Complexation of steroid hormones: prednisolone, ethinyloestradiol and estriol with β-cyclodextrin. An aqueous ^{1}H NMR study

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The inclusion complexes of β-cyclodextrin (β-CD) with prednisolone **1**, ethinyloestradiol **2** and estriol **3** in aqueous solution were investigated using ^{1}H NMR and molecular modelling. The NMR spectra of the steroids studied in the presence of β-CD are fully assigned and interpreted by means of 2D GCOSY and NOESY spectra. The parallel interpretation of β-CD chemical shift changes and dipolar contacts allows the mode of binding to be established. On the basis of ROESY data, the "low resolution" β-cyclodextrin complexes of **1**, **2** and **3** have been determined by multistep restrained molecular dynamics calculations. Calculated structures of β-cyclodextrin complexes with **1**, **2** and **3** fully agree with experimental data. Combined approaches allow the distinction of weak nonspecific binding for **1** as compared to stronger, "through cavity", inclusion established for **2** and **3**.

Introduction

Cyclodextrins (CDs) are α-1,4-linked cyclic oligomers of D-glucose which possess remarkable properties in forming inclusion complexes with a variety of molecules of appropriate size *via* noncovalent interactions. CDs have received considerable attention in pharmacy because of improved water solubility, chemical stability and bioavailability of various drug molecules through the preparation of inclusion complexes.[1–5] The solubility of steroid hormones can also be largely enhanced due to the complexation with β-cyclodextrin (β-CD).[6]

Formation of inclusion compounds by prednisolone **1**, ethinyloestradiol **2** and estriol **3** (Fig. 1) and β-CD has been

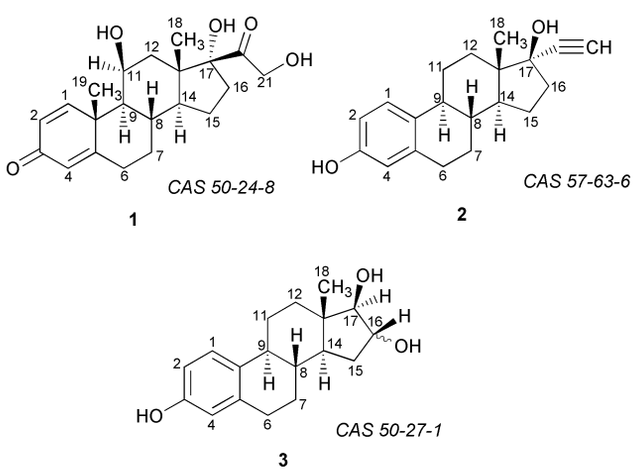


Fig. 1 Structural formulas of steroids: prednisolone **1**, ethinyloestradiol **2** and estriol **3**

previously studied by HPLC.[7–11] Uekama *et al.* have studied inclusion complexation of 18 steroid hormones with CDs by different techniques (**1** was the sole molecule in common with those studied here). It was stated that the A-ring (Fig. 2) of the

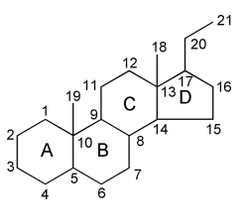
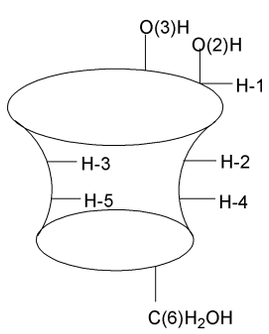


Fig. 2 Conventional representation of the steroid ring system, showing the letters used to designate the rings and the numbers used to identify carbon atoms.



Scheme 1 Atomic numbering and schematic representation of relative positions of β-CD protons.

steroid molecule was predominantly included in the cavity of the CDs (Scheme 1).[6]

As a result of HPLC investigations, it was also found that the structural features of ring A had a great influence on the stability of the complexes, **1**, **2** and **3**.[11] In a methanol–water mixture (20 : 80 v/v) the association constants of estrogens, having a phenolic A ring, were one order of magnitude larger than the association constants of other steroids, having one or two double bonds conjugated with the keto group (cyclohexenone or cyclohexadienone-like structure of ring A). Nevertheless, the

properties of ring D, which should not be in contact with β-CD, also influenced the complex stability.

