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^{1}H NMR structural and thermodynamical analysis of the hetero-association of daunomycin and novatrone in aqueous solution

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Abstract

The complexation of antitumour antibiotics novatrone (NOV) and daunomycin (DAU) in aqueous solution has been studied by one- and two-dimensional ^{1}H-NMR spectroscopy (500 MHz) in order to elucidate the probable molecular mechanism of the action of aromatic antitumour drugs in combination chemotherapy.

The equilibrium reaction constants, thermodynamical parameters (ΔH, ΔS) of hetero-association of NOV with DAU and the limiting values of proton chemical shifts of the molecules in the hetero-complexes have been determined from the experimental concentration and temperature dependences of proton chemical shifts of the aromatic molecules. The most favourable structure of the 1:1 NOV–DAU hetero-association complex has been determined using both the molecular mechanics methods (X-PLOR software) and the limiting values of proton chemical shifts of the molecules. The obtained results have shown that intermolecular complexes between NOV and DAU molecules are mainly stabilized by stacking interactions of the aromatic chromophores. It is likely that there is an additional stabilization of the NOV–DAU hetero-complexes by intermolecular hydrogen bonds. It is concluded that aromatic molecules of antibiotics may form energetically stable hetero-association complexes in aqueous solution and hence effect their medical–biological (and probably toxic) activity.

Keywords: Antitumour antibiotic; Novatrone; Daunomycin; Hetero-association; NMR spectroscopy

1. Introduction

The synthetic antitumour antibiotic mitoxantrone (novatrone) has proven to be very effective in a combinational therapy, in particular when used in combination with other antitumour drugs, e.g. doxorubicin, bleomycin, etc. [1].

Results of many investigations, carried out up to the present time, show a substantial effect of the process of hetero-association of aromatic biologically active compounds on the efficacy of both their complexation with DNA and the medical–biological activity [2–5]. At the same time, the quantitative analysis of the chemical composition and the physical–chemical properties of the pharmaceutical preparation containing two or more components in the mixture, without any preliminary chemical separation step, represents a rather difficult practical task. Thus, for example, classical spectrophotometrical methods, due to overlapping of absorption bands of drug molecules in a multicomponent solution, are generally not applicable for a quantitative analysis of the relative content of biologically active compounds in the mixture containing three or more different molecular components [6]. For these purposes may be used chemometric methods of computer processing of absorption spectra of biologically active compounds in multicomponent mixture [6]. However, the most effective experimental method, which enables to obtain the structural and thermodynamic parameters of interacting molecules in a multicomponent solution, is the NMR spectroscopy ([5] and references therein).

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Different mathematical models have been proposed for interpretation of experimental data of hetero-association of aromatic drug molecules in aqueous solution [2–5,7–10]. Dimer models are mostly used to interprete spectrophotometric data, i.e. they take into account the formation of self-aggregates and hetero-association complexes with not more than two molecules in the stack [3,7]. When comparatively large concentrations of molecules are used in the NMR experiment, more general models are considered as they take into consideration indefinite self- and hetero-association of aromatic molecules in solution [8–10]. Such models consider formation of hetero-complexes with not more than two hetero-stacks in the dynamic equilibrium in solution. At the same time investigation of different models of hetero-association of aromatic molecules based on NMR data enables to conclude [10], that detailed calculation of such hetero-complexes results in essential complication of the procedure of transformation of the initial equations to the final analytical form for the observed proton chemical shifts of the molecules in the NMR experiment. The analysis has shown that introduction of new reactions to the generalized model [10] to describe the dynamic equilibrium in solution considerably complicates the form of the initial equations and, in general, it is impossible to get finite analytical expressions for the obtained experimental parameter. As a result, the probability (stochastic) model of hetero-association of aromatic compounds has been developed [11,12], which is not associated with functional-analytical modelling of dynamic equilibrium in solution and, hence, may be used for analysis of multicomponent systems of any dimension.

In this work, the analytical statistical-thermodynamic model of hetero-association of molecules, taking into account formation of associated complexes of different dimensions for reactions of self- and hetero-association of aromatic molecules in two-component system [8,9], has been used for analysis of reactions of complexation of antitumour antibiotics novatrone (NOV) and daunomycin (DAU) in aqueous salt solution. Hetero-association of antibiotics DAU and NOV have been investigated by 1D and 2D ^{1}H NMR-spectroscopy (500 MHz); parameters of complexation of aromatic molecules have been determined from concentration and temperature dependences of proton chemical shifts of the interacting molecules [5,8,9]. The self-association of NOV and DAU molecules has been investigated earlier by 1D- and 2D-^{1}H NMR-spectroscopy under the same experimental conditions [13,14].

