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ORIGINAL ARTICLE

NMR as a tool for simultaneous study of diastereoisomeric inclusion complexes formed by racemic mixture of 4′-hydroxyflavanone and heptakis-(2,6-*O*-dimethyl)-*β*-cyclodextrin

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Abstract The complexes formed by (±)-4′-hydroxyflavanone (OHFL) and heptakis-(2,6-*O*-dimethyl)-*β*-cyclodextrin (DM-*β*-CD) were obtained using the racemic mixture of OHFL. These complexes were able to be studied due to their enantiodifferentiation by ^{1}H-NMR spectroscopy. Stoichiometry, association constants and thermodynamic parameters were obtained from these NMR data, and inclusion geometries were proposed from ROESY and docking experiments. The results show that diastereoisomeric complexes can be studied even when they are formed by enantiomeric mixtures.

Keywords Cyclodextrin · NMR · Enantiomeric differentiation

Introduction

Cyclodextrin inclusion complexes have been studied for a long time due to their promising applications as medicinal agents in the pharmaceutical industry; they can increase solubility, bioavailability and stability [1]. They can also act as catalysts [2, 3], chemzymes [4] and have been used to drive asymmetric synthesis [5], applications which are possible due to the selectivity for substrate, reaction and stereo selectivity of cyclodextrins.

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Cyclodextrins are cyclic oligosaccharides formed by 6, 7 or 8 α-D-glucopyranose units, and their conformation in aqueous system is assumed to approach a truncated cone (Fig. 1). They have a hydrophobic internal surface and hydrophilic outer surface, and hence they can form inclusion complexes, especially with aromatic substrates. The complex formation is due to weak physical interactions, and it is believed that Van der Waals and hydrophobic interactions are the most important, although hydrogen bonding and steric effects are important as well [6]. The chirality, characteristic of cyclodextrins, has been used to include chiral molecules that when are complexed could exhibit different physical properties, such as different chemical shifts and/or association constants [7, 8].

There have been several techniques used to study enantiomeric differentiation using cyclodextrin as chiral selectors, including fluorescence anisotropy [9–11], HPLC [12] and NMR [8]. These techniques allow the study of diastereoisomeric complexes through the determination of the association constant, but NMR has an advantage over the other techniques in that the inclusion geometry can be estimated. In NMR the differentiation between two diastereoisomeric complexes depends on the intrinsic chemical shift of each complex as well as the magnitude of their association constant. These complexes are in equilibrium with their free parts, and the chemical shift is a contribution of the free and complexed forms [8]. On the other hand, the Nuclear Overhauser Effect (NOE), which is a through-space effect, provides direct evidence of the portion of the guest molecule which enters into the CD torus. This effect leads to the mutual enhancement of proximate guest and host resonances. One and two dimensional NOE techniques have been applied but the most effective is the ROESY (Rotating frame Overhauser Effect SpectroscopY) experiment which

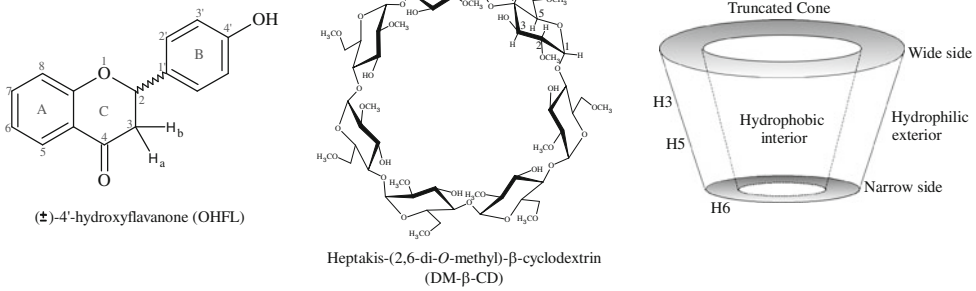

Fig. 1 OHFL and DM-β-CD structures

overcomes the problem of the small NOE magnitude of the most CD inclusion complexes [8, 13].

NMR is a useful tool to evaluate the characteristics of diastereoisomeric complexes, allowing the determination of the stoichiometry, association constants and inclusion geometries in racemic mixtures.

