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ORIGINAL ARTICLE

Spectroscopic (fluorescence, 1D-ROESY) and theoretical studies of the thiabendazole and β-cyclodextrin inclusion complex

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Abstract The inclusion complex between the anti-helminthic drug thiabendazole (TBZ) and the β-cyclodextrin (βCD) was characterized in solution using fluorescence and ^{1}H-Nuclear Magnetic Resonance spectroscopy and studied theoretically by semi empirical PM3 and density functional theory (DFT) quantum mechanical calculations. Thermodynamic stability associated with the formation of the TBZ:βCD inclusion complex in aqueous solution was determined treating the drug's fluorescence enhancement in the presence of cyclodextrin by a non-linear model, which indicated a moderate host–guest affinity at equilibrium (K 150 ± 31 at 25 °C). Its supramolecular structure in solution was studied through the 1D-ROESY NMR experiment, which produced evidence that the guest molecular encapsulation occurs preferably via the drug's benzimidazole group. Theoretical study employing molecular optimization with the semi empirical PM3 method provided two energetic-equivalent complex structures that are in accordance with the NMR experimental evidences. Single point energy calculations with DFT at the B3LYP/6-31G (d,p) level suggest the most stable structure of the inclusion complex and further comprehension on the interactions and conformational strains involved in its formation.

Keywords Thiabendazole · β-cyclodextrin · Fluorescence · 1D-ROESY · PM3 · DFT

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Introduction

Thiabendazole (TBZ, 2-(4-thiazolyl)-1*H*-benzimidazole) is a drug derived from benzimidazole that was synthesized in 1961 at Merck Laboratories. TBZ has a wide applicability in the pharmaceutical and agricultural fields due to its anti-helminthic, fungicide, and bactericide activities [1]. TBZ is a weak alkali (pKa 5.92 ± 0.12) [2], has an orthorhombic unit cell, m.p. 297–303 °C [3], and low solubility in water (60 μg mL^{-1}) [4].

Cyclodextrins (CDs) are cyclic polysaccharides with 6(α), 7(β), or 8(γ) D-(+)-glucopyranose units linked by α-1,4 bonds, resulting in a molecule with a toroidal geometry. This structure creates two regions with different polarities: an inner hydrophobic cavity with high electron density, and a hydrophilic external surface, due to hydroxyl groups. Poorly soluble guests can interact with the CD cavity, generally by van der Waals forces, leading to the formation of inclusion complexes. CDs have wide applications in the food and pharmaceutical industry, due to its biocompatibility and easy production from starch. Besides, guests generally become protected from degradation and have their water solubility increased when associated with CDs [5].

Thermodynamic characterization of inclusion compounds with CDs can be done through guest fluorescence emission in guest-CD solutions [5]. The apparent stability constant, K, can be obtained modeling the dependence of the guest fluorescence with the molar CD concentration in solution. Both linear and non-linear models have already been developed to predict the value of K [5–9].

Structural information on guest:CD inclusion complexes can be obtained by Nuclear Magnetic Resonance (NMR) spectroscopy, employing more sophisticated experiments beyond just simple chemical shift change analysis [10].

The nOe-based ROESY (Rotating frame Overhauser Enhancement SpectroscopY) experiment can provide internuclear correlations between guest and CD protons closely apart (0.4 nm), and the rOe values will be always positive in this experiment [11]. If these measurements involve the guest and the CD protons inside its cavity, the supramolecular organization of the guest:CD inclusion complex in solution can be elucidated [10, 12, 13].

Theoretical calculations with the quantum mechanical semi empirical PM3 method have been widely exploited for investigating inclusion complexes with CDs, when more detailed information on host–guest system are required. This level of calculation is suitable when applied to molecular optimization of guest:CD inclusion geometry, providing reliable results with less expensive computing time. However, this semi empirical method fails to estimate the interaction energies accurately [14] and further comprehension on the conformation strains and intermolecular interactions have to be treated by ab initio methods. Recalculation of energies of semi empirically optimized inclusion complex structures with ab initio methods at a single point overcomes this limitation and is a general approach employed for the theoretical investigation of CDs inclusion complexes [15–17].

