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Analysis by Means of ^{1}H NMR Spectroscopy of Heteroassociaion in Water Solution of Antitumor Antibiotics Daunomycin and Actinomycin D

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Abstract—Heteroassociation of aromatic antitumor antibiotics daunomycin (DAU) and actinomycin D (AMD) was investigated using 1D and 2D ^{1}H NMR spectroscopy (at 500 MHz) and molecular mechanics procedure with the goal of establishing the mechanism of the combined action of antibiotics in the system AMD–DAU. The experimental data were processed applying a modified statistical and thermodynamic model of the molecules heteroassociation. Proceeding from this model the values were obtained of induced proton chemical shifts, equilibrium constant and thermodynamic parameters of complexing reaction between DAU and AMD. By means of molecular mechanics with the use of X-PLOR software and of the analysis results of the induced proton chemical shifts in the molecules the most probable spatial structure, 1:1, was established for the heterocomplex of DAU and AMD. Heterocomplexes of daunomycin and actinomycin D form due to stacking interaction between the aromatic chromophores with possible additional stabilization of the complexes by an intermolecular hydrogen bond.

The results of numerous investigations carried out hitherto demonstrated significant effect of heteroassociation process of aromatic biologically active compounds on the efficiency of their complexing with DNA and on their medicobiological potency [1–4]. A quantitative analysis of the chemical composition and evaluation of physicochemical properties of pharma-ceuticals containing a mixture of two and more components without their separation by chemical procedures is a difficult problem. For instance, the spectrophotometric methods are not suitable for estimating the quantitative composition of a mixture of three and more molecular components because of overlapping of absorption bands of molecules in a multicomponent solution [5]. To these ends chemometric procedures of computer processing of absorption spectra from a multicomponent system can be applied [5]. However the most efficient experimental method for evaluation of structural and thermodynamic characteristics of interacting molecules in a multicomponent solution is the NMR spectroscopy [4].

Different models were advanced for interpretation of experimental data on heteroassociation of aromatic bioactive compounds in water solution [2–4, 6–9]. As a rule in the spectrophotometric studies the dimeric model of heteroassociation is used where two interacting molecules are present in an aggregate with a single contact (heterojunction) between them [2, 6]. In NMR investigations of aromatic molecules heteroassocoiation the models applied are of infinite dimensions with no limitations concerning the size of the aggregates, and formation of heterocomplexes in solution is taken into consideration when they contain no more than two heterojunctions [7–9]. The study of various heteroassociation models of aromatic molecules basing on NMR spectral data leads to a conclusion [9] that strict consideration of heterocomplexes with more than two heterojunctions results in considerably complicated transformation procedure of the initial equations into the final analytical form of registering the chemical shifts of protons observed in the NMR experiment. The analysis shows that including new reactions into the generalized model [9] for treating the dynamic equilibrium in solution significantly complicates the construction of initial equations and prevents deducing the final analytical expressions for the parameter measured in the experiment. This problem can be solved by probability

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(algorithmic) model of the heteroassociation of aromatic substances [10] that is not limited to the functionally analytical simulation of the dynamic equilibrium in solution and thus can be applied to the analysis of multicomponent systems of any dimensionality.

In the present study we used an analytical statistical and thermodynamic model of molecules heteroassociation accounting for aggregate formation of any dimensions both due to self-association and heteroassociation in a two-component system [7, 8]. This model was applied to the analysis of heteroassociation of antitumor antibiotics actinomycin D (AMD) and daunomycin (DAU) in water-salt solutions.

Actinomycin D was among the first antitumor drugs to find clinical application. The antibiotic molecule is built of two pentapeptide lactone rings attached to a planar polycyclic phenoxazone chromophore (Fig. 1a). Daunomycin belongs to the antitumor drugs of anthracycline antibiotics group having in the molecular structure a tetrahydrotetracenequinone chromophore that contains three coplanar six-membered rings, and a positively charged hexosamine ring whose plane is approximately perpendicular to that of the chromophore (Fig. 1b). The chemotherapeutic effect of the antitumor antibiotics under study originates from their ability to interact with DNA and to inhibit the synthesis of DNA and RNA [11, 12].