Previous ^{1}H NMR studies[12,13] of the **1**–β-CD complex has shown that the complex is of 1 : 1 stoichiometry, and according to the authors' classification, is of type II,[13] *i.e.*, that inclusion of the steroid molecule occurs from the direction of the wide secondary rim. These studies also showed the presence of the stabilizing hydrogen bonds in the **1**–β-CD complex.

On the other hand, we have not found any data regarding the ^{1}H NMR investigation of the other two steroid hormones, **2** and **3**, and so we aimed to study thoroughly the dipolar interactions and spatial proton proximities of the three steroids with β-CD, using 2D ROESY experiments.

The stoichiometry and binding constants of the studied complexes have been previously established in water–methanol mixtures by other techniques, *i.e.*, **1**, by Uekama *et al.*[6] and **2** and **3** by Sadlej-Sosnowska.[11] Therefore we did not attempt to reevaluate these values very thoroughly in water by NMR, assuming that the stoichiometry would be the same as established in earlier studies, but rather we tried to make an estimate verifying our expectation that the binding constants should be much larger in water than in water–methanol mixtures. This is mainly due to the very poor solubility of these steroid hormones in water that makes the binding constant determination difficult to conduct accurately. Instead, we have attempted to identify a model for the interaction of β-CD with the studied compounds, which was not discussed in previous approaches. The complex **1**–β-CD was also studied to provide additional information to that presented earlier.[6,12–14] In contrast to this previous work we attempted to use in parallel both sources of available experimental information, *i.e.*, the chemical shift changes of β-CD and the intermolecular contacts manifested by 2D ROESY crosspeaks.

The current research on the host–guest complexation of small molecules with CD's is largely concerned with the mode and depth of the inclusion into the hydrophobic core of the host, the kinetics of exchange and the differences in the binding of the stereoisomers or enantiomers.[15] The drawbacks involved in these studies are concerned with the high mobility of the guest, which is revealed by the breadth of the complex signals,[16] possible deformations of the included compounds,[17] or the strong dependence of the ROESY pattern and inferred complex structure on the minor variations of the guest structure.[18] Nevertheless, despite these difficulties, some generalizations seem to emerge: the complex stability is enhanced by an increase in the guest binding site electron density, it is enhanced by an increase in binding site polarizability and it is decreased by an increase in binding site polarity.[19] In cases where the sizes of guest molecules are adequate for the "goodness of fit", aromatic molecules are bound tightly over the aromatic ring inside the cavity of β-CD.[18,20–23] Generally, the β-CD cavity is more hydrophobic than the surrounding polar solvent,[19,24,25] and binds tightly hydrophobic compounds and the more nonpolar parts of compounds that possess polar and nonpolar moieties. This hydrophobic effect is reflected in a very good correlation of association constants with log *P* (partition coefficient, diethyl ether–water) for 27 acyclic alcohols[24] and some linear correlations between water solubility and complex stability.[26,27] The more or less planar guests are frequently tilted inside the β-CD cavity and this arrangement allows the guest molecule to occupy most of the available cavity space while keeping the polar substituents close to the hydroxy groups on the β-CD cavity rim.[18,28] The polar substituents often protrude from the cavity as they are in contact not only with the hydroxy groups of the CD rim but also with the solvent.[19]

One of the challenges in this field is the problem in distinguishing between various modes of binding[29] or excluding the averaging of the multiple binding modes. We have addressed this problem in the present work by means of generating the theoretical geometries of complexes, using experimental restraints derived from dipolar contacts observed in 2D ROESY NMR spectra. This approach allows the clear distinction between the unique binding geometry and the average of multiple complex geometries.

Experimental

NMR measurements

^{1}H NMR measurements were performed at 303 K in D_2O solutions using a VARIAN INOVA 500 MHz spectrometer. For accurate determination of ^{1}H chemical shifts, sodium 3-trimethylsilyltetradeuteriopropionate (TSP-d_4) was used as an external reference. Signal assignments were made with the aid of the 2D GCOSY[30] and TOCSY[31] spectra. These spectra were acquired by using spectral widths of 4600 Hz in both dimensions, an acquisition time of 0.223 s, 4 transients per increment for the 256 increments, and 2048 data points in the F2 domain. For TOCSY an 8 kHz spin lock field in CW pulse mode centered at the water resonance with 20 ms and 130 ms mixing times were applied.