The enantiomeric differentiation is a common problem in biological systems as well. Such is the case of 11β-HSD1 reductase, an enzyme with an asymmetric active site [14] which is involved in diabetes. This enzyme is inhibited by the (±)-4′-hydroxyflavanone (OHFL), a chiral flavonoid which was studied on the 11β-HSD1 reductase as a racemic mixture [15]. Although the chiral nature of the active site points to a difference in the activity between both enantiomers, the only activity data available corresponds to the racemic mixture.

A large number of cyclodextrin derivatives have been used for NMR enantiomeric differentiation, and heptakis-(2,6-*O*-dimethyl)-β-cyclodextrin (DM-β-CD) (Fig. 1) is one of them. In this work NMR chemical shifts were used to study the diastereoisomeric complexes formed between DM-β-CD and OHFL, and the results are further explained with molecular modeling techniques. Thus, this work constitutes a first step toward the separation of the racemic mixtures [16], separation that would be consequentially performed with HPLC or other technique.

Experimental

Apparatus

NMR spectra were recorded at 298 K on a Bruker Avance DRX 300 spectrometer operating at 300.13 MHz for ^{1}H. Chemical shifts were measured relative to DHO signal at 4.7 ppm. 1D spectra were collected by co-addition of 128 scans.

Rotating-frame Overhauser Effect SpectroscopY (ROESY) spectra were acquired in the phase sensitive mode with the same spectrometer and Bruker standard parameters (pulse program roesygpph19). Each spectrum consisted of a matrix of 16 K (F2) by 8 K (F1) points covering a spectral width of 3000 Hz. Spectra were obtained with a spin-lock mixing time of 400 ms, relaxation delay 2 s, and 32 scans were recorded.

Materials

2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one (OHFL) and heptakis-(2,6-*O*-dimethyl)-β-cyclodextrin (DM-β-CD) were purchased from Aldrich (USA). D_2O and CD_3OD employed in the NMR analyses were of spectroscopic reagent grade, from Aldrich and Merck, respectively.

Methods

All the samples used in this work were performed using the appropriate amounts of (±)-4′-hydroxyflavanone (OHFL) and heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) in CD_3OD:D_2O, stirring for 24 h in a thermostatic water bath at constant temperature. Several methanol:water ratios were tested, finding that the best methanol amounts were the used in this study.

Solutions were made in methanol due to the low solubility of the OHFL in water, and for its low affinity for binding with cyclodextrins [17, 18].

Stoichiometry determination

A reliable determination of the complex stoichiometry is provided by the continuous variation technique (Job's plot). ^{1}H-NMR spectra were performed for a series of OHFL/DM-β-CD mixtures, obtained dissolving OHFL and DM-β-CD in CD_3OD:D_2O, 50:50, and maintaining the total concentration of guest and host molecules constant (1 mM), but varying the molar ratio fraction of each component from 0 to 1. Each solution was done at 30 °C. H2′ proton chemical shifts changes between free and complexed OHFL were obtained and the stoichiometry was determined plotting $\Delta\delta_{obs}$·[OHFL] vs. χ_{OHFL}, where $\Delta\delta_{obs}$ is the chemical shift difference, [OHFL] is the concentration of OHFL and χ_{OHFL} is the mole fraction of OHFL. The

equilibrium between the complex and its components is given by Eq. 1

$$\text{OHFL} + n(\text{DM-}\beta\text{-CD}) \Leftrightarrow \text{OHFL}/n\text{CD} \quad (1)$$

n is given by Eq. 2

$$\chi_{\text{Max.}} = (n+1)^{-1} \quad (2)$$

Association constant

Solutions of OHFL and DM-β-CD were obtained with a constant concentration (1 mM) of OHFL and incremental concentrations (1–10 mM) of DM-β-CD in CD_3OD:D_2O, 40:60. With the chemical shifts differences ($\Delta\delta_{\text{obs.}}$) between the free and complexed OHFL, it was possible to obtain the association constants for each enantiomer through the non-linear procedure in agreement with Eq. 3 [19, 20].

$$\Delta\delta_{obs.} = \frac{\Delta\delta_{\max}}{2\cdot[FL]}\left[\left([FL]+[CD]+\left(\frac{1}{Ka}\right)\right) - \left[\left([FL]+[CD]+\left(\frac{1}{Ka}\right)\right)^2 - 4[FL][CD]\right]^{1/2}\right] \quad (3)$$

where $\Delta\delta_{\text{obs}}$ is the observed chemical shift difference between the free and complexed guest, $\Delta\delta_{\max}$ is the difference in chemical shift between the free and completely complexed guest, and [FL] and [CD] are the OHFL and DM-β-CD concentrations, respectively. The non-linear procedure is an iterative method which has to be started with approximated initial parameters of $\Delta\delta_{\max}$ and Ka. In order to obtain these initial parameters, the Eq. 4 which corresponds to the Benesi-Hildebrand method for a 1:1 stoichiometry [21, 22], was employed to obtain Ka and $\Delta\delta_{\max}$ averages.

$$\frac{1}{\Delta\delta_{obs.}} = \frac{1}{K_a\cdot[CD]_T\cdot\Delta\delta_{\max}} + \frac{1}{\Delta\delta_{\max}} \quad (4)$$

The plot of $[CD]^{-1}$ vs. $\Delta\delta_{\text{obs}}^{-1}$ gives approximate values of Ka and $\Delta\delta_{\max}$ which are used to initiate the iterative process of the non-linear method.

Inclusion thermodynamics

Thermodynamic parameters were obtained for each enantiomer, determining the association constants at different temperatures and using the Van't Hoff Equation (Eq. 5) [6, 23].

$$\ln(Ka) = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (5)$$

ΔH and ΔS are the enthalpy and entropy changes, and R the gas constant (8.314 J/K mol). Free energy (ΔG) was obtained at 298 K in agreement with Eq. 6

$$\Delta G = \Delta H - T\Delta S \quad (6)$$

The relation $\Delta G = RT \cdot \text{Ln}(Ka)$ was also employed to calculate the ΔG values due to its better accuracy, however the results were the same as using Eq. 6.

ROESY experiment

A solution of OHFL (3 mM) and DM-β-CD (3 mM) was obtained in CD_3OD:D_2O, 40:60, at 30 °C.

Molecular Modeling

DM-β-CD was built with the Builder module of the InsightII software (Insight II, MSI, San Diego, California) and optimized as previously described [24]. The OHFL enantiomers were built with the Gaussview software of the Gaussian 98 package [25] and optimized at the BP86 [26, 27] level of theory with the Resolution of the identity method for the approximation of the two-electron integrals [28], as implemented in the TURBOMOLE 5.9 program [28]. Quasi relativistic Stuttgart effective core potentials (ECP) was used for carbon and oxygen atoms. Double-zeta basis set was used for all atoms, with a d polarization function for carbon and oxygen and a p polarization function for hydrogen [29].

Autodock 3.05 [30] with Lamarkian genetic algorithm (LGA) was used for the docking with very stringent parameters: a maximal number of evaluations of 15,000,000 and a maximal number of generations of 150,000. Values for elitism, mutation rate and crossover rate of 1, 0.2 and 0.08, respectively, were used. The best solutions obtained with these parameters were further refined by a local search algorithm such as pseudo Solis and Wets (PSW).

For the docking procedure, 200 runs were carried out for each OHFL enantiomer with DM-β-CD. As there were no significant differences between the solutions in both cases (see the Results section), comparison with the experimental data was directly carried out with the whole ensemble of structures for each OHFL enantiomer. Since the Autodock results correctly reproduced the experimental data, no further optimizations were carried out.

Results and discussion

In order to characterize the OHFL and DM-β-CD molecules, ^{1}H-NMR were acquired, and their chemical shifts are shown in Table 1.

OHFL spectrum shows signals on the aliphatic zone for H2, H3a and H3b protons whose chemical shifts are 5.5, 2.9 and 3.2 ppm, respectively. OHFL aromatic protons are between 6.8 and 7.9 ppm. The ^{1}H chemical shift range of

Table 1 Chemical shifts displacements (ppm) for OHFL and DM-β-CD in D_2O:CD_3OD 60:40

	H2	H3a	H3b	H5	H6	H7	H8	H2′	H3′
OHFL	5.51	2.89	3.20	7.84	7.12	7.62	7.05	7.38	6.81
	H1	H3	H5	H6	H2-OMe	H6-OMe			
DM-β-CD	5.04	3.79	3.69	3.60	3.46	3.28			

cyclodextrins is narrow (3.2–5.1 ppm), and consequently the signals do not overlap the aromatic protons of OHFL which were used for obtaining the stoichiometry and association constants. Cyclodextrins have a characteristic NMR pattern which does not depend on cyclodextrin concentration, and where H3 and H5 internal protons have strategic positions to report host/guest interactions. It has been widely described the truncated-cone shape of cyclodextrins and the location of these protons, H3 near to the wider rim of the cyclodextrin, H6 near to the narrower side of the cyclodextrin, and H5 in between them. In addition to the location of these protons, their chemical shifts are well known and their changes indicate the inner inclusion of the guest into the cyclodextrin cavity [31]. Therefore, in order to confirm the inclusion, chemical shifts changes of the H3, H5, H6 internal protons of the DM-β-CD were analyzed (Fig. 2).

When the complexes are formed, H3, H5, and H6 protons of DM-β-CD have an upfield chemical shift displacement due to the anisotropic effect of the aromatic fraction of the OHFL [32]. This anisotropic effect is due to

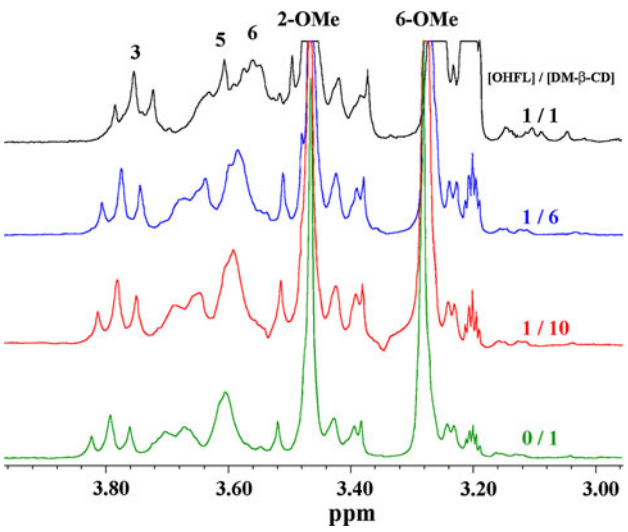

Fig. 2 DM-β-CD ^{1}H-NMR spectra extension at constant OHFL concentration (1 mM), and variable DM-β-CD concentrations (1, 6, 10 mM). Complexes formed at 25 °C

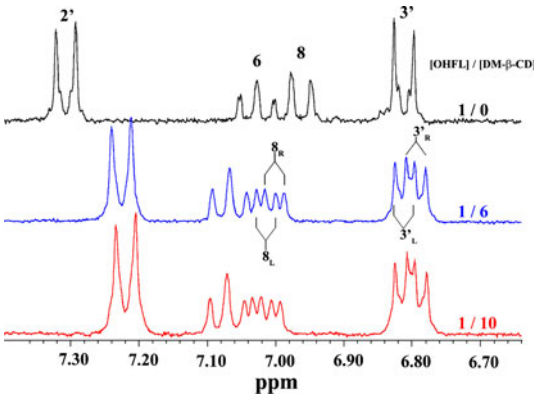

Fig. 3 ^{1}H-NMR spectra at constant OHFL concentration (1 mM) and variable DM-β-CD concentration (0, 6, 10 mM). *L* and *R* subscripts are left and right respectively. Complexes formed at 25 °C

the included aromatic rings rich in π electrons, indicating that OHFL is included into the cyclodextrin cavity. Likewise, signals of H8 and H3′ protons of OHFL split in the presence of DM-β-CD in the ^{1}H-NMR spectrum indicating enantiomeric differentiation, having signals at the left ($H8_L$, $H3'_L$) and at the right ($H8_R$, $H3'_R$) sides referring to the spectrum (Fig. 3). The observed enantiomeric differentiations on these protons allowed us to estimate the association constants for both enantiomers. In the spectra, we observed that protons H3′ and H2′, for both enantiomers, have an upfield displacement, whereas H8 and H6, for both enantiomers, have a downfield displacement.

Since the H8 protons have the largest chemical shifts changes for each enantiomer, these protons will be used for determining their association constants. Relative to the spectrum, the left and right side signals will be labeled $H8_L$ and $H8_R$, respectively. Accordingly, the $H8_L$ signal enantiomer will be named left enantiomer, as well as the $H8_R$ signal enantiomer be named right enantiomer.

Inclusion stoichiometry

To evaluate the stoichiometry for both complexes, Job's plot was performed. Chemical shift differences between the free and complexed OHFL were observed for proton H2′. The plot of $\Delta\delta_{obs}\cdot[\text{OHFL}]$ vs. χ_{OHFL} is shown in Fig. 4.

The maximum value of the parabolic curve in the X coordinate gives the stoichiometry. A maximum value of 0.5 for χ_{OHFL} was found, meaning that the stoichiometry of the mixture of diastereoisomeric complexes is 1:1 (Eq. 2). Likewise, $\Delta\delta_{obs}^{-1}$ vs. $[\text{DM-}\beta\text{-CD}]^{-1}$ plots (data not shown) for each enantiomer are in agreement with the Job's plots results, supporting the 1:1 inclusion stoichiometry of each enantiomer.

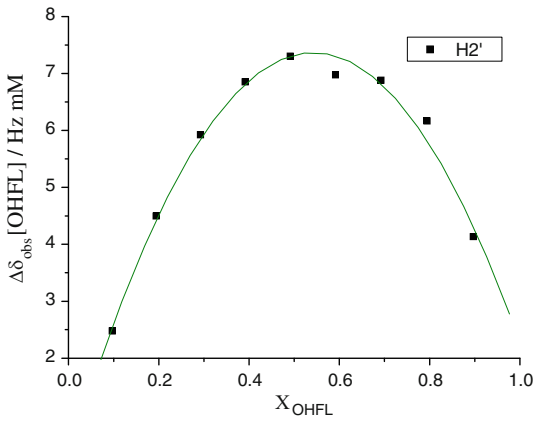

Fig. 4 Job's plot for OHFL/DM-β-CD diastereoisomeric complexes

Association constants

Benesi-Hildebrand is the most common method for determining Ka, and it needs to obey the requirement that one of the complex components concentration has to be larger than the other component, with a minimum of $\geq$10 [19, 33]. The experimental conditions of this work do not accomplish the requirement above described, and to avoid a large excess of one of the components, the non-linear procedure was employed in this study.

The non-linear procedure is an iterative method which needs to be started with approximated initial parameters of $\Delta\delta_{max}$ and Ka. In order to obtain these initial parameters, Benesi-Hildebrand (Eq. 4) was employed obtaining a Ka average of 500 M^{-1} and a $\Delta\delta_{max}$ average of 25 Hz. These values were used to begin the iterative process. Plots of [DM-β-CD] vs. $\Delta\delta_{obs.}$ were performed (Fig. 5), and the non-linear method was used to obtain the association constants of each diastereoisomeric complex at three different temperatures, as shown in Table 2.

Table 2 shows that $\Delta\delta_{max}$ values do not display significant changes with temperature. This might be due to temperature values being too similar to reflect variations between these values. This means that there are no significant changes in the guest environment at the used temperatures. The association constants can also be seen to decrease with increasing temperature. The right enantiomer has the largest Ka when compared with the left enantiomer at each temperature, which means that the right enantiomer is more favored than the left enantiomer at the used conditions.

It has been described [34] that complexes with association constant ratios larger than 1.05 are possible to be separated through HPLC. Considering this evidence, enantiomeric separation through HPLC should be possible even with the low values shown in Table 2. Likewise, Ka^{H8_R}/Ka^{H8_L} ratio values indicate that the enantiomeric differentiation is better at lower temperatures, which also means that the enantiomeric separation through HPLC should be better as well.

Inclusion Thermodynamics

According to the association constants tendency with temperature, the inclusion process has an exothermic behavior which is reflected in the thermodynamic parameters obtained using the Van't Hoff equation, as is shown in Table 3 and Fig. 6.

The ΔH values for each complex are negative, indicating that formation of host/guest inclusion complexes is exothermic. The negative value of standard Gibbs energy change, given by enthalpy and entropy changes, shows the spontaneous formation of host/guest inclusion complexes. It has been reported that Van der Waals and hydrophobic interactions constitute the major driving forces for cyclodextrin complexation, whereas electrostatic interactions and hydrogen bonding can significantly affect the conformation of a particular inclusion complex [35]. According to the thermodynamic values, there is a pronounced enthalpy–entropy compensation for both enantiomers, the ΔH values being larger than the $\Delta S \cdot T$ values for both complexes which means that Van der Waals interactions are important on the complexation process, but ΔS is not negligible and

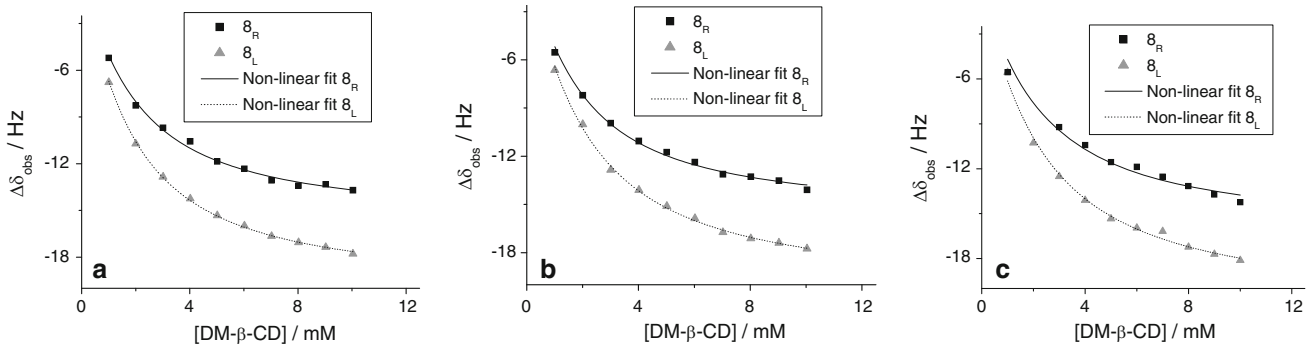

Fig. 5 Non-linear procedure for $H8_L$ and $H8_R$ protons at **a** 25 °C, **b** 30 °C, and **c** 35 °C

Table 2 Association constants, $\Delta\delta_{max}$ values, and association constant ratio between the right and left enantiomers (Ka^{H8_R}/Ka^{H8_L}) obtained at different temperatures

Temperature (°C)	$H8_L$		$H8_R$		Ka^{H8_R}/Ka^{H8_L}
	Ka (M^{-1})	$\Delta\delta_{max}$ (Hz)	Ka (M^{-1})	$\Delta\delta_{max}$ (Hz)	
25	8.02×10^2	−20.66	8.53×10^2	−16.13	1.064
30	6.60×10^2	−20.97	6.99×10^2	−16.32	1.059
35	5.42×10^2	−20.83	5.71×10^2	−16.32	1.054
Average		−20.82		−16.26	
SD		0.16		0.11	

Table 3 Thermodynamic parameters for diastereoisomeric complexes formed by OHFL and DM-β-CD

Thermodynamic parameter	Left enantiomer $H8_L$	Right enantiomer $H8_R$
ΔH (kJ mol^{-1})	−30.0 ± 0.3	−30.7 ± 0.5
ΔS (J (mol K)$^{-1}$)	−44.8 ± 1.0	−46.7 ± 1.6
$\Delta S{\cdot}T$ (kJ mol^{-1})	−13.4 ± 0.3	−13.9 ± 0.5
ΔG (kJ mol^{-1})	−16.6 ± 1.0	−16.8 ± 1.0

