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Molecular recognition of steroid isomers by cyclodextrins and cucurbiturils: thermodynamic contributions and structural determinants

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Binding of *cis*- and *trans*-steroids with cyclodextrins (**CD**s) and cucurbiturils (**CB**s) comprised of seven and eight monomeric subunits was investigated to clarify the effects of gonane isomerism and functionalisation on binding stoichiometry and thermodynamic complexation parameters ($K°$, $\Delta_r X°$ where X = G, H, S, C_p). While *trans*-steroids formed both 1 : 1 and 1 : 2 (guest : host) complexes with β-cyclodextrin, the binding of V-shaped *cis*-androsterone resulted solely in the formation of a 1 : 1 complex, revealing that steroid isomerism dictates the binding stoichiometry with this receptor. By contrast, all guests formed exclusively 1 : 1 complexes with **CB7**, **CB8**, and **γ-CD**, whereby the former receptor showed pronounced selectivity for the *trans*-steroids combined with negligible affinity for the *cis*-isomer. Complexation with hosts containing seven monomeric units and with **CB8** was predominantly enthalpically driven, while the favourable entropic term dominated over the enthalpic one in the case of γ-cyclodextrin. Due to steric compatibility, and enthalpically unfavourable hydration of its cavity, the larger cucurbituril outperformed all other studied hosts. A clear correlation between the cyclodextrin affinity for the bile salt anions and the number of OH groups attached to the gonane backbone was observed. In accord, the ROESY NMR spectra of the complexes revealed that this polar group prefers to remain hydrated rather than to engage in interactions with the hosts, in turn shifting the guest binding site from rings A and B towards the non-polar side chain and the ring D. The obtained results demonstrate how the steroid isomerism and functionalisation, as well as cyclodextrin and cucurbituril size determine the inclusion complex stoichiometry and its stability, providing a basis for the rational use of these receptors in recognition, solubilisation, and separation of steroids and their derivatives.

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Introduction

Steroids are a class of compounds present in fungi, plants, and animals, where they act as hormones, structural and signalising membrane constituents, and participate in digestion of fats.[1] Their synthetic derivatives are used as androgen hormone substituents and to achieve contraception,[2] while some of them exhibit powerful anti-inflammatory[3] and antimicrobial properties.[4,5] Anabolic steroids are also illicitly employed to enhance the physical performance of athletes.[6]

All steroids share a common tetracyclic skeleton (gonane or sterane) comprised of four fused hydrocarbon rings (Fig. 1), three of which (rings A to C) incorporate six carbon atoms, while the fourth (ring D) comprises five C atoms. They can exist as either 5α (*trans*) quasi-planar or 5β (*cis*) bent (V-shaped) stereoisomers (Fig. 1). The presence of polar or charged groups attached to the basic structure increases their solubility in water, which at best reaches millimolar values (bile salts). The amphiphilic nature of charged derivatives makes them prone to micellization,[7–10] while the extreme hydrophobicity of

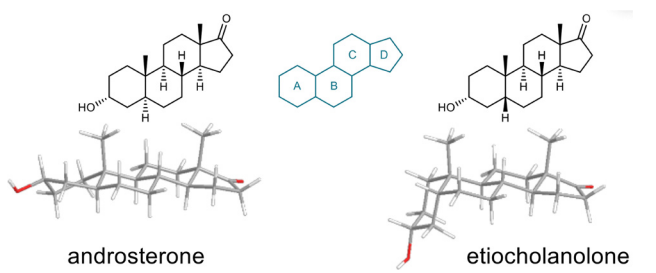

Fig. 1 3D structures of androsterone (5α, *trans*-steroid) and etiocholanolone (5β, *cis*-steroid).

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non-polar cyclic core enables their incorporation in membranes and inclusion within the hydrophobic cavities of cyclodextrins (**CD**s) and cucurbiturils (**CB**s).[11–17] The formed complexes are usually more soluble than the guests, a feature that can be used to control steroid bioavailability.[15,16,18–22] On the other hand, bile salts secreted from the gall bladder commonly displace hydrophobic and amphiphilic pharmaceutically active ingredients from the cyclodextrin cavities, thereby affecting the kinetics of drug absorption from the cyclodextrin-containing formulations in the intestines.[23–27] **CD**s are also used to remove cholesterol from margarine, dairy products, and other complex mixtures.[28–31]

Due to the mentioned solubility issues, the majority of published work deals with the hosting of bile-salt anions by sterically compatible cyclooligomeric receptors comprised of seven or eight monomeric subunits. In the early 1990s, Tan and Lindenbaum reported that such steroids form 1 : 1 complexes with β-cyclodextrin, whereby the inclusion of the ring D and the attached chain within the smaller receptor was noticed, while the guests resided more deeply within the cavity of the larger macrocycle.[32] Intriguingly, the complex stabilities increased with the number of hydrophilic OH groups attached to rings B and C (positions 7 and 12). In contrast, Ramos-Cabrer *et al.* recounted that bile salt anions form 1 : 1 and 1 : 2 complexes (steroid to host ratio) with **β-CD**,[33,34] whereby the first binding site corresponded to that reported earlier,[32] while the additional one encompassed ring A and peripherally ring B. On the other hand, **γ-CD** formed exclusively 1 : 1 complexes.

According to subsequent studies,[26,35,36] bile salt anions formed exclusively 1 : 1 complexes with **β-CD** while the strongly exothermic inclusion suggested the release of high-energy cavity water as the main complexation driving force.[11,14,37–41] Molecular modelling of host–guest systems indicated that the **β-CD** bulkiness prevented the formation of 1 : 2 (anion : host) complexes with a V-shaped anion and the performed NMR studies revealed that the host resides on the C and D rings of the gonane skeleton. That said, the binding of 5α (*trans*) steroids, whose quasi-planar structure does not prevent the formation of higher stoichiometry complexes with **β-CD**, remained unexplored, as did the impact of gonane isomerism on the binding stoichiometry and the complexation thermodynamics with larger **γ-CD**.