2D ROESY[32] measurements were made using the standard Varian software with the experimental conditions as follows: spectral width of 4600 Hz, acquisition time 0.223 s, 128 transients per increment for the 256 increments, a 2 kHz spin lock field in CW pulse mode centered at the water resonance, with a 300 ms mixing time duration and 2048 data points in the F2 domain. All spectra were processed with a π/2 shifted squared sine-bell filter in both dimensions.

Determination of binding constants

The association constants were determined using the 1 : 1 binding isotherm[33] which has the hyperbolic form. Its linearization produces the double reciprocal (Benesi–Hildebrandt) plot;

$$\frac{1}{\Delta} = \frac{1}{\Delta_1 K_1[\mathrm{CD}]} + \frac{1}{\Delta_1} \qquad (1)$$

where $\Delta = \delta_{AV} - \delta_S$, $\Delta_1 = \delta_{S\text{–}CD} - \delta_S$, δ_S is the steroid observed chemical shift before the addition of β-CD to the steroid solution, δ_{AV} is the steroid observed chemical shift after the β-CD has been added and $\delta_{S\text{–}CD}$ is the chemical shift of the complex.

Regression of $1/\Delta$ *vs.* 1/[CD] allowed the determination of K_1 iteratively, using known total concentrations of [CD] instead of the unknown concentrations of the free ligand S. With this preliminary estimate of K_1 the required [CD] values could be calculated. This process was repeated until the K_1 values converged.

Having been aware of the limitations concerned with the uncertainty of the binding constants determined by NMR[34] due to low solubility of the studied compounds, we have used several steroid protons to follow the changes of their chemical shifts on titration with β-CD. These were H1, H2, H11, CH_3-18, CH_3-19 for **1** and H1, H2 for **2**. The average values of binding constants *K* were calculated as 3000 M^{-1} and 50 000 M^{-1} for **1** and **2**, respectively.

Computational methodology

The ligand–β-cyclodextrin complex structures were determined with the aid of the Molecular Simulations Insight II(2000) package. In all calculations the cvff forcefield and charges[35] were used. For steroid molecules ESP charges were obtained by Dmol (MSI) using BLYP functional and DNP atomic basis set with frozen inner core orbitals. In the calculations with solvent represented by a polarizable continuum, the relative permeability value ε was set distance dependent as 4.5**r*, whereas during calculations with explicit water molecules ε was set to 1 and the cubic periodic boundary box of dimension 30 Å

Table 1 Chemical shifts, δ (ppm), of prednisolone **1** protons in D_2O solution with ~5 mM β-CD and a qualitative score of the size of ROE effects

Protons	δ (ppm)	2D ROE Interaction with H(3′)	2D ROE Interaction with H(5′)
1	7.61	++	++
2	6.33	++	++
4	6.16	++	++
6^{α}	2.58	++	+
6^{β}	2.81	+	−
7^{α}	1.22	++	−
7^{β}	2.36	+	−
8	2.33	−	−
9	1.08	++	+
$19CH_3$	1.59[a]	++	++
11	4.61	++	−
12^{α}	2.05	++	+
12^{β}	1.81	++	+
$18CH_3$	0.97	++	++
14	1.72	++	−
15^{α}	1.94	++	+
15^{β}	1.57[a]	++	++
16^{β}	2.68	−	++
16^{α}	1.57[a]	++	++
21^{a}	4.40	−	++
21^{b}	4.73	−	+

[a] Overlap for crosspeaks of protons occurs.

Table 2 Chemical shifts, δ (ppm), of ethinyloestradiol **2** protons in D_2O solution with ~1 mM β-CD and the qualitative score of the size of the ROE effects

Protons	δ (ppm)	2D ROE Interaction with H(3′)	2D ROE Interaction with H(5′)
1	7.17	−	++
2	6.70		
4	6.69	−	++
6^{α}	2.81	+	++
6^{β}	2.81	+	++
7^{α}	1.46	+	−
7^{β}	2.11	++	−
8	1.38	++	−
9	2.19[a]	++	+
11^{α}	2.60	++	−
11^{β}	1.54[a]	++	−
12^{α}	2.08	++	−
12^{β}	1.92	++	−
$18CH_3$	1.04	++	−
14	1.86	+	−
15^{α}	1.99	++	−
15^{β}	1.54[a]	++	−
16^{α}	2.47	−	−
16^{β}	2.19[a]	++	+
CH	3.14	++	–

[a] Overlap for crosspeaks of protons occurs.

filled with standard INSIGHT waterbox water model. All molecular dynamics calculations were based on a 1 fs time step.

