

Thermodynamic study on inclusion complex formation of riboflavin with hydroxypropyl- β -cyclodextrin in water

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Abstract The inclusion complex formation of riboflavin (RF) with hydroxypropyl- β -cyclodextrin (HP- β -CD) in water was investigated by ^1H NMR, UV-vis spectroscopy, and solubility methods. A 1:1 stoichiometry and thermodynamic parameters of complex formation (K , $\Delta_c G^0$, $\Delta_c H^0$, and $\Delta_c S^0$) were determined. Complexation was characterized by negative enthalpy and entropy changes due to prevalence of van der Waals interactions and hydrogen bonding between polar groups of the solutes. A partial insertion of RF into macrocyclic cavity was revealed on the basis of ^1H NMR data and molecular mechanics calculation. Location of benzene ring of RF molecule inside the hydrophobic cavity of HP- β -CD results in an increase of aqueous solubility of the former.

Keywords Inclusion complex formation · Hydroxypropyl- β -cyclodextrin · Riboflavin · Thermodynamics

Introduction

Riboflavin (RF) (7,8-dimethyl-10-(1'-D-ribityl)isoalloxazine) is vitamin B₂ that is required for a wide variety of cellular processes. Especially, it is involved in numerous

redox processes and biological electron transport. Moreover, RF is widely used in pharmaceutical and food supplement industries. It is well known that RF is slightly soluble in aqueous solutions and is a highly photosensitive compound [1, 2]. Photodegradation and poor solubility result in reduction of biological and pharmacological activity of RF [3].

Some attempts have been made to improve the photostability and solubility of RF by various methods. One of them is based on the incorporation of RF into liposomes [4–6]. An alternative method can be the encapsulation by cyclodextrins (CDs). CDs are cyclic oligosaccharides obtained by the enzymatic disintegration of starch. Owing to hydrophilic exterior and hydrophobic interior, CDs are capable of forming water-soluble inclusion complexes with different organic compounds [7–9]. Inclusion complexes of CDs have a significant scope of applications [10, 11]. With regard to RF, the changes of redox properties and enhancement of aqueous solubility upon inclusion in CD cavity can be observed.

In the literature there are some publications dealing with complex formation of RF and its derivatives with CDs [12–17]. A spectroelectrochemical study of the interactions occurring between RF and β -CD in water containing the supporting electrolyte of 0.10 M HCl + 0.10 M KCl (pH 1.1) was performed by Wang et al. [12]. These authors also investigated the surface-enhanced Raman spectroelectrochemical behavior of RF in the absence and presence of β -CD on a silver electrode [13]. It was shown that β -CD exerts a stabilizing effect on the reduction process due to inclusion complex formation. Roy et al. [14] revealed the formation of both 1:1 and 1:2 β -CD/RF complexes in water at 303 K by means of fluorimetric and absorption study. They obtained relatively large binding constants denoting the high complex stability. Several kinetic investigations of photodegradation

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of RF-5/(dihydrogen-phosphate monosodium salt) in the presence of different CDs were performed by Loukas et al. [15–17]. A pronounced stabilizing effect of CDs on RF photodegradation was detected. This effect was explained by inclusion complex formation of RF with CDs. It was found that RF forms 1:1 inclusion complexes with hydroxypropyl- β -cyclodextrin (HP- β -CD) [15] and γ -CD [16, 17], whereas 1:2 composition ratio was observed for complexation with α -CD [16, 17]. The stability of 1:2 complexes was considerably lower than for 1:1 complexes. It should be noted that in all these works only stability constants were determined whereas the other thermodynamic parameters of complex formation such as the enthalpy and entropy changes were not considered.

Thus, the aim of this study was to obtain a complete set of thermodynamic parameters of complex formation (K , $\Delta_c G^0$, $\Delta_c H^0$, and $\Delta_c S^0$) of RF with HP- β -CD in water at 298.15 K and to propose the binding mode and driving forces of interaction.

Experimental

Materials

HP- β -CD and RF were purchased from Sigma-Aldrich and were used without further purification. The average substitution degree of HP- β -CD was 0.6 per glucose unit. All solutions were prepared by weight on the basis of double-distilled, degassed water.