2. Experimental

Antibiotics novatrone (1,4-dehydroxy-5,8-bis [[[2-(2-hydroxyethyl) amino] ethyl] amino]-9,10-antracenedion) and DAU (Fig. 1) were purchased from 'Sigma' and used without further purification. The samples were lyophilized

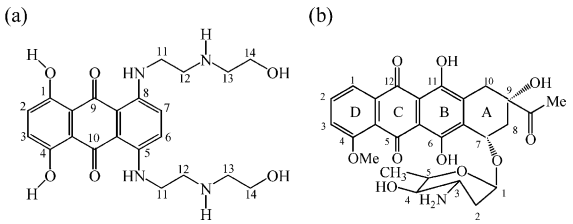

Fig. 1. Structures of novatrone (a) and daunomycin (b).

from D_2O and re-dissolved in 0.1 M phosphate buffer in 99.95% D_2O, pD = 7.1, containing 10^{-4} M EDTA. The concentrations of the stock solutions of the aromatic molecules were measured spectrophotometrically on appropriate dilution using the following molar absorption coefficients: $\varepsilon = 8360\ M^{-1}\ cm^{-1}$ ($\lambda = 682$ nm) for NOV [15] and $\varepsilon = 11,500\ M^{-1}\ cm^{-1}$ ($\lambda = 477$ nm) for DAU [16,17]. 1D and 2D ^{1}H NMR spectra were recorded on a Bruker DRX spectrometer (500 MHz). NOV showed limited solubility in aqueous buffered solution and, therefore, chemical shifts measurements of non-exchangeable protons of the aromatic molecules were made as a function of concentration of DAU at relatively high temperature $T = 318$ K (from 3.33 to 0.25 mM), keeping NOV concentration constant ($C_{NOV} = p_0 = 0.68$ mM). Temperature dependences of proton chemical shifts were measured at constant concentrations of drug molecules in the temperature range 312–351 K. All NMR measurements were made in the fast-exchange condition on the NMR time-scale. Chemical shifts were measured relative to an internal reference tetramethylammonium bromide (TMA) and recalculated with respect to sodium 2.2 dimethyl 2-silapentane-5-sulphonate (DSS), i.e. $\delta_{DSS} = \delta_{TMA} + 3.178$ (ppm).

Signal assignments of the non-exchangeable protons of the drugs were obtained using two-dimensional homonuclear TOCSY, NOESY and ROESY experiments. The methods of preparation of samples and performance of the NMR experiments are described in detail elsewhere [5,14].

3. Results

The 2D-ROESY spectrum of DAU–NOV mixed solution (Fig. 2) obtained at initial drugs concentrations exhibits intermolecular ROE contacts between protons of novatrone and DAU: H6/H7 NOV-4CH3 DAU and H6/H7 NOV-H1/3 DAU (shown by arrows), which provide unambiguous evidence of the mutual arrangements of DAU and NOV chromophores in 1:1 hetero-complex, i.e. aromatic ring D of DAU (Fig. 1) is situated above an aromatic ring of novatrone chromophore, containing H6/H7 protons.

Structural and thermodynamical parameters of hetero-association between DAU and NOV have been determined,