The thermodynamically far more favourable binding of a majority of hydrophobic guests with cucurbiturils[15,37,38,42–46] usually enables the determination of the associated equilibrium constants even in the case of quite low guest solubilities. In accordance, Nau *et al.*[15] investigated the hosting of principal sex hormones, contraceptives, cholesterol, and steroid-based muscle relaxants by **CB7** and **CB8** *via* dye-displacement titrations, relying on X-ray crystal diffraction, NMR, and DFT data for structural insights. The complexation with **CB8** was also studied calorimetrically, as was the binding of nandrolone with **CB7**. The complex stability constants (1 : 1 stoichiometry) encompassed a wide range of values ($10^3 < K^\circ < 10^{10}$), revealing a clear preference of all guests for the larger host, which proved to be the best synthetic receptor of quasi-planar steroids reported to this day. Concerning the complex structure, either rings C and D or C and B were present within the **CB7** cavity, while the **CB8** provided enough space for the inclusion of ring A as well. As expected, the examined dications formed the most stable complexes, and the inclusion bore the fingerprint of non-classical hydrophobic effect.[15,37,38,47–50] Nevertheless, the cucurbituril ability to discern between the quasi-planar and bent gonane stereoisomers was not addressed. Namely, despite the fact that **CB**s exhibit much larger affinities for smaller, size-compatible hydrophobic guests than more flexible **CD**s,[14,37,38,51–53] their rigidity could be a disadvantage when hosting sizeable *cis*-steroids, especially in the case of **CB7**.

Based on the presented literature survey and considering the biological importance of steroids, we decided to examine in detail the impact of gonane isomerism on the stoichiometry and thermodynamics of the complexation reactions involving cyclodextrins and cucurbiturils comprised of seven and eight monomeric subunits as hosts. Apart from that, the influence of temperature, position, and the number of attached polar functionalities on the complex stability was (re)addressed. To achieve these goals, the guest library encompassed androstanedione (**A2**), androsterone (**A1**), and its *cis*-isomer etiocholanolone (**E**), quasi-planar testosterone (**T**), and bile salts with varying lipophilicity (Fig. 2). It should be noted that some of the chosen host–guest systems have been studied before (Table 1). As can be seen, the complex stabilities, obtained over a range of conditions vary considerably, and even different binding stoichiometry was obtained in some cases. Moreover, the inclusion was seldom characterised calorimetrically, leaving the complexation thermodynamic underexplored, while the binding of lithocholate anion was not investigated at all.

The herein presented study hence relied on ITC for complete characterisation of the binding event, and the spectroscopic investigations (ROESY NMR) and molecular modelling, using the combination of conformer-rotamer ensemble sampling tool (CREST) based on the GFN methods and DFT computations, jointly provided structural insights into the complexes formed. More specifically, complex geometry screening was implemented though CREST by using the iterative meta-dynamic sampling for non-covalently bound complexes and the analytical linearised Poisson–Boltzmann solvation water model. The best found complex geometries were refined at the SMD(water)–r^{2}SCAN-3c level of theory and the structures were then utilised for qualitative comparison with the 2D NMR spectra. Such a comprehensive approach resulted in a deeper understanding of

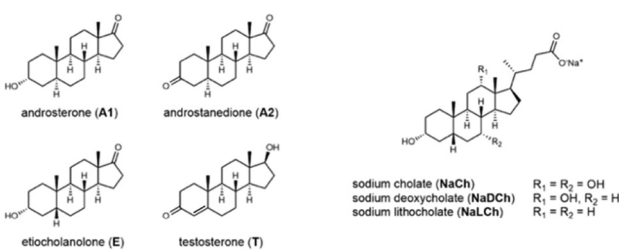

Fig. 2 Structures of investigated steroids and bile salts.

Table 1 Thermodynamic parameters of steroids complexation at 298 K: literature survey

GUEST	HOST	$\log K_i$[a]	Technique, conditions, add. data[a]	Ref.
A1	**β-CD**	2.37	^{1}H NMR titration in D_2O; apparent constant (low guest solubility)	54
T	**CB7**	<3	ITC, H_2O, no appreciable binding, Δ_rH° and Δ_rS° not determinable	15
	CB8	8.04	Competitive ITC in H_2O; $\Delta_rH^\circ = -40.5$ kJ mol^{-1}; $-T\Delta_rS^\circ = -5.4$ kJ mol^{-1}	15
NaCh	**β-CD**	3.50	Flow microcal. in H_2O; $\Delta_rH^\circ = -40.5$ kJ mol^{-1}; $-T\Delta_rS^\circ = -5.4$ kJ mol^{-1}	32
		3.90	^{13}C NMR in D_2O, K from complexation-induced shifts	33
		2.97	Determined from **β-CD**-induced shift of the surfactant CMC	55
	γ-CD	3.51	^{13}C NMR, D_2O, 1 : 1; no calorimetric data	34
NaDCh	**β-CD**	4.79 (GH) 2.86 (GH_2)	Flow microcal. in H_2O; Δ_rH°/kJ mol^{-1} = −21.8 (GH); −19.1 (GH_2); $-T\Delta_rS^\circ$/kJ mol^{-1} = −5.53 (GH); 2.78 (GH_2)	32
		n.a.	^{13}C NMR, D_2O; modelled as a 1 : 2 (guest : host) complex (not directly comparable)	33
		3.48	Determined from **β-CD**-induced shift of the surfactant CMC	55
	γ-CD	3.30	^{13}C NMR, D_2O, 1 : 1, K from complexation-induced shifts	34

[a] The listed successive thermodynamic reaction parameters $\left(K_i^\circ, \Delta_rH_i^\circ, -T\Delta_rS_i^\circ\right)$ correspond to the following processes: G + H ⇄ GH (G : H = 1 : 1; i = 1) and GH + H ⇄ GH_2 (G : H = 1 : 2; i = 2).

the structure–affinity relationship, which should enable far more effective utilisation of cyclodextrins and cucurbiturils as steroid hosts.