In this work the stability of the TBZ:βCD inclusion complex was investigated exploiting drug emission fluorescence changes in aqueous solution. Molecular structures of the complex in D_2O solution were elucidated through NMR 1D-ROESY and theoretical study with semi empirical molecular structure optimizations and calculations of ab initio energies in the SP were done for detailed comprehension of its supramolecular system.

Experimental

Materials and methods

TBZ (lot M200242) was a gift from EMS Sigma Pharma Co.(Hortolândia, SP). βCD (99.5 %) was acquired from ISP Technologies, Inc. (São Paulo, SP). Acetonitrile and ethanol (HPLC grade) were purchased from Tedia (Rio de Janeiro, RJ). Sodium chloride (99.5 %) was purchased from Chemco Ind. & Com. Ltd. (Hortolândia, SP), potassium di-hydrogenphosphate (99.5 %) was from Art Lab (Rio de Janeiro, RJ) and sodium hydroxide (99.5 %) from Casa de Química Ind. e Com. Ltd. (Diadema, SP). Deuterium oxide (99.9 % D) was purchased from Cambridge Isotope Laboratories, Inc. Deionized water was obtained from a Milli-Q equipment.

Preparation of TBZ:βCD inclusion complex

The TBZ:βCD inclusion complex was prepared by co-precipitation [4], followed by lyophilization. 2.5×10^{-4} mol of TBZ and βCD were dissolved separately in 20 mL of ethanol and deionized water, respectively. Both solutions were mixed in a round bottom flask, and the ethanol was eliminated under reduced pressure at Rotavapor Büchi 461a, leading to TBZ:βCD precipitation. The resulting suspension was kept under mild agitation for 24 h at room temperature and protected from light, until solubility equilibrium was achieved. Finally, the suspension was freezed under liquid nitrogen and lyophilized for 3 days, giving a dry powder.

Fluorescence measurements

βCD solutions were prepared at 1.0, 2.0, 3.0, 5.0, 9.0, 10.0, and 12.0 mM concentrations, with KH_2PO_4 (50 mM)/NaOH buffer solution pH 6.80 ± 0.05. For each βCD solution, 10 μL of standard TBZ solution (1.0×10^{-3} M in acetonitrile) was added resulting in 10.0 mL of TBZ:βCD solution (TBZ 1.0×10^{-6} M). The blank TBZ solution was prepared without the addition of βCD. The spectra of the TBZ solutions (in duplicate) were acquired at 25.0 ± 0.5 °C, in a Varian Cary Eclipse fluorescence spectrometer with a temperature controller, using excitation wavelength at 298 nm, 5.0 nm excitation slit, 10 nm emission slit, and 600 nm min^{-1} scan speed.

NMR spectroscopy

The sample was prepared dissolving 5 mg of TBZ:βCD complex in 700 μL of D_2O in an Eppendorf tube in an ultrasound bath, to get a saturated solution that was filtered (Millipore pore diameter of 0.45 μm) before the experiment. The spectra were acquired on the Varian INOVA 500 MHz NMR spectrometer (^{1}H 499.886 MHz), at 24.6 °C, with signals referenced to HOD resonance (δ 4.67 ppm). 1D-ROESY experiments were carried out applying the 180°–90° sel.-spinlock—FID pulse sequence, 1,500 μs mixture time and 1,024 scans. The pulses were applied separately on each of the selected TBZ ^{1}H signals.

Computational methodology

All the calculations were done using the Gaussian 2003 revision D.01 [18] software. The molecular structures of TBZ and βCD were obtained from Drug Bank Database and Protein Database (*file 1DMB2.pdb*), respectively, and each one optimized. βCD was optimized at the PM3 level, whereas for TBZ, DFT was employed with the hybrid base B3LYP/6-31G (d,p). The methodology used for determination of the lowest energy TBZ:βCD structure is detailed: first, βCD was fixed on the center of the *XY* plane and TBZ was placed along the *Z* axis. TBZ was allowed to pass entirely through the βCD cavity with all orientation, and the resulting complex structure optimization was done at

each 1 Å guest displacement, referenced by the thiazolyl C atom linked to the benzimidazole group. The calculations were carried out in vacuum, with no symmetry restriction.