The initial structure of the β-cyclodextrin was adopted from the crystallographic data of the β-cyclodextrin complexed with *E. coli* D-maltodextrin-binding protein[36] (Protein Data Bank[37] 1DMB entrance at http://www.rcsb.org/pdb/). Molecular mechanics calculations were based on the structure of free β-cyclodextrin to avoid any influence by any conformational changes arising from any specific host–guest interactions. We believe that the conformation of the β-cyclodextrin complexed with a large protein was closer to the aqueous one than that obtained from pure β-cyclodextrin crystals because of a large amount of water existing in protein crystals. Ligand molecules were initially optimised by *in vacuo* molecular mechanics calculations. The standard interactive insight docking procedure was used to generate an initial complex structure. The complex buildup procedure was driven to satisfy most of experimentally determined interresidual NOE contacts. Raw refinement was made by restrained molecular dynamics calculations. For each intermolecular crosspeak found in the 2-D 300 ms mixing time ROESY spectrum, a 6 Å upper distance constraint with 10 kcal mol^{-1} $Å^{-2}$ force constants was used. Because of the high symmetry of the β-cyclodextrin molecule the commonly used pseudoatom correction for magnetically equivalent protons was not applicable.[38] In consequence all upper limit constraints derived from intrerresidual NOE's were arbitrarily assigned to the closest of the magnetically equivalent β-cyclodextrin protons. During calculations constraint assignment was updated after every 1000 steps of Molecular Dynamics simulations analogous to the floating chirality protocol.[39] In order to improve calculations before the final optimisation, the complex structure was tuned by 300 ps restrained MD calculations at 400 K with the fixed conformation of β-cyclodextrin, 300 ps restrained MD calculations were performed at 300 K proceeding 300 ps unrestrained MD calculations. Finally, the β-cyclodextrin–ligand system was placed in a water box and after a short 1000 step minimisation 40 ps unrestrained Molecular Dynamics in the water filled box with periodic boundary conditions were applied.

Table 3 Chemical shifts, δ (ppm), of estriol **3** protons in D_2O solution with ~0.8 mM β-CD and the qualitative score of the size of the ROE effects

Protons	δ (ppm)	2D ROE Interaction with (H3′)
1	7.22	−
2	6.64	−
4	6.62	−
6^{α}	2.84	+
6^{β}	2.93	+
7^{α}	1.48	+
7^{β}	2.06[a]	++
8	1.38	+
9	2.26	+
11^{α}	2.54	++
11^{β}	1.55	++
12^{α}	2.07[a]	++
12^{β}	1.48	+
$18CH_3$	0.94	+
14	1.69	−
15^{α}	1.76	+
15^{β}	2.00	+
16	4.27	–
17	3.67	Not determined

[a] Overlap for crosspeaks of protons occurs.

Results and discussion

The contour plots of the 2D ROESY experiments are displayed in Fig. 3 (a and b) for **1** and **2**, respectively. The ^1H chemical shifts of the steroid protons, as well as dipolar contacts between the β-CD methine protons H3′ and H5′ and those of steroids are tabulated in Tables 1–3. In the case of **3**, the interactions with H5′ were not determined due to overlap of the signals.