Results and discussion

Complexation of neutral steroids

Hosts containing seven monomeric units. The microcalorimetric titrations of neutral steroids with β-cyclodextrin and cucurbit[7]uril (Fig. 3, S1, S2 and S4–S8, SI) allowed for detailed thermodynamic characterisation of the complexation reactions. As evident from data shown in Fig. 3 and S1 (SI) and listed in Table 2, androsterone and testosterone form 1 : 1 and 1 : 2 (guest : host) complexes with **β-CD**, while the reaction of etiocholanolone (*cis*-androsterone) leads solely to the formation of a 1 : 1 complex (Fig. S2, SI). This finding finally resolves the controversies concerning the steroid-binding stoichiometry with this host[32,33] and clearly pinpoints that steroid isomerism dictates the number of coordinating β-cyclodextrin molecules. Specifically, the V-shaped structure of *cis*-gonane (Fig. 1) does not support the attachment of a second **β-CD** molecule, whereas the elongated and open structure of *trans*-steroid isomers readily results in both 1 : 1 and 1 : 2 complexes. However, the conclusion applies solely to guests with peripherally attached polar groups (rings A and D). As will be evident from the data presented in continuation, the presence of polar groups on the central part of the gonane skeleton (rings C and B) strongly affects the binding thermodynamics and can alter the binding stoichiometry as well.

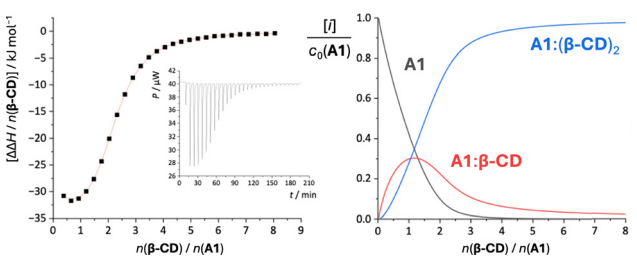

Fig. 3 Microcalorimetric titration of **A1** (c = 5.51 × 10^{-5} mol dm^{-3}, V_0 = 1.45 cm^3) with **β-CD** (c = 2.14 × 10^{-3} mol dm^{-3}) at 298 K (left) and the corresponding distribution diagram (right).

Concerning the complexation thermodynamics, the binding of **β-CD** molecules to the *trans*-isomers and nearly planar testosterone is strongly exothermic (more so for androsterone) and entropically unfavourable or with $\Delta_rS^\circ \approx 0$ (testosterone). This is typical for the formation of **β-CD** inclusion complexes and can be explained by the exothermic release of high-enthalpy cavity water on one hand, and the large loss of translational entropy upon complexation (the formation of one particle out of two) on the other.[13,14,52,53,56,57] It should be noted that the attachment of the first **β-CD** molecule results in somewhat lower enthalpy and entropy changes than the attachment of the second.

Our attempts to deduce the structure of 1 : 1 or 1 : 2 (guest : host) complexes of *trans*-steroids with **β-CD** from the NMR spectra of corresponding mixtures were in vain, as mixing at concentrations higher than those used for ITC experiments led to complex precipitation (no presence of steroids was observed in the supernatant). This is in line with the formation of an insoluble cholesterol complex with **β-CD**, a well-known phenomenon used for cholesterol removal from dairy products.[30,31] Nevertheless, we managed to gain insight into the possible structure of the **A1**:**β-CD** and **A1**:(**β-CD**)$_2$ complexes from the performed computations (Fig. 4, Table S1, SI). As can be seen in Fig. 4, rings C and D partially reside within the **β-CD** cavity in the optimised structure of the adduct, while the attachment of an additional host molecule to the gonane results in the burial of all four rings inside the two hosts. In that sandwich-like structure the macrocycles are facing each other and are therefore able to realise multiple contacts between the hydroxylic groups, thus providing additional system stabilisation.

Regarding the structure of the **E**:**β-CD** complex, both NMR spectra (Fig. S3, SI) and the results of computational investigations (Fig. 4) suggest the burial of rings C and D within the host cavity. Once the structure of the optimised 1 : 1 adduct is examined more closely, one notices that the cavity of β-cyclodextrin cannot readily accommodate the bulky steroid region encompassing rings A and B of this *cis*-gonane. Instead, those rings stay near the secondary rim of the host and the hydroxyl group of ring A interacts with the cyclodextrin alcohol groups *via* hydrogen bonding. This is in

Table 2 Thermodynamic parameters of complexation of **A1**, **E**, **A2**, and **T** with cyclodextrins and cucurbiturils containing seven or eight monomeric subunits at 298 K. Uncertainties of the last digit are given in parentheses as standard errors of the mean (N = 3–5)

GUEST	HOST	G : H	$\log K_i^\circ$	$\Delta_r G_i^\circ$ / kJ mol^{-1}	$\Delta_r H_i^\circ$ / kJ mol^{-1}	$-T\Delta_r S_i^\circ$ / kJ mol^{-1}
A1	**β-CD**	1 : 1	5.19(2)	−29.62(9)	−33.6(2)	4.0(3)
		1 : 2	4.74(1)	−27.04(2)	−46.47(6)	19.43(5)
	CB7	1 : 1	5.45(2)	−31.1(1)	−47.9(4)	16.8(4)
	γ-CD	1 : 1	4.86(1)	−27.76(8)	−8.33(8)	−19.4(2)
	CB8	1 : 1	7.44(8)	−42.5(5)	−46.6(9)	4(1)
E	**β-CD**	1 : 1	4.72(1)	−26.91(3)	−36.97(3)	10.05(1)
	CB7	—[a]	—[a]	—[a]	—[a]	—[a]
	γ-CD	1 : 1	4.76(3)	−27.2(2)	−11.18(4)	−16.0(2)
	CB8	1 : 1	7.38(4)	−42.1(3)	−47.4(5)	5.2(7)
A2	**β-CD**	—[b]	—[b]	—[b]	—[b]	—[b]
	CB7	1 : 1	5.59(4)	−31.9(2)	−44.4(3)	12.4(2)
	γ-CD	1 : 1	4.6(1)	−26.1(6)	−6.1(8)	−20(1)
	CB8	1 : 1	7.49(3)	−42.7(2)	−45.6(2)	3(1)
T	**β-CD**	1 : 1	4.56(2)	−26.0(1)	−25.6(4)	−0.4(5)
		1 : 2	3.85(1)	−21.95(1)	−29.6(3)	7.6(3)
	CB7	1 : 1	5.22(2)	−29.8(1)	−38.9(3)	9.0(4)
	γ-CD	1 : 1	4.51(1)	−25.72(7)	−7.10(2)	−18.62(7)
	CB8	1 : 1	7.3(2)	−42(1)	−37.9(5)	−4(1)

