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NMR investigation of the complexation and chiral discrimination of pyrazole sulfonamide derivatives with cyclodextrins

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ABSTRACT

The complexes formed between six original chiral diaryl-pyrazole sulfonamide derivatives, displaying poor solubility, and various CDs (native α-, β- and γ-CDs, hydroxypropylated HP-β-CD, methylated Me-β-CD or amino NH_2-β-CD) were studied by 1D and 2D ^{1}H NMR at physiological pH in order to determine their apparent binding constant, stoichiometry and structure of the supramolecular assembly. For some complexes, the spectra obtained for free racemic compound and for racemic compound in presence of CD indicate a splitting of signal(s). Additional experiments with pure enantiomer and enriched enantiomer allow us to attribute this behavior to chiral discrimination. The complexing ability of the native β-CD towards our compounds appears the most promising since binding values around 7×10^2 M^{-1} are obtained. The two-dimensional ROESY (^{1}H–^{1}H) experiments prove the inclusion of the aliphatic part of the compound in the CD cavity. It is noteworthy that this inclusion occurs via the smaller opening of the cavity.

1. Introduction

Cyclodextrins (CDs) are macrolytic compounds with several D-glucopyranose units linked by α-1,4-glycosidic bonds. The common α-CD, β-CD and γ-CD are composed of 6, 7 and 8 glucose units, respectively. The shape of CDs is a truncated cone with a central cavity due to the chair conformation of the glucopyranose units. Their exterior surface is hydrophilic due to the presence of hydroxyl groups whereas the central cavity is lined by skeletal carbons and ethereal oxygens of the glucose residues which gives it a relatively lipophilic character. These properties make them complexing agents of first interest since they are able to form inclusion complexes with a great variety of molecules of appropriate polarity and size (Duchêne, 1987). Their pharmaceutical applications rely mainly on their abilities to enhance the solubility, the stability and the bioavailability of drug molecules (Loftsson & Duchêne, 2007).

CDs are optically active and offer the potential discrimination of enantiomeric substances. They are widely used for the enantioseparation of drugs using high-performance liquid chromatography (Subramanian, 1994) and capillary electrophoresis (Chankvetadze, 1997). The formation of the diastereoisomeric species (between the CD and the enantiomers) is generally described as a result of the inclusion of an apolar part of the guest in the hydrophobic cavity and polar interactions with the outer hydrophilic rim of the CD. To permit enantiodiscrimination, the substituents of the stereogenic center must interact with the hydroxyl groups at the mouth of the CD or/and with the functional group of a derivatized CD (Xiao & Armstrong, 2004). Numerous functionalized CD derivatives are available to modify their complex forming ability and enantioselectivity as methylated-, hydroxypropylated-, sulfated- or amino-CDs for example.

Previously, a new class of human carbonic anhydrase (hCA) inhibitors, diaryl-pyrazole sulfonamide derivatives, has been synthesized and pharmacologically evaluated (Rogez-Florent et al., 2013). These compounds have a very limited water solubility which can limit their pharmaceutical development. Various formulation techniques can be applied to overcome the low aqueous solubility of drugs. Among them, the complexation with CDs offers the possibility to improve their solubility without affecting their original structure and have proved to be one of the most effective (Loftsson & Duchêne, 2007). Moreover, these compounds having a chiral center, it was essential to separate their enantiomers and verify their

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optical purities before envisaging the study of their pharmacological activity. In a previous work, a capillary electrophoretic method was developed in a dual CD mode using the native β-CD and the cationic NH_2-β-CD (Rogez-Florent et al., 2014). Thus, two distinct objectives in the development of our original compounds requires the use of CDs: the enhancement of their aqueous solubility and the separation of their enantiomers. For these objectives, the study of the complexes formed between our compounds and various CDs is essential, either to choose the most appropriate CD or to improve the knowledge on enantiodiscrimination mechanism of our compounds.