The used temperature to obtain $\Delta S{\cdot}T$ and ΔG is 298 K

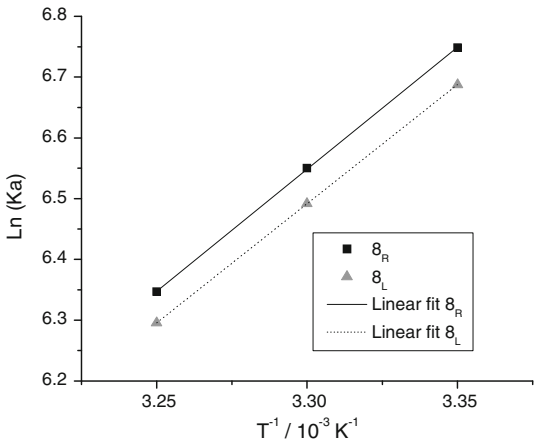

Fig. 6 Van't Hoff plot for diastereoisomeric complexes formed by OHFL and DM-β-CD. For both linear fits R = 0.999

then hydrophobic interactions could be important as well. On the other hand, Table 3 shows that both enantiomers have the same kind of exothermic behavior having similar values for the three thermodynamic parameters. This is in agreement with the association constant values, which are similar between both diastereoisomeric complexes.

Inclusion geometry

In order to obtain the inclusion geometry, ROESY experiments were performed and analyzed qualitatively. In agreement with the 1D spectra, H3, H5 and H6 cyclodextrin protons interact with the OHFL mixture due to their chemical shift displacement. Figure 7 shows a partial contour plot of 2D-ROESY spectra of the inclusion complex. There are several intermolecular cross-peaks between H5 and H2′ OHFL protons with H3 and H5 of DM-β-CD. We also observe, with a minor intensity, dipolar interactions of the H2′ OHFL proton with H6 and H6OMe DM-β-CD protons. To explain the interactions of the H2′ OHFL proton with the four cyclodextrin protons, each enantiomer should have different inclusion geometries. On the other hand, the

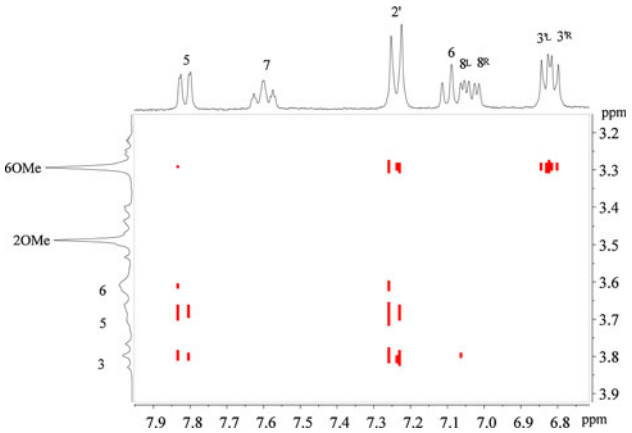

Fig. 7 ROESY spectrum for diastereoisomeric complexes formed by OHFL racemic mixture and DM-β-CD

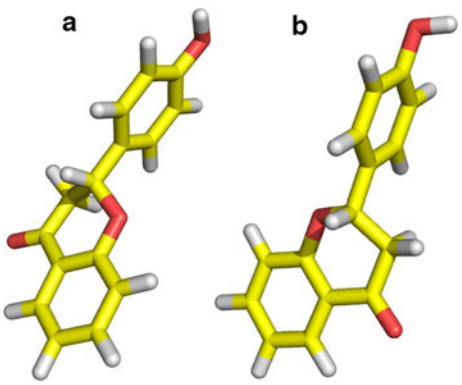

Fig. 8 Optimized geometries of flavanones. **a** and **b** are the R and S enantiomers, respectively

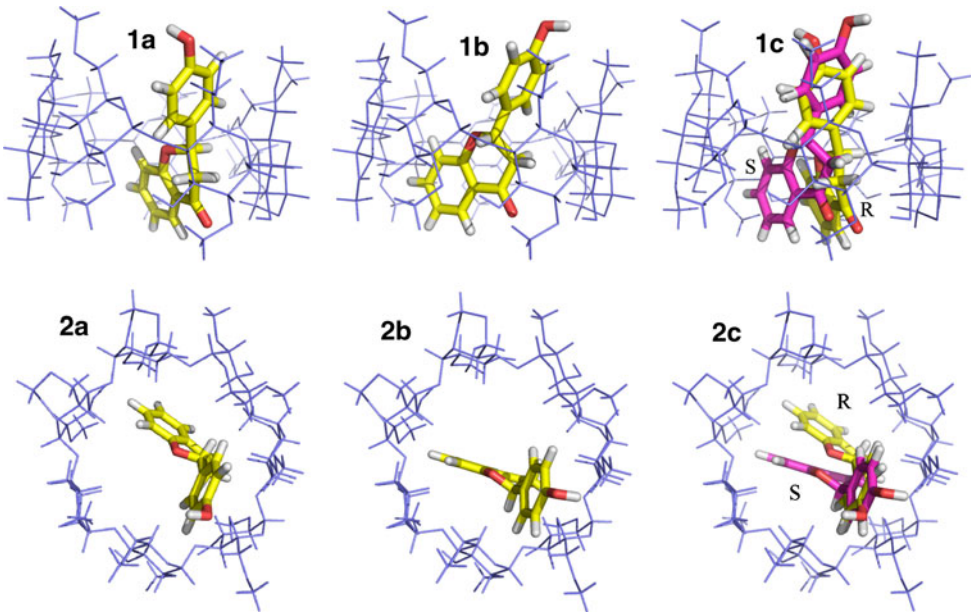

Fig. 9 Docking results for complexes formed by OHFL enantiomers and DM-β-CD. Structures labeled as **1** and **2** are the side and top views of the complexes, respectively. Structures labeled as **a** and **b** are the complexes formed by the R, and S enantiomers, respectively. Structures labeled as **c** correspond to the R and S enantiomers comparison

H5 OHFL proton correlates with H3 and H5 DM-β-CD protons which suggests that the inclusion geometries could be similar between them, because these DM-β-CD protons are next to the wide side of the DM-β-CD. In addition, H3′ OHFL proton has cross peaks that are in agreement with a subtle difference between enantiomers, since this proton shows interaction only with the H6OMe DM-β-CD protons. This means that H3′ OHFL protons have to be near to the H6OMe DM-β-CD protons, indicating that H3′ OHFL protons have to be by the narrow side of the cyclodextrin.

In order to rationalize these results, docking studies were performed on optimized geometries of flavanones and cyclodextrin. The ab initio optimized geometries of the flavanones are shown on Fig. 8.

The docking studies revealed that a preferred final relative orientation for both diastereoisomeric complexes occur in spite of the different initial configurations arbitrarily imposed. It is interesting to note that although no fixed distances were imposed during the docking calculations, the results are in good agreement with the results obtained by the ROESY spectrum. In both complexes (Fig. 9) the OHFL is inserted into the DM-β-CD cavity with the B ring inserted into the narrow side, and the A ring inserted into the wide side of the DM-β-CD. However, the complexes present subtle differences. Referring to the H6OMe DM-β-CD protons, the entire OHFL S enantiomer is closer to these protons than the OHFL R enantiomer. On the other hand, the S enantiomer is more reclined than the R enantiomer. According to these results, these geometric differences should be consequence of different interactions for each enantiomer, which implies different magnetic environments and therfore, the enantiomeric differentiation in the 1D-NMR spectra.

In addition, the docking calculations revealed that no hydrogen bonds are present; therefore the complexes interactions should mostly be Van der Waals and hydrophobic.

Conclusions

This work demonstrates that OHFL enantiomers can be studied as a racemic mixture when DM-β-CD is used. Stoichiometry, association constants, thermodynamic parameters and inclusion geometry were obtained. The result showed a 1:1 stoichiometry for both enantiomers, and the thermodynamic parameters revealed that the enthalpy energy would be the main contribution to the inclusion interaction for both enantiomers.

On the other hand, the ratio between the association constants would be in agreement with a possible separation through HPLC.

The docking results showed similar results to the experimental data, which allowed to rationalize the experimental results, and obtained accurate tridimensional geometries.

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