Methods

UV-vis spectroscopy

Absorption spectra of freshly prepared RF solutions with and without the addition of HP- β -CD were recorded in the range 200–600 nm on a UV-2401 PC UV-vis Recording Spectrometer (Shimadzu, Japan) equipped with a TCC-240 A temperature controlled cell holder. All measurements were carried out in 1-cm quartz cuvettes at different temperatures (298.15, 303.15, 308.18, and 313.15 K). The absorption spectra of RF in the presence of HP- β -CD are shown in Fig. 1.

The stoichiometry of the complexes was determined using Job method (continuous variation method) [18, 19]. According to this method, 0.1 mmol kg⁻¹ solutions of RF and HP- β -CD were mixed at different concentration ratios ($R = c_{\text{HP-}\beta\text{-CD}}/(c_{\text{HP-}\beta\text{-CD}} + c_{\text{RF}})$) to constant volume. The stoichiometric ratio was obtained by plotting $\Delta A \cdot R$ against R (where ΔA is the difference of absorbance of RF with and without HP- β -CD) and finding the R value corresponding to the extreme of this dependence. A Job plot for RF/HP- β -CD complexation is given in Fig. 2.

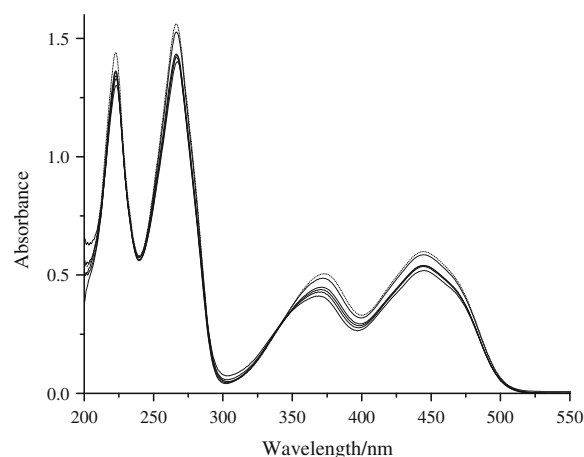


Fig. 1 Absorption spectra of riboflavin in water (dash lines) and in the presence of variable amounts (0.01 ÷ 0.1 mol kg⁻¹) of HP- β -CD (straight lines) at 298.15 K

To calculate the binding constant, the RF concentration was kept constant (5.6×10^{-5} mol kg⁻¹) and HP- β -CD concentration was varied from 0 to 0.1 mol kg⁻¹. The HP- β -CD solutions of the corresponding concentration were used in the reference cuvettes. The influence of HP- β -CD concentration on RF absorption is illustrated in Fig. 3. The binding constant (K) was evaluated by the nonlinear curve fitting procedure based on the following equation:

$$A = A_0 + (\Delta\varepsilon \cdot K \cdot C_{\text{RF}} \cdot C_{\text{HP-}\beta\text{-CD}}) / (1 + K \cdot C_{\text{HP-}\beta\text{-CD}}) \quad (1)$$

where A and A_0 are the absorbance of RF in the presence and absence of HP- β -CD, respectively; C_{RF} and $C_{\text{HP-}\beta\text{-CD}}$ are the initial concentrations of RF and HP- β -CD, respectively; $\Delta\varepsilon$ is the difference in molar absorptivities between

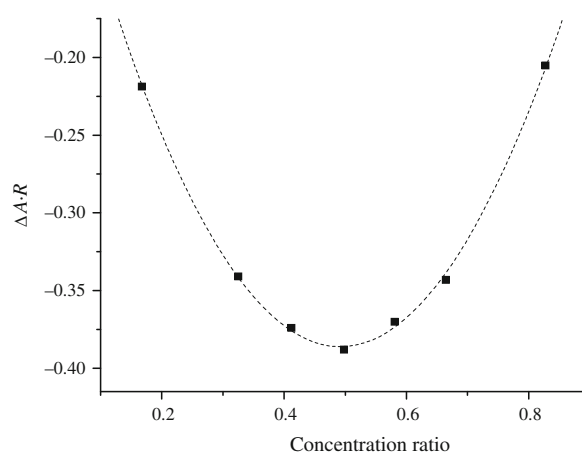


Fig. 2 Job plot for complex formation of riboflavin with HP- β -CD at 298.15 K ($\lambda = 445$ nm)