The NMR spectroscopy is one of the most useful techniques to study interactions of cyclodextrins with guest compounds (Bakkour et al., 2006; Chankvetadze, Endresz, Schulte, Bergenthal, & Blaschke, 1996; Chankvetadze et al., 1999, 2000a,b; Hellriegel et al., 2001; Zhou et al., 2003a,b; Danel et al., 2006, 2008, 2011; Brun Malta et al., 2008; Bednarek, Bocian, & Michalska, 2008; Marçon et al., 2009; Negi & Singh, 2013; Srinivasan & Stalin, 2014; Yuan, Jin, & Xu, 2012). This technique permits a better description of the supramolecular assembly, especially the right orientation of the guest molecule inside the cavity, and also provides information on the stoichiometry and binding constant of the host:guest complexes (Fielding, 2000).

In this paper, we investigate by 1D and 2D ^{1}H NMR the complexes formed between six original chiral diaryl-pyrazole sulfonamide derivatives and six CDs (native α-, β- and γ-CDs, hydroxypropylated HP-β-CD, methylated Me-β-CD or amino NH_2-β-CD) at physiological pH. The experiments are performed using the racemic or the pure enantiomers of the compounds in order to obtain information on the chiral discrimination. Whereas the stoichiometry is investigated for one complex (using the continuous variation method), the apparent binding constants are determined for the eleven complexes (using the titration method) in order to select the most promising CD. Last, the structure of the supramolecular assembly is investigated with the selected CD.

2. Experimental

2.1. Chemicals

Deionized water was obtained from Milli-*Q* system (Millipore, Saint-en-Yvelines, France). Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merck (Nogent-sur-Marne, France). Deuterium oxide (100%) and dimethylsulfoxide-*d6* (99.8%) were purchased from Euriso-top (Gif sur Yvette, France).

2.2. Studied compounds

The diaryl-pyrazole sulfonamide derivatives (**1**–**6**, Fig. 1) were designed and synthesized by some of us (Rogez-Florent et al., 2013). The enantiomers of **1** were resolved and prepared by chiral SFC (supercritical fluid chromatography) coupled to a diode array detector using an AD-H column (Daicel®, 250 mm × 10 mm; 5 μm) (others experimental conditions: 30% methanol, 35 °C, 150 bars). The optical rotation of solutions (10 mg/mL in methanol/dichloromethane—33%/67%) were measured using the Na D line (589 nm): $[\alpha]_D^{20}$ = +18 and −18.

2.3. Cyclodextrins

α-CD and γ-CD were purchased from Wacker Chimie (Lyon, France) and 6-monodeoxy-6-monoamino-β-CD (NH_2-β-CD) from Cyclolab (Budapest, Hungary). β-CD, HP-β-CD and Me-β-CD were kindly supplied by Roquette Laboratories (Lestrem, France). The HP-β-CD and Me-β-CD represent multicomponent mixtures with molar substitution (MS) of 0.61 and 0.57 per glucose unit, respectively. Their molar concentration was calculated taking into account their averaged molecular weight.

2.4. Nuclear magnetic resonance

The NMR experiments were realized on a Bruker AVANCE 500 with a TXI probe operating at 500.13 MHz for proton and 125.8 MHz for carbon.

Attributions of the ^{1}H signals of the compounds **1**–**6** were realized by standard NMR experiment spectra: COSY (homonuclear scalar correlation ^{1}H–^{1}H), HSQC (heteronuclear correlation ^{1}H–^{13}C) and HMBC (long range correlation ^{1}H–^{13}C). ROESY (homonuclear dipolar correlation ^{1}H–^{1}H) experiments spectra were recorded with a mixing time of 500 ms. Five hundred microliter of solutions were introduced into standard 5 mm NMR tubes and the experiments were realized at 298 K. The 67 mM phosphate buffer pD 7.4 was prepared by mixing appropriate amounts of both sodium salts in D_2O. Due to the poor solubility of our compounds, all solutions were prepared adding 30% (v/v) of DMSO-*d6*. The **1**–**6** compounds were racemate, equimolar mixture of both enantiomers (otherwise specified).