Heteroassociation of antibiotics DAU and AMD was investigated by 1D and 2D ^{1}H NMR spectroscopy at operating frequency 500 MHz. The association parameters of aromatic molecules were estimated from concentration and temperature dependences of the proton chemical shifts of the interacting molecules in the same fashion as had been done with the other molecular systems [4, 7, 8]. The self-association of AMD and DAU was previously studied by 1D and 2D ^{1}H NMR spectroscopy under similar experimental conditions [13, 14].

In the two-dimensional spectra (2D-NOESY and 2D-ROESY) of the mixed solutions AMD–DAU no intermolecular cross-peaks were observed even at the maximum concentrations studied. The negligibly low intensity of intermolecular cross-peaks is caused first of all by low solubility of actinomycin D in the water-salt solution (0.1 M phosphate buffer) and therefore by the relatively low initial concentration of AMD in the solution containing DAU. Besides various heteroassociates of AMD and DAU presumably formed with relatively small content of each type complex in solution [4].

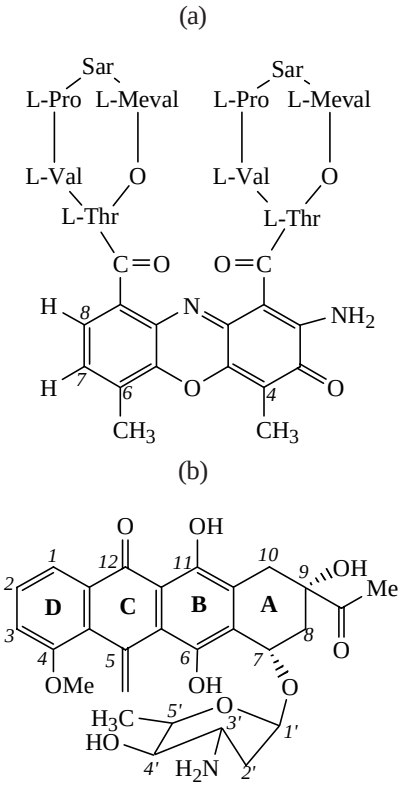

Fig. 1. Structural formulas of actinomycin D (**a**) and daunomycin (**b**) molecules.

The structural and thermodynamic parameters of AMD and DAU heteroassociates were estimated as for the previously studied molecular systems [4, 8] by analysis of the dependence of chemical shifts of nonexchanging protons in both aromatic compounds on concentration (Fig. 2a) and temperature (Fig. 2b). The equilibrium constant of self-association for AMD molecules is twice as large as that for DAU (K_{DAU} = 720, K_{AMD} = 1440 l mol^{-1} at 298 K), therefore in measuring the concentration dependences of the proton chemical shifts of the studied aromatic molecules the changes in AMD concentration considerably stronger affect the distribution of the aggregates than variation of the DAU content. Unlike the previous experiments on the heteroassociation of biologically active aromatic molecules [4, 7, 8] we kept in the system AMD–DAU a constant AMD concentration ($C_0 = p_0$ = 0.26 mmol l^{-1}) at variation of DAU content in the solution (Fig. 2a) because of significantly lower solubility of actinomycin D compared to daunomycin.

A qualitative analysis of the concentration dependences of proton chemical shifts of AMD and DAU in the course of homo- [13, 14] and heteroassocia-tion of the antibiotics (Fig. 2a) reveals somewhat greater shielding of protons