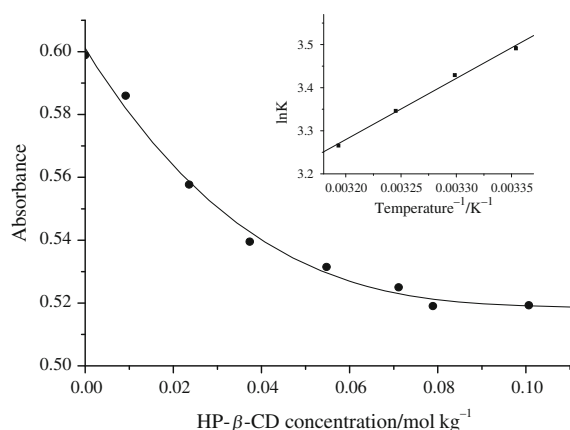


Fig. 3 Dependence of riboflavin absorbance from HP- β -CD concentration at 298.15 K and $\lambda = 445$ nm. *Inset*: van't Hoff graph plotted on the basis of UV-vis spectroscopy data

free and complexed RF and together with K it was adjusted parameter in Eq. 1.

^1H NMR

^1H NMR spectra were recorded on a Bruker-AV-500 spectrometer operating at 500 MHz in the temperature range $293.15 \div 318.15$ K. Cyclohexane was used as external reference. The solutions containing a constant concentration of RF (0.3 mmol kg^{-1}) and variable concentration of HP- β -CD ($0 \div 0.03 \text{ mol kg}^{-1}$) were prepared in deuterated water of 99.9% isotopic purity. ^1H NMR spectra of RF shown in Fig. 4 were analyzed.

Determination of the binding constant (K) and complexation-induced chemical shifts ($\Delta_c\delta$) was based on the variation of $\Delta\delta$ in the presence of different amounts of HP- β -CD. Concentration dependences of $\Delta\delta$ are given in Fig. 5. The following equation was used for calculation of K :

$$K = \frac{C_{\text{RF}} \cdot \Delta\delta / (\Delta_c\delta \cdot (C_{\text{RF}} - C_{\text{RF}} \cdot \Delta\delta / \Delta_c\delta)) \cdot (C_{\text{HP-}\beta\text{-CD}} - C_{\text{RF}} \cdot \Delta\delta / \Delta_c\delta)}{\quad} \quad (2)$$

where C_{RF} and $C_{\text{HP-}\beta\text{-CD}}$ are the concentrations of RF and HP- β -CD, respectively; $\Delta\delta$ is the experimentally observed chemical shift change of RF protons that is expressed as $\Delta\delta = \delta_{\text{complexed}} - \delta_{\text{free}}$.

Solubility study

An excess amount of RF was added to 2 ml of water or HP- β -CD solutions of different concentrations ($0.005 \div 0.04 \text{ mol kg}^{-1}$). The solutions were placed in plastic vials which were covered with aluminum foil in order to prevent degradation of RF by light. The vials were mechanically

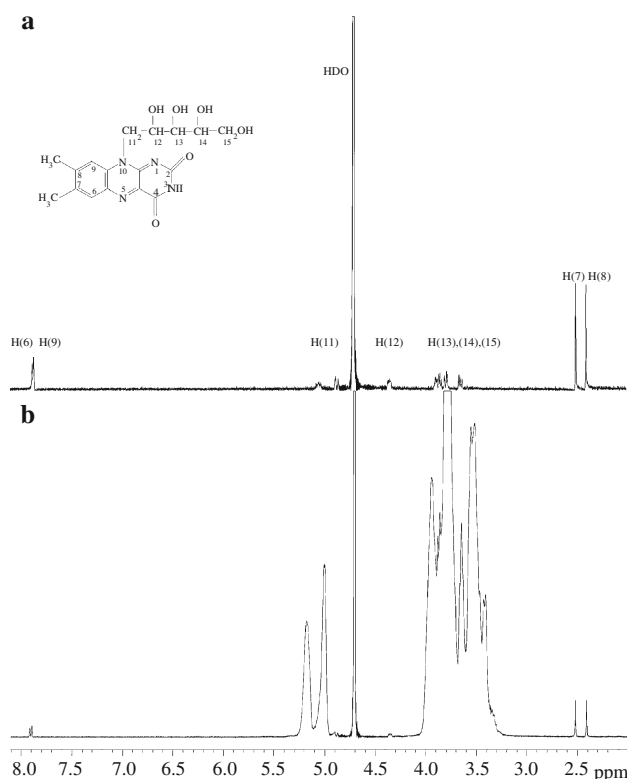


Fig. 4 ^1H NMR spectrum of riboflavin alone (a) and in the presence of $0.005 \text{ mol kg}^{-1}$ HP- β -CD (b) at 298.15 K