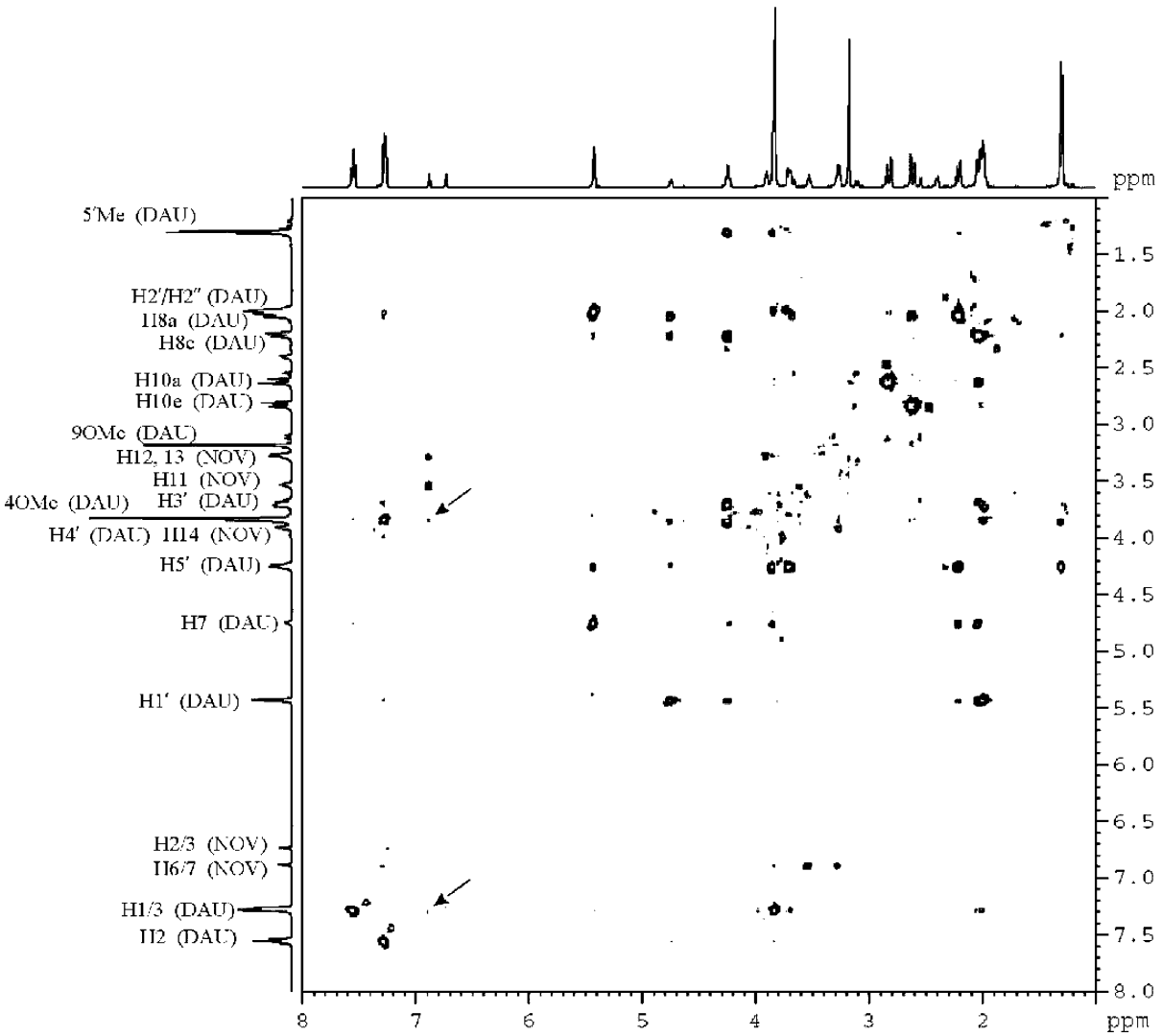

Fig. 2. 2D-ROESY spectrum (500 MHz, $\tau_m = 240$ ms) of DAU–NOV mixed solution at $C_{DAU} = a_0 = 3$ mM, $C_{NOV} = p_0 = 0.6$ mM ($T = 310$ K, pD = 7.1, 0.1 M NaCl). Intermolecular cross-peaks between protons of DAU and NOV are shown by arrows.

as made previously for other molecular systems [5,8,9], using chemical shifts changes of both molecules in mixed solution as a function of concentration (Fig. 3a) and temperature (Fig. 3b).

The equilibrium self-association constant of NOV is many times greater than that of DAU ($K_{DAU} = 580\ M^{-1}$, $K_{NOV} = 22000\ M^{-1}$ at $T = 303$ K) at the same temperature and therefore changes of concentration of NOV affect the equilibrium distribution of the aggregates more than keeping it constant and varying the concentration of DAU in solution. However, in contrast to previous NMR experiments [5,8,9] of hetero-association of biologically active aromatic molecules, in NOV–DAU system the concentration of NOV was kept constant ($p_0 = 0.68$ mM), whereas the relative content of DAU was changed in aqueous solution (Fig. 3a). Such performance of the NMR experiments was made due to substantially lower solubility of novatrone compared with DAU.