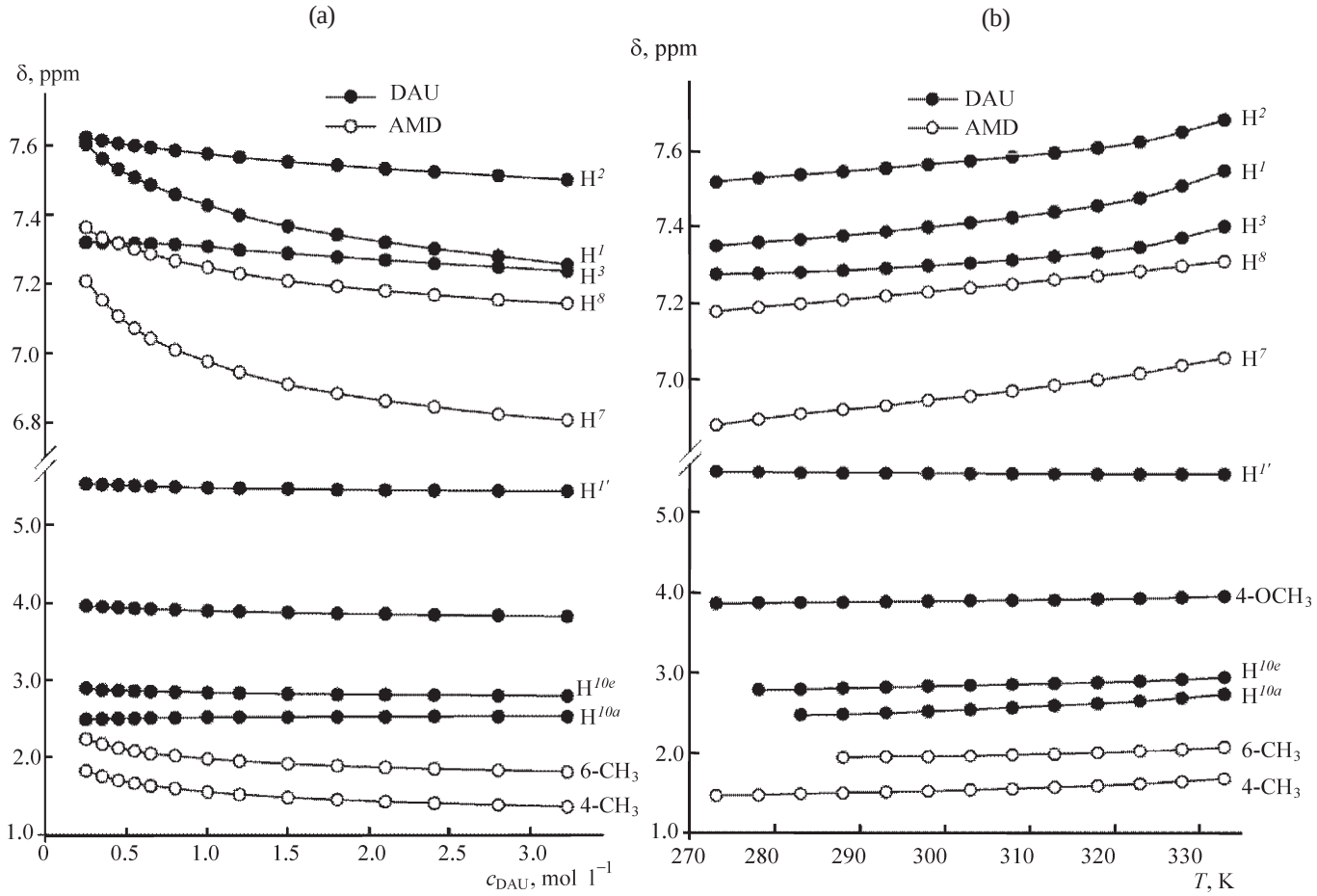

Fig. 2. Dependence of proton chemical shifts in DAU and AMD in solution (a) on concentration at T = 298 K, c_{AMD} 0.26 mmol^{-1}; (b) on temperature at c_{DAU} 1.20 mmol^{-1}, c_{AMD} 0.26 mmol^{-1}.

in the heterocomplexes. The chemical shift of H^{10a} proton in daunomycin in contrast to its other protons shifts downfield at increasing DAU concentration in the mixture with AMD. This fact is apparently caused by significantly stronger shielding of the H^{10a} proton in the DAU–AMD complex as compared with the dimer aggregate of daunomycin and by redistribution of the associated forms of DAU at its growing concentration in the solution.

The following scheme of aromatic molecules interaction in solution was considered in the analysis of the experimental data [7, 8]:

The equilibrium constants of homoassociation of DAU

$$A_1 + A_i \overset{K_A}{\longleftrightarrow} A_{i+1}\,(a),\ P_1 + P_j \overset{K_P}{\longleftrightarrow} P_{j+1}\,(b),$$
$$A_i + P_j \overset{K_h}{\longleftrightarrow} A_i P_j\,(c),\ P_j A_i + P_l \overset{K_h}{\longleftrightarrow} P_j A_i P_l\,(d), \quad (1)$$
$$A_k + P_j A_i \overset{K_h}{\longleftrightarrow} A_i P_j A_k\,(e).$$