2.4.1. Determination of the stoichiometry

The stoichiometry of the **1**-(+)/NH_2-β-CD complex was studied using the continuous variation method (Job, 1928). The total concentration of the interacting species was kept constant at 1 mM. The molar fractions r (r = [**1**-(+)]/([**1**-(+)] + [NH_2-β-CD])) varied in the range 0–1.

2.4.2. Determination of the apparent binding constants

The apparent binding constants were determined for eleven complexes: the complexes formed between **1** and the 6 CDs and the complexes forms between β-CD and the 6 compounds. To be in accordance with the Scott's model (Scott, 1956) and take into account that the equilibrium molar concentration of the CD

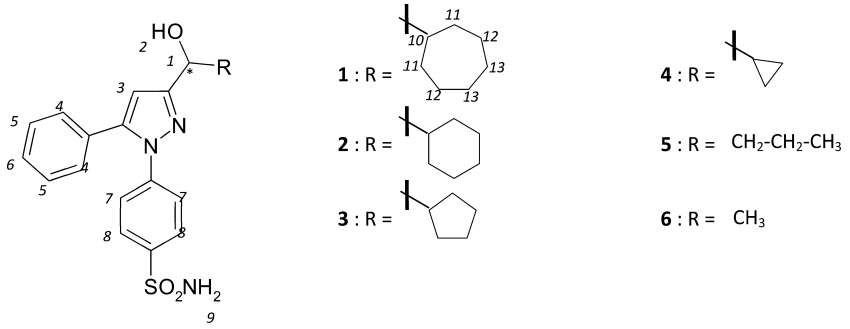

Fig. 1. Structure and assignments of the hydrogen atoms of the studied compounds.

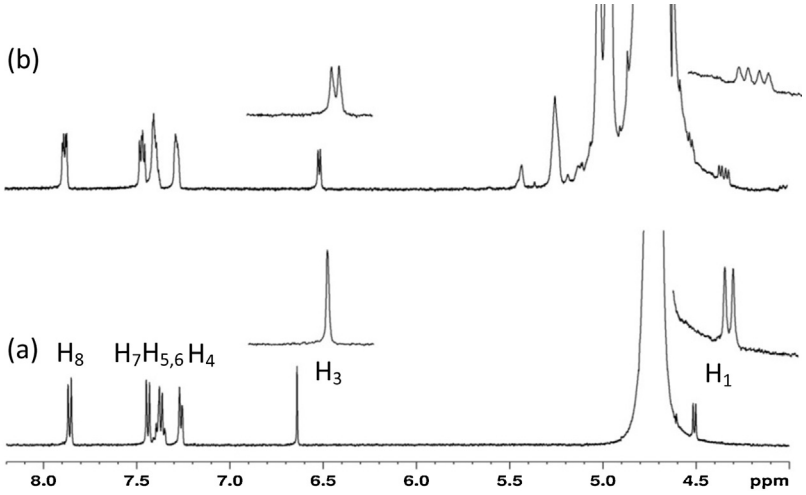

Fig. 2. Extracts of the ^{1}H NMR spectra of **1** (a) in the absence of CD ([**1**] = 0.6 mM) (b) with NH_2-β-CD ([**1**] = 0.6 mM and [NH_2-β-CD] = 3.0 mM)—assignments of the ^{1}H signals of **1**.

is equal to the total introduced CD concentration, the concentration of the CD in the complexed form must be insignificant. The [CD]/[compound] ratios were progressively increased from 5 to 25 for the β-CD and from 5 to 35 for the modified CDs, more soluble than the β-CD.

*2.4.3. Study of the structure of the **1**/β-CD complex*

To study the structure of the complex by 2D ROESY experiments, the choice of the concentration of **1**-(+) and β-CD results from a compromise between a relative high analyte concentration (for better signal/noise ratios) and a good separation of analyte resonance signals; the optimal condition was obtained for an equimolar mixture (0.5 mM for **1** and 0.5 mM for β-CD).