The N-value (in the range of 1.00 ± 0.04) was fitted in cases where an inflection point was observed close to 1 : 1 host : guest ratio. For all other reactions with 1 : 1 binding stoichiometry N was set to 1 as fitting the data with 1 : 1 and 1 : 2 binding model did not yield good agreement with the experimental data. The sequential binding model was used when indicated by an inflection point at approximately 1 : 2 (guest : host) molar ratio. [a] No binding was observed/binding too weak. [b] Results obtained from microcalorimetric titrations of **A2** with **β-CD** (Fig. S4 in the SI) suggest formation of GH and GH_2 complexes but could not be adequately fitted. The listed successive thermodynamic reaction parameters (K_i° and $\Delta_r X_i^\circ$ where $X = G, H, S$) correspond to the following processes: G + H ⇄ GH (G : H = 1 : 1; i = 1) and GH + H ⇄ GH_2 (G : H = 1 : 2; i = 2).

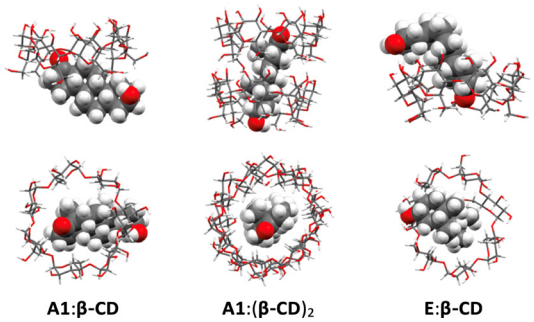

Fig. 4 Representations (side and top views) of the minimised geometries of studied androsterone (**A1**) and etiocholanolone (**E**) complexes computed using the SMD(water)–r^{2}SCAN-3c method. Colour coding: C, grey; H, white; O, red.

agreement with (calorimetrically) observed differences in binding stoichiometries of herein explored *cis*- and *trans*-steroids with **β-CD**.

Unlike the β-cyclodextrin, the barrel-shaped **CB7** forms only 1 : 1 complexes with *trans*-steroid isomers (Fig. S5–S7 in the SI, Table 2) and exhibits extremely low affinity for the *cis*-isomer **E** (the associated equilibrium constant could not be deduced from calorimetric titration experiments (Fig. S8, SI). Furthermore, no significant changes were observed in the ^{1}H NMR spectrum of the mixture containing **E** and **CB7** compared to the **E** solution (Fig. S9, SI). The latter finding is evidently due to the incompatibility of bent etiocholanolone and host geometries, whereas the former one implies that the *trans*-steroid backbone does not support the binding of two **CB7** molecules, whose cavity is considerably deeper than that of **β-CD** (9.1 Å *vs.* 7.8–7.9 Å).[58–60] Accordingly, the optimised geometries of **A1**:**CB7** complex (Fig. S10), and the correlation signals between H-18 and H-19 androsterone methyl groups atoms and H3′ atoms of **CB7** in the ROESY NMR spectra (Fig. 5) confirm the presence of all four rings (B and C completely, A and D peripherally) within the cavity. Observed correlations are consistent with the guest not being fully buried inside the cavity. Specifically, the methyl group (H-18) resides near the portal region, within the NOE distance of the portal-facing CH protons (H-1′ and H-2′), resulting in very weak but observable correlations. It should be noted that the **CB7** ability to discern between *cis*- and *trans*-steroid isomers is of importance not just from the fundamental point of view; rather, it also allows for the aimed recognition of planar gonanes in a solution containing both isomeric forms. This feature can be used to determine the concentration of *trans*-steroid isomers in a multicomponent steroid mixture, or for chromatographic separation of isomers using a carrier with covalently attached **CB7** or a cucurbituril-based eluent, analogous to strategies already implemented for smaller molecules.[61–65]

Turning our attention to the binding thermodynamics, all reactions of *trans*-steroids with **CB7** are enthalpically driven (non-classical hydrophobic effect) and characterised by rather similar $\Delta_r H^\circ$ and $\Delta_r S^\circ$ values. This finding implies a similar structure of all *trans*-gonane complexes with **CB7**. Curiously, when the enthalpies for androsterone and testosterone complexation with **β-CD** (1 : 1 complex) are compared against those for **CB7**, one observes that the inclusion into the cavity of the latter host is not as strongly enthalpically preferred as is in the case of smaller guests containing only one polar group.[37,38,43,51–53,66] This can be attributed to the bulkiness of the gonane backbone, requiring the dehydration of both cucurbituril portals, which is, due to particularly strong hydrogen-

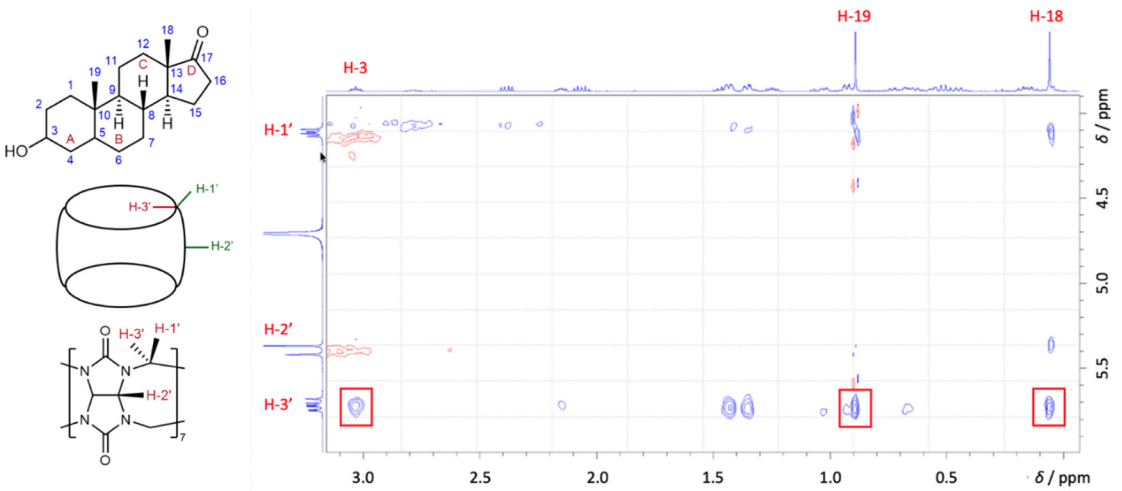

Fig. 5 Partial contour plot of the 2D ROESY NMR spectrum of a mixture containing **A1** (m = 0.15 mg) and **CB7** (m = 0.76 mg) in 500 μL of D_2O at 298 K.