The CD region of the ^1H NMR spectra of the β-CD alone and the three studied complexes are shown in Fig. 4. The shape of the ^1H NMR spectra is different for **1**, with respect to **2** and **3**. The spectra of **2** and **3** show broadening of the H5′ and H6′ protons of the CD, which is reduced on heating the sample and, therefore, gives information about the dynamic processes occurring in the sample. The most probable, and expected, physical process in the systems studied is inclusion, which in the case of **2** and **3** is apparently in the intermediate exchange

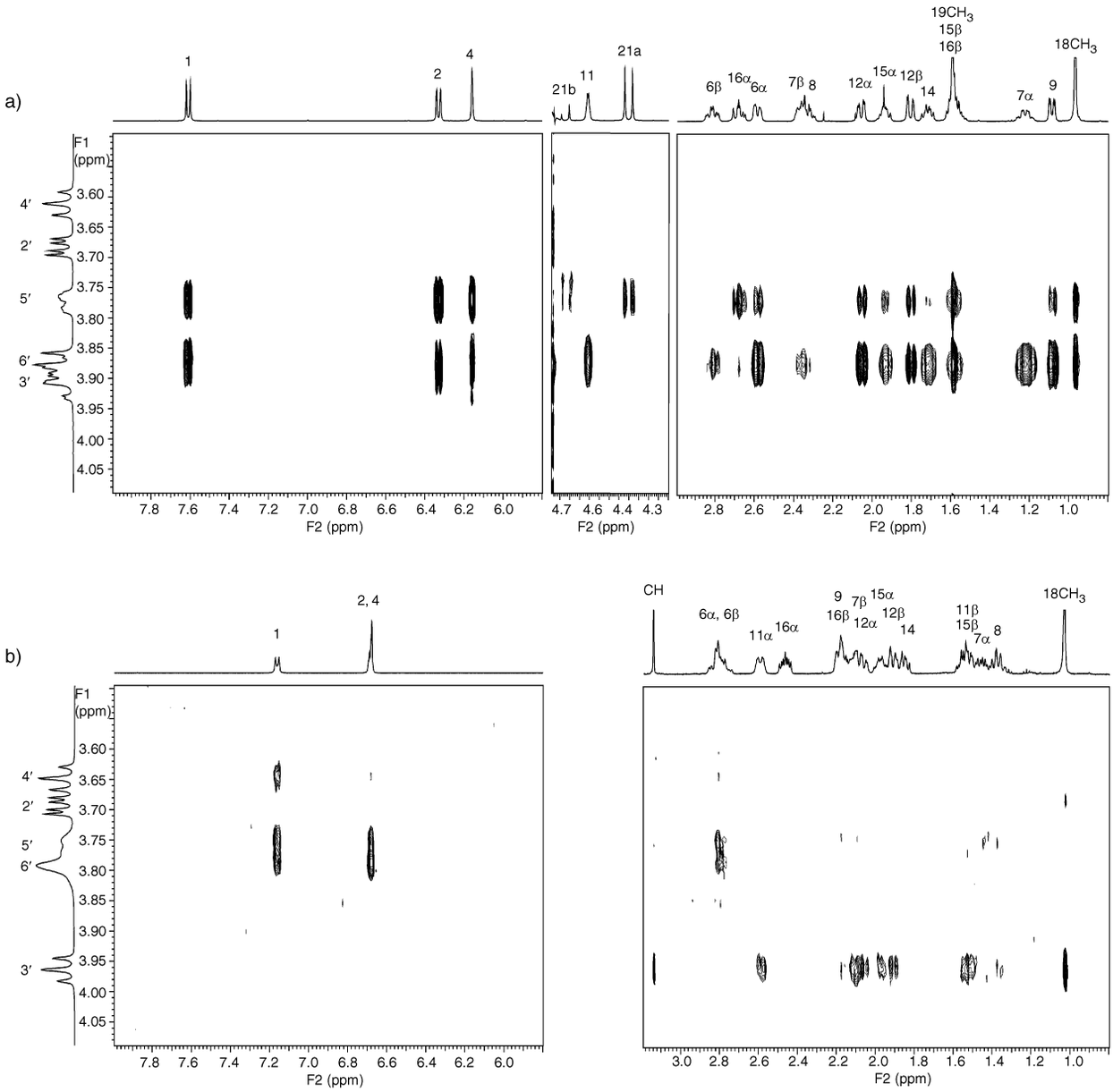


Fig. 3 (a) 2D ROESY NMR spectra of prednisolone **1** with β-cyclodextrin in D_2O; (b) 2D ROESY NMR spectra of ethinyloestradiol **2** with β-cyclodextrin in D_2O.

regime. However, the ^1H spectrum of **1** shows sharp lines indicating that the process of inclusion is in the fast exchange limit. This qualitatively agrees with the binding constants of the compounds studied, as the association constants for **1** are an order of magnitude smaller than for **2** and **3**[11] as established earlier in water–methanol mixtures and confirmed in this work in water, the respective values of K for **1** and **2** being 3000 M^{-1} and 50000 M^{-1}.