3. Results and discussion

3.1. Chiral discrimination

The first experiments performed in presence of both partners (one racemic diaryl-pyrazole sulfonamide derivative and one CD) displayed splittings for numerous signals as illustrated in Fig. 2 for the complex **1**-(±)/NH_2-β-CD. According to the resolution of the signals, the splittings are more or less pronounced but peak modifications (by splitting or widening) are observed for numerous signals of **1**. This phenomenon can principally be of two origins: (a) the exchange between the bound and unbound forms is slow on the NMR time scale and both forms are visualized, (b) a chiral discrimination occurs by different diastereomeric nature of the complexes either or by different association constants, more often a combination of these two effects (Smith et al., 2003; Chankvetadze et al., 1996). The exchange between free and complexed species being commonly too fast on the NMR time-scale, the first hypothesis seems less probable but additional experiments were performed to confirm the second hypothesis. Fig. 3 illustrates the spectra obtained for the complexes formed between the NH_2-β-CD and (a) the racemic **1**, (b) an enriched enantiomeric mixture **1**-(+) 33%/**1**-(−) 67% and (c) the pure enantiomer **1**-(+). No splitting of signals are observed in the spectra of the complex formed between the enantiopure **1**-(+) and the NH_2-β-CD. Then, the splittings are a result of chiral discrimination, i.e. nonequivalence of complexation-induced chemical shifts for both diastereoisomeric complexes, **1**-(+)/NH_2-β-CD and **1**-(−)/NH_2-β-CD. It is worth mentioning that

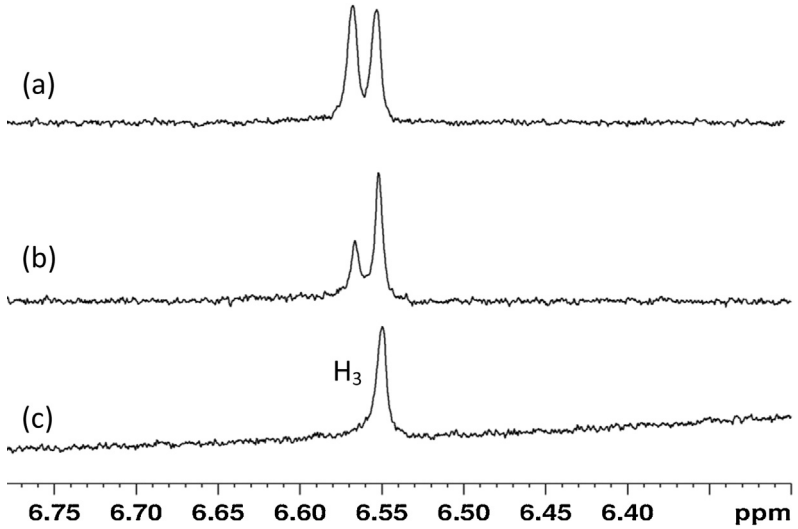

Fig. 3. Extracts of the ^{1}H NMR spectra of **1**: H_3 signal for various enantiomeric compositions (a) **1**-(±), (b) **1**-(+)/**1**-(−)—33%/67% and (c) **1**-(+) ([**1**] = 0.6 mM and [NH_2-β-CD] = 3.0 mM).

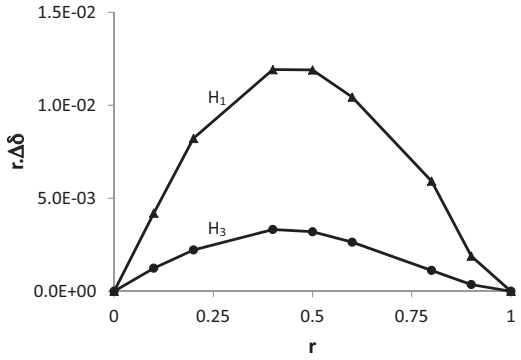

Fig. 4. Job's plots for the complex **1**-(+)/NH_2-β-CD: signals H_1 and H_3 of **1**-(+).

this phenomenon enables the use of the racemic compound for the study of the enantiospecific binding parameters. For the complexes showing chiral discrimination, two apparent binding constants are determined.