bonding between water and the carbonyl groups, a particularly enthalpically detrimental process.[67,68]

When the herein determined stability constants are compared against the corresponding literature values (Tables 1 and 2), one can observe that our results indicate considerably larger affinity of **CB7** and **β-CD** for androsterone and testosterone. The discrepancy is most likely due to the fact that the previous investigations predominantly relied on NMR experiments for equilibrium constant determination. Namely, this experimental method is far less sensitive than ITC which allows the characterisation of the binding event in the case of quite high complex stabilities ($\log K^\circ \approx 7.5$ for 1 : 1 complexes). Apart from that, our results, unlike earlier findings, clearly demonstrate that these quasi-planar guest form both 1 : 1 and 1 : 2 (G : H) complexes with **β-CD**.

Hosts containing eight monomeric units. The larger cavity of **γ-CD** accommodates only one steroid molecule regardless of the isomeric form (Fig. S11–S18 in the SI, Table 2). Their inclusion is in all cases both enthalpy and entropy driven, whereby the $-T\Delta_rS^\circ$ outperforms the favourable enthalpic contribution to the Δ_rG°. Such thermodynamic signature complies with both classical and non-classical hydrophobic effect, and can be explained by the expulsion of high-energy cavity water and realised host–guest interactions ($\Delta_rH^\circ < 0$) on one hand, and the stronger association of water surrounding the steroid backbone compared to the bulk ($-T\Delta_rS^\circ < 0$) on the other. The weakly exothermic and entropically favourable steroid binding to **γ-CD** *vs.* exclusively enthalpy-driven binding to **β-CD** agrees with the results of previous comparative investigations[14,38,53] and the computational investigation regarding the organisation of the CD cavity water,[37,38] which both imply the far more exothermic dehydration of the seven-membered ring receptor. Namely, although the number of enthalpically-rich (''frustrated'') water molecules is higher in the case of a larger receptor, the hydrogen-bonding patterns of the included water resemble much more those present in bulk water compared to receptors containing seven monomeric subunits. An additional increase in reaction enthalpy and entropy is expected to arise from the dehydration of the classically hydrated steroid backbone, in line with the herein obtained data.

Our results reveal that **γ-CD** poorly discriminates between the steroid isomeric forms ($4.5 < \log K^\circ < 4.9$), suggesting that both the quasi-planar and the V-shaped steroids fit fairly well within the relatively flexible cavity of the larger cyclodextrin. Still, the binding of bulkier *cis*-steroid isomers is somewhat enthalpically preferred, while the opposite holds when comparing the accompanying entropy changes. This implies that the accommodation of the former guests leads to more extensive dehydration of **γ-CD** interior, hence also more favourable host–guest interactions.

Correlation signals between the γ-cyclodextrin cavity and the **A1** steroid backbone and methyl group protons in the ROESY NMR spectra suggest that the atoms of all four guest rings contribute to its binding with **γ-CD** (Fig. 6). Due to the overlap of the cyclodextrin proton signals (H-3′ and H-6′), as well as the overlap of cyclodextrin H-4′ signal and the most deshielded proton H-3 from the androsterone ring A, the orientation of the guest within the cavity could not be experimentally determined. Given the **γ-CD** size, and the unspecific nature of possible intermolecular interactions, it might well be that the **A1** assumes different orientations within the cavity. A computationally found complex geometry depicted in Fig. S19 (SI) elucidates one feasible guest placement: ring A situated on the side of the primary **γ-CD** rim, rings B and C located in the middle cavity, while ring D is consequently placed closer to the secondary rim. Similar conclusions can be drawn for **E**:**γ-CD** complex from the ROESY NMR spectrum (Fig. S20, SI), which is also in line with computationally obtained structures (Fig. S21, SI). It should be noted that compared to **A1**, which assumed a tilted position inside the cavity, leading to elongation of the macrocycle, the inclusion of **E** resulted in more symmetric **γ-CD** conformation.

The binding of all four steroids with **CB8** also results in the formation of 1 : 1 inclusion complexes, which are notably more stable ($\log K^\circ \approx 7$) compared to those formed with **γ-CD**