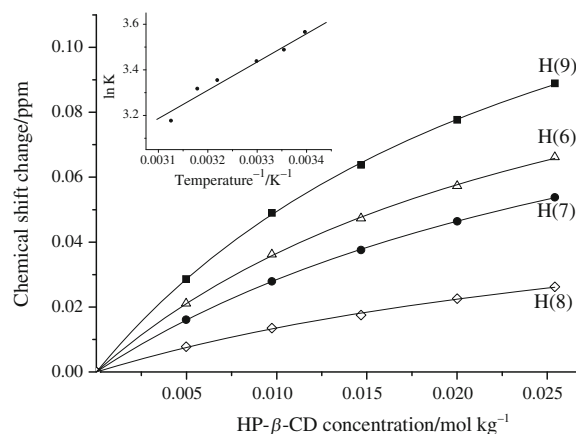


Fig. 5 Dependences of chemical shift changes of riboflavin protons from HP- β -CD concentration at 298.15 K. *Inset*: van't Hoff graph plotted on the basis of ^1H NMR results

shaken during 3 days. After equilibration, the solutions were centrifugated at the corresponding temperature and analyzed spectrophotometrically using a UV-2401 PC UV-vis Recording Spectrometer and 0.1-cm quartz cuvettes. The experiment was performed at different temperatures ($298.15 \div 318.15$ K). Each measurement was prepared in duplicate.

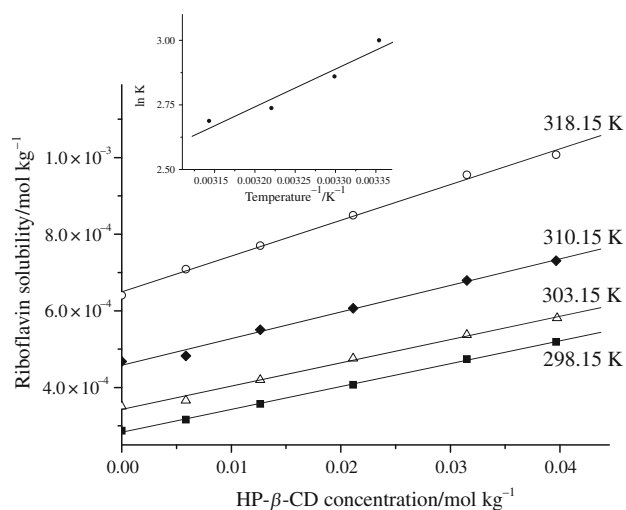


Fig. 6 Solubility diagrams at different temperatures. *Inset:* van't Hoff graph plotted on the basis of solubility data

According to the Higuchi and Connors method [20], the stability constant of RF/HP- β -CD complexes was estimated from the phase-solubility diagrams presented in Fig. 6:

$$S = S_0 + K \cdot S_0 \cdot C_{\text{HP-}\beta\text{-CD}} \quad (3)$$

where S_0 and S are the solubility of RF in pure water and HP- β -CD solutions, respectively; $C_{\text{HP-}\beta\text{-CD}}$ is the concentration of HP- β -CD; K is the stability constant.

Molecular modeling

Calculations were performed by OPLS force field using HyperChem 7.0 software. Molecular mechanic simulations started from crystallographic host and guest geometries which are minimized using conjugate algorithm with the convergence criteria $0.001 \text{ kcal mol}^{-1}$.

Results and discussion

The thermodynamic study on complex formation of RF with HP- β -CD was based on determination of K from the concentration dependences of the experimentally measured property (A , $\Delta\delta$, and S) with the consequent calculation of the free energy change:

$$\Delta_c G^0 = -RT \ln K \quad (4)$$

The enthalpy and entropy changes were obtained from the temperature dependence of the stability constant:

$$\ln K = -\Delta_c H^0 / RT + \Delta_c S^0 / R \quad (5)$$

It was not possible to derive the thermodynamic parameters directly from calorimetry because RF is poorly soluble in water.