Experimental data were analyzed in terms of statistical thermodynamical model of molecular hetero-association of two components A and P [8,9]. It is assumed in this model that there is a dynamic equilibrium that includes indefinite self-association of both A and P as well as indefinite hetero-association reactions of different types, as shown in Scheme 1:

$$A_1 + A_i \overset{K_A}{\leftrightarrow} A_{i+1} \quad P_1 + P_j \overset{K_P}{\leftrightarrow} P_{j+1} \quad P_j + A_i \overset{K_h}{\leftrightarrow} P_j A_i$$
$$P_j A_i + P_l \overset{K_h}{\leftrightarrow} P_j A_i P_l \quad A_k + P_j A_i \overset{K_h}{\leftrightarrow} A_k P_j A_i \tag{1}$$

where A_1 and P_1 correspond to the monomers of DAU and NOV, and A_i, A_k, P_j and P_l are the aggregates containing i, k monomers of DAU and j, l monomers of the NOV, respectively. Analysis of the scheme of reactions (1) leads to the following expression for the observed proton

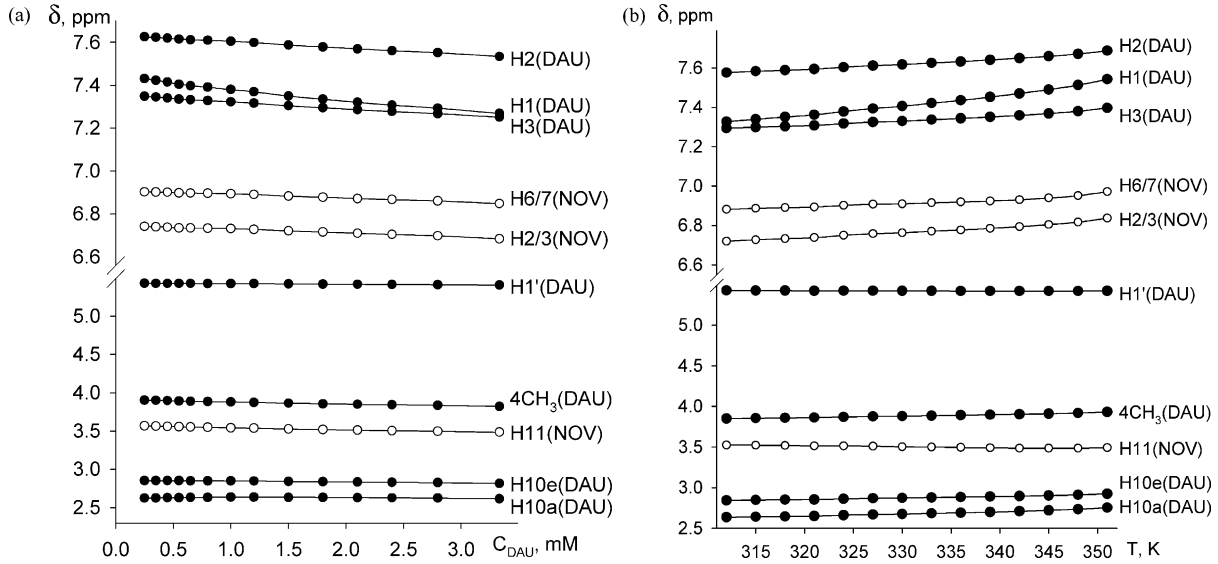

Fig. 3. Dependence of proton chemical shifts of DAU and NOV on: (a) concentration of DAU ($T = 318$ K, $C_{NOV} = p_0 = 0.68$ mM); (b) temperature ($C_{DAU} = a_0 = 2.01$ mM, $C_{NOV} = p_0 = 0.48$ mM) in aqueous solution, 0.1 M NaCl, pD = 7.1.

chemical shift of the component A [8,9]:

$$\delta_A = \frac{a_1}{a_0}\left[\delta_{mA}\left(2(1+K_Aa_1) - \frac{1}{(1-K_Aa_1)^2}\right) + 2\delta_{dA}\left(\frac{1}{(1-K_Aa_1)^2} - 1 - K_Aa_1\right) + \delta_{hA}\frac{K_hp_1}{(1-K_Aa_1)^2(1-K_Pp_1)} \times\left(1 + \frac{K_hp_1}{2(1-K_Pp_1)} + \frac{K_ha_1}{1-K_Aa_1}\right)\right] \quad (2)$$

As Scheme 1 is symmetrical with respect to the interacting drugs A and P, the values of chemical shifts for the protons of the P component can be obtained from Eq. (2) by means of substitution of indexes a for p and vice versa. The values of δ_{mA}, δ_{dA}, δ_{hA}, and δ_{mP}, δ_{dP}, δ_{hP} are the proton chemical shifts of DAU/NOV in the monomer, dimer and hetero-complex, respectively.