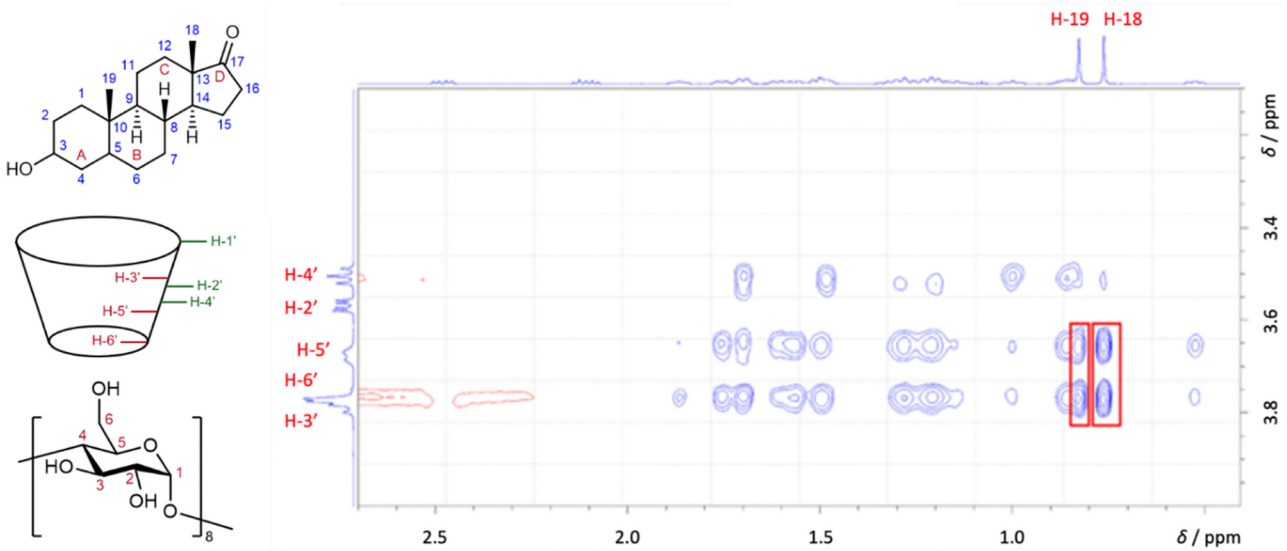

Fig. 6 Partial contour plot of the 2D ROESY NMR spectrum of a mixture containing **A1** (m = 0.15 mg) and **γ-CD** (m = 0.75 mg) in 500 μL of D_2O at 298 K.

(Table 2). This is a consequence of much lower $\Delta_r H^\circ$ values for the binding with the glycoluril-based receptor (the $\Delta_r S^\circ$ for hosting by **CB8** is much lower than for **γ-CD**), attributable to poorer organisation of its cavity water.[38] Similarly to **γ-CD**, the complexation of stereoisomers with **CB8** does not result in notably different standard thermodynamic complexation parameters. The cavity of cucurbit[8]uril is obviously large enough to accommodate both gonane stereoisomers readily, however, the somewhat more exothermic binding of etiocholanolone implies the tighter fit of the bulkier *cis*-isomer. Additionally, it is worth noting that the herein determined standard thermodynamic reaction parameters for the binding of testosterone agree very well with those reported by Nau *et al.*[15]

Due to the very low solubility of **CB8**, the structure of the corresponding steroid complexes could not be deduced by means of ROESY NMR. Insight into the likely positions of androsterone and etiocholanolone (*cis*-androsterone) was again provided by computational investigations (Fig. S19 and S21, SI). In both optimised complex geometries rings A and B were immersed into the **CB8** cavity, however, guest **E** was embedded somewhat deeper, in line with the proposed better fit assumption.

It is interesting to compare the much larger **CB8** affinities for the explored neutral *trans*-steroids with those of **γ-CD** *vs.* rather similar affinities of their counterparts containing seven monomeric subunits. There are two reasons for this trend. First, the hosting of these bulky guests requires the enthalpically unfavourable dehydration of both cucurbituril portals. This process is much less demanding for the larger host. Second, the *trans*-gonane is by its size and shape almost perfectly suited to the **CB8** cavity and less so to that of a shallower **γ-CD**. However, as illustrated in Fig. 4 and S10 (SI), the opposite holds for the smaller hosts. Specifically, the incorporation of *trans*-steroids inside the narrow, elongated **CB7**, in which this large guest cannot assume a tilted conformation, results in its less extensive cavity dehydration compared to the wider and more flexible **β-CD**.

Complexation of bile salts with cyclodextrins

The data listed in Table 3 and compared in Fig. 7 demonstrate that the host affinity for bile salt anions decreases with the number of OH groups attached to gonane backbone. This finding suggests that the favourably hydrated –OH group ($\Delta_{hyd}H^\circ = -36.38$ kJ mol^{-1}, $-T\Delta_{hyd}S^\circ = 10.43$ kJ mol^{-1})[69] prefers to remain solvent-exposed rather than incorporated within the cavity of the examined receptors. The ROESY NMR spectra of the bile salt anion complexes with both cyclodextrins (Fig. S31–S34, SI) corroborate the calorimetrically deduced conclusions.

Namely, the lithocholate side chain, rings C and D, and in part ring B occupy the **β-CD** cavity, whereas no interactions of H-19 protons from the methyl group at the A/B ring boundary with cyclodextrin protons could be observed in the complex with the cholate anion. Furthermore, the strong cross peak between the H-21 protons from the methyl group on the side chain with the H5′ protons of **β-CD** indicates that the carboxyl group protrudes through the primary rim. Concerning the structure of the lithocholate anion complex with **γ-CD**, the spectral data reveal that all three methyl guest groups occupy the cavity. On the other hand, the absence of cross peaks between the H-19 protons from the cholate anion methyl group

Table 3 Thermodynamic parameters of bile salts complexation with **β-CD** and **γ-CD** at 298 K. Uncertainties of the last digit are given in parentheses as standard errors of the mean (N = 3–4)

GUEST	HOST	$\log K^\circ$	$\Delta_r G^\circ$/kJ mol^{-1}	$\Delta_r H^\circ$/kJ mol^{-1}	$-T\Delta_r S^\circ$/kJ mol^{-1}
NaLCh	**β-CD**	6.23(2)	−35.6(1)	−39.9(3)	4.3(4)
	γ-CD	6.29(2)	−35.92(9)	−15.76(3)	−20.2(1)
NaDCh	**β-CD**	3.85(1)	−21.99(3)	−34.6(1)	12.6(1)
	γ-CD	4.44(6)	−25.3(3)	−7.3(3)	−18.0(6)
NaCh	**β-CD**	3.64(2)	−20.8(1)	−28.2(4)	7.5(5)
	γ-CD	3.77(1)	−21.50(2)	−10.98(6)	−10.53(8)