3.2. Determination of the stoichiometry

The continuous variation method, Job's method (Job, 1928), based on the ^{1}H NMR chemical shifts of both analyte and/or CD, is used to determine the stoichiometry of the **1**-(+)/NH_2-β-CD complex. Since the ^{1}H NMR spectrum of the monosubstituted CD are not well resolved due to overlaps of unequivalent protons of the substituted and non-substituted glucose units, information about the stoichiometry are deduced solely on the basis of the chemical shift variations ($\Delta\delta$) of the drug signals. According to the ^{1}H NMR spectra obtained for the complex, only some hydrogen atoms of **1**-(+) can be selected for measurements of their chemical shifts variations. The protons of the cycloheptyl ring are not clearly resolved to be precisely followed. The aromatic protons (H_4 to H_8) display well resolved signals but their chemical shift change are insufficient to be taking into account. Then, the Job's plots are established for both protons displaying the highest $\Delta\delta$: H_1 and H_3. Similar Job's plots are obtained with maxima observed for a 0.5 molar ratio and indicate the 1:1 stoichiometry of the complex (Fig. 4).

3.3. Determination of apparent binding constants

Benesi–Hildebrand's method (Benesi & Hildebrand, 1949) and Scott's modified equation (Scott, 1956) allow, for analyte/CD complex of assumed 1:1 stoichiometry, the calculation of apparent binding constants from the three following linear equations obtained after mathematical rearrangements (*y*-reciprocal (1), double-reciprocal (2) and *x*-reciprocal (3)):

$$\frac{[CD]}{\delta_i - \delta_f} = \frac{1}{\delta_c - \delta_f}[CD] + \frac{1}{(\delta_c - \delta_f)K} \quad (1)$$

$$\frac{1}{\delta_i - \delta_f} = \frac{1}{(\delta_c - \delta_f)K}\frac{1}{[CD]} + \frac{1}{(\delta_c - \delta_f)} \quad (2)$$

$$\frac{(\delta_i - \delta_f)}{[CD]} = -K(\delta_i - \delta_f) + K(\delta_c - \delta_f) \quad (3)$$

where K is the apparent binding constant, [CD] is considered to be the total concentration since the complexed CD concentration must be insignificant, δ_i is the chemical shift observed and δ_f and δ_c are the chemical shifts of the analyte in their free and complexed forms, respectively. In our study, the three linear equations are used since differences due to relative uncertainties of the variables before and after transformation for plotting can occur (Rundlett & Armstrong, 1997; Plätzer, Schwarz, & Neubert, 1999). Moreover, deviations from linearity can be more easily observed using *x*- or double-reciprocal plot than the *y*-reciprocal plot (Rundlett & Armstrong, 2001). It is worth mentioning that the binding constants are apparent because the concentrations are considered (instead of activities) and averaged when the complexation occurred with modified CDs of averaged degree of substitution.

Table 1a
Apparent and averaged binding constants for the complexes formed between **1** and the six CD studied.

Complex	$K(M^{-1})$
1/α-CD	nd
1/β-CD	764
1/γ-CD	139
1/NH_2-β-CD	335/426*
1/HP-β-CD	588/668*
1/Me-β-CD	nd

nd: not determined due to insufficient chemical shift differences.
* *K* values of both enantiomers due to chiral discrimination.