The equilibrium self-association constants K_A and K_P as well as δ_{mA}, δ_{dA} and δ_{mP}, δ_{dP} have been determined independently under the same experimental conditions [13,14]. It follows that the observed concentration dependences of proton chemical shifts of DAU and NOV in mixed solutions (Fig. 3a) are a function of two unknown quantities δ_h and K_h, which have been determined using the computational procedure described previously [8,9]. The magnitudes of the calculated parameters K_h, δ_{hA} and δ_{hP} at $T = 318$ K are summarized in Table 1.

4. Discussion

4.1. Hetero-association of novatrone with daunomycin

In spite of the fact that the magnitude of hetero-association constant K_h for complexation of NOV with DAU is intermediate between the values of self-association constants of the interacting aromatic drug molecules (Table 1), K_h value is much higher than those found for most of the systems of aromatic molecules studied previously [5,13]. Taking into account different chemical structures of DAU and NOV chromophores, it may be assumed, that increased K_h value results not only from dispersive

Table 1
Hetero-association parameters of DAU (A) with novatrone (P) in 0.1 mol l^{-1} phosphate buffer solutions, pD 7.1, $T = 318$ K

Protons of A	δ_{hA} (ppm)	δ_{mA} (ppm)	Protons of P	δ_{hP} (ppm)	δ_{mP} (ppm)	$K_h \times 10^3$ M^{-1}	$-\Delta G_{het}$ (kJ/mol)	$-\Delta H_{het}$ (kJ/mol)	$-\Delta S_{het}$ (J/(mol K))
$K_A = (307 \pm 60)$ M^{-1}, $K_P = (12,400 \pm 3800)$ M^{-1}						3.33 ± 0.35	21.4 ± 0.3	59.5 ± 6.2	95 ± 10
H2	7.42	7.83	H6/7	6.84	7.68				
H1	6.95	7.78	H2/3	6.69	7.30				
H3	7.12	7.55	H11	3.51	3.96				
H1′	5.33	5.52							
$4OCH_3$	3.73	4.02							
H10*e*	2.69	3.05							
H10*a*	2.54	2.81							

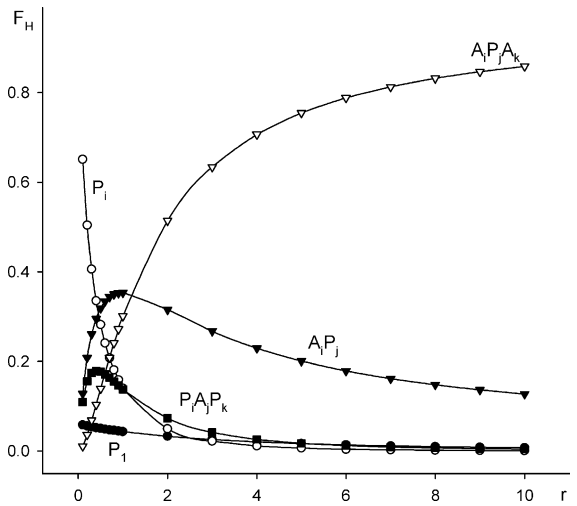

Fig. 4. Relative content (F_H) of self-aggregates of NOV and its hetero-complexes with DAU as a function of ratio of initial concentrations of DAU to NOV, $r = a_0/p_0$, $p_0 = 1.0$ mM = const.

interactions of aromatic chromophores, but also from additional stabilization of NOV–DAU hetero-complex due to formation of intermolecular hydrogen bonds [9].

