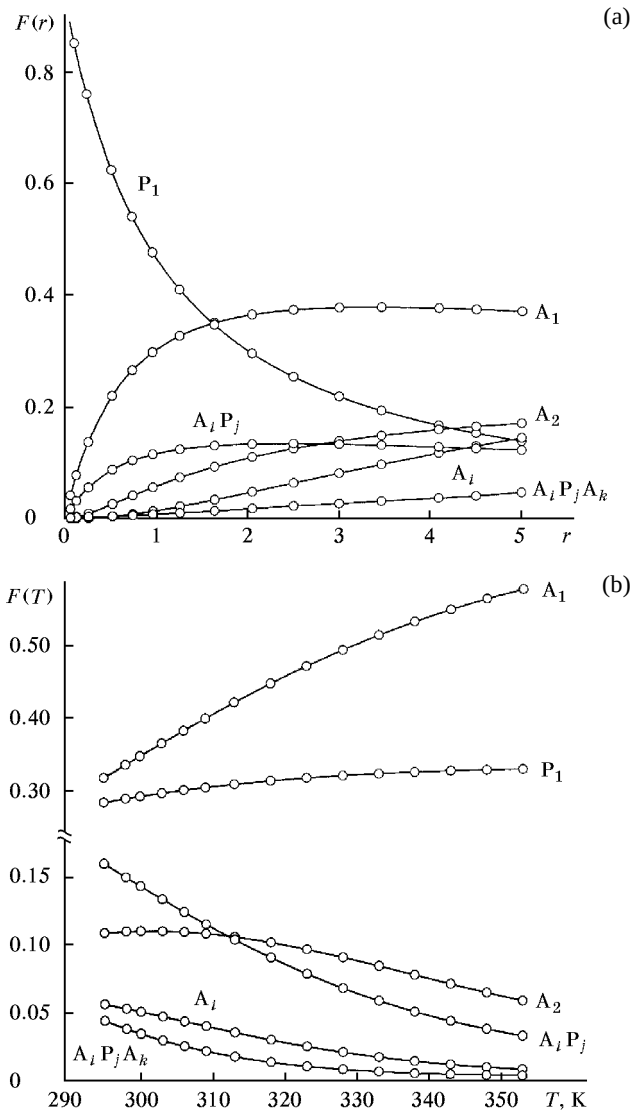

Fig. 3. Relative concentrations of self-associates and heteroassociates of Daunomycin and Propidium iodide versus (a) DAU–PI initial concentration ratio $r = a_0/p_0$ and (b) temperature, $p_0 = 0.79$, $a_0 = 1.61$ mM.

various medicobiological properties [16, 22–25], the equilibrium parameters of formation of heterocomplexes in solution strongly depend on the structure of both chromophore and side chains. As a rule, the heteroassociation constants were intermediate between the equilibrium self-association constants of the components [16, 22–25]. However, the heteroassociation constants for the acridine dye Proflavine (PF), and phenanthridine dye Ethidium bromide (EB) with Daunomycin [17, 19] were considerably greater than the self-association constants of the dyes and DAU. Taking into account the most probable structures of heterocomplexes and thermodynamic parameters for complex formation, this fact unambiguously indicates an additional stabilization of heteroassociates by intermolecular hydrogen bonds formed between the amino groups of the dyes and the 9-MeCO group of DAU. It should be noted that in the two systems, PF–DAU and EB-DAU, where heterocomplexes are stabilized by hydrogen bonds, the self-association constants of the components were of the same order of magnitude: $K_{PF} = 540$, $K_{DAU} = 580$ [17], and $K_{EB} = 270$ l/mol [19] at 303 K. The corresponding self-association constants for the system PI–DAU differ by more than an order of magnitude: $K_{DAU} = 580$, $K_{PI} = 46$ l/mol at 303 K. Large differences between the self-association constants were also observed while studying heteroassociation of Daunomycin and Proflavine with caffeine [23, 24], but in these cases the heteroassociation equilibrium constants had intermediate values between the corresponding self-association constants. In our experiments [22, 25] on the interactions of Acridine Orange and Novanthrone with caffeine the self-association constants differed, respectively, by two and three orders of magnitude. Nevertheless, in these systems the heteroassociation constants also had intermediate values between the corresponding self-association constants. We can thus presume that the relatively high DAU–PI heteroassociation constant, as well as in the system EB–DAU, results from additional stabilization of the heterocomplex via formation of intermolecular hydrogen bond between the 3- or 8-amino group of the dye and 9-acetyl group of the antibiotic.