The absorption spectra of RF recorded in pure water and in HP- β -CD solutions are shown in Fig. 1. As can be seen, four absorption maximum wavelengths were observed at 223, 267, 374, and 445 nm. The presence of HP- β -CD induces a small hypsochromic shift only for the third maximum, reaching 369 nm at the highest concentration of HP- β -CD used in this study. The RF absorbance is decreased upon the addition of HP- β -CD. These spectral changes are similar to those revealed for inclusion complex formation of RF with parent β -CD [12]. The appearance of an isosbestic point at 341 nm testifies to the formation of only one equilibrium:



The 1:1 stoichiometry of the complexes is confirmed by the Job method. The Job plot presented in Fig. 2 is symmetrical, and it shows the predominance of one type of complexes. The minimum of this dependence corresponds to a 1:1 stoichiometric ratio. Therefore, all thermodynamic parameters summarized in the Table 1 are related to 1:1 complexation.

Thermodynamic parameters of complex formation derived from spectroscopic and solubility methods were calculated using Eqs. 1–3 for stability constants and Eq. 5 for enthalpy and entropy determination. The temperature dependences of $\ln K$ are given in the insets to Figs. 3, 5 and 6.

The thermodynamic parameters of complex formation of RF with HP- β -CD are reported in the Table 1. A good agreement of the results obtained by different experimental techniques should be emphasized in this case. As can be seen from the Table 1, complex formation of RF with HP- β -CD is characterized by the negative enthalpy and entropy changes. The negative value of free energy and complex stability are determined by the enthalpic contribution, and therefore, the complexation process is enthalpy-driven.

It is well known that the binding of CDs with guest molecules is realized via noncovalent interactions such as hydrophobic, electrostatic, and van der Waals interactions as well as the hydrogen bonding [21]. Dehydration of solutes and the release of water molecules from the CD cavity also take place. It should be mentioned that dehydration together with hydrophobic interactions are

Table 1 Thermodynamic parameters of 1:1 complex formation of riboflavin with HP- β -CD in water at 298.15 K obtained by different experimental methods

Method	K / kg mol^{-1}	$\Delta_c G^0$ / kJ mol^{-1}	$\Delta_c H^0$ / kJ mol^{-1}	$T\Delta_c S^0$ / kJ mol^{-1}
$^1\text{H NMR}$	33 ± 1	-8.7	-10.3 ± 1.0	-1.6
Solubility	21 ± 2	-7.5	-12.2 ± 1.1	-4.7
UV-vis	29 ± 2	-8.3	-11.8 ± 0.6	-3.5

accompanied by positive enthalpy and entropy changes [21, 22]. The negative $\Delta_c H^0$ and $\Delta_c S^0$ obtained in our case are mainly determined by dominant contribution from van der Waals interactions and possible hydrogen bonding between the polar side group of RF and the hydroxypropyl- and hydroxyl-groups surrounding the HP- β -CD cavity. Due to van der Waals interactions, RF should be in close contact with the HP- β -CD cavity, and a considerable number of water molecules should be released in the bulk solvent giving positive enthalpic and entropic contributions. However, this positive contribution that can occur is predominated by the negative contribution from van der Waals interactions and H-bonding which are characterized by high exothermicity. These interactions also result in limited conformational flexibility and a decrease of the entropy. Thus, the attractive interactions are more important than solvent effects and make the complexation process enthalpically favorable.

^1H NMR spectra of RF in the presence of HP- β -CD were examined in order to propose the probable structure of the complexes. ^1H NMR spectra of RF in pure water and in aqueous solution of HP- β -CD are shown in Fig. 4. The spectrum of HP- β -CD and chemical shift changes of CD protons were not considered because of a negligible influence of RF being due to its very low solubility in water. The ^1H NMR spectrum of RF in deuterated water consists of the signals from protons H(6), H(7), H(8), H(9), H(11), H(12), H(13), H(14), and H(15) [15]. Unfortunately, these protons belong to only one benzene ring and ribityl side chain and hence it was impossible to obtain any information on the participation of another part of RF molecule in the binding with HP- β -CD. Moreover, in the presence of HP- β -CD the peaks of H(11)–H(15) protons are overlapped with the peaks of HP- β -CD protons. Therefore, the signals of H(6), H(7), H(8), and H(9) protons that are clearly visible in the spectrum were used to calculate the stability constants reported in the Table 1.