Using the values of equilibrium self-association constants of novatrone [13] and DAU [14] and their hetero-association (Table 1), obtained in this work, the relative content of each type of molecular complex in mixed solution has been calculated as a function of r ($= a_0/p_0$, the ratio of the initial concentrations of DAU and NOV). The results are presented in Fig. 4. It is seen from Fig. 4 that there is an increase of the content of hetero-complexes (A_iP_j), ($A_iP_jA_1$) and ($P_iA_jP_i$) of DAU and NOV with increasing concentration of DAU in solution. At $r > 0.3$ hetero-complexes become predominant in the mixed solution (Fig. 4). The latter, obviously, leads to decrease or alteration of the efficacy of medical–biological action of antitumour drugs.

4.2. Structure of 1:1 DAU–NOV hetero-association complex in aqueous solution

The calculated values of chemical shifts δ_h for DAU and NOV protons (Table 1) were used for determination of the most probable structure of 1:1 hetero-complex between novatrone and DAU in aqueous solution. The mutual orientation of the molecules in the hetero-complex has been determined by comparison of the obtained induced proton chemical shifts, $\Delta\delta_h = \delta_m - \delta_h$, and their theoretical values from quantum-mechanical calculations of iso-shielding curves for novatrone and DAU [18]. It should be noted that comparative analysis of induced proton chemical shifts of drugs in hetero-complex $\Delta\delta_h$ and in self-associated dimers of NOV/DAU, $\Delta\delta_d = \delta_m - \delta_d$ enables to make a certain conclusion about the structure of 1:1 DAU–NOV hetero-complexes being formed in aqueous solution. Thus, approximately proportional distribution of protons shielding is observed in both the hetero-complex and NOV/DAU dimers but $\Delta\delta_h$ value is essentially higher in NOV–DAU complex than $\Delta\delta_d$ in NOV–NOV and DAU–DAU dimers [13,14]. Hence, it may be concluded that the distance between aromatic chromophores in 1:1 DAU–NOV hetero-complex is somewhat lower compared with that in NOV/DAU dimers [13,14].

Intermolecular cross-peaks in 2D-ROESY spectra and the values of induced proton chemical shifts of drug molecules in NOV–DAU hetero-complex (Table 1), enable to make an unambiguous conclusion about the arrangement of aromatic ring of NOV, containing H6/H7 protons above the aromatic ring D of DAU with H1, H2, and H3 protons. Calculation of spatial structure of 1:1 NOV–DAU complex has been made by the methods of molecular mechanics using X-PLOR software (version 3.851) [19]. Modelling of aqueous environment was performed by water molecules in the form of TIP3P [20], placed in rectangular box (1100 molecules). Topology of DAU and novatrone molecules and parametrization of their valent interactions have been obtained with the help of XPLO2D-software [21] using crystal structures from PDB databank [22]. Parameters of non-valent interactions between atoms corresponded to force field MM3 [23].

The most probable calculated spatial structure of 1:1 NOV–DAU hetero-complex in aqueous solution is presented in Fig. 5. In the calculated structure, the planes of the chromophores of NOV and DAU molecules in 1:1 hetero-complex are parallel to each other at a distance of about 0.32 nm which is somewhat lower than that in NOV/DAU dimers (0.34 nm) [13,14]. Intensive overlap of the aromatic parts of the chromophores has been found for NOV–DAU hetero-complex, which indicates substantial stacking interactions of the drug molecules.

At the same time, the calculated structure of 1:1 NOV–DAU complex shows the probability of formation of hydrogen bonds between 5NH and 8NH groups of side chains of NOV and 5O, 12O atoms of DAU (shown by dotted lines). It should be noted that the calculated structure of 1:1 NOV–DAU complex is consistent with both the limiting proton chemical shifts for this molecular system, and the minimum value of its potential energy determined by the methods of molecular modelling.

4.3. Thermodynamics of hetero-association of novatrone with daunomycin in aqueous solution

The thermodynamical parameters ΔH^0_{het} and ΔS^0_{het} of NOV–DAU hetero-association were determined from measurements of the proton chemical shifts of the molecules in the mixed solution as a function of temperature using the additive model for the observed proton chemical shifts and van't Hoff's formalism [8]. Qualitatively similar character of the experimental dependences $\delta(T)$ for all non-exchangeable protons of DAU and NOV (Fig. 3a) enables to assume, that experimentally observed changes of chemical shifts