Chemical shift changes of the individual protons in β-CD accompanying complexation with the three steroids yields supplementary information on the β-CD–steroid interactions compared to that found with the 2D ROESY experiments (Fig. 4). For β-CD resonances, large differences are observed upon inclusion of the steroid. The most diagnostic are the upfield shifts for H5′ and H3′ *i.e.* the signals of the hydrogens located on the inner side of the cyclodextrin cavity, as has been previously observed for **1**,[14] and for a very similar compound—hydrocortisone.[40]

The signal of the H3′ proton has the largest shift for **1**, whereas it is only slightly affected by **2** and **3**. The shifts of the H5′ proton signal are large and comparable for the three complexes. Fig. 4 shows that the signal of hydrogen H6′, at the "narrow" (primary) cyclodextrin rim is slightly shifted for **1**, but its shift is much larger for **2** and **3** (this larger shift of the H6′ proton signal is accompanied by a lower shift of H3′). In contrast, the chemical shift changes of the H2′ and H4′ "external" protons are negligible as expected.

The detailed interpretation of the ROESY contacts in complexes **1**–**3** suggests that the mode of complexation is similar for **2** and **3**, whereas the complexation of **1** differs significantly. The NMR 2D ROESY spectrum of the ethinyloestradiol-complex (Fig 3(b)) exhibits a number of intermolecular crosspeak patterns. The most significant are the existence of ethinyloestradiol H(1), H(2), H(4), H(6) to β-CD H5′ and H6′ crosspeaks with no effect on the β-cyclodextrin H3′. This ROESY pattern is plausibly confirmed by the computed structure (*vide infra*). According to this the closest calculated distance between the H3′ of the CD to the nearest aromatic proton of ring B in **2** is *ca.* 4.6 Å, but the aromatic proton distance to the H5′ proton of the CD is, on average, *ca.* 2.5 Å. The remaining protons of the molecule interact only with the β-CD H3′. This clearly indicates that the ethinyloestradiol A ring penetrates the β-cyclodextrin cavity narrow rim. This is also confirmed by the relatively large upfield shift of both the H6′ and H5′ β-CD

protons due to magnetic anisotropy of the neighbouring A ring. A negligible change in the β-CD H3′ proton chemical shift upon complexation is observed. Also broadening of the H6′ resonance indicates a lowering of the rotational flexibility of the C6′H_2OH exocyclic group due to interaction with the ligand molecule. Almost all of the rest of the ethinyloestradiol protons were found to interact solely with the β-CD H3′ proton. This indicates that the ethinyloestradiol D ring is located close to the β-CD wide rim. Finally, taking into account a 1 : 1 complex stoichiometry,[12] the conclusion is drawn that ethinyloestradiol in the complex is crossing the β-cyclodextrin internal cavity with ring A, pointing towards the narrow rim, and ring D being exposed to the solvent on the opposite side of the β-CD molecule. As mentioned above, this conclusion seems to be plausibly supported by the observed significant, 0.15 ppm, low frequency shift of H5′ protons which could be induced by the A ring current anisotropy on these protons rather than on the H3′ proton. This suggests then a deep "transverse" immersion of the guest inside the cavity. Analogous, but not so evident analysis, due to severe broadening of the H5′ proton resonance, could be presented for the β-CD complex of estriol **3**.

The β-CD chemical shift changes upon complexation with prednisolone **1** exhibit significantly different tendencies. Both H3′ and H5′ protons are shifted upfield, whereas no significant change of the H6′ chemical shift is observed (Fig. 4). This indicates that ring A is localised inside the cavity, close to both the H3′ and H5′ β-cyclodextrin protons. The narrow resonance lines of H6′ indicate that complexation preferentially affects the wide rim protons of the cavity. Unfortunately, due to overlap of the H3′ and the H6′ signals, it is not possible to distinguish between crosspeaks due to H3′ and H6′, which are crucial for the discrimination of the binding mode. Almost all of the prednisolone protons exhibit interresidual crosspeaks due to both the H3′ and H5′ β-cyclodextrin protons.