Results and discussion

Determination of an apparent stability constant, *K*, by fluorescence spectroscopy

Average fluorescence spectra of TBZ obtained from the TBZ:βCD solutions (TBZ 1.0×10^{-6} M) at 25.0 ± 0.5 °C are shown in Fig. 1. The increase in βCD concentration resulted in enhancement of the drug emission band and a gradual blue of the spectra, which can be attributed to the migration of TBZ molecules toward the non-polar βCD cavity [4]. The increase on TBZ fluorescence emission can be correlated with the apparent association constant $K_{1:1}$ through the non-linear Eq. 1 [6, 19, 20], considering the TBZ:βCD 1:1 most stable molar ratio.

$$I/I_o = \left(1 + \left(\varphi_{guest:CD}/\varphi_{guest}\right) K_{1:1}[\beta CD]\right) / \left(1 + K_{1:1}[\beta CD]\right) \quad (1)$$

where I and I_o are the TBZ fluorescence emissions for each βCD solution and without βCD, respectively. φ_{guest} and $\varphi_{guest:CD}$ are the quantum yields for pure TBZ and the corresponding inclusion complex, in solution, respectively. I/I_o were plotted against (βCD) (Fig. 2), and the fit suggests TBZ:βCD 1:1 molar stoichiometry, accordingly with it was already reported [1, 4]. The apparent formation constant $K_{1:1}$(25 °C) obtained was 150 ± 31, which indicates a moderate thermodynamic stability for this complex for this aqueous solution conditions. The non-linear fit was performed using the Levenberg–Marquardt algorithm implemented on QtiPlot v. 0.9.7 software—free for Linux.

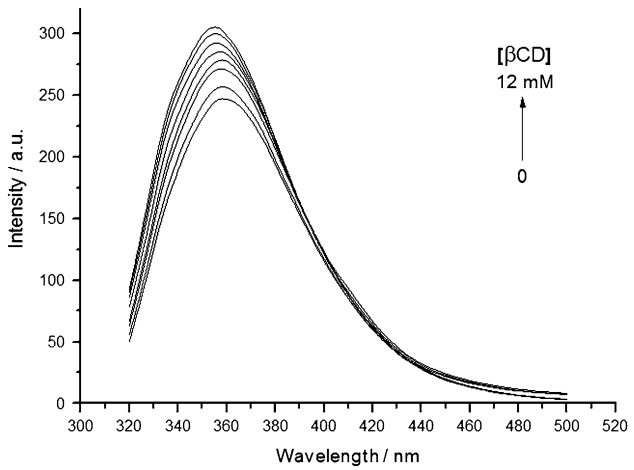

Fig. 1 Fluorescence spectra of TBZ in βCD solutions at 25.0 ± 0.5 °C

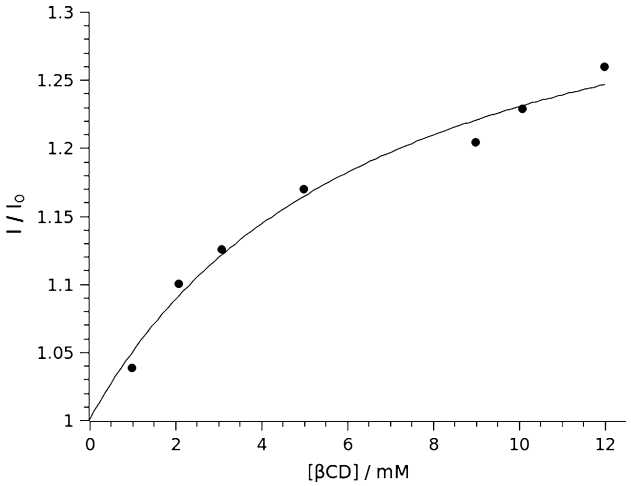

Fig. 2 Non-linear fitting and determination of the $K_{1:1}$ (25 °C) for the TBZ:βCD complex (R 0.980)