(K_A), AMD (K_P), and hetroassociation of the molecules (K_h) in the statistical and thermodunamic model are assumed to be independent of the number of molecules in the aggregates and complexes [7, 8]. However sedimentation studies of actinomycin D antibiotic in water solutions [15] demonstrated that AMD aggregates larger than dimers did not virtually form in solution even at concentrations close to saturation. Apparently the formation of higher associates from AMD antibiotic possessing bulky peptide lactones is hampered by steric reasons. Taking into account these features of aggregate formation from actinomycin D molecules we used in experimental data treatment dimer models of AMD self-association and its heteroassociation with DAU, i.e. in scheme (1) j, l = 1, 2 in formation of heterocomplexes (1*c*–*e*). In scheme (1) A_1 and P_1 correspond to monomers DAU and AMD, A_i, A_k, P_j, P_l to homoassociates containing i, k daunomycin molecules and j, $l \le 2$ actinomycin D molecules.

Taking into account the law of mass action for reactions (1) the dependences of the observed chemical shifts of protons in DAU and AMD on the concentration

$$\delta_A = \frac{a_1}{a_0}\left[\delta_{mA}\left(2(1 + K_A a_1) - \frac{1}{(1 - K_A a_1)^2}\right) + 2\delta_{dA}\left(\frac{1}{(1 - K_A a_1)^2} - 1 - K_A a_1\right)\right.$$
$$\left. + \delta_{hA}\frac{K_h(p_1 + K_h p_1^2)}{(1 - K_A a_1)^2}\left(1 + \frac{K_h(p_1 + K_h p_1^2)}{2} + \frac{K_h a_1}{1 - K_A a_1}\right)\right] \quad (2)$$

$$\delta_P = \frac{p_1}{p_0}\left[\delta_{mP} + 2\delta_{dP}K_P p_1 + \delta_{hP}\frac{K_h a_1(1 + 2K_p p_1)}{1 - K_A a_1}\left(1 + \frac{K_h a_1}{2(1 - K_A a_1)} + K_h(p_1 + K_p p_1^2)\right)\right] \quad (3)$$

of the molecules in solution are expressed respectively by equations (2) and (3) [16].

Here a_0, p_0 and a_1, p_1 are initial and monomer concentrations of daunomycin and actinomycin D respectively; values δ_{mA}, δ_{dA}, δ_{hA} and δ_{mP}, δ_{dP}, δ_{hP} are respectively the proton chemical shifts for DAU and AMD in monomers, dimers, and heteroassociates. The proton chemical shift values δ_{mA}, δ_{dA}, δ_{mP}, δ_{dP} and equilibrium constants K_A, K_P were established in special experiments on homoassociation of the molecules under investigation in identical experimental conditions [13, 14]. The calculation procedure for parameters of heteroassociation of the molecules was described in detail before [7, 8]. The calculated parameters of associates formation between AMD and DAU at 298 K are presented in a table.

The equilibrium value of DAU and AMD heteroassociation constant at T 298 K exceeds the equilibrium constants of their homoassociation. In the most other molecular systems already studied [4, 7] the constants of heteroassociation of molecules were of intermediate value between the equilibrium constants of self-association. The constant of heteroassociation exceeding the constants of homoassociation was observed only in systems where the heterocomplexes were additionally stabilized by intermolecular hydrogen bonds [8]. This suggests that in the system DAU–AMD the intermolecular hydrogen bonds contribute to stabilization of the heterocomplexes of the aromatic molecules.

The values of equilibrium constants of self-association for AMD [13] and DAU [14] and of their heteroassociation (see table) established in this work were used for calculation of relative content of different complex types in water solution as a function of the ratio of the initial concentrations of DAU and AMD ($r = a_o/p_o$). As seen from Fig. 3, with increasing DAU concentration in solution grows the relative content of heterocomplexes (A_iP_j), $(A_iP_jA_l)$ $(P_iA_jP_i)$ of actinomycin D with daunomycin. At $r > 2$ the heterocomplexes prevail in solution, and this may affect the efficiency of the medicobiological activity of antibiotics actinomycin D and daunomycin.