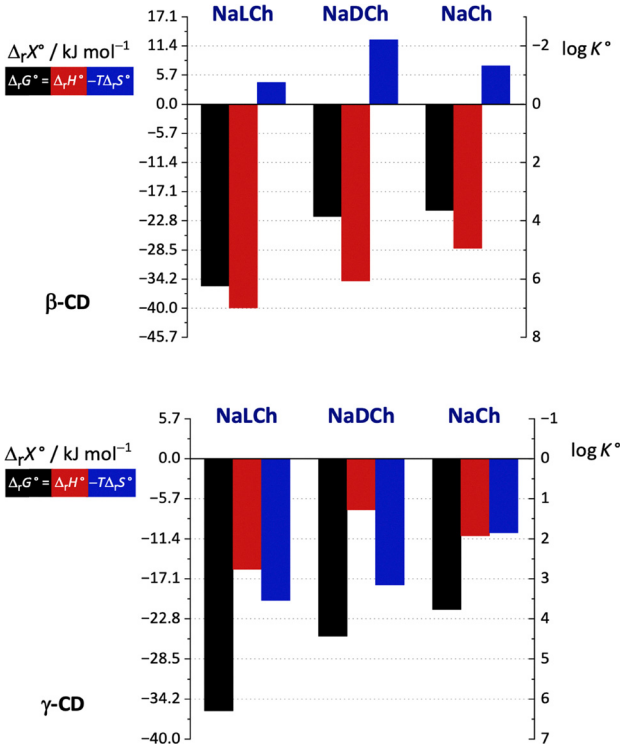

Fig. 7 Thermodynamic parameters for complexation of **NaLCh**, **NaDCh**, and **NaCh** with **β-CD** (above) and **γ-CD** (below) at 298 K.

at the A/B ring boundary with H5′ and H3′ protons of γ-cyclodextrin signals confirms that the hydroxyl group at the C12 atom of this anion prevents the inclusion of rings A and B. The results of computational investigation agree well with the experimental findings and the obtained complex geometries (Fig. S35 and S36, SI) successfully capture the main host–guest interactions found by the NMR analysis. Note that in all optimised structures of the corresponding complexes, the ring A hydroxyl groups reside outside the cyclodextrin cavities and can form hydrogen bonds with the –OH groups at the host rim. Although the performed computations of the host–guest systems with implicitly included water solvation most likely exaggerate the importance of host–guest hydrogen bonds for complex stabilisation, they nevertheless clearly indicate that the guest polar groups avoid the lipophilic cavity of cyclodextrins.

Noteworthy, herein reported stability constants for $\mathbf{Ch}^-$ and $\mathbf{DCh}^-$ complexes with both cyclodextrins are consistent with reported literature values (Table 1). Apart from that, the standard thermodynamic parameters for the hosting of cholate anion by **β-CD** agree well with those for taurocholic and glycocholic acid reported by Holm *et al.*[26] The same holds true for deoxycholate anion and therein investigated analogues,[26] further indicating that the affinity is rather poorly affected by the hydrophilic substituents attached to the sidechain of ring D.

When the Δ_rG° for hosting of bile anions within both **β-** and **γ-CD** are compared (Table 3 and Fig. 7), a correlation between the obtained values and the number of guest OH groups emerges. The same holds for the corresponding dominant contributions (Δ_rH° for **β-CD** and $-T\Delta_rS^\circ$ for **γ-CD**) to standard reaction Gibbs energy. This clearly identifies the hydrophobic effect as the main complexation driving force, simultaneously revealing that the increase in the number of polar groups primarily affects the number of expelled high-energy water molecules and the included area of classically hydrated gonane backbone. Nonetheless, the recent investigations of cyclodextrin and cucurbituril complexations[52,53,56,66,70–72] have pointed out that temperature strongly affects the binding thermodynamics, particularly in the case of cyclodextrins, where even a shift from completely classical (entropy driven binding) to non-classical hydrophobic effect (exothermic, entropy opposed binding) was observed.[52] With this in mind, we performed a series of ITC experiments involving androsterone and the cholate anion as guests and **γ-CD** as the host over the 5–45 °C temperature range (Fig. S28–S30, Tables S3 and S4, SI). The obtained results are described in continuation.

The temperature effect on the binding thermodynamics

To gain insight into the temperature effect on the inclusion of steroids, a neutral *trans*-steroid functionalised with only one peripherally attached hydroxyl group (**A1**) and a *cis*-bile salt anion containing two additional hydroxyl groups on rings B and C (cholate anions) were chosen as guests, while the larger and more soluble **γ-CD**, which accommodates both guests readily, served as a host. In line with previous investigations, the inclusion of both guests[26,52,53,66,70] was characterised by a strong decrease of Δ_rH° and Δ_rS° with temperature ($\Delta_rC_p^\circ/\mathrm{J\ K^{-1}\ mol^{-1}} = -335$ (**A1**) and -328(**Ch**$^-$)), resulting in weak Δ_rG° (T) (enthalpy–entropy compensation). As we demonstrated earlier,[52] the described effect is primarily due to the temperature induced "melting" of guest hydration spheres, which also accounts for the well documented correlation of the $\Delta_rC_p^\circ$ magnitude and the size of dehydrated non-polar surface.[26,52,53,66,70] Since the formation of **A1**:**γ-CD** and **Ch**$^-$:**γ-CD** complexes leads to a considerable dehydration of the host cavity, it is useful to compare the herein obtained $\Delta_rC_p^\circ$ values with those for the binding of bulkier apical and medial diamantane-based alcohols.[53] Namely, significantly more negative values were observed for the inclusion of diamantan-4-ol (-607 J K^{-1} mol^{-1}), assuming a somewhat tilted conformation within the receptor (OH group protruding into the bulk), and for diamantan-1-ol (-715 J K^{-1} mol^{-1}), realising a particularly snug fit. Conversely, the complexation of diamantan-4,9-diol was characterised by substantially higher $\Delta_rC_p^\circ$ value (-440 J K^{-1} mol^{-1}), much closer to those determined for androsterone and cholate anion. This implies that the inclusion of both steroids is accompanied by a partial dehydration of guest polar groups and/or that their hydration spheres are considerably less temperature responsive compared to that of diamantane (*i.e.*, that the organisation of water depends on the structure of non-polar part of the guest). The latter argument is supported by differences in experimental and estimated hydration heat capacities of hydrocarbons of similar size.[69,73–75] This implies that the size of the buried nonpolar surface is by no means the only factor determining the magnitude of

reaction heat capacities for the association of hydrophobic species in water.