However, enhanced affinity of aromatic DAU and PI molecules for each other did not give rise to intermolecular cross peaks in the 2M-ROESY spectrum even at their maximal concentrations. The negligible intensity of intermolecular cross peaks may be due to displacement of equilibrium on addition of Propidium iodide which promotes formation of different heteroassociates between DAU and PI with relatively low concentrations of complexes of each type in solution. It should be emphasized that the same factor is likely to be responsible for the absence in the 2M-ROESY spectrum of even intermolecular cross peaks from self-associates of DAU, whereas such peaks were observed at the same antibiotic concentrations in solution containing no dye [21].

The equilibrium constants obtained in the present work (Table 1) were used to calculate the relative concentrations of heterocomplexes against the concentration of DAU and temperature (Fig. 3). Figure 3a shows that the overall contribution of heterocomplexes ($A_i P_j$ and $A_i P_j A_k$) to dynamic equilibrium in solution is approximately equal to the contribution of

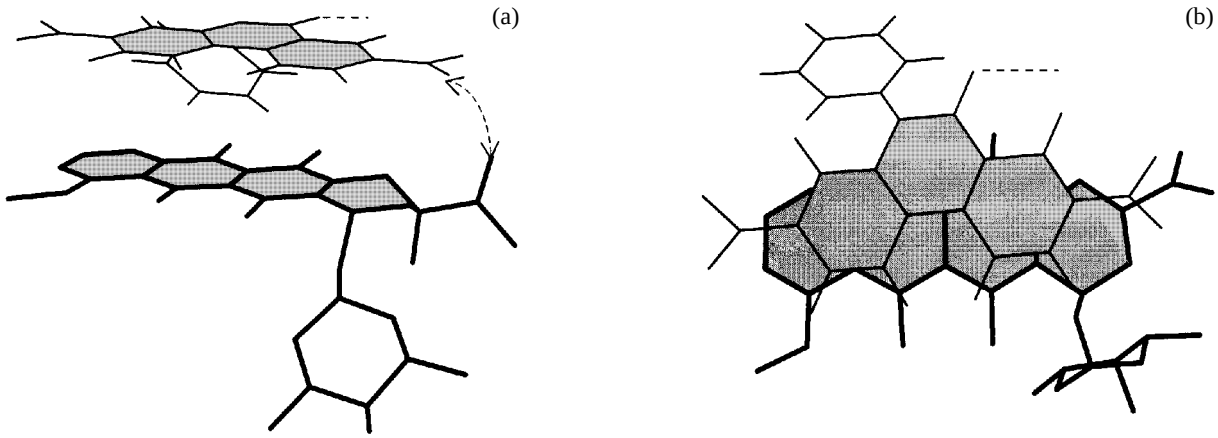

Fig. 4. Calculated structure of the 1:1 Propidium iodide–Daunomycin complex: (a) side view: the chromophore planes of the PI and DAU molecules are shaded; the aminoalkyl side chain of PI is shown as broken line; the hydrogen bond between the 3-amino group of PI and the 9-acetyl group of DAU is shown as dashed line; (b) top view: mutual arrangement of the chromophore planes of PI and DAU in the heterocomplex is illustrated.

DAU self-associates (A_2, A_i). This is explained by the fact that the self-association constant of DAU coincides within the error of determination with the DAU–PI heteroassociation constant. Propidium iodide exist in solution mostly in the monomeric form; therefore, heterocomplexes $A_iP_jA_k$ consist mainly of PI monomers flanked by aggregates of DAU. This conclusion is supported by the temperature dependence of the relative concentration of complexes of each type in a mixed solution (Fig. 3b). At low temperature, the fraction of DAU and PI molecules in the associates is fairly large, and the contribution of almost all associates gradually decreases as the temperature rises, thus increasing the concentration of monomeric forms (P_1 and A_1) of the dye and antibiotic in solution.