First, the apparent binding constants are determined for the six complexes formed between **1** and the 6 CDs (native α-, β- and γ-CDs, HP-β-CD, Me-β-CD and NH_2-β-CD) in order to select the most appropriate CD for the complexation of our compounds. For higher accuracy of the results, the calculations must be based on the chemical shifts variations of the signals which present the highest $\Delta\delta$. For the **1**/NH_2-β-CD complex, the $\Delta\delta$ are 76 Hz for H_1 and 64 Hz for H_3 for a ratio [NH_2-β-CD]/**1** equal to 30 although $\Delta\delta$ remains inferior to 10 Hz for other protons. Then, the signals H_1 and H_3 are selected. However, due to interferences of the H_1 signal with the signal of residual water for some complexes, the H_3 signal is preferred. All the calculations of apparent binding constants are achieved taking into account the H_3 signal. The apparent binding constants for the complexes **1**/α-CD and **1**/Me-β-CD cannot be determined due to insufficient chemical shifts variations. For the other complexes, linear Scott's plots are obtained with determination coefficient values higher than 0.99 using the three Eqs. (1–3) and prove the adequacy of the model for 1/1 complexes. For all complexes, the three values obtained are in good agreement. Only the values obtained with the y-reciprocal model are displayed in Table 1a. Higher apparent binding constants are obtained for the native or substituted β-CDs (except the Me-β-CD): the β cavity seems more adapted to the fit of the compound in the cavity; the α- and γ- cavities may be too small and too large to favor the inclusion by host-guest interactions, respectively. The highest binding constant is determined with the native β-CD (764 M^{-1}). Its substitution by hydroxypropyl, amino or methyl groups greatly affects the interaction since the association constant decreases of 13% for HP-β-CD and 44% for NH_2-β-CD and is too low to be quantified for Me-β-CD. Whereas no chiral discrimination is observed for the complex **1**/β-CD, the apparent binding constants have been determined for both enantiomers for the complexes **1**/HP-α-CD and **1**/NH_2-β-CD and should be considered as significantly different with accepted RDS error around 5–10% (Danel et al., 2006, 2008). It is worth mentioning that even if the interaction between **1** and Me-β-CD is too low to be quantified, it is sufficient to allow chiral discrimination since splittings of the H_1 signals are observed for this complex (see Supplementary data). Then, these results confirm that if the binding of the racemic compound with the CD is a prerequisite for enantioselectivity, no direct proportional dependence may necessarily exist between the binding strength and the stereoselectivity (Chankvetadze et al., 1996).

Second, the complexes formed between the six compounds and the β-CD are studied. The β-CD is chosen since its complexing ability towards the compound **1** appears the most promising with a binding value around 7×10^2 M^{-1}. The results are displayed in Table 1b. The comparison of the results obtained for the various compounds, showing structural modifications of the aliphatic