NMR spectroscopy: structure elucidation of the TBZ:βCD complex by 1D-ROESY

Since the fluorescence experiments gave a small value of $K_{1:1}$ for the complex, the 1D-ROESY experiment was selected due to its better sensitivity over the corresponding 2D experiment [11]. The 1D-ROESY spectra for TBZ protons are shown in Fig. 3a–d. Ha (δ7.302 ppm) and Hb (δ7.637 ppm) are benzimidazole group protons, while Hc (δ8.236 ppm) and Hd (δ9.076 ppm) are the protons of the thiazolyl group (Fig. 4) [1]. The βCD protons which shows internuclear correlation in the spectra are named according Fig. 4 [10].

The major rOe was observed for Ha, where internuclear correlation was detected for all βCD protons. The most intense correlation with βCD is observed for H5 and H6 (except the correlation with HOD at 4.67 ppm), and approximately the same rOe intensities were detected for other nuclei (Fig. 3a). Only a very weak inter-nuclear correlation with the H5 and H6 was observed for the others TBZ nuclei from the benzimidazole group (Hb). Therefore small interactions between this nucleus and the CD's narrow aperture were detected (Fig. 3b). The same experiment on the Hc and Hd (thiazolyl group) TBZ nuclei gave only a single rOe signal with the βCD, between Hd and H1 (Fig. 3d). This shows that no inter-nuclear correlations are detected between this part of the TBZ molecule and the cavity of βCD. These experiments suggest TBZ encapsulated through its benzimidazole group. As higher rOe signal is observed for Ha and Hb with H5 and H6 instead of H3 in their spectrum, it suggests that TBZ encapsulation must occur through the narrow βCD aperture and/or the drug molecule is located deeply inside the cavity through the large aperture (Fig. 5).

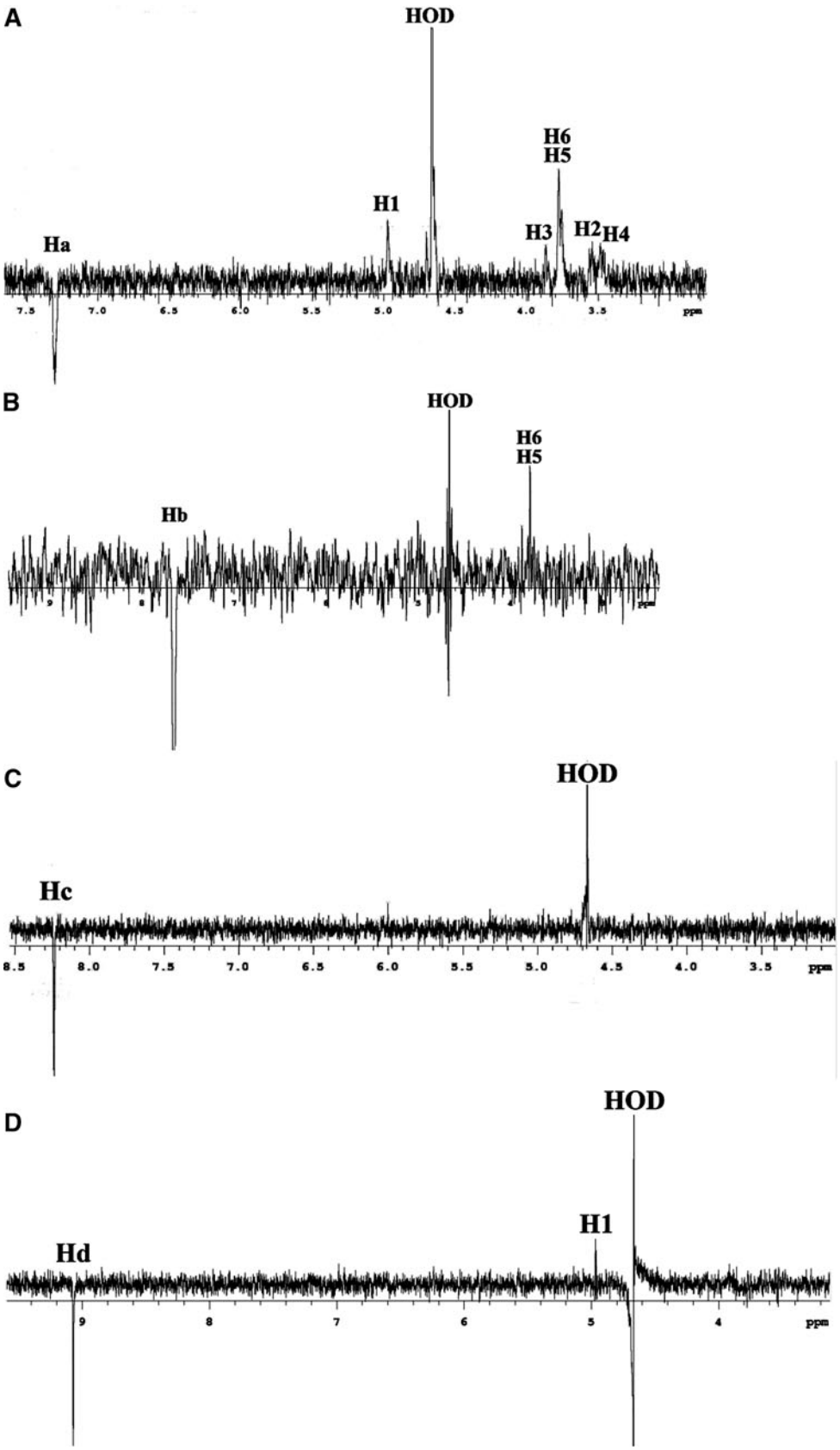

Fig. 3 1D-ROESY spectra after selective pulse of TBZ nucleus from benzimidazole (Ha, Hb) and thiazolyl (Hc, Hd) groups

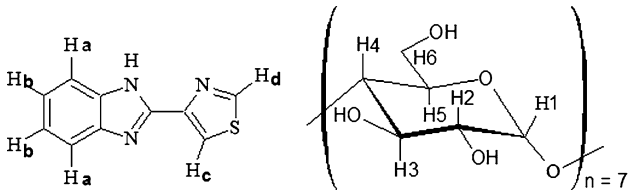

Fig. 4 NMR protons for TBZ (*left*) and βCD (*right*)

Theoretical calculations

The energies of TBZ:βCD inclusion complex calculated semi empirically, according to the TBZ position from the center of βCD, are graphed in Fig. 6. Orientation-1 corresponds to TBZ penetration through the wider aperture of βCD by the benzimidazole group, whereas in Orientation-2, encapsulation occurs by the thiazolyl group.

The minimum energy structures of TBZ:βCD complex for both drug orientations were obtained in Z 4.6 Å (Orientation-1) and Z −4.7 Å (Orientation-2). In the corresponding molecular structures, named **A** and **B,** respectively, TBZ's benzimidazole group is inserted into the CD cavity (Fig. 7), resulting in similar structures that were suggested based on the 1D-ROESY experiment. Molecular optimization also revealed a discrete energy difference (ΔE) between these TBZ:βCD optimized geometries (ΔE 1.57 kJ mol^{-1}, with E(**A**) < E(**B**)). Due to these energy values, **A** and **B** had their energies recalculated at a single-point through DFT, employing the hybrid functional B3LYP/6-31G (d,p). Binding energies of the complex (ΔE_{bind}) were calculated according Eq. 2 at both the PM3 and DFT levels, based on the energies of optimized geometries at a single-point of the complex, E(TBZ:βCD), and isolated host and guest, $E(\beta\text{CD})_{\text{free}}$ and $E(\text{TBZ})_{\text{free}}$, respectively. Interaction (ΔE_{int}) and deformation (ΔE_{def}) energies related to the formation of the complex were only calculated via DFT (Eq. 3 and 4, respectively [21]).

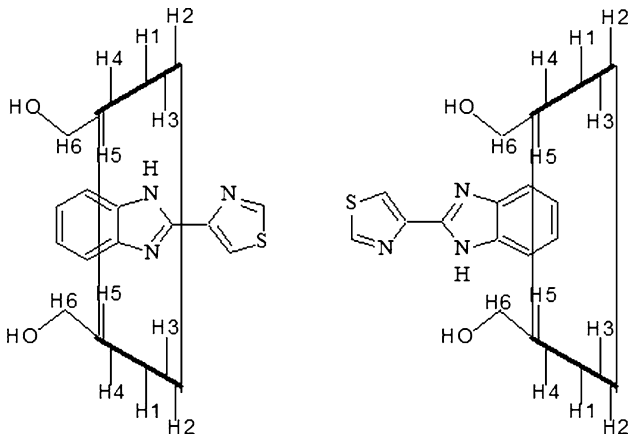

Fig. 5 Possible TBZ:βCD complex molecular structures based on the 1D-ROESY spectra

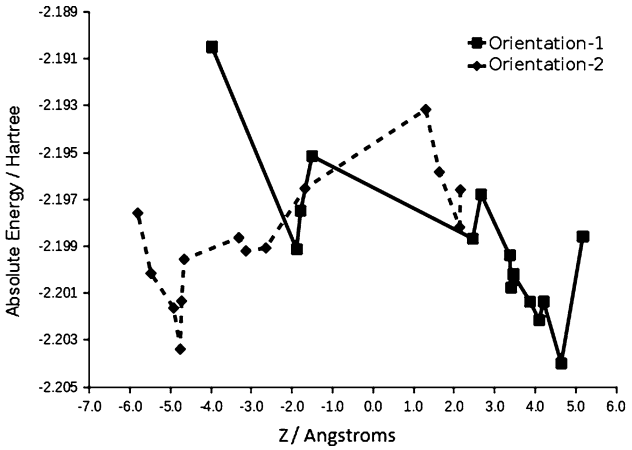

Fig. 6 Energies of TBZ:βCD complex as a function of the TBZ position from the center of the CD cavity

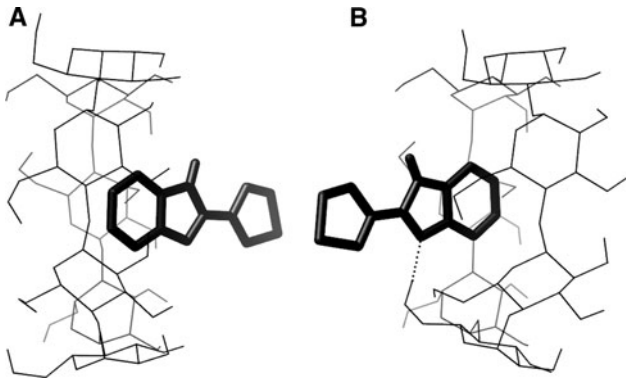

Fig. 7 Molecular structures of the most stable TBZ:βCD **A** and **B** conformations

$$\Delta E_{\text{bind}} = E(\text{TBZ} : \beta\text{CD}) - \left[E(\text{TBZ})_{\text{free}} + E(\beta\text{CD})_{\text{free}}\right] \quad (2)$$

$$\Delta E_{\text{def}}X = E(X)_{\text{complex}} - E(X)_{\text{free}} \quad (3)$$

$$\Delta E_{\text{int}} = E(\text{TBZ} : \beta\text{CD}) - \left[E(\text{TBZ})_{\text{complex}} + E(\beta\text{CD})_{\text{complex}}\right] \quad (4)$$

In Eq. 3, $\Delta E_{\text{def}}X$ is defined as the difference of the energies of each component X (TBZ or βCD) in their optimized conformations in the complex ($E(X)_{\text{complex}}$) and the isolated ($E(X)_{\text{free}}$) form. The interaction energy (Eq. 4) is calculated as the difference between E(TBZ:βCD) and the sum of each component's energy in the inclusion complex, $E(\text{TBZ})_{\text{complex}}$ and $E(\beta\text{CD})_{\text{complex}}$. All the energy results from the PM3 and DFT calculations are in Table 1.

A significant difference between the optimized **A** and **B** complex geometries were observed for the DFT calculations, wherein **B** (TBZ into the narrow βCD aperture) is the more stable complex (ΔE_{bind} (**B**) < 0) (Table 1). Since

Table 1 Formation and binding energies comparatione from PM3 and DFT calculations, deformation of host and guest and interaction energies (only by DFT), for optimized complex geometries **A** and **B**

Energy	PM3 (kJ mol^{-1})	B3LYP/6-31G (d,p)[a]
E(TBZ)	−358.70	−947.748118
E(βCD)	−6,101.49	−4,275.215389
E(TBZ:βCD) (**A/B**)	−5,786.57/−5,785.00	−5,222.94079/−5,222.970710
ΔE_{bin} (**A/B**)	673.61/675.19	59.64/−18.91
ΔE_{def}TBZ (**A/B**)	–	24.58/25.69
$\Delta E_{def}\beta$CD (**A/B**)	–	10.84/0.33
ΔE_{int} (**A/B**)	–	24.21 /−44.93

[a] In B3LYP/6-31G(d,p), E are expressed in Hartree and ΔE in kJ mol^{-1}

there is no solvent influence, the driving force for the negative ΔE_{bind} (**B**) value was studied by analyzing both ΔE_{int} (**A/B**) and ΔE_{def} (**A/B**), the last one defined as the overall deformation energies in each **A** and **B** geometry separately, ΔE_{def} (**A/B**) = ΔE_{def} TBZ (**A/B**) + ΔE_{def} βCD (**A/B**). As seen in Table 1, the DFT calculations showed a negative value for ΔE_{int} (**B**) that demonstrates, according to Song et al. [21], predominantly attractive interactions between host and guest for this complex geometry. The same was not observed in **A**, where a large repulsion forces were observed for this geometry (ΔE_{int} (**A**) > 0). The negative ΔE_{int} (**B**) can be ascribed to the hydrogen bond between the *N* atom of the TBZ benzimidazole group and OH6 from βCD ($R_{OH\cdots N}$ = 1.835 Å, see Fig. 7), that occurs exclusively in this conformation. Due to the significative difference of ΔE_{int} (**A/B**), i.e,. ΔE_{int} (**A**)−ΔE_{int} (**B**) = + 69.14 kJ mol^{-1} for B3LYP/6-31G(d,p) calculations, and the qualitative analysis purpose of optimized complexes stabilities, the basis set superposition error was not taken in account for further energies correction. **A** and **B** have positive values of ΔE_{def} for both TBZ and βCD, which shows non-favored conformation strains for host and guest in the complex. As ΔE_{def} (**B**) is less positive than ΔE_{def} (**A**) the complex formation toward conformation **B** is favoured. The DFT calculations showed that the formation of the most stable TBZ:βCD inclusion complex (**B** geometry) is favored by host–guest attraction forces that overcome the strain destabilization, while for **A** the positive values of ΔE_{bind} (**A**) and ΔE_{int} (**A**) left this conformation less stable.

The presence of polar groups on TBZ and βCD changes their hydration pattern differently on the formation of **A** or **B** complex structures, which could modify their respective stability energies. However, the solvent effect is very complex and was not considered in this study, once PM3/DFT theoretical calculation on vacuum could complement satisfactorily the aqueous 1D-ROESY results and provide interesting information about the host–guest interactions.

Conclusions

Fluorescence and NMR spectroscopies are suitable techniques for investigating the thermodynamic stability and molecular structural characterization of the TBZ:βCD inclusion complex in aqueous solution. Molecular structure optimization with the PM3 method reinforces the experimental evidence that were obtained from 1D-ROESY experiment. The treatment of this system by DFT provided a deeper understanding on the interaction and conformational strains energies involved during the inclusion complex formation.

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