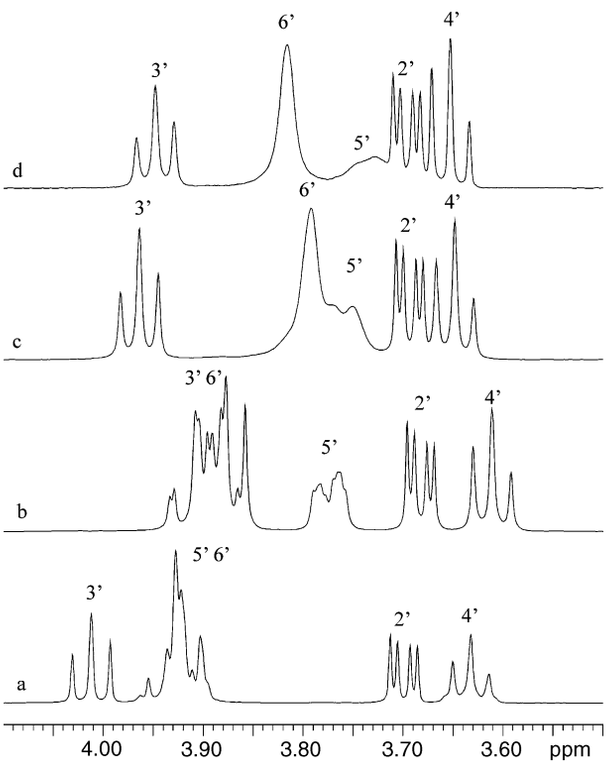


Fig. 4 Partial ^{1}H NMR spectra (δ 3.5–4.1) of the steroid-β-cyclodextrin system in D_2O: (a) β-cyclodextrin alone; (b) β-cyclodextrin with prednisolone **1**; (c) β-cyclodextrin with ethinyloestradiol **2**; (d) β-cyclodextrin with estriol **3**.

In Fig. 5 the molecular modelling derived stereostructures of the studied complexes are displayed. Initial structures of the β-CD complexes for **2** and **3** were built according to the presented interpretation of NMR 2D-ROESY data. NMR restraints were introduced in the form of potentials taking into account the existing ROE crosspeaks. Both ethinyloestradiol and estriol complexes were generated with steroid molecules crossing the β-CD internal cavity. Ring A was solvent exposed in the narrow rim. The complex was found to be stable during all types of calculations. In the final structure of the complex,the ethinyloestradiol O(3) and O(17) and estriol O(3), O(16) and O(17) OH oxygen functions are solvent exposed, whereas the apolar central B and C rings are protected from the solvent by the β-cyclodextrin molecule. During the final 30 ps molecular dynamics calculations, with explicit water molecules, no trends of movement outside the cavity were observed. Reorientation of the steroid molecule inside the cavity was accompanied by small changes of β-cyclodextrin conformation.

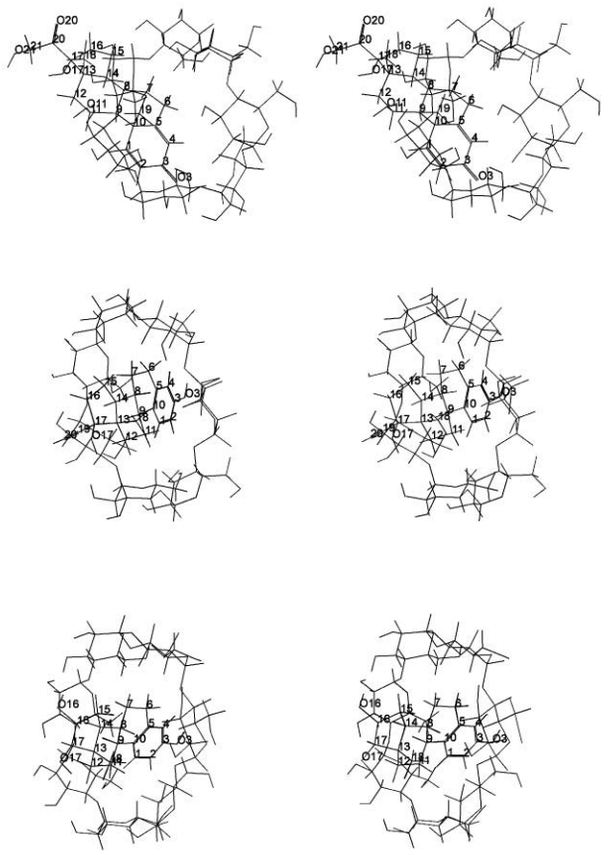


Fig. 5 Stereoview of computed structures of studied complexes: **1**, **2** and **3** from top to bottom.