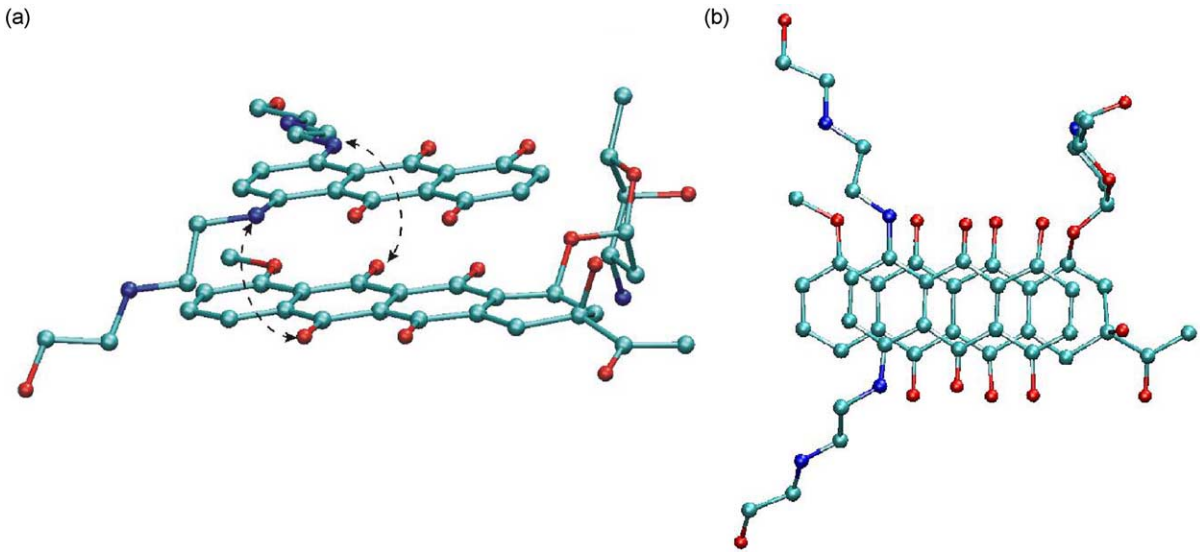

Fig. 5. The calculated NMR structure of the 1:1 hetero-association complex of NOV with DAU: (a) side view of the hetero-complex (intermolecular hydrogen bonds between 5NH and 8NH groups of side chains of NOV and 5O, 12O atoms of DAU are shown by arrows); (b) view looking perpendicular to the planes of the chromophores of aromatic molecules.

with increasing temperature are mainly due to the displacement of the molecular equilibrium in solution.

Relation (2) was used for calculations of thermodynamical parameters in which the influence of temperature on the values of $\delta(T)$ is determined by the temperature dependence of the equilibrium constants of self- and hetero-association of molecules in solution

$$K_i(T) = \exp(\Delta S_i^0/R - \Delta H_i^0/RT), \quad (3)$$

assuming that values of ΔS_i^0 and ΔH_i^0 do not change substantially with temperature in the range studied. As a result, the following mean values of thermodynamical parameters of NOV–DAU hetero-association were obtained: $\Delta G_{het}^0 = -(21.4 \pm 0.3)$ kJ/mol, $\Delta H_{het}^0 = -(59.5 \pm 6.2)$ kJ/mol, $\Delta S_{het}^0 = -(95 \pm 10)$ J/(mol K).

Rather large negative values of enthalpy and entropy of hetero-association of NOV–DAU aromatic molecules enable to conclude that dispersive interactions play an essential role in formation of hetero-complexes of these molecules. Dispersive van der Waals interactions are characterized both by negative enthalpy and negative entropy [24]. The quantitative analysis of thermodynamic parameters of NOV–DAU hetero-complex formation confirms the above made assumption about stabilization of hetero-complex of aromatic drug molecules in aqueous solution by intermolecular hydrogen bonds. Hydrogen bond formation in NOV–DAU hetero-complex also results in negative values of ΔH and ΔS; the magnitude of enthalpy of hydrogen bond formation in aqueous solution is estimated to be from -8 up to -13 kJ/mol [24].

In conclusion, it should be emphasized that aromatic antitumour drugs, e.g. novatrone and DAU, may form energetically stable hetero-association complexes in aqueous solution and hence affect their medical–biological (and probably toxic) activity. Such investigations are important for elucidation of interrelations of antitumour antibiotics in combination chemotherapy.

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