The observed proton chemical shifts δ_c (Table 1) of PI and DAU were used to calculate the most probable structure of a 1:1 complex of Propidium iodide with Daunomycin in aqueous solution. As previously [16, 20], mutual orientation of the PI and DAU molecules in the complex was determined by establishing a relation between the induced proton chemical shifts ($\Delta\delta = \delta_m - \delta_c$) and the theoretical shielding curves which were calculated by quantum-chemical method for aromatic molecules [26]. The $\Delta\delta$ values for the heterocomplex PI–DAU (Table 1) almost coincide with those characterizing proton shielding in the system EB–DAU [19].

Figure 4 shows the most probable steric structure of the 1:1 PI–DAU heterocomplex in aqueous solution, which was simulated by the calculations (the structure was plotted with the aid of Mathematica 2.2 program, Wolfram Res. Inc.). The planes of the PI and DAU chromophores in the 1:1 heterocomplex are coplanar and are arranged at a distance of 0.34 nm from each other. Such a complex can be stabilized by both stacking interaction of the aromatic chromophores and hydrogen bond between the 3-amino group of the dye and 9-MeCO group of the antibiotic (the hydrogen bond is shown as dashed line in Fig. 4a). The calculated structure of the 1:1 PI–DAU complex is well consistent with the structure of the EB–DAU heterocomplex in aqueous solution [19].

The stabilization of the heterocomplex in aqueous solution by hydrogen bond is qualitatively confirmed by analysis of thermodynamic parameters for complex formation of PI with DAU (Table 2). Relatively high negative ΔH and ΔS values for the system DAU–PI

Table 2. Thermodynamic parameters of self-association of Daunomycin and Propidium iodide and their heteroassociation in 0.1 M phosphate buffer (pD 7.1)

Compound	$-\Delta G^0_{303}$, kJ/mol	$-\Delta H^0$, kJ/mol	$-\Delta S^0_{303}$, J mol^{-1} K^{-1}
	Self-association		
DAU[a]	16.5±0.5	34±6	59.4±14.5
PI[b]	9.6±0.2	26±6	54±17
	Heteroassociation		
DAU+PI	15.9±0.4	37±7	70±15

[a] Data of [21].
[b] Data of [16].

are determined to a considerable extent by van der Waals dispersion interactions, as follows from the strong overlap of the aromatic chromophores in the complex (Fig. 4). Van der Waals dispersion interactions are characterized by negative enthalpy and entropy [27]. The formation of hydrogen bond in the DAU–PI complex is also characterized by negative ΔH and ΔS values [27, 28]. According to different sources, the enthalpy of hydrogen bond formation in aqueous solution ranges from –8 to –13 kJ/mol [30]. The data in Table 2 show that the thermodynamic parameters of heteroassociation of DAU and PI molecules are greater in absolute values than the corresponding parameters for self-association of these compounds. A qualitatively similar pattern was observed for the heterocomplexes PF–DAU [17] and EB–DAU [19] stabilized by intermolecular H-bond.

EXPERIMENTAL

Propidium iodide (Sigma) and Daunomycin (Fluka) were dissolved in D_2O (isotope purity 99.95%, Sigma), and the solution was subjected to lyophilization. Solutions were prepared by adding a required amount of samples in 0.1 M deuterated phosphate buffer (pD 7.1) containing 10^{-4} mol/l of EDTA. The concentration of aromatic molecules in aqueous solution was determined by spectrophotometry: PI, λ_{max} = 493 nm (ε = 5900 l mol^{-1} cm^{-1}) [29]; DAU, λ_{max} = 477 nm (ε = 11500 l mol^{-1} cm^{-1}) [30, 31].

The ^{1}H NMR spectra (1M and 2M) were measured on a Bruker DRX spectrometer at 500 MHz. The chemical shifts were measured at 303 and 313 K in the range of DAU concentrations from 3.93 to 0 M; the temperature dependences of proton chemical shifts were determined in the range from 295 to 353 K. The chemical shifts were measured relative to 2,2-dimethyl-2-silapentane-5-sulfonic acid using tetramethylammonium bromide as internal reference. The signals were assigned using two-dimensional homonuclear TOCSY and ROESY techniques [16, 20].

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