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Interactions between glycyl-*L*-phenylalanine and β-cyclodextrin from diffusion, spectroscopic and computational studies

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ABSTRACT

The transport and association of the dipeptide glycyl-*L*-phenylalanine (Gly-L-Phen) in solutions containing cyclodextrins are of prime importance given its relevance in different areas, from biochemistry to wastewater treatment. We have investigated the interaction between aqueous Gly-*L*-Phen and β-cyclodextrin (β-CD) by NMR, DFT calculations in parallel with UV/visible absorption, luminescence spectral measurements and diffusion experiments. Ternary mutual diffusion coefficients of aqueous {Gly-L-Phen + β-CD} solutions have been measured by using the Taylor dispersion technique. The results show that the association of Gly-L-Phen and β-CD has a significant effect on the diffusion coefficients, consistent with an association constant of about 50 to 100, assuming a 1:1 (Gly-L-Phen):(β-CD) stoichiometry. This stoichiometry has been assessed by the continuous variation (Job's plot) method and the association constant was determined by using the Hildebrand-Benesi equation modified for NMR applications. The association constant was estimated to be equal to 40. This association constant is in close agreement with that obtained by fluorescence measurements ($K = 43$). Additionally, from NMR, detailed structural information on the complex has been obtained. This has been complemented by DFT and TD-DFT studies.

1. Introduction

Glycyl-*L*-phenylalanine (Gly-L-Phen) is a dipeptide formed from glycine and L-phenylalanine (see Scheme 1a), of relevance for different fields. For example, glycyl-*L*-phenylalanine has been used for the synthesis of biocompatible and non-toxic metal organic frameworks (MOFs), by forming coordination polymers with metal ions (e.g., Zn (II)), with applications in the biomedical and pharmaceutical fields [1]. The ability of dipeptides to complex metal ions is also used to better understand the hydrolysis processes of proteins [2] and the removal of metal ions from wastewaters [3].

Peptides can also be used as substrates for drug encapsulation and release, acting as effective anchors between the carrier and the drug [4], which can be cleaved by enzymes, thus increasing the concentration of released drug in the target [5,6]. As a consequence of the hydrolysis of dietary proteins, knowledge of the transmembrane transport of peptides becomes relevant [7]. Gly-L-Phen has been used, for example, as a model peptide for the study of tracheal permeation [8], highlighting the role of peptides on drug deliver via the respiratory tract. Another approach relates the importance of membrane transport in analytical chemistry. Muthiac et al. [9] have reported that transport through liquid membranes can be used as an efficient tool for the recognition/separation of peptides. They have also highlighted the importance of crown ethers, and their complexation with peptides, for an efficient separation process [10].

The formation of peptides-containing supramolecular complexes has also attracted the attention of researchers. These complexes can be applied in various areas as, for example, sensing [11] and catalysis [12]. The interaction of Gly-L-Phen with different macrocycles has been reported. H.J. Buschmann et al. [13] have shown that the interaction between hexasodium *p*-sulfonatocalix[4]arene and Gly-L-Phen leads to the formation of a 1:1 complex with association *K* constant equal to 1698. They also reported an increase in the size of the macrocycle ring for hexasodium *p*-sulfonatocalix[6]arene leads to an association constant of 1905. This relatively small change in *K* suggests that the interaction of the phenyl group with the macrocyle ring does not play the main role on the complexation process. This is supported by the fact the complexation is enthalpy-driven [13]. The complexation

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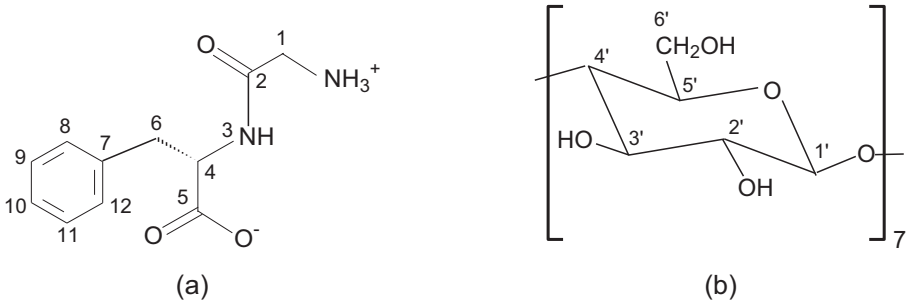

Scheme 1. Structures of the Gly-L-Phen (a) and the β-CD (b).

of Gly-L-Phen with other guests, such as cucurbit[6]uril and α-cyclodextrin [14,15], has also been reported [16]. The experiments involving the cucurbit[6]uril were carried out in aqueous solutions of formic acid (50 v/v %), leading to a complex formation with association constant 1122, also an enthalpy-driven process. However, no association constant was computed for (α-CD)/(Gly-L-Phen), for which only small enthalpy changes were measured by isothermal titration calorimetry. This might be related to the very weak association of the peptide and the cyclodextrin consistent with the very small association constant ($K = 10.9$ [17]) for α-CD and phenol.

Cyclodextrins are cyclic oligosaccharide composed of six, seven or eight α-glucopyranose units, for α-, β- or γ-CD, respectively (the most common CDs), forming a torus-shaped structure. As a consequence of its spatial arrangement, the cavity is relatively hydrophobic while the external surface is hydrophilic. Because of the nonpolar character of the cavity compared with the polar exterior, CDs can form inclusion complexes with a wide variety of guest molecules, predominantly due to hydrophobic interactions [18]. The ability to promote water solubilization of poorly soluble compounds is of particular interest in the development of pharmaceutical formulations, promoting interest in the investigation of CDs and their interaction with biologically relevant compounds. Moreover, the low toxicity of CDs and their increasingly economic availability makes these host compounds particularly amenable for drug protection and delivery [19–21].

Recently, we carried out a comprehensive study of the diffusion of l-3,4-dihydroxyphenylalanine (L-dopa) in aqueous solutions containing β-cyclodextrin as a carrier [22]. Specifically, we measured ternary mutual diffusion coefficients for coupled transport in aqueous solutions of L-dopa + β-CD, using the Taylor dispersion technique, complemented with structural studies using ^{1}H NMR spectroscopy. In this paper we extend that study by addressing two different issues: the transport by mutual diffusion of Gly-L-Phen and the effect of presence of β-CD (see Scheme 1b) in the transport, thermodynamic and structural properties of the complex. This will be discussed on the basis of intermolecular diffusion coefficients, NMR and fluorescence spectroscopies and DFT analysis.

2. Materials and methods

2.1. Reagents

Glycyl-*L*-phenylalanine (Gly-L-Phen) (*pa*, ≥99%) and β-cyclodextrin (β-CD) (>97% and a water mass fraction of 13.1%) were purchased from Sigma-Aldrich, Germany. Aqueous solutions were prepared using Millipore-Q water (specific resistance = 1.82×10^5 Ω m, at 298.15 K) and D_2O (Sigma-Aldrich) for NMR spectroscopy. All solutions were freshly prepared at 298.15 K (±0.01 K) before each experiment.

2.2. Diffusion measurements

The Taylor method for measuring diffusion coefficients used in the present study is based on monitoring the dispersion of solution samples injected into laminar carrier streams of slightly different composition flowing through long capillary tubes. Taylor dispersion experiments are described in detail in the literature [23–29]. Only a brief summary of the equipment and procedure is given here.

At the start of each run, a 6-port Teflon injection valve (Rheodyne, model 5020) was used to introduce 0.063 cm^3 of solution into a laminar carrier stream of slightly different composition. A flow rate of 0.23 cm^{3-} min^{-1} was maintained by a metering pump (Gilson model Minipuls 3) to give retention times of about 1.1×10^4 s. The dispersion tube (length 3279.9 (±0.1) cm, internal radius 0.03222 (±0.00003) cm) and the injection valve were kept at 298.15 K (±0.01 K) in an air thermostat. Dispersion of the injected samples was monitored by a differential refractometer (Waters model 2410) at the outlet of the dispersion tube. Detector voltages were measured at 5 s intervals with a digital voltmeter (Agilent 34401 A).

2.2.1. Binary diffusion

Mutual diffusion in aqueous Gly-L-Phen solutions is described by the Fick equation.

$$J(\text{Gly}-\text{L}-\text{Phen}) = -D\nabla C \tag{1}$$

D, J and ∇C are the binary diffusion coefficient, the molar flux and the gradient in the concentration of Gly-L-Phen. To measure D by the Taylor technique, samples of solution containing Gly-L-Phen at concentration $C + \Delta C$ were injected into laminar carrier streams of composition C.

Binary diffusion coefficients D were evaluated by fitting the dispersion equation

$$V(t) = V_0 + V_1 t + V_{\max}(t_R/t)^{1/2} \exp\left(-\frac{12D(t-t_R)^2}{r^2 t}\right) \tag{2}$$

to the measured detector voltages, $V(t)$. V_0 is the baseline voltage, V_1 is the baseline slope, $V_{\max}$ is the peak height, and t_R is the mean sample retention time.

2.2.2. Ternary diffusion

Mutual diffusion in aqueous {Gly-L-Phen (1) + β-CD (2)} solutions is described by the coupled Fick equations [26]

$$J_1(\text{Gly}-\text{L}-\text{Phen}) = -D_{11}\nabla C_1 - D_{12}\nabla C_2 \tag{3}$$

$$J_2((\beta-\text{CD}) = -\text{D}_{12}\nabla C_1 - \text{D}_{22}\nabla C_2 \tag{4}$$

with ternary mutual diffusion coefficients D_{11}, D_{12}, D_{21} and D_{22}. Main diffusion coefficients D_{11} and D_{22} give the flux of each solute (Gly-L-Phen (1) or β-CD (2)) produced by its own concentration gradient. Cross-diffusion coefficients D_{12} and D_{21} give, respectively, the flux of Gly-L-Phen caused by the β-CD concentration gradient (∇C_2) and the flux of β-CD caused by the Gly-L-Phen concentration gradient (∇C_1).

Ternary dispersion profiles were prepared by injecting {Gly-L-Phen (1) + β-CD (2)} solution samples of composition $C_1 + \Delta C_1$, $C_2 + \Delta C_2$

into carrier streams of composition C_1, C_2. The ternary D_{ik} coefficients were evaluated by fitting the ternary dispersion equation [28,30].

$$V(t) = V_0 + V_1 t + V_{max}(t_R/t)^{1/2}\left[W_1 \exp\left(-\frac{12D_1(t-t_R)^2}{r^2 t}\right) + (1-W_1)\exp\left(-\frac{12D_2(t-t_R)^2}{r^2 t}\right)\right] \quad (5)$$

D_1 and D_2 are the refractive index profiles. W_1 and $(1 - W_{1)}$ are the normalized pre-exponential factors.

2.3. NMR experiments

Solutions of Gly-L-Phen and β-CD were prepared in D_2O (mass fraction purity 0.999, Aldrich) and the pH* values quoted are the direct pH-meter readings after standardization with aqueous (H_2O) buffers [31]. The ^1H NMR spectra were obtained on a Bruker Avance III HD 500 MHz NMR spectrometer (at 499.843). The methyl signal of *tert*-butyl alcohol was used as internal reference (δ 1.3) relative to TMS.

2.4. Fluorescence spectroscopy

Absorption and steady-state fluorescence measurements were recorded in a Cary 5000 UV–Vis-NIR and Horiba-Jobin-Yvon Fluoromax 4 spectrometers, respectively. The fluorescence emission spectra of the inclusion complexes were collected in similar experimental conditions.

2.5. Computational details

The intial structures for Gly-L-Phen and β-CD were obtained from the Cambridge Crystallographic Data Centre, CCDC [32]. These structures were then optimized at the density functional theory (DFT) level with the B3LYP functional [33–35] (Becke's three-parameter exchange functional [33] and the Lee, Yang, and Parr correlation functional [34]). The standard 3-21G(d) basis set was used in these calculations. Two intial models of the inclusion complex (Gly-L-Phen):(β-CD) were used. The models were built by placing the guest molecule at the centre of the β-CD cavity oriented along the perpendicular to the plane defined by the glycosidic oxygen atoms. In one of the models, the phenyl group of the guest molecule points towards the primary hydroxyl groups of β-CD (model 1); in the second model, the phenyl group of the guest molecule points towards the secondary hydroxyl groups of β-CD (model 2). These two models were then optimized at the DFT B3LYP/3-21G (d) level of theory. All the calculations were carried out taking into account the bulk solvent (water) effects through the polarizable continuum model (PCM), using the integral equation formalism variant (IEFPCM) [36,37] The harmonic vibrational frequencies were calculated at the same theory level for all the optimized geometries to confirm that the stationary points found in the potential energy surface are true minima. All the calculations were performed with the GAUSSIAN 16 program [38].

3. Results and discussion

3.1. Binary diffusion coefficient of aqueous Gly-L-Phen solutions

The binary diffusion coefficients measured for aqueous solutions of Gly-L-Phen at 298.15 K and concentrations from (0.0010 to 0.0100) mol dm^{-3} are summarized in Table 1. The concentration dependence of the binary diffusion coefficient of Gly-L-Phen shows a linear relationship (Eq. (6)), as expected for dilute nonelectrolyte solutions. The limiting diffusion coefficient D^0 at zero concentration is 7.66×10^{-10} m^2 s^{-1}.

$$D/\left(10^{-9}\ m^2\ s^{-1}\right) = 0.766 - 1.126C/\left(mol\ dm^{-3}\right) \quad (6)$$

Table 1
Binary diffusion coefficients of Gly-L-Phen (1) in aqueous systems.

Flow solution C_1/(mol dm^{-3})	$(D \pm S_D)/(10^{-9}\ m^2\ s^{-1})$
0.0000	0.766[a]
0.0010	0.765 ± 0.003
0.0025	0.763 ± 0.004
0.0050	0.760 ± 0.003
0.0075	0.757 ± 0.002
0.0100	0.755 ± 0.004

[a] Extrapolated limiting value.

Additionally, it can be observed that, taking into account the standard deviation, diffusion coefficients do not change with the concentration (a variation of ca. 1.3% for the whole concentration range). Diffusion measurements at higher concentrations were not made because of the low solubility of β-CD (0.016 mol.dm^{-3} at 298.15 K).

Table 2 gives the diffusion coefficients for β-CD in aqueous solutions at 298.15 K [39] measured for the same Taylor equipment. From the data showing in Tables 1 and 2, it can be that Gly-L-Phen molecules diffuse more rapidly than the larger and less-mobile β-CD molecules.

3.2. Ternary diffusion coefficients of aqueous {Gly-L-Phen (1) + β-CD (2)} solutions

The interaction of Gly-L-Phen and β-CD was investigated by measuring ternary mutual diffusion coefficients (D_{11}, D_{12}, D_{21} and D_{22}) for aqueous {Gly-L-Phen (1) + β-CD (2)} solutions (see Table 3).

The reproducibility of the main and cross-diffusion coefficients are: ± (0.02×10^{-9}) m^2 s^{-1} and ± (0.04×10^{-9}) m^2 s^{-1}, respectively. The values of D_{22} are smaller than the D_{11} values, indicating the β-CD molecules diffuse more slowly than the smaller and mobile Gly-L-Phen molecules. Also, D_{11} (D_{22}) increases (decreases) with increasing solute fraction of Gly-L-Phen, defined as $X_1 = C_1/(C_1 + C_2)$. Cross-coefficient D_{12} is negative, indicating that β-CD (2) concentration gradients produce counter-current coupled flows of Gly-L-Phen (1). In the limit $X_1 \to 0$, however, D_{12} is zero because β-CD concentration gradient cannot drive coupled flows of Gly-L-Phen in solutions that do not contain Gly-L-Phen. The values of D_{21}, the other cross coefficient are significantly smaller and generally indistinguishable from zero within the precision of the measurements.

The ratio D_{12}/D_{22} gives the number of moles of Gly-L-Phen counter-transported per mole of β-CD. The measured diffusion coefficients show that a mole of diffusing β-CD counter transports at most 0.33 mol of Gly-L-Phen. The values of D_{21}/D_{11} indicate that a mole of diffusing Gly-L-Phen co-transports up to 0.02 mol β-CD.

It is interesting to consider the effects of complex formation [40–44].

$$Gly-L-Phen\ (A) + \beta-CD\ (B) \rightleftarrows (Gly-L-Phen) : (\beta-CD)\ (AB) \quad (7)$$

on coupled diffusion in {Gly-L-Phen + β-CD} solutions. Using C_A for the concentration of free Gly-L-Phen molecules, C_B for the concentration of

Table 2
Binary diffusion coefficients of β-CD (2) in aqueous systems [39].

Flow solution C_2/(mol dm^{-3})	$(D \pm S_D)/(10^{-9}\ m^2\ s^{-1})$
0.0000	0.326[a]
0.0020	0.324 ± 0.003
0.0040	0.323 ± 0.001
0.0060	0.321 ± 0.003
0.0080	0.318 ± 0.002

[a] Extrapolated limiting value.

Table 3
Ternary mutual diffusion coefficients of aqueous {Gly-L-Phen (1) + β-CD (2)} solutions at 298.15 K[a,b].

C_1	C_2	X_1	D_{11}	D_{12}	D_{21}	D_{22}
0.000	0.0100	0.00	0.589 ± 0.001	−0.045 ± 0.015	0.036 ± 0.001	0.460 ± 0.002
0.0025	0.0075	0.25	0.680 ± 0.009	−0.136 ± 0.027	0.002 ± 0.003	0.499 ± 0.004
0.0050	0.0050	0.50	0.678 ± 0.011	−0.180 ± 0.017	0.012 ± 0.006	0.543 ± 0.007
0.0075	0.0025	0.75	0.736 ± 0.024	−0.156 ± 0.020	0.004 ± 0.009	0.540 ± 0.004
0.0100	0.0000	1.00	0.745 ± 0.008	−0.122 ± 0.039	0.013 ± 0.004	0.530 ± 0.016

[a] C_1 and C_2 in units of (mol dm^{-3}).
[b] ($D_{ik} \pm S_D$) in units of (10^{-9} m^2 s^{-1}).

free β-CD molecules, and C_{AB} for the concentration of the (Gly-L-Phen):(β-CD) complex, the equilibrium constant K for the A + B ⇄ AB association reaction is

$$K = \frac{C_{AB}/C_{AB,s}}{(C_A/C_{A,s})(C_B/C_{B,s})} \tag{8}$$

assuming activity coefficients are equal to unit and $C_{AB,s}$, $C_{A,s}$ and $C_{B,s}$ are the corresponding standard concentrations (1 mol dm^{-3}).

Previous work [38] has shown the ternary mutual diffusion coefficients for dilute solutions of nonelectrolyte components that associate to form a 1:1 complex are given by

$$D_{11} = D_A\left(\frac{\partial C_A}{\partial C_1}\right)_{C_2} + D_{AB}\left(\frac{\partial C_{AB}}{\partial C_1}\right)_{C_2} = \frac{C_A(C_B + C_{AB})D_A + C_B C_{AB} D_{AB}}{C_A C_B + (C_A + C_{AB})C_{AB}} \tag{9}$$

$$D_{12} = D_A\left(\frac{\partial C_A}{\partial C_2}\right)_{C_1} + D_{AB}\left(\frac{\partial C_{AB}}{\partial C_2}\right)_{C_1} = \frac{C_A C_{AB}}{C_A(C_B + C_{AB}) + C_B C_{AB}}(D_{AB} - D_A) \tag{10}$$

$$D_{21} = D_B\left(\frac{\partial C_A}{\partial C_1}\right)_{C_2} + D_{AB}\left(\frac{\partial C_{AB}}{\partial C_1}\right)_{C_2} = \frac{C_A C_{AB}}{C_A(C_B + C_{AB}) + C_B C_{AB}}(D_{AB} - D_B) \tag{11}$$

$$D_{22} = D_B\left(\frac{\partial C_B}{\partial C_2}\right)_{C_1} + D_{AB}\left(\frac{\partial C_{AB}}{\partial C_2}\right)_{C_1} = \frac{C_B(C_A + C_{AB})D_B + C_A C_{AB} D_{AB}}{C_A C_B + (C_A + C_{AB})C_{AB}} \tag{12}$$

D_A and D_B are the diffusion coefficients of free A and free B molecules. D_{AB} is the diffusion coefficient of the AB complex.

Eqs. (9) to (12) are helpful for understanding transport in {Gly-L-Phen + β-CD} solutions because they relate ternary mutual diffusion D_{ik} coefficients measured for the total Gly-L-Phen and β-CD components to the diffusion coefficients of the actual diffusing species: free Gly-L-Phen molecules (A), free β-CD molecules (B), and the (Gly-L-Phen):(β-CD) complex (AB) (Table 4).

The diffusion coefficients of free Gly-L-Phen and free β-CD molecules can be assigned the values $D_A = 0.745 \times 10^{-9}$ m^2 s^{-1} (from D_{11} at $X_1 = 1$) and $D_B = 0.460 \times 10^{-9}$ m^2 s^{-1} (from D_{22} at $X_1 = 0$), respectively, taken from Table 2. The diffusion coefficient of the (Gly-L-Phen):(β-CD) complex ($D_{AB} = 0.428 \times 10^{-9}$ m^2 s^{-1}) is estimated by using the Stokes-Einstein approximation (Eq. (13)) that the diffusion coefficient of a solution species is inversely proportional to its effective radius and therefore inversely proportional to the cube root of its molecular volume [45], which gives

$$D_{AB} = \left(D_A^{-3} + D_B^{-3}\right)^{-1/3} \tag{13}$$

Fig. 1 shows a representative graph of the predicted values when compared with experimental data, for different equilibrium constants (K = 50, 100, 200 and 500).

From Fig. 1, we can conclude that the D_{11} coefficients predicted by Eq. (9) are in reasonably good agreement (to within 7% or better) with the measured D_{11} values for equilibrium constants from about 50 to 100. This result suggests that the interaction between Gly-L-Phen and β-CD is weak, in agreement with values obtained for similar guests [22,46,47].

Eq. (9) shows that ternary mutual diffusion coefficient D_{11} for the Gly-L-Phen component is an average of diffusion coefficient D_A for free Gly-L-Phen molecules and diffusion coefficient D_{AB} for the (Gly-L-Phen):(β-CD) complex, weighted in proportion to $\partial C_A/\partial C_1$ and $\partial C_{AB}/\partial C_1$, respectively. For the diffusion of trace amounts of Gly-L-Phen in β-CD solutions, however, the expression for D_{11} in Eq. (9) reduces to the simple concentration-weighted average of the diffusion coefficients D_A and D_{AB}.

$$^{tracer}D_{11}(X_1 = 0) = \frac{C_A D_A + C_{AB} D_{AB}}{C_A + C_B} = \frac{D_A + KC_2 D_{AB}}{1 + KC_2} \tag{14}$$

In the limit $X_1 \to 0$, where $C_B \to C_2$, equilibrium of the association reaction (Eq. (8)) gives $C_{AB}/C_A = KC_2$ for the relative amounts of

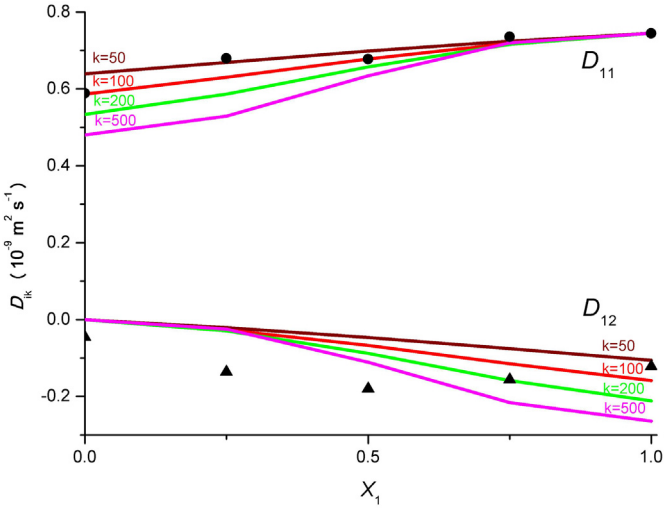

Fig. 1. Ternary mutual diffusion coefficients of aqueous {Gly-L-Phen (1) + β-CD (2)} solutions at 298.15 K plotted against the solute fraction of Gly-L-Phen, X_1. D_{ik} values predicted by Eqs. (9) and (10) for different values of association constant K (Eq. (8)), ————. Measured values: ● (D_{11}); ▲ (D_{12}).

Table 4
Diffusion coefficients for solution species, $D_{species}$.

	$D_{species}/(10^{-9}$ m^2 s$^{-1})$
Gly-L-Phen (D_A)	0.745[a]
β-CD (D_B)	0.460[a]
(Gly-L-Phen):(β-CD) (D_{AB})	0.428

[a] These values are the estimations of the diffusion coefficients of free Gly-L-Phen and β-CD, respectively, obtained from D_{11} at $X_1 = 1$ and D_{22} at $X_1 = 0$.

complexed and free Gly-LPhen molecules. As shown in Eq. (14), this raises the interesting possibility of calculating the equilibrium constant for the association of Gly-L-Phen and β-CD from tracer diffusion measurements. For example, using the estimates of D_A and D_{AB} in Table 2 together with the Gly-L-Phen tracer diffusion coefficient $D_{11}(X_1 = 0) = 0.589 \times 10^{-9}\ m^2\ s^{-1}$ measured at β-CD concentration $C_2 = 0.0100$ mol dm^{-3}, Eq. (14) gives $K = 97$ for equilibrium constant K.

β-CD forms the complex (Gly-L-Phen):(β-CD) with Gly-L-Phen molecules and therefore acts as a carrier for the peptide. As a result, diffusing β-CD might be expected to co-transport Gly-L-Phen. But cross-coefficient D_{12} is negative, indicating the coupled flux $-D_{12}\nabla C_2$ of the Gly-L-Phen component driven by the β-CD concentration gradient is counter-current to the flux $-D_{22}\nabla C_2$ of the β-CD component. To understand this counterintuitive behavior, it is helpful to consider transport of the solution species produced by the gradient ∇C_2 in the concentration of the β-CD component, holding the concentration of the Gly-L-Phen component constant along the diffusion path ($\nabla C_1 = 0$). Under these conditions, because $C_1 = C_A + C_{AB}$ is fixed, the β-CD concentration gradient produces concentration gradients in free and complexed Gly-L-Phen of equal magnitude but opposite sign: $\nabla C_A = -\nabla C_{AB}$. This means the flux $-D_{AB}\nabla c_{AB}$ of complexed Gly-L-Phen molecules diffusing "down" the β-CD concentration gradient is accompanied by the flux $-D_A\nabla c_A$ of free Gly-L-Phen molecules diffusing "up" the β-CD concentration gradient. Because the diffusion coefficient D_A of the free Gly-L-Phen molecules is larger than the diffusion coefficient D_{AB} of the larger and less mobile (Gly-L-Phen):(β-CD) complex, the counter-current flux of free Gly-L-Phen "up" the β-CD concentration gradient is larger than the co-current flux of complexed Gly-L-Phen "down" the β-CD concentration gradient, leading to a net counter-current coupled flow of Gly-L-Phen ($D_{12} < 0$).

The proposed mechanism for the coupled diffusion of Gly-L-Phen is supported by the fact the values of D_{12} predicted by Eq. (10) are proportional to ($D_{AB} - D_A$). Similarly, cross-coefficient D_{21} for the coupled flow of β-CD driven by Gly-L-Phen concentration gradients is proportional to ($D_{AB} - D_{B)}$ (see Eq. (11)). The free β-CD molecules and the (Gly-L-Phen):(β-CD) complex are both relatively large and have similar diffusion coefficients (see Table 3). Consequently, the predicted D_{21} values are significantly smaller than the predicted D_{12} values, in qualitative agreement with the experimental results.

3.3. Spectroscopic characterization of aqueous (Gly-L-Phen)/(β-CD) solutions

Further information on the structure and host-guest interactions between Gly-L-Phen and β-CD is given by ^{1}H NMR spectroscopy and fluorescence measurements.

Fig. 2 shows the ^{1}H NMR spectra for solutions of β-CD, Gly-L-Phen and the mixture of these components, respectively. Scheme 1 shows the structures and the numbering of Gly-L-Phen and β-CD, while the complete ^{1}H spectral parameters are shown in Tables S1 and S2 (in Supplementary information).

The ^{1}H NMR spectra of β-CD in the presence of Gly-L-Phen (10 mmol dm^{-3}: 10 mmol dm^{-3}, pH 6.3) display significant shifts in the signals for H-3′ and H-5′ towards lower frequencies, accompanied by the appearance, at lower frequencies, of a new signal for H-6′. The signals for the other protons show negligible chemical shift or linewidth changes. This supports the presence of some interaction between the two species. Conformational changes in β-CD in the presence of Gly-L-Phen are expected to be minor, and this is supported by the observation of only slight alterations of the coupling constants. The observation of a single signal for protons H-1′ to H-5′, for both the free and bound species Gly-L-Phen and β-CD, confirms the small conformational changes

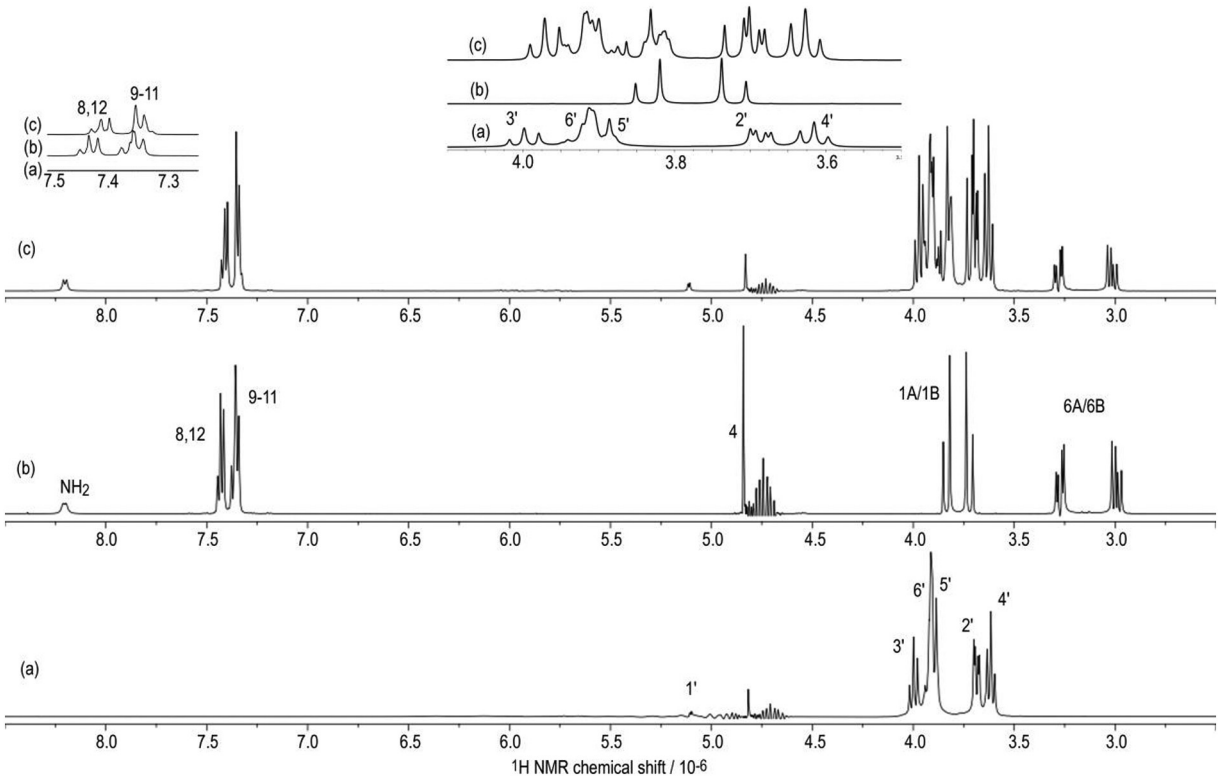

Fig. 2. ^{1}H NMR spectra of solutions in H_2O/D_2O of (a) β-CD 10 mmol dm^{-3}, pH* 6.5 [30]; (b) Gly-L-Phen 10 mmol dm^{-3}, pH* 6.2 [30]; (c) (Gly-L-Phen)/(β-CD) 10: 10 mmol dm^{-3}, pH* 6.3 [30]; insets containing expansions: from 3.40 to 4.10 for better visualization of signals for H-2′, H-3′, H-4′, H-5′ and H-6′ protons of β-CD alone and in the presence of Gly-L-Phen, and from 7.25 to 7.50 ppm for better visualization of signals for H-8, H-9, H-10, H-11 and H-12 protons of Gly-L-Phen alone and in the presence of β-CD at 298.15 K.

of these positions for cyclodextrin (β-CD). For the H-6′ protons, the observation of a new signal ($\delta = 3.81$ ppm) can indicate, specifically for this position, a slower rate, in the NMR time scale, of the dynamic processes in the bound peptide to β-CD (complex), suggesting a higher rigidity of the region.

Marked changes are also observed for the ^{1}H NMR signals of the phenyl group of Gly-L-Phen in the presence of β-CD. Although displaying slight shifts to lower frequencies, a visible narrowing of the profile of the signals is observed, particularly for signals of protons H-9, H-10 and H-11. The signals for the other protons show negligible chemical shift and linewidth changes and no significant modifications are seen for the coupling constants. The shifts observed for the H-3′ and H-5′ signals inside the β-CD cavity as well as the observation of a new signal for the H-6′ protons, together with the corresponding changes for the phenyl protons of Gly-L-Phen in the ^{1}H NMR spectra of mixtures of Gly-L-Phen and β-CD, support the formation of a complex between Gly-L-Phen and β-CD, and by analogy with previous studies [22,47] points to the inclusion of the phenyl moiety of Gly-L-Phen into the hydrophobic cavity of β-CD. Additional support comes from the NOE cross peaks detected (highlighted by the arrow) in the bi-dimensional spectrum NOESY (Fig. 3). From the 2D-NOESY, after which through-space connectivities can be traced out, we have the indications that the distances between protons H-3′ and H-5′ of β-CD and the protons of the phenyl moiety H-9, H-10 and H-11 of Gly-L-Phen are less than 5 Å [48].

The stoichiometry of host-guest complex was determined by the continuous variation (Job's plot) method [49]. It involves the preparation of a series of solutions containing both the host (Gly-L-Phen) and the guest (β-CD) in varying proportions so that a complete range of mole ratios is sampled ($0 < C_1 / (C_1 + C_2) < 1$), where C_1 and C_2 are the total concentrations of Gly-L-Phen and β-CD, respectively, and where the total concentration ($C_1 + C_2$) is kept constant for each solution. The observed parameter is the chemical shift for H-3′ of β-CD, which is sensitive to complex formation (Fig. S1 - Supplementary information). The plot of $\Delta\nu$ (H-3′) × C_2 against the mole fraction of β-CD (X_2) is shown in Fig. 4. The plot shows a maximum value at $X_2 = 0.5$ and a highly symmetrical shape, which demonstrates the existence of (Gly-L-Phen):(β-CD) complex with a 1:1 stoichiometry.

To obtain information on the extent of the interaction, ^{1}H NMR spectra were obtained of solutions containing 2 mmol dm^{-3} β-CD with Gly-L-Phen concentrations ranging from (0.5 to 5) mmol dm^{-3} (Fig. S2 - Supplementary information). From the chemical shifts of H-3′ of β-CD, it is possible to measure $\Delta\nu = \nu - \nu^0$, the difference between the frequency observed for H-3′ for each of the solutions (Gly-L-Phen)/(β-CD) and the solution of β-CD alone. From this, the association constant for complexation of Gly-L-Phen with β-CD was estimated following the method of Sompornpisut et al. [46], through the relationship of $C_1/\Delta\nu$ as function ($C_1 + C_2$). A value of 40 was estimated for association constant K.

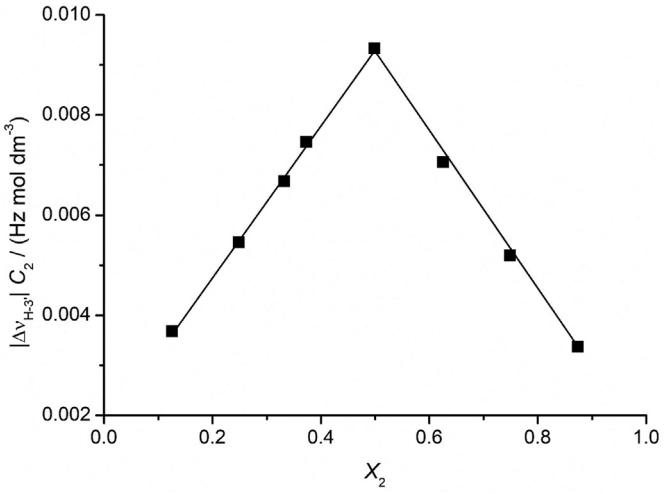

Fig. 4. Job's plot (continuous variation method) of (Gly-L-Phen):(β-CD) inclusion complex showing 1:1 stoichiometry ($\Delta\nu = \nu$(complex) - ν(free)).

The formation of the inclusion complex between Gly-L-Phen and β-CD was further investigated through steady-state fluorescence measurements. Fig. 5A shows the fluorescence emission spectra of a 0.5 mmol dm^{-3} aqueous solution of Gly-L-Phen collected with excitation at 255 nm in the absence and presence of β-CD. In the absence of β-CD only the characteristic emission spectra of Gly-L-Phen is observed with maxima at 290 nm. While with the addition of β-CD a new emission band appears at higher wavelengths and an overall increase in fluorescence intensity was observed, see

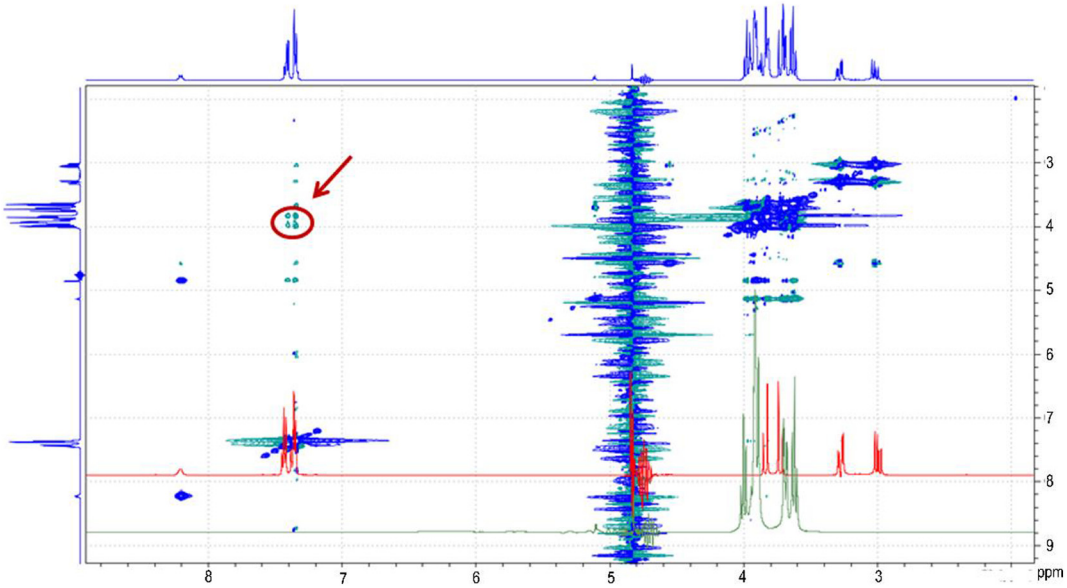

Fig. 3. 2D-NOESY NMR spectrum of a solution in H_2O/D_2O of (Gly-L-Phen)/(β-CD) at the concentration ratio 10: 10 mmol dm^{-3}, pH* 6.3 and 298.15 K.

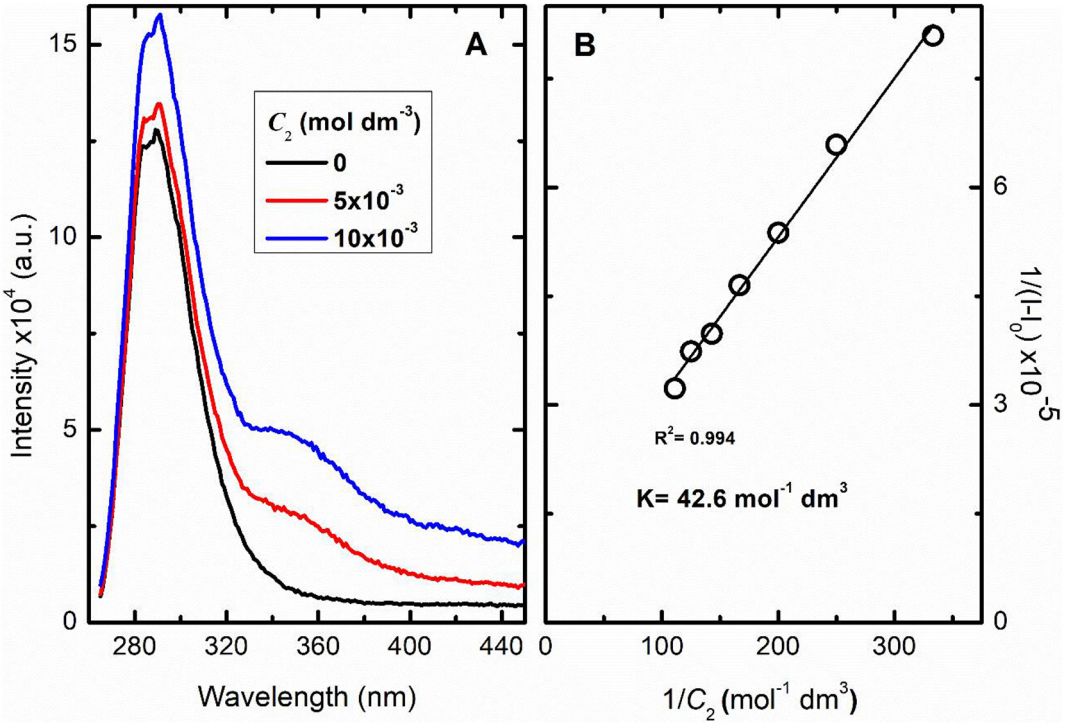

Fig. 5. A) Dependence of the fluorescence emission spectra of Gly-L-Phen on the β-CD concentration. B) Benesi-Hildebrand plot (collected at 360 nm) for the 1:1 inclusion complex formed between Gly-L-Phen and β-CD in aqueous solution at 298.15 K. For the meaning of parameters please see the text.

Fig. 5A. Indeed, cyclodextrins are known to form inclusion complexes with a wide range of guest molecules, protecting the included guest molecule from quenchers and restrict the twisting of certain molecules thus enhancing fluorescence intensity [50].

The enhancement of the fluorescence intensity of the emission band at 360 nm measured as a function of β-CD concentration was used to obtain the binding constant, *K*, for the complex by means of the Benesi-Hildebrand approach [51].

$$\frac{1}{I-I_0} = \frac{1}{I_1-I_0} + \frac{1}{K(I_1-I_0)C_2} \tag{15}$$

where C_2 is the concentration of the total β-CD component, I and I_0 are the fluorescence intensities of Gly-L-Phen in the presence and absence of β-CD, respectively, and I_1 is the expected fluorescence intensity when all the guest molecules are included in a complex.

The linear relationship with a good determination coefficient ($R^2 = 0.994$) obtained for the double reciprocal plot in Fig. 5B supports the formation of a 1:1 complex between Gly-L-Phen and β-CD. From the ratio between the intercept and the slope of the Benesi-Hildebrand plot a binding constant of ~43 was obtained. These values are in close agreement with that obtained by NMR spectroscopy and that qualitatively estimated by diffusion coefficients.

3.4. DFT and TD-DFT studies

Density functional theory (DFT) calculations were used in order to analyze the stability of the inclusion complexes formed in aqueous {Gly-L-Phen + β-CD} solutions. Based on the experimental results which suggest a 1:1 stoichiometry, two types of models for the inclusion complex were considered as starting structures. These structures were built as described in the computational details section and were then optimized at the DFT (B3LYP) level of theory. Model 1, in which the phenyl group of the guest molecule is oriented towards the primary hydroxyl groups of β-CD (Fig. 6, left), was found to be more stable than model 2 (in which the phenyl group of the guest molecule is oriented towards the secondary hydroxyl groups of β-CD) (Fig. 6, right) by 34.8 kcal mol^{-1}. In both optimized geometries, the peptide hydrophobic phenyl group is located inside the cavity and the hydrophylic groups are located outside the β-CD cavity. A significant difference between the two optimized structures is that in complex 1 the N and O atoms of the peptide are involved in an extensive hydrogen bond network with the secondary hydroxyl groups of β-CD, while in complex 2, probably due to unfavorable contacts, the only significant hydrogen bond interactions present are intramolecular (in the peptide and in β-CD).

The binding energies of the inclusion complexes, calculated from equation $E_{binding} = E_{complex} - (E_{Gly\text{-}L\text{-}Phen} + E_{\beta\text{-}CD})$ were found to be −52.58 kcal mol^{-1} for model 1 and −17.79 kcal mol^{-1} for model 2. The high stability of complex 1 is due in part to the energetic stabilization caused by the intermolecular (Gly-L-Phen)/(β-CD) hydrogen bond interactions established in this complex (Fig. 7). These results show that Gly-L-Phen and β-CD form stable complexes in aqueous solutions. In the optimized structure of complex 1, the distances between the phenyl H-9, H-10 and H-11 protons of Gly-L-Phen and the closest H-3′ and H-5′ protons of the β-CD are 2.02, 2.21, 2.73 and 2.99 Å for H-9 and H-11, and 3.60 and 4.11 Å for H-10, therefore in accordance with the observation of NOE cross peaks for these protons.

The DFT optimized structure for the (Gly-L-Phen):(β-CD) complex 1 suggests that the CH_2OH fragments in β-CD may adopt a variety of slightly different conformations, some of these induced by the interactions with the peptide inside the β-CD cavity. This is in conformity with the finding in the NMR spectra of the inclusion complex of an additional broad signal for the H-6′ CH_2 protons.

To obtain further insight on the excited state behavior of inclusion complex 1, TD-DFT calculations were carried out to obtain its vertical excitation energies, oscillator strengths and main contributions to the excited states. Table S3 (Supplementary information) summarizes the results obtained for the UV–Visible region up to 174 nm. The first

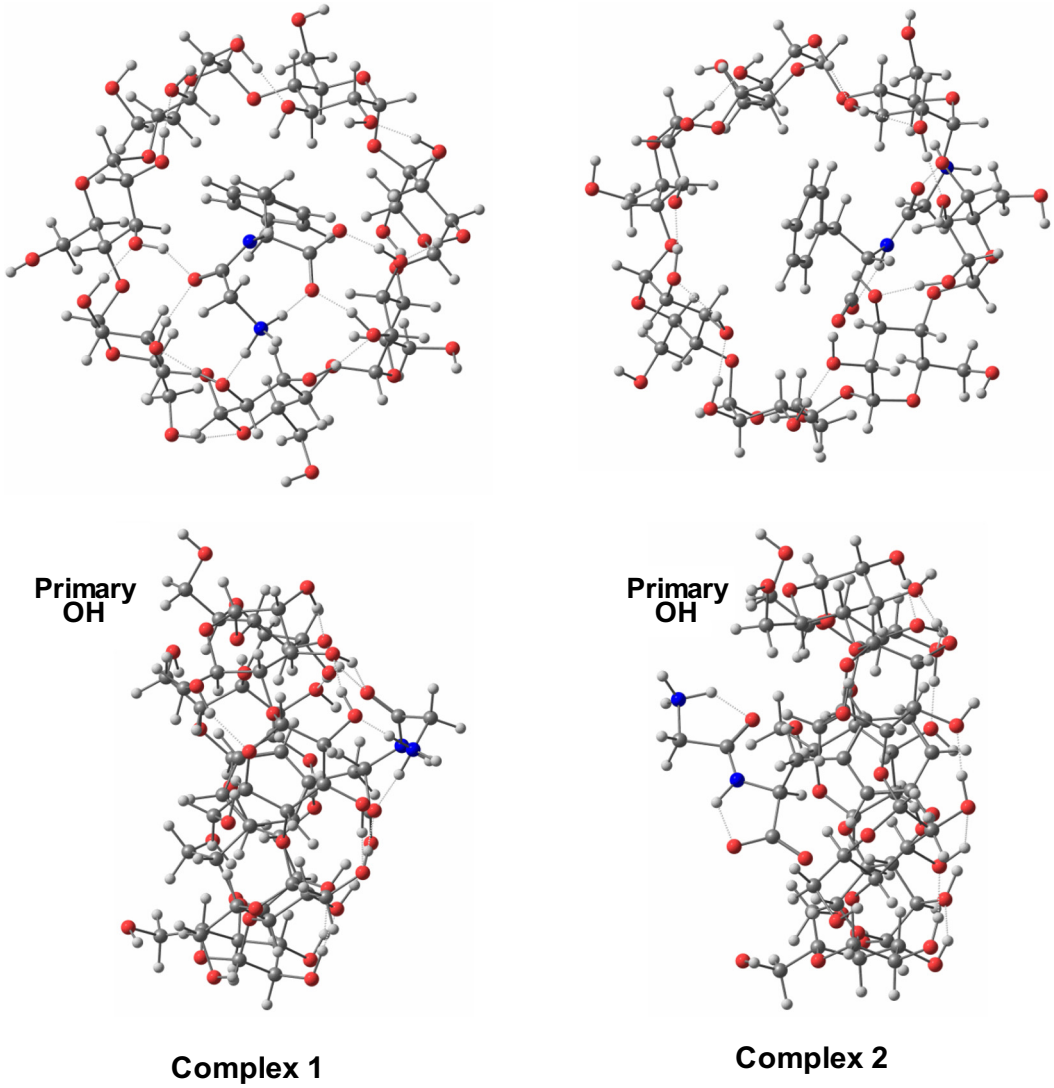

Fig. 6. DFT B3LYP/3-21G(d) optimized geometries for the inclusion complexes 1 and 2 of (Gly-L-Phen):(β-CD).

excited state, predicted at 224 nm, has a very low oscillator strength and involves contributions from a HOMO-7 to LUMO+1 excitation, together with HOMO-3 to LUMO+2 and HOMO-3 to LUMO excitations. The orbitals involved in these transitions shown in Fig. 8 reveal that the S_1 excited state of the complex involves charge transfer between different regions of the β-CD and the peptide. The TD-DFT calculations indicate that the absorption spectrum of the complex in the region up to 190 nm will show two stronger bands at 195 and 197 nm (S_7 and S_6 states, respectively), together with two weaker bands at 212 and 221 nm (S_4 and S_3 states, respectively). The two stronger bands involve transitions between the orbitals HOMO-7 and HOMO-3 to orbitals LUMO, LUMO+1 and LUMO+2. These orbitals also describe the main contributions to the S_1 excited state (Fig. 8), and we can conclude that the two bands at 195 and 197 nm also correspond to charge transfer between different regions of the β-CD and the peptide.

4. Conclusions

We have investigated the interaction between Gly-L-Phen and β-CD using diffusiometry, NMR and fluorescence spectroscopy, and DFT calculations. From Taylor diffusion technique we have found that the limiting diffusion coefficient of aqueous Gly-L-Phen is 7.66×10^{-10} m^2 s^{-1} at 298.15 K. Concerning the ternary systems, the main diffusion coefficient of Gly-L-Phen, D_{11}, increases with increasing Gly-L-Phen solute fraction. Cross-coefficient D_{12} is negative, indicating counter-current coupled flows of Gly-L-Phen. This behavior also shows that the diffusion of Gly-L-Phen is affected by the association with β-CD, consistent with an association constant lower than 100. This qualitative assessment has been confirmed by NMR and fluorescence studies, where association constants of 40 and 43 were obtained, respectively. From NMR spectroscopy, it was also concluded that the stoichiometry of interaction is 1:1. Structural analysis of the complex was achieved by NMR and DFT analysis. From NMR spectra, it can be observed a shielding of cavity protons of β-CD and the slight shielding accompanied by a visible narrowing of the profile of aromatic protons of Gly-L-Phen, which suggest the complexation between β-CD and Gly-L-Phen. Support comes from the cross peaks observed in the 2D-NOESY spectrum and also from the DFT calculations which predict the phenyl ring of Gly-L-Phen inside of the torus cavity of β-CD. The DFT optimized geometry of the complex is in accordance with the observation of NOE cross peaks for the phenyl protons, and the intermolecular peptide/β-CD hydrogen bond interactions are also relevant, as indicated by DFT, contributing to the high stability of the complex.

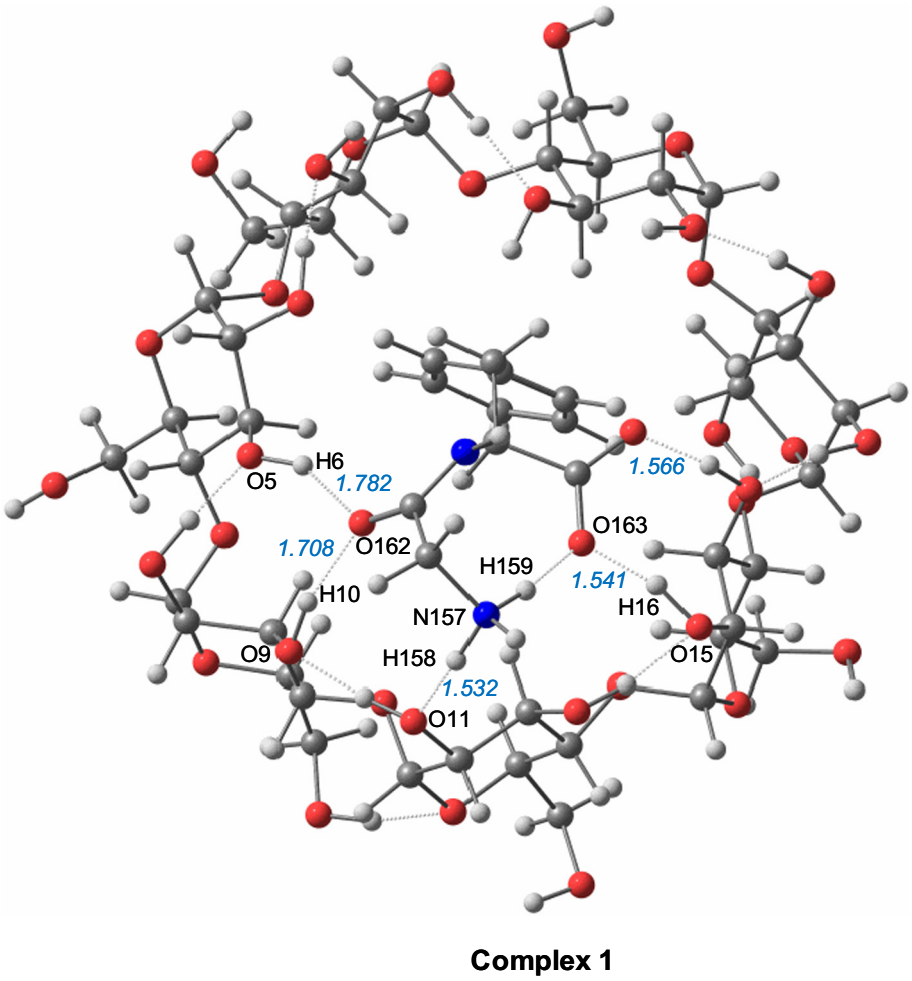

Fig. 7. Intermolecular hydrogen bond network established in complex 1 (between Gly-L-Phen and β-CD) obtained at the DFT B3LYP/3-21G(d) level.

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CRediT authorship contribution statement

M.L. Ramos: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition. **D.C. Dias:** Investigation, Resources. **L.L.G. Justino:** Software, Investigation, Resources, Data curation. **L.M.P. Verissimo:** Software, Investigation. **A.J.M. Valente:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **M.A. Esteso:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. **A.C.F. Ribeiro:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **D.G. Leaist:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Visualization. **J. Pina:** Investigation, Data curation. **A.M.T.D.P.V. Cabral:** Writing - review & editing. **M.M. Rodrigo:** Investigation, Data curation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2020.113704.

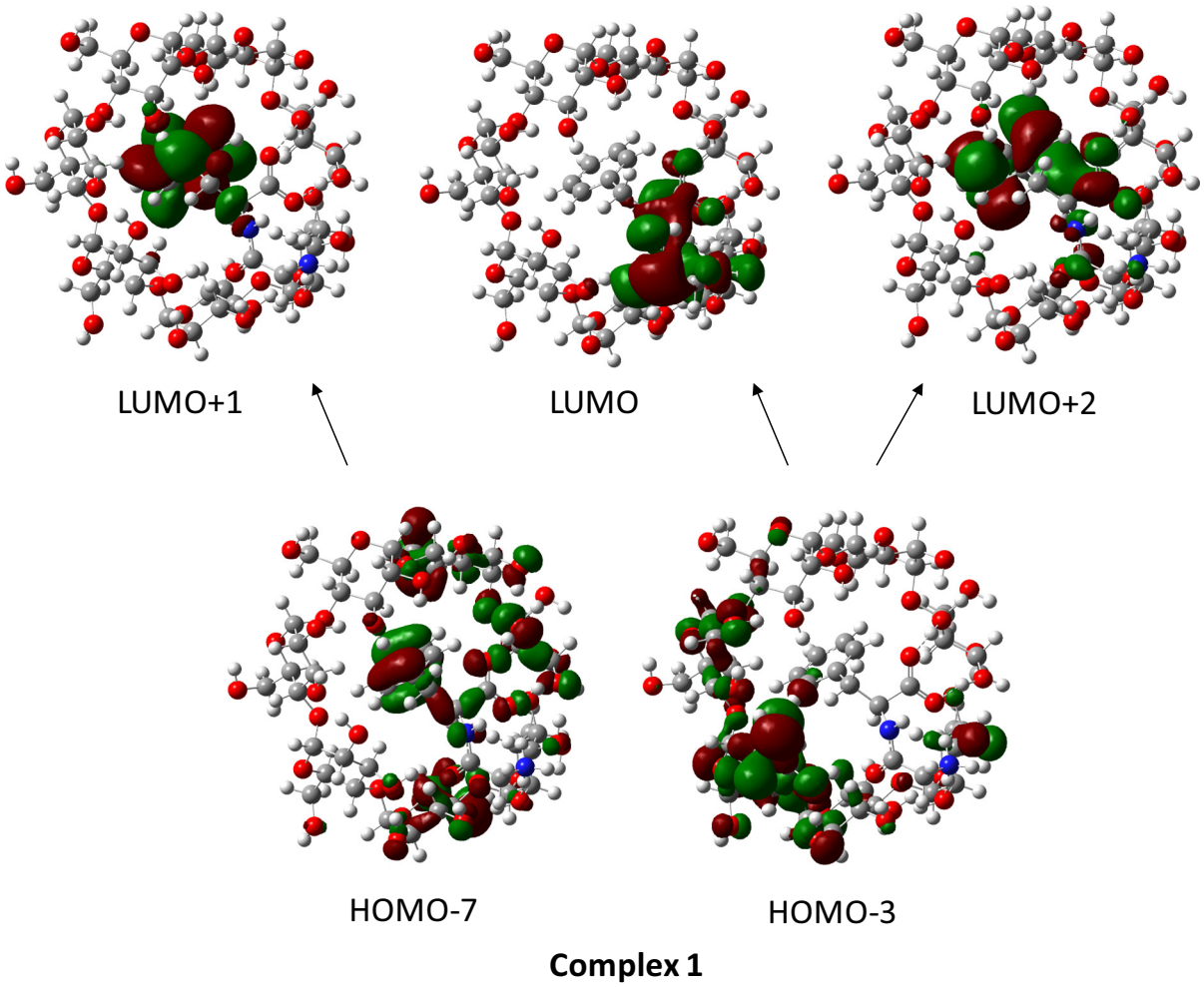

Fig. 8. Diagram showing the main transitions contributing to the S_1 excited state of the inclusion complex 1.

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