Structure of hetroassociate (1:1) DAU+AMD in water solution. A comparative analysis of induced chemical shifts of protons in the heterocomplexes ($\Delta\delta_h = \delta_m - \delta_h$) and self-associates $\Delta\delta_d = \delta_m - \delta_d$ (see table) suggests some conclusions on the structure of heterocomplexes obtained. It follows from the data that for the chemical shifts of all protons of DAU and AMD an approximately proportional increase is observed in $\Delta\delta_h$

Calculated values of heteroassociation parameters for daunomycin (**À**) and actinomycin **D** (Ð) in 0.1 M phosphate buffer, pD 7.1, T 298 K

Protons A (DAU)	δ_{hA}, ppm	δ_{dA}, ppm	δ_{mA}, ppm	Protons P (AMD)	δ_{hP}, ppm	δ_{dP}, ppm	δ_{mP}, ppm	$K \times 10^3$, l mol^{-1}
H^2	7.42	7.53	7.83	H^8	7.07	7.41	7.51	K_A 0.72 ± 0.13
								K_P 1.42 ± 0.13
								K_h 2.75 ± 1.10
H^1	7.20	7.33	7.78	H^7	6.74	7.41	7.51	
H^3	7.18	7.26	7.55	6-CH_3	1.75	2.39	2.60	
$H^{1'}$	5.56	5.43	5.52	4-CH_3	1.27	1.69	2.28	
4-OCH_3	3.89	3.83	4.02					
H^{10e}	2.61	2.86	3.05					
H^{10a}	2.25	2.64	2.81					

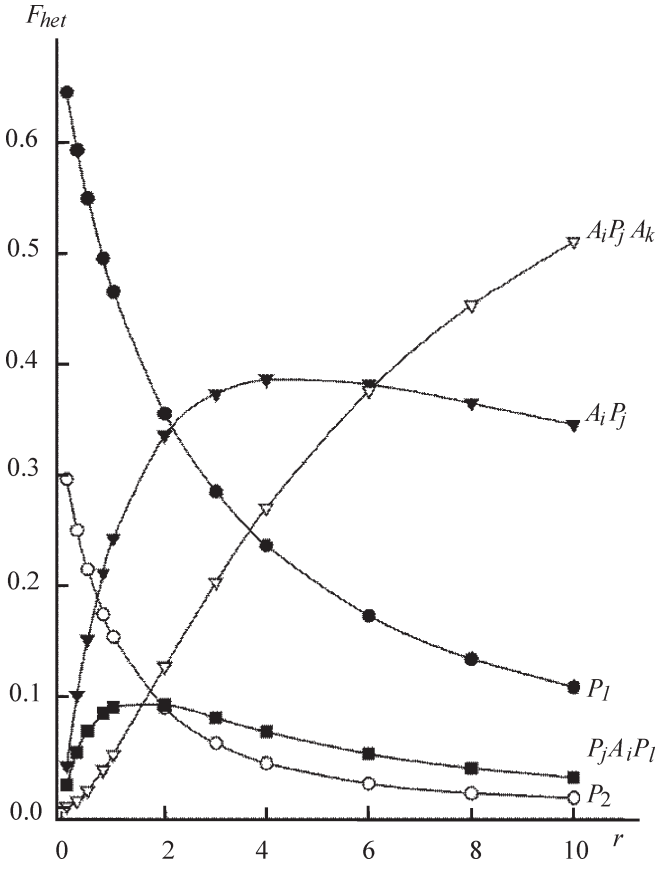

Fig. 3. Relative content (F_{het}) of heterocomplexes of actinomycin D as a function of the ratio $r = a_0/p_0$ of initial concentration DAU (a_0) and AMD (p_0), p_0 0.25 mmol l^{-1} const), see text.

with respect to $\Delta\delta_d$ presumably due to some decrease in the distance between chromophores in the heterocomplexes as compared with the self-associates [13, 14].

The obtained chemical shift values δ_h for protons of DAU and AMD (see table) were used in calculation of the most probable structure of a complex 1:1 from actinomycin D and daunomycin in a water solution. The reciprocal position of the molecules in the heteroassociate was established from the comparison of the values of induced proton chemical shifts ($\Delta\delta_h = \delta_m - \delta_h$) with the theoretical shielding curves obtained by quantum-chemical calculations for actinomycin D and daunomycin [17]. The values of induced chemical shifts of protons of the molecules in the heterocomplex AMD–DAU (see table) unambiguously indicate that the quinoid ring of the chromophore of the AMD molecule containing 4-CH_3 protons is located above the aromatic ring D of daunomycin containing protons H^1, H^2, and H^3. The spatial structure of the complex AMD–DAU (1:1) was calculated by molecular mechanics procedure using X-PLOR software (version 3.851) [18]. The water environment was simulated by water molecules placed into a rectangular box (1100 molecules) TIP3P [19]. The topology of daunomycin and actinomycin D molecules and parametrization of their valence interactions were obtained applying XPLO2D software [20] with the use of crystalline structures from the PDB Database [21]. The parameters of nonvalence interactions between atoms corresponded to the force field MM3 [22].