Conclusion

The results obtained by means of microcalorimetric titrations, NMR spectroscopy, and molecular modelling demonstrate that the interplay between gonane geometry and functionalisation, as well as the host size and cavity hydration, plays a decisive role in the inclusion of steroids and bile salts into cyclodextrins and cucurbiturils.

Steroid isomerism was found to be a key factor for complexation with hosts containing seven monomeric units (**β-CD** and **CB7**). Namely, while quasi-planar *trans*-steroids formed 1 : 1 and 1 : 2 (guest : host) complexes, the V-shaped *cis*-isomer formed exclusively the 1 : 1-type complex. This rationalises the previously reported differences in steroid:**β-CD** complex stoichiometries and implies that the formation of higher-order complexes requires an elongated, quasi-planar steroid backbone. By contrast, **CB7** formed 1 : 1 complexes with all *trans*-steroids and displayed very low affinity for the *cis*-isomer. Such pronounced discrimination between quasi-planar and bent steroid backbone could serve in selective recognition and/or separation of steroid isomers in mixtures.[61–64] Importantly, the microcalorimetric experiments revealed far larger affinity of **CB7** and **β-CD** for quasi-planar steroids than earlier findings.

The binding with receptors comprising eight monomeric subunits was significantly less affected by the steroid isomerism, whereby all investigated guest molecules formed exclusively 1 : 1 complexes with **CB8** and **β-CD**. As anticipated, the complexes with **CB8** were (significantly) more stable, due to exceptional host–guest size and shape compatibility and the far more exothermic dehydration of the cucurbituril cavity.

The thermodynamic fingerprint of the studied supramolecular processes was a function of the receptor cavity size, and characteristic of the two receptor families. That is to say, the binding to **β-CD** and cucurbiturils was predominantly enthalpically driven (non-classical hydrophobic effect) and entropically opposed, whereas in the case of **γ-CD**, the complexation was predominantly entropically controlled (classical hydrophobic effect).

Concerning the hosting of bile salt anions by the larger cyclodextrin, a correlation between complex stability constants and the number of hydroxyl groups in the gonane backbone was observed, suggesting that polar functionality prefers to stay hydrated rather than buried within the cavity. This in turn resulted in shallower inclusion of the less lipophilic guests and shifted the binding site towards the D-ring and the cavity.

Thermodynamic investigations performed from 5 to 45 °C revealed pronounced enthalpy–entropy compensation and typically negative $\Delta_r C_p^\circ$ values. The observed selectivity of **β-CD** and **CB7** toward specific steroid isomers, together with the high affinity of cucurbit[8]uril, provide opportunity for further implementation of these compounds in solubilisation of steroids, their recognition, sensing, separation, and related application particularly in pharmaceutical and food industry.

Materials and methods

Materials

Androsterone (**A1**, ≥98%), testosterone (**T**, ≥99%), lithocholic acid (**HLCh**, ≥95%), deoxycholic acid (**HDCh**, ≥99.0%), etiocholanolone (**E**, ≥98%) and macrocyclic receptors: β-cyclodextrin (**β-CD**, ≥98%), γ-cyclodextrin (**γ-CD**, >99.0%) and cucurbit[7]uril (**CB7**, hydrate) were purchased from Sigma Aldrich, sodium cholate (**NaCh**, ∼98%) was purchased from Fluka and used as received. All receptors were dried at 150 °C for 3 h prior to use. Androstanedione (**A2**) was synthesised through the oxidation of **A1**, whereas sodium deoxycholate (**NaDCh**) was synthesised from **HDCh**. Sodium hydroxide (Gram-Mol, *pro analysi*) was used for the deprotonation of bile acids. MiliQ water was used as a solvent for microcalorimetric experiments and deuterium oxide (Sigma Aldrich, >99.9% D) for NMR experiments. The above-mentioned solvents were used without further purification.

Methods

Microcalorimetry. ITC measurements were performed using Malvern Microcal VP-ITC (V_{cell} = 1.43 or 1.45 cm^3) and PEAQ-ITC (V_{cell} = 0.205 cm^3) calorimeters. All titrations were carried out by stepwise addition of the host (titrant) solution to a solution of the steroid guest (titrand), the only exception being **CB8** due to its low solubility. The concentration (c_0 from 8.30 × 10^{-4} to 1.10 × 10^{-2} mol dm^{-3}) and volume ($V_{addition}$ from 10 to 20 μL (VP-ITC), 1 to 2 μL (PEAQ-ITC)) of the titrant were varied depending on the concentration of the titrand solution (c_0 from 4.86 × 10^{-5} to 5.57 × 10^{-4} mol dm^{-3}). Constant stirring was applied and, depending on temperature, the time between additions varied from 350 to 600 s (VP-ITC) or 100 to 150 s (PEAQ-ITC). Blank experiments were carried out for each experiment, and the heats of the titrant dilution were subtracted from those measured in the titration experiments.

Microcal OriginPro 7.0, Microcal PEAQ-ITC Control Software, Microcal PEAQ-ITC Analysis Software (all supplied by the manufacturer), and HypDH from HYPERQUAD program package[76] were used for data acquisition and processing. The experimental data were fitted to a 1 : 1 or 1 : 2 (guest : host) complex stoichiometry. All ITC titrations were conducted at least in triplicate, and the determined thermodynamic parameters are reported as mean values with the standard errors of the mean provided as a measure of uncertainty. Isobaric reaction heat capacities ($\Delta_r C_p^\circ$) were determined by weighted linear regression analysis of $\Delta_r H^\circ$ *vs.* T dependence.

NMR spectroscopy. NMR experiments were performed in D_2O at 298 K using a Bruker Avance Neo 600 MHz/54 mm spectrometer equipped with a 5 mm inverse broadband room temperature probe, PA BBI 1H/D-BB. The structure of the complexes was investigated using 2D ROESY NMR spectra with water suppression using the standard Bruker pulse sequence ROESYPHPR. For the assignment of correlation signals in the ROESY spectra, COSY, HSQCed, and HMBC spectra of the complexes and individual components in deuterium oxide were used. Standard Bruker pulse