Calculations performed for prednisolone **1** were based on the set of structures, in which the steroid A ring enters the cavity from the wide rim side. During NMR-constrained, vacuum molecular dynamics, a reorganisation of the complex was observed. In a few cases the molecule left the cavity and a side-to-side complex on the wide rim was formed. When the NMR-restraints were removed, the prednisolone molecule left the cavity in all simulations. Even during molecular dynamics with explicit water molecules the prednisolone molecule showed a systematic tendency to escape the cavity.

It is concluded that ethinyloestradiol and estriol form with β-CD stable "transverse" complexes, in which the apolar B and C rings are protected from the solvent. In such complexes, the β-CD H4′ proton is within the 5–6 Å distance range to the steroid H1 and H4 protons, which explains the observed crosspeaks to the "external" β-CD H4′ proton.

In the case of prednisolone **1**, the "transverse" form of the complex would have kept the O(11) OH group inside the cavity, which would be unfavourable in terms of free energy. In consequence, only rings A and B or C and D may penetrate the β-cyclodextrin cavity separately. Moreover, both the calculation

and the NMR experiments yielded evidence that the complex does not have a rigid structure. The side-to-side conformation, derived from molecular modelling (as presented in Fig. 5) also seems to be populated. In the latter case there is less reduction in the hydrophobic surface area, but all the steroid polar groups are exposed to solvent.

The results presented here also allow conclusions to be made regarding the distinction between the two extreme cases in the host–guest interactions of small molecules in the CD cavity, *i.e.*, single, rigid geometry, as observed for **2** and **3** *vs.* average of multiple geometries, as seen in **1**. In the first case the experimental ROESY restraints are rare but specific for a single geometry of the complex. Moreover, none of the experimental restraints is violated in a theoretical geometry calculation procedure. In the studied cases, the intermediate exchange regime for the host–guest interaction is observed, as shown by the severe line broadening of the CD resonances. This precludes the quantitative use of the chemical shift changes on complexation for the binding constant evaluation.

In the case of **1** another extreme is observed. Nearly all prednisolone protons have dipolar contacts with the CD protons H3′ and H5′ and the 1D spectrum gives the complexation shifts and the narrow lines showing fast exchange. This strongly suggests a multiple exchange process, therefore the 2D ROESY spectrum gives the pattern of overlapped cross-peaks from multiple discrete geometries. This is also confirmed by the theoretical calculations which yield several geometries. In this case even the discrimination of the predominant mode of binding is not a trivial goal as it requires the back calculation of the crosspeak volumes for the given, theoretically predicted, geometry and a comparison with the experimental ones.

Conclusions

The ^{1}H NMR experiments showed that the inclusion mode of prednisolone, **1**, into the β-cyclodextrin cavity in D_2O differs markedly from that established for other estrogens: ethinyloestradiol **2** and estriol **3**. The results of molecular modelling are in agreement with the experimental data; notably a connection was found between the interactions shown by the ^{1}H NMR chemical shift changes, the 2D ROESY contacts and the proximity of the corresponding protons shown in the computer generated complex structures. Prednisolone **1**, binds weakly and nonspecifically; the process of inclusion is in the fast-exchange regime on the NMR timescale used. Different modes of binding, *i.e.* through the wide rim, or, side-to-side, may be rationalised on the basis of molecular modelling results, which is in contradiction to previous work concerning this molecule. In contrast to **1**, ethinylestradiol **2** and estriol **3**, bind more strongly and the inclusion process is in the intermediate-exchange regime. Guest molecules penetrate deeply into the β-cyclodextrin cavity forming the "transverse" arrangement.

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