The most probable spatial structure of the heterocomplex AMD–DAU (1:1) in water solution obtained by calculations is shown on Fig 4. In this kind of complex aromatic rings of the interacting molecules are relatively strong overlapped (stacking-interaction) suggesting a significant role of dispersion forces in heteroassociate formation. At the same time the calculated structure of AMD–DAU (1:1) evidences the possibility of a hydrogen bond formation between the O^{12} atom in DAU and the group NH in D–Val pentapeptide ring of AMD attached to the C^1 atom of the chromophore (Fig. 4, the hydrogen bond is shown by a dotted line).

The calculated structure of the complex AMD–DAU (1:1) is consistent both with the limiting chemical shifts of protons of this molecular system and with the minimum of its potential energy estimated by molecular mechanics simulation.

Thermodynamics of heteoassociation of daunomycin and actinomycin D molecules in water solution. Thermodynamic parameters ΔH^0_{het} and ΔS^0_{het} for heteroassociation of AMD and DAU were established from the experimental temperature dependences of chemical shifts of protons using in describing the observed chemical shift of protons an additive model and van't Hoff formalism [7]. Inasmuch as the experimental dependencies $\delta(T)$ are qualitatively alike for all nonexchanging protons of DAU and AMD (Fig. 2b) it is presumable that the experimentally obseved variations in the chemical shifts at raising the temperature originate mainly from the displacement of the molecular equilibrium in the solution.

We use for estimation of the thermodynamic parameters expressions (2) and (3) where the effect of the temperature on the $\delta(T)$ value is determined by the relation (4) describing the temperature dependence of the equilibrium constants of homo- and heteroassociation of the molecules.