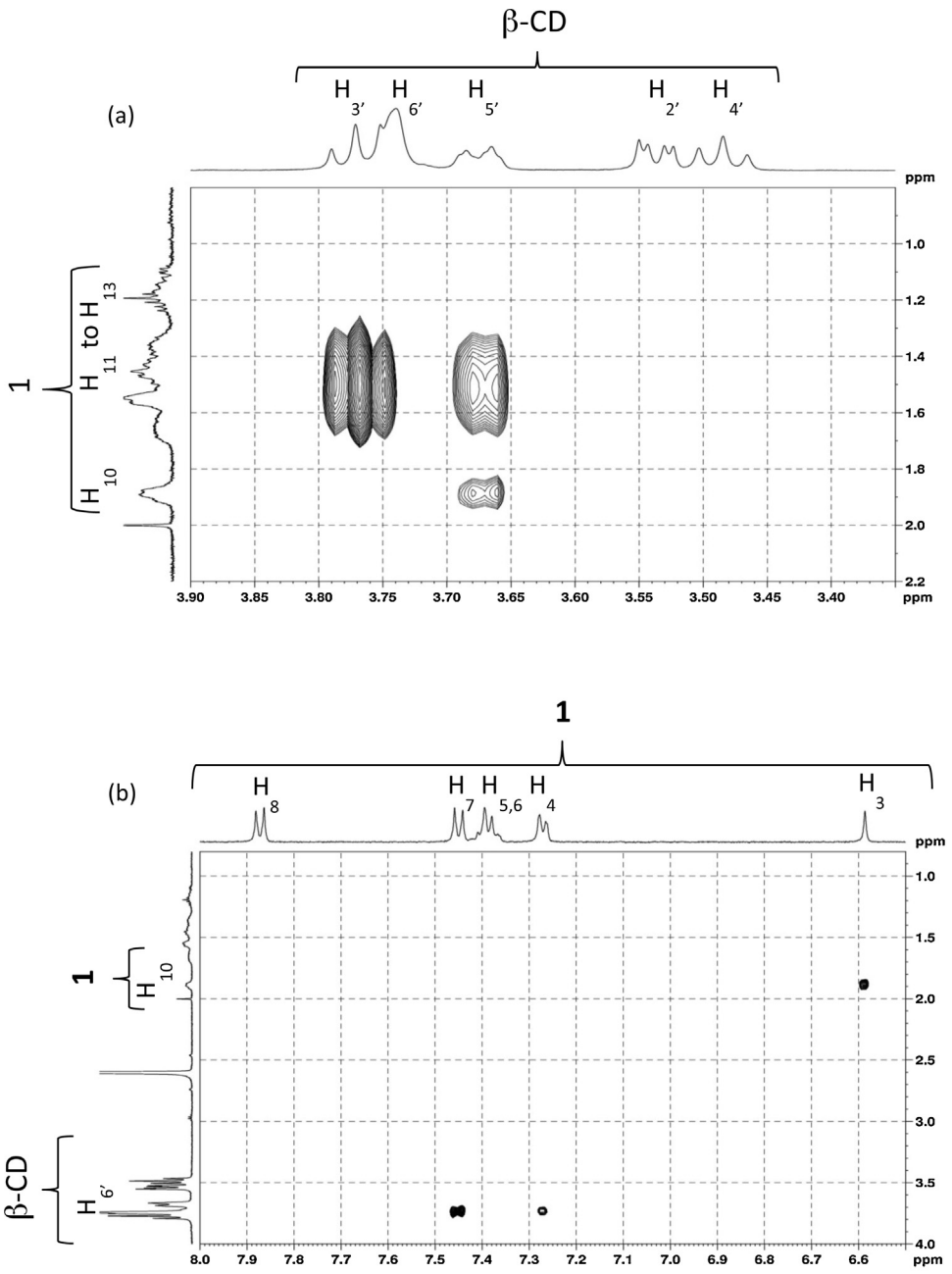

Fig. 5. 2D-ROESY spectra of the **1**-(+)/β-CD complex ([**1**-(+)] = 0.5 mM and [β-CD] = 0.5 mM).

substituent on the asymmetrical carbon, allows us to study the relation between the nature of this substituent and the affinity towards the β-CD. A direct correlation appears between the bulk of the substituent and the apparent binding constants: *K* are 764 M^{-1}, 561 M^{-1} and 199 M^{-1} for the cycloheptyl (**1**), cyclohexyl (**2**) and cyclopentyl (**3**) substituents, respectively. For smaller substituents (cyclopropyl (**4**), propye (**5**) and methyl (**6**)), the chemical shifts variations are too low to quantify the interaction. For the complexes **2**/β-CD and **3**/β-CD the chiral discrimination observed allows the determination of the binding constants for each enantiomer. However, the difference between the binding constants obtained for both enantiomers of **3** and the β-CD is insignificant since the RSD of apparent binding constants determined by NMR using the Scott's method is usually around 5–10% (Danel et al., 2006, 2008). The interaction between the aliphatic part of the compounds and the CD seems to greatly contribute in the binding. Moreover, up-field chemical shifts were observed for the protons H_1 and H_3 of **1** in complexed state compared with free state ($\Delta\delta = \delta_i - \delta_f < 0$) whereas for the other followed protons (aromatic protons H_4 to H_8), very slight down-field chemical shifts were observed. These results indicate that both protons H_1 and H_3 are surrounded by electron

Table 1b
Apparent and averaged binding constants for the complexes formed between the β-CD and the six compounds studied.