As it follows from Fig. 5, the addition of HP- β -CD induces a downfield shift of RF protons H(6)–H(9). This indicates that the benzene ring of RF molecule is located inside the macrocyclic cavity and inclusion complex formation takes place. To confirm the proposed binding mode the molecular modeling of complex formation was performed. The structure of the RF/HP- β -CD complex obtained using HyperChem program is illustrated in Fig. 7. The results of computer modeling show that one benzene ring of RF is included into the cavity of HP- β -CD, whereas its bulky ribityl side chain and the remaining two aromatic rings are placed outside the cavity. Insertion is realized in the direction from the wide rim of the macrocyclic cavity. Dimensions of the HP- β -CD cavity are similar to β -CD.

In particular, the internal diameter and the depth of the cavity are equal to $6.0 \div 6.5$ Å and 7.9 Å, respectively

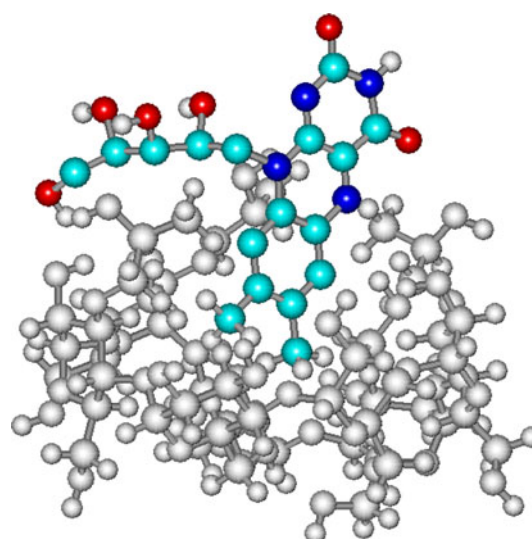


Fig. 7 Stereo view of computed structure of riboflavin/HP- β -CD complex

[22, 23]. Therefore, the whole molecule of RF is unable to penetrate the cavity and only a partial inclusion takes place. According to this binding mode, the ribityl side chain containing several OH-groups can form hydrogen bonds with hydroxyl- and hydroxypropyl-groups surrounding the rim of the HP- β -CD cavity. Moreover, two nitrogen atoms of the second aromatic ring are located near the external polar groups of HP- β -CD and can be involved in the hydrogen bonding with them. Thus, hydrogen bonding plays an important role in complexation and determines the negative values of $\Delta_c H^0$ and $\Delta_c S^0$.

As it was concluded above, one benzene ring of RF molecule penetrates the HP- β -CD cavity. This should be favorable for enhancement of the aqueous solubility of vitamin. For this purpose, the solubility of RF in pure water and in aqueous solutions of HP- β -CD was measured in the temperature range 298.15–318.15 K. The solubility of RF in pure water at 298.15 K was found to be $(2.88 \pm 0.01) \times 10^{-4}$ mol kg $^{-1}$, which is in accordance with the data obtained by Frost (2.92×10^{-4} mol kg $^{-1}$) [24]. An approximately 2-fold increase in RF solubility was obtained at different temperatures and over the HP- β -CD concentration range studied (Fig. 6). Solubility diagrams depicted in Fig. 6 are linear, and therefore, they are classified as A_L -type according to the Higuchi and Connors method [20]. The A_L diagram corresponds to 1:1 binding and it additionally proves the 1:1 stoichiometry of the complexes revealed in this study by other methods. Thus, the solubilizing effect of HP- β -CD is determined by placing the apolar part of the RF molecule inside the hydrophobic cavity and by the hydrophilic character of the external surface of the inclusion complexes.

Conclusions

A thermodynamic study of complex formation of RF with HP- β -CD performed by spectroscopic and solubility methods revealed that the 1:1 binding is enthalpy-driven due to the prevalence of van der Waals interactions and hydrogen bonding. The ^1H NMR data that are in agreement with the results of molecular modeling showed the insertion of the benzene ring of RF molecule into the hydrophobic cavity of HP- β -CD. The proposed inclusion complex formation induces the enhancement of RF solubility in aqueous medium.

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