$$K_i(T) = \exp(\Delta S^0_i/R - \Delta H^0_i//RT) \quad (4)$$

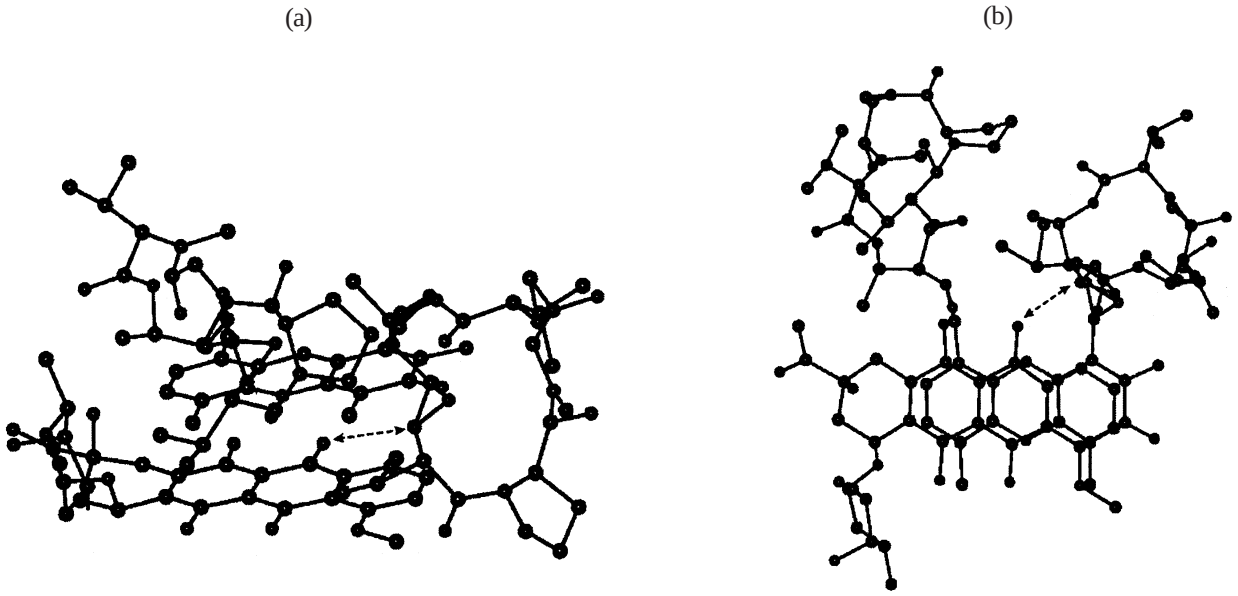

Fig. 4. Structure of heterocomplex DAU–AMD (1:1). (a) side view of the complex; (b) top view of the complex demonstrating the reciprocal position of the chromophore planes of DAU and AMD molecules. The intermolecular hydrogen bond is shown by a dotted line.

It was assumed that ΔS_i^0 and ΔH_i^0 values do not essentially depend on the temperature in the temperature range under investigation. The calculations gave the following mean values of Gibbs energy, enthalpy, and entropy of AMD and DAU heteroassociation in solution: ΔG_{het}^0 –(19.6 ± 1.2) kJ mol^{-1}, ΔH_{het}^0 –(38.8 ± 4.3) kJ mol^{-1}, ΔS^0_{het} –(53 ± 12) J mol^{-1} K^{-1}. Relatively large negative values of enthalpy and entropy for heteroassociation of aromatic molecules AMD and DAU permit a conclusion that the dispersion forces play an essential role in formation of these heterocomplexes. The dispersion interactions are known to be characterized by negative enthalpy and entropy values [23]. The quantitative analysis of the thermodynamic parameters of formation of the AMD–DAU heterocomplex provides a qualitative confirmation of the complex stabilization in water solution by intermolecular hydrogen bonds. The hydrogen bond arising in the heterocomplex AMD–DAU also results in the negative ΔH and ΔS values [23, 24]. The enthalpy of formation of a hydrogen bond in a water solution according to various estimation is in the range from –8 to –13 kJ mol^{-1} [23].

Thus it is possible to conclude that the aromatic molecules of the antitumor antibiotics, in particular, actinomycin D and daunomycin, can form energetically strong heteroassociates in water solution which can regulate their medicobiological activity. This kind studies may be important in revealing the mutual influence of antitumor antibiotics and their compatibility in the course of chemotherapy.

It should be noted here that the concentration of DAU and AMD in the human serum during the chemotherapeutic treatment is as a rule too low to expect their considerable interaction at combined application. However it is important that the initial concentrations C_0 in the introduced dose are close to those used in the NMR experiment. For instance, for AMD $C_0 \approx 0.4$ mmol l^{-1}, for anthracycline antibiotics $C_0 \approx 1$–4 mmol l^{-1}, for other antibiotics the initial concentrations can be 10-100 times larger [25]. Note in conclusion that at present the combined chemotherapy is considered to be far more efficient than the monotherapy [25].

EXPERIMENTAL

Commercial antitumor antibiotics daunomycin and actinomycin D (Fig. 1) purchased from Sigma were dissolved in D_2O (isotope purity 99.95%, Sigma) and lyophilized. The solutions were prepared by adding a weighed amount of a sample to the deuterated 0.1 M phosphate buffer (pD 7.1) containing 10^{-4} mol l^{-1} of EDTA. The concentration of aromatic substances in water solution was measured spectrophotometrically. The extinction coefficients for AMD and DAU, l mol^{-1} cm^{-1}, are respectively ε 24500 (λ 440 nm) [26] and ε 11500 (λ 477 nm) [27]. 1D and 2D ^{1}H NMR spectra were

registered on spectrometer Bruker DRX at operating frequency 500 MHz. The concentration dependences of the proton chemical shifts were measured at 298 K in the range of DAU concentrations from 3.22 to 0.25 mmol l^{-1} in a solution containing AMD in a constant concentration ($C_{AMD} = p_0 = 0.26$ mmol l^{-1}). The temperature variations of chemical shifts of protons were measured in the temperature range from 273 to 333 K. The chemical shifts were determined with respect to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), internal reference tetramethylammonium bromide.

The assignment of signals in the ^1H NMR spectra, attribution of chemical bonds and spatial arrangement was performed applying two-dimensional homonuclear NMR experiments TOCSY, NOESY, and ROESY. The procedure of samples preparation and experimental technique was described in [4].

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