Complex	*K*(M^{-1})
1/β-CD	764
2/β-CD	379/561*
3/β-CD	178/199*
4/β-CD	nd
5/β-CD	nd
6/β-CD	nd

nd: not determined due to insufficient chemical shift differences.
* *K* values of both enantiomers due to chiral discrimination.

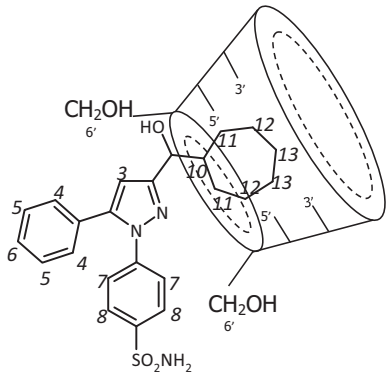

Fig. 6. Proposed model for the inclusion of **1** in the CD cavity.

density of the CD when the complexation occurs and can suggest that the inclusion does not occurs by the aromatic rings of **1**. The sign of $\Delta\delta$ for the aliphatic protons would have been very informative but unfortunately their signals were not sufficiently resolved to be clearly followed. Since enantiorecognition is observed, the host-guest inclusion is proved. An inclusion by this aliphatic substituent is suggested and is further studied by ^{1}H NMR 2D ROESY experiments.

3.4. Study of the structure of the *1*/β-CD complex

The structure of the complex formed between **1**-(+) and β-CD is studied through ^{1}H NMR 2D ROESY experiments. The ROESY is a powerful tool for investigating intra- and inter-molecular interactions. The presence of cross-peaks between the protons of two different species, which are generated by Nuclear Overhauser Effects (NOE) is an indication that they are in a spatial contact within 3–5 Å (Ribeiro, Carvalho, Ferreira, & Veiga, 2005). The complex **1**/β-CD is especially studied since the β-CD allows the highest apparent binding constant and then is the best candidate to envisage the development of a new formulation. Moreover, the ^{1}H signals of the β-CD are well resolved and assigned, compared to modified CD, and could provide more detailed information about the geometry of the complex. The partial ROESY spectra obtained are displayed in Fig. 5 and reveal both intra- and inter- molecular interactions. The single intramolecular interaction visible on the figure occurs between H_3 and H_{10} of the analyte. All other cross-peaks observed are attributed to intermolecular interactions. The major cross-peaks observed concern the signals $H_{3'}$ and $H_{5'}$ of the β-CD, both protons located into the CD cavity, and the protons H_{10} to H_{13} of the cycloheptyl ring of **1**. This suggests the inclusion of the aliphatic ring of **1** in the β-CD cavity. Even more interesting is the lack of cross-peak between H_{10} (borne by the asymmetrical carbon of **1**) and $H_{3'}$ of the β-CD. This surprising result suggests an inclusion of the cycloheptyl ring by the smaller opening of the toroid presenting the primary hydroxyl groups. Fig. 5(b) shows two additional intermolecular cross-peaks between the external $H_{6'}$, attached to the primary rim, with H_4 and H_7 of the aromatic rings of **1**. This result confirms our hypothesis and leads us to propose an inclusion model via the smaller opening of the cavity (Fig. 6).

4. Conclusion

All the information obtained could be used for the purpose of a new formulation development and for understanding the chiral discrimination mechanisms. The complexing ability of the native β-CD towards our compounds appears the most promising since binding values around 7×10^2 M^{-1} are obtained. After the preparation of solid inclusion complexes with the β-CD by grinding or/and a less common technique using the supercritical CO_2 fluid (Banchero & Manna, 2011), studies of the solubility will be performed. Due to the structure of our compounds, a classical inclusion of one of the aromatic rings inside the CD cavity via the larger opening side could have been envisaged. The 2D ROESY experiments have proved that the inclusion takes place by the aliphatic part of the compound via the smaller opening of the cavity.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.09.046.

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