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ORIGINAL ARTICLE

Affinity and enantioselectivity of Rifamycin SV towards low molecular weight compounds

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Abstract Association of amino acids and some other low molecular weight compounds with rifamycin SV in water has been studied by ^{1}H NMR titrations. Rifamycin binds aromatic amino acids with pronounced enantioselectivity in favor of L-enantiomers and forms complexes with heterocyclic compounds but does not interact with simple benzene derivatives. Binding constants correlate with LUMO energies and hydrophobicities (expressed as log P values) of guest molecules indicating contributions to the binding free energy from aromatic stacking interactions with the naphthohydroquinone fragment of rifamycin SV and from hydrophobic interactions. Proposed mode of binding is supported by semiempirical calculations of structures of host–guest complexes.

Keywords Rifamycin · Enantioselectivity · Amino acid · Binding constant

Introduction

Using of natural compounds as macrocyclic receptors has an advantage of avoiding laborious synthetic macrocyclization procedures. Previously we demonstrated that some natural macrocyclic compounds such as iso-quinoline alkaloids [1] and peptide antibiotics [2] can be successfully employed for recognition of low molecular weight guests. Continuing this line of research we undertook a study of recognition properties of rifamycin SV.

Rifamycins are macrocyclic antibiotics belonging to a type of molecules known as naphtalenic ansamycins, which exert their activity against a large variety of organisms, such as bacteria, eukaryotes, and viruses by specific inhibition of bacterial DNA-dependent RNA polymerase [3]. The molecule is constituted by a naphtoquinonic system condensed to a furanone ring (chromophore) spanned by a 17-membered ansa chain connecting two opposite sides of the chromophore. Many studies on the relationship of structural features with the activity of these antibiotics have been carried out [3–11]. From these studies, the antibacterial activity can be explained by a phenomenological model that involves formation of hydrogen bonds between the enzyme and four rifamycin hydroxyl groups (located at C1, C8, C21 and C23, see Fig. 1), which must be arranged in a specific favorable three-dimensional pattern. Recently, crystal structures of rifampicin [12], rifabutin and rifapentin complexed with RNA polymerase have been determined [13]. Also a distance-restrained docking study of rifampicin and rifamycin SV to RNA polymerase has been reported [14].

Rifamycin SV (Fig. 1) contains nine stereogenic centers, five hydroxyl groups, two aromatic rings, one amide bond, ethyl ester and methyl ether on the aliphatic chain which are capable of providing multiple interactions necessary to achieve chiral recognition of enantiomers. Rifamycin SV has been used as a chiral selector in capillary electrophoresis to enantioselectively resolve negatively charged solutes containing sufficiently voluminous aromatic fragments such as dansyl aspartic acid, hexobarbital and glutethimide [15–18]. Hassan and co-workers [19] demonstarted that rifamycin SV has an excellent electrochemical response for potassium ion and developed a sensor based on it displaying

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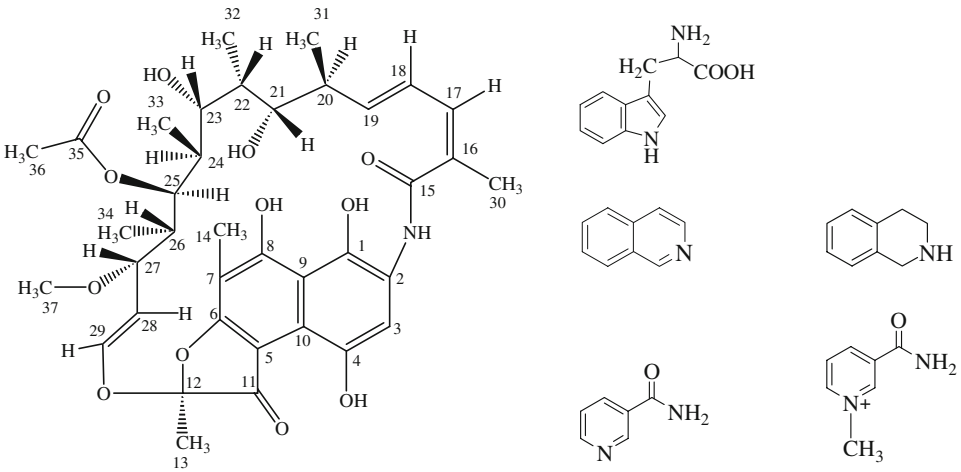

Fig. 1 Chemical structures of rifamycin SV and molecules employed as guests

a potentiometric selectivity in the order $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$. Other ansamycins also were proposed as possible chiral selectors for various separation techniques [15–18]. At the same time the binding of potential guest molecules to ansamycins was never studied quantitatively. Such study is of interest since it can provide valuable information on specificity and enantioselectivity of interactions of ansamycins with low molecular weight compounds, which will create a rational approach to choosing suitable analytes and also could contribute to better understanding of their interactions with proteins. This paper presents the studies of binding of amino acids and simple organic molecules to rifamycin SV by ^{1}H-NMR in water aimed to establish the selectivity of molecular recognition by this macrocycle.

Experimental section

Materials and instrumentation

All reagents were purchased from Aldrich and used as received. ^{1}H NMR spectra were obtained in a 400 MHz Varian UNITY INOVA spectrometer.

Methodology

For ^{1}H NMR titrations, the solutions of guests and rifamycin SV were prepared in D_2O buffered with 0.067 M boric acid and NaOD at pH 9.0. The ^{1}H-NMR titrations were performed by adding aliquots of guest stock solutions prepared at maximum possible concentrations allowed by their solubilities to 2 mM rifamycin SV. From 5 to 10 signals of different protons of rifamycin SV were used for fitting and obtained binding constants were averaged. The experimental data were fitted using non-linear least-squares regression with Microcal Origin 5 program.

The structure of the complexes of rifamycin SV with selected guests were obtained by molecular simulations performed with Gaussian 03 package, using the semiempirical pm3 mm basis set which corrects pm3 deficiences in description of the amide linkage. The structures of rifamycin–guest complex were obtained by placing the guest in a position maximally close to the rifamycin protons which undergo the largest complexation-induced shifts in ^{1}H-NMR spectra followed by minimization in vacuo of the potential energy with Berny algorithm using redundant internal coordinates [20]. In all cases the rifamycin macrocycle structure resembled the reported X-ray structure very accurately, also in complexes. Electronic properties (frontier orbitals) of rifamycin and guest molecules were calculated using density functional theory (DFT) at the B3LYP level with 6-311 + G(d,p) basis set., which combines the GGA exchange three-parameter hybrid functional developed by Becke [21] and the correlation functional of Lee-Yang-Parr [22], as implemented in the Gaussian 03 suite of programs [23].

Results and discussion

In solution rifamycin SV behaves as a strong acid with pK_a 1.8 [24] which means that in neutral and basic solutions it exists as an anion and therefore is expected to interact electrostatically with cationic species. The exact site of rifamycin deprotonation is unknown, but most probably this is one of the hydroxyl groups of the naphthohydroquinone fragment. Successful application of rifamycin SV for chiral discrimination of anionic analytes [15–18] indicates that the negative charge is strongly delocalized within the naphthohydroquinone fragment and electrostatic interactions do not play any essential role in complex formation with rifamycin SV. Also, hydrogen bonding, which is a predominant type of interaction of rifamycin SV with proteins, hardly can

contribute significantly to interactions with low molecular weight compounds in water, but the naphthohydroquinone fragment may be important for stacking and/or charge-transfer interactions with aromatic molecules. In addition an extensive portion of the macrocycle is hydrophobic and may provide significant contribution of hydrophobic interactions with organic guests.

Since rifamycin SV possesses an intensively absorbing naphthohydroquinone chromophore we attempted first to follow its interactions with other molecules spectrophotometrically, but observed spectral changes were small and poorly reproducible. Therefore all studies were performed by ^{1}H-NMR technique, which also has an advantage of providing some information on the structure of complexes. The assignments of ^{1}H signals were based on reported NMR studies of rifamycins [25].

The observed chemical shifts δ_{obs} of rifamycin protons were analyzed as a function of guest concentration by using Eq. 1 for a 1:1 stoichiometry, where δ_H is the chemical shift of a given proton in free rifamycin, δ_∞ is the chemical shift of the proton in complexed rifamycin at saturation, $[G]_T$ is the total concentration of guest and K is the binding constant [26].

$$\delta_{obs} = \frac{\delta_H + \delta_\infty K[G]_T}{1 + K[G]_T} \quad (1)$$

Preliminary testing of interactions between rifamycin SV and amino acids (Ala, AlaGly, N-Ac-Ala. Ala-OMe, Phe, Phe-OMe, Trp) showed that only aromatic amino acids formed detectable complexes in solution. Binding of L-Phe-OMe was detectable, but complexation-induced shifts in NMR signals were too small for accurate determination of K (an estimate gave $K < 10\ M^{-1}$). Additions of D-Phe-OMe did not produce any detectable changes in NMR spectra. Significantly higher affinities were observed for Trp as a guest. Titration plots of rifamycin by L- and D-Trp at selected protons are shown in Fig. 2 and the average values of binding constants calculated using several rifamycin protons are $K = 34 \pm 4\ M^{-1}$ and $18 \pm 8\ M^{-1}$ for L- and D-Trp respectively. Evidently with both amino acids rifamycin SV prefers L-enantiomers. Complexation-induced shifts in signals of rifamycin protons on interactions with both enantiomers of Trp are up-field as is typical when protons are in contact with aromatic groups producing the shielding effect due to the π-system ring current. The importance of the presence of an aromatic group in the guest molecule points to the stacking interaction with the naphthohydroquinone fragment as a principal contribution to the complex stability.

In order to rationalize the origin of L/D enantioselectivity the molecular mechanics calculations of possible complex structures were performed with both Trp enantiomers. The

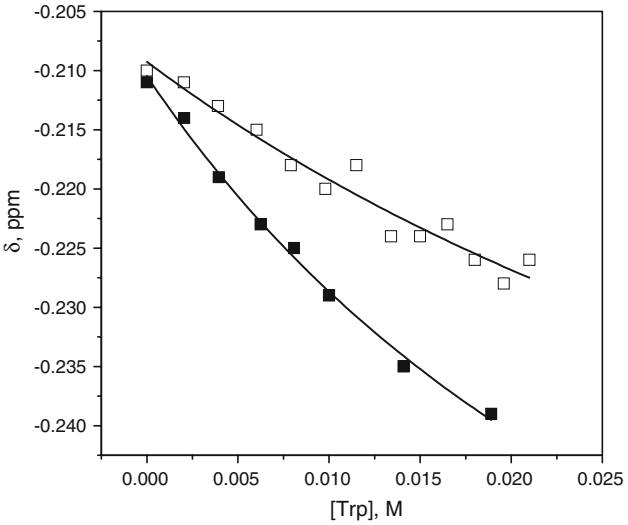

Fig. 2 Typical ^{1}H NMR titration plots of rifamycin SV with Trp enantiomers: L-Trp (solid squares) and D-Trp (open squares). Changes in chemical shifts of H34 signals are shown

X-ray crystal structure of rifamycin SV deprotonated at one of the hydroxil groups of the naphthohydroquinone fragment was taken as the initial structure of the macrocycle. Binding of Trp induces the largest shifts in NMR signals of protons (H13, H24, H29, H33, H34), which are positioned in the ansa chain in front of the naphthohydroquinone fragment. This indicates that the aromatic ring of Trp approaches the naphthohydroquinone fragment from the side of the ansa ring. Therefore the initial structure of the complex was obtained by placing Trp zwitterion in a position with an approximately parallel arrangement of the aromatic group of amino acid in respect of the naphthohydroquinone fragment from the side of the ansa ring where the guest molecule can be in contact with protons undergoing largest complexation induced shifts. The subsequent energy minimization lead to structures shown in Fig. 3 for both enantiomers.

In both complexes the ammonium group the amino acid forms an ion-pair contact with the deprotonated hydroxyl of the macrocycle with practically same $O^-\cdots NH_3^+$ distances of 2.89 and 2.83 Å for L-Trp and D-Trp respectively. Calculated distances between amino acid carboxylate group and deprotonated hydroxyl of the macrocycle also are similar: 5.26 and 5.22 Å. Thus the balance of electrostatic attractive and repulsive interactions is the same for both enantiomers. There is, however, a significant difference in the distances between aromatic planes of the host and guest molecules: 4.43 Å for L-Trp and 5.23 Å for D-Trp. Therefore a stronger interaction with L-enantiomer can be attributed to a better fit of this molecule, which allows a closer contact and stronger stacking interactions between aromatic host and guest moieties.

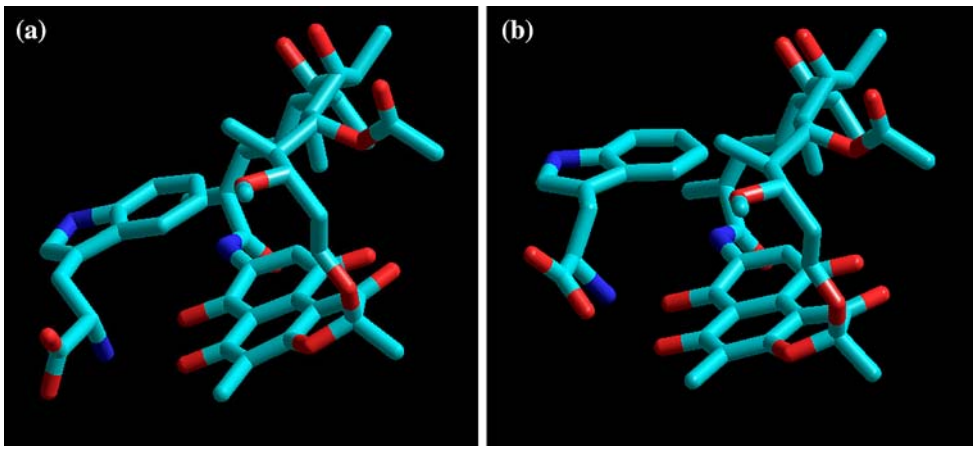

Fig. 3 Simulated structures of the rifamycin SV complexes with (**a**) L-Trp and (**b**) D-Trp

These results prompted us to see in more details interactions of rifamycin SV with purely aromatic guests. No interaction was observed with simple benzene derivatives like phenol or aniline, but heterocyclic compounds formed detectable complexes. Structures of tested guests are given in Fig. 1. Typical titration plots for iso-quinoline, tetrahydro-iso-quinoline and nicotinamide are shown in Fig. 4.

The binding constant for iso-quinoline $K = 74 \pm 17$ M^{-1} is larger than for L-Trp, but its non-planar tetrahydro derivative in spite of being positively charged at the pH of experiment ($pK_a = 9.66$) shows a much weaker binding with $K = 10 \pm 2$ M^{-1}. Nicotinamide interacts weakly with $K = 7 \pm 1$ M^{-1}, but its N-methylated derivative binds rifamycin SV strongly and shows rather unusual titration behavior. Several NMR signals (H13, H14, H30) behave "normally", that is the titration plots have typical hyperbolic shape, as illustrated in Fig. 5a, and can be fitted to Eq. 1 with average $K = 150 \pm 20$ M^{-1}. However, for signals of protons H19, H23, H25, H27, H31, H32, H37 the titration plots pass through a maximum about 0.03 M and than go down, as shown in Fig. 5b. Such behavior clearly indicates formation of a second host–guest complex in which the position of the signal moves to the opposite direction as compared with the complexation induced shift in the first complex. Plots at concentrations below maximum can be fitted to the Eq. 1 for 1:1 complexation, but with larger K values about 250 M^{-1}. The reason for this is that the opposite shift of signals due to the formation of the second complex leads to a more rapid "saturation" of the titration plot and apparently larger binding constant. With signals of protons, H21, H28, H29, H33 titration curves pass through a more pronounced maximum shifted to lower concentrations, Fig. 5c. Such behavior can be observed if the complexation of the first guest molecule induced very small shift of the signal of a given proton, but binding of the second guest molecule induces large and opposite shift. Finally, for protons H3 and H34 one observes a nearly linear dependence, Fig. 5d, typical for a case of very weak binding.

The titration plots for a case when both 1:1 and 1:2 complexes are formed should be analyzed with Eq. 2, where δ_{obs} is the observed chemical shift, δ_0, δ_1, δ_2 are the chemical shifts of a given proton in free host and its 1:1 and 1:2 complexes respectively, K_1 and K_2 are the

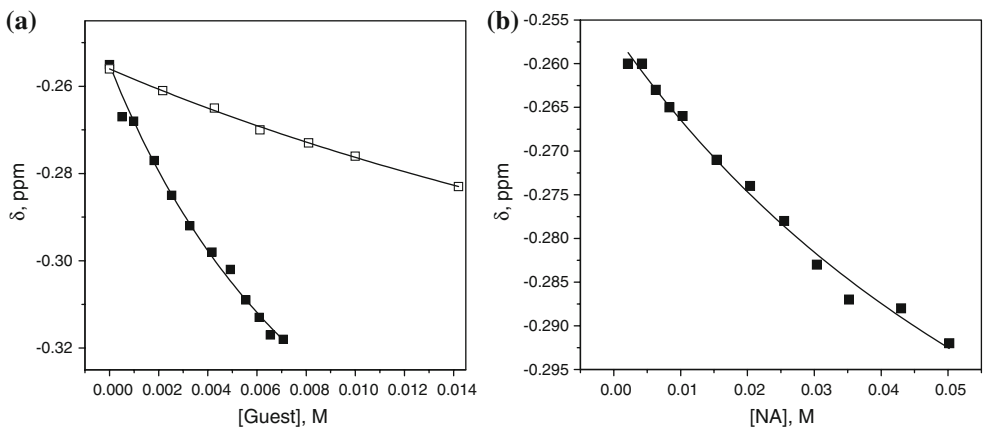

Fig. 4 Typical ^{1}H NMR titration plots of rifamycin SV with heterocyclic compounds. Changes in chemical shifts of H34 signals are shown. **a** Titrations with iso-quinoline (*solid squares*) and tetrahydro-iso-quinoline (*open squares*) H34a, **b** titration with nicotinamide H34b

corresponding stepwise formation constants and $[G]_T$ is the total concentration of the guest.

$$\delta_{obs} = \frac{\delta_0 + \delta_1 K_1[G]_T + \delta_2 K_1 K_2[G]_T^2}{1 + K_1[G]_T + K_1 K_2[G]_T^2} \quad (2)$$

However, a practical application of Eq. 2 is rather problematic because it involves too many adjustable parameters: two equilibrium constants K_1 and K_2 and two chemical shifts δ_1 and δ_2 for protons of complexes 1:1 and 1:2. Titration plots with clearly observed "saturation", like that in Fig. 5a, can be observed in situations when δ_2 is close to δ_1 and the transformation of the 1:1 complex into the 1:2 complex at increasing guest concentrations is not seen for a given proton. However, if one sets $\delta_2 = \delta_1$ in the Eq. 2 it takes the form (3), which is not equivalent to (1) and it is not evident that formal fitting of these plots to the Eq. 1 indeed will give a correct value of K_1.

$$\delta_{obs} = \frac{\delta_0 + \delta_1 K_1[G]_T(1 + K_2[G]_T)}{1 + K_1[G]_T(1 + K_2[G]_T)} \quad (3)$$

On the other hand, titration plots passing through maxima (Fig. 5b, c) or going monotonically up-field (Fig. 5d) do not even tend to "saturate" at high guest concentrations when the transformation of the first complex of the 1:1 stoichiometry into the second 1:2 complex takes place. This means that K_2 is small and therefore the term $K_2[G]_T$ is always much less than unity. With this simplification the Eq. 3 indeed reduces to the Eq. 1 and so a value of K_1 can be obtained. In order to prove that this K_1 value is consistent with results obtained for other protons we applied Eq. 2 for the fitting the titration plots like those shown in Fig. 5b–d with fixed value of $K_1 = 150\ M^{-1}$ and also fixed value of K_2 arbitrary chosen to be equal $1\ M^{-1}$ (one cannot leave K_2 as an adjustable parameter because of strong interdependence between parameters; when we attempted to use Eq. 2 with three adjustable parameters, δ_1, δ_2 and K_2 we observed either divergence or physically meaningless values of K_2 about $0.01\ M^{-1}$ together with inexplicably very large differences between δ_1 and δ_2 about 10 ppm). The solid lines in Fig. 5b–d show thus obtained fitting curves. For the plot in Fig. 5b one obtains δ_1 larger than δ_0 by 0.03 ppm, like in the case of Fig. 5a, and δ_2 lower than δ_1 by 0.3 ppm; for the plot in Fig. 5c the difference between δ_1 and δ_0 is 0.025 ppm and the difference between δ_2 and δ_1 is −0.49 ppm; for the plot in Fig. 5d the respective differences are −0.01 and −1.02 ppm. We see from this analysis that all results

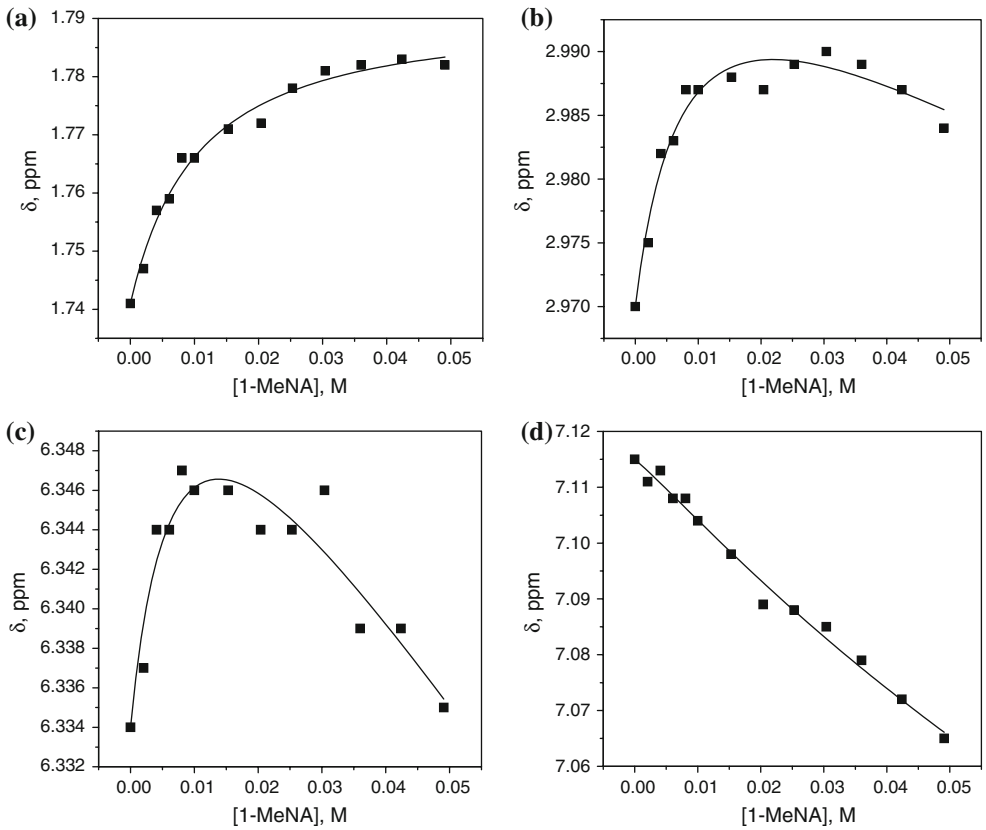

Fig. 5 Selected ^{1}H NMR titration plots of rifamycin SV with 1-methylnicotinamide observed for signals of different protons: (**a**) H13, (**b**) H37, (**c**) H29, (**d**) H3

agree with a model in which the first complexation is strong, but produces small shifts in positions of the signals, and the binding of second guest molecule in contrast is weak, but indices much larger shifts in signal positions.

The titration results with 1-methylnicotinamide perhaps are not very common, but are rather instructive. They clearly demonstrate that even very weak binding of the second guest species may strongly affect the shape of the titration plot and completely mask much stronger binding of the first species. Thus, the analysis of titration plots must be performed by using all host or guest signals which undergo changes on complexation. Another interesting point, which follows from the above results is that the condition of $K_2[G]_T << 1$ is obviously valid for titration plots obtained with any proton. Nevertheless the term $K_2[G]_T$ cannot be excluded from the fitting equation when the spectral change induced by formation of the second complex is significantly larger than that due to formation of the first complex.

In contrast to all other guests 1-methylnicotinamide induces mainly down-field shifts of the signals of the host protons. This can be due to strong electronacceptor character of this guest producing deshielding effects on adjacent protons. Strongly increased binding of N-methylnicotinamide as compared to neutral nicotinamide was reported for a highly preorganized aromatic molecular clip in water and attributed to contribution of cation-π interactions [27]. Such interactions are manifested also in tight binding of tetraalkylammonium cations [27, 28], but we did not observe any binding of $(n\text{-}Pr)_4\ N^+$ and $Et_4\ N^+$ cations to rifamycin SV. Also a decreased affinity of tetrahydro-isoquinoline as compared to isoquinoline points to importance of having planar aromatic structure for the guest. It seems therefore that stacking interactions are the most plausible type of binding of guests to rifamycin SV.

Molecular mechanics calculations of structures of the complexes of rifamycin SV with nicotinamide and 1-methylnicotinamide, Fig. 6, indicate significant differences in the mode of binding of these two guests. Nicotinamide forms an edge-to-face contact with the naphthohydroquinone fragment observed in several cyclophane inclusion complexes with aromatic guests [29], but 1-methylnicotinamide turns into a more coplanar orientation favorable for stacking interaction with the distance 5.32 Å between centroids of aromatic rings of host and guest.

Stacking interactions have generally a complex nature and involve different types of intermolecular forces related to polarizability of aromatic π-systems and charge transfer interactions [26, 29]. The last type of interactions is related to the electron transfer between frontier orbitals and can be analyzed by comparison of energies of LUMO and HOMO of host and guest molecules [29, 30]. As one can see from the data given in Table 1, the energies of HOMO for all guests are far from the energy of LUMO of rifamycin SV, but HOMO of rifamycin is in the range of LUMO of guests which means that the host can participate as an electrodonating component and this implies a possibility of host-to-guest charge transfer. Notably, LUMO of 1-methylnicotinamide, which is the most tightly bound guest, is lower than for all other guest molecules and even lower than HOMO of rifamycin. Stronger binding of isoquinoline as compared to tetrahydroisoquinoline and both enantiomers of Trp also can be attributed to lower LUMO energy of the former molecule. It should be noted that stacking interactions with nicotinamide [31] and charge transfer interactions with 1-methylnicotinamide [32] are well documented in literature.

On the other hand binding of nicotinamide is much weaker than that of isoquinoline in spite of similar LUMO energies. This may be related to lower hydrophobicity of nicotinamide. To test a possible contribution of hydrophobic interactions

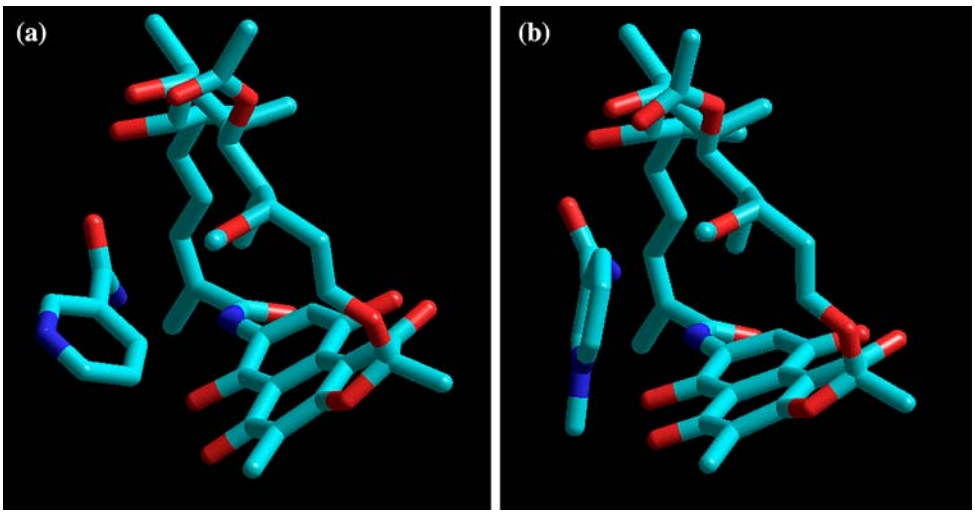

Fig. 6 Simulated structures of the rifamycin SV complexes with (**a**) nicotinamide and (**b**) 1-methylnicotinamide

Table 1 Energy (Hartree) of frontier orbitals of rifamycin SV and the guests studied in this work. Calculations performed at DFT theory level with 6-311 + G(d,p) basis set

Molecule	HOMO	LUMO	Log K	Log P	α-CD
Rifamycin SV	−0.071	0.038			
Trp	−0.207	−0.018	1.54 (L-Trp) 1.25 (D-Trp)	2.14	21.2 (L)
Isoquinoline	−0.241	−0.065	1.9	2.08	22.7
Tetrahydroisoquinoline	−0.229	−0.016	1.0	1.82	
Nicotinamide	−0.261	−0.057	0.84	−0.37	
1-Methylnicotinamide	−0.410	−0.253	2.2	(−0.37)	

we attempted to correlate the results for all guests in terms of a two parameter Eq. 4 where E_{LUMO} is the energy of the guest LUMO and log P is the logarithm of partition constant of the guest between octanol and water [33].

$$\log K = a + b \cdot E_{LUMO} + c \cdot \log P \quad (4)$$

For the cationic guest 1-methylnicotinamide log P is unknown and by analogy with other cationic solutes it should have a large negative value [34] resulting from the strong hydration of ionic species. However, one can see from the simulated structure in Fig. 6b that the positively charged N-methyl ammonium center of the guest is directed outside the host cavity and may conserve its hydration in the complex. Therefore we assigned the same log P value for both nicotinamide and its methylated derivative. For the same reason for Trp the value of log P used in the correlation corresponds to indole since the zwitterionic fragment is directed outside the macrocycle cavity, see Fig. 3. The multiparameter regression of the results in Table 1 shows that all coefficients in the Eq. 4 are significant and have the values of $a = 0.5 \pm 0.2$. $b = -7.5 \pm 1.5$ and $c = 0.4 \pm 0.1$ with R = 0.89615. A positive coefficient at log P may reflect either contribution of hydrophobic interactions to aromatic stacking or some additional contribution due to contacts of guest molecules with hydrophobic fragments of rifamycin SV (Fig. 7).

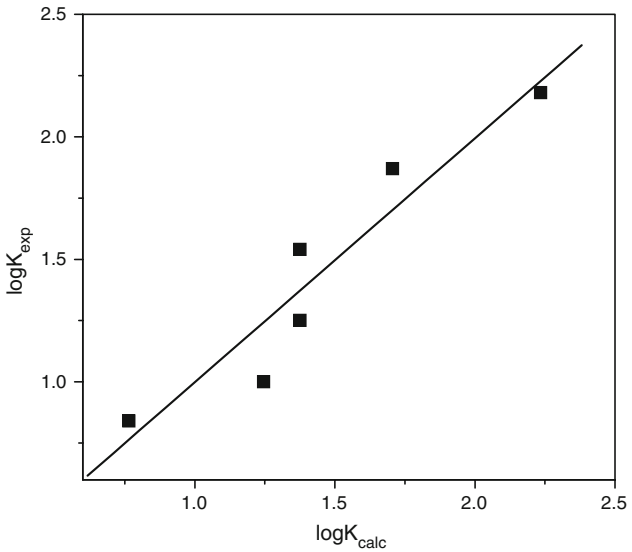

Fig. 7 Correlation between experimental and calculated with Eq. 4 stability constants of complexes with rifamycin SV

Conclusions

Simulated structures of rifamycin SV complexes clearly show that binding of guest molecules cannot be described as "inclusion" because of too small size of the macrocycle cavity. Nevertheless affinity of rifamycin SV to amino acids and heterocyclic guests is similar to that reported for cyclodextrins, classical hosts for formation of inclusion complexes. Thus binding constants of tryptophane and phenylalanine to α- and β-cyclodextrins are about 20 M^{-1} and 10 M^{-1} respectively with practically no difference for L- and D-enantiomers [35]. In contrast, rifamycin SV shows a significant enantioselectivity with K_L/K_D ratio of 1.9 for tryptophane. The largest affinity of rifamycin SV is expected towards electronaccepting hydrophobic guests like nitrogen heterocycles, in particular N-substituted nicotinamide derivatives. Therefore this macrocycle may be recommended for analytical applications with such compounds as NAD^+, NADP or tryptophan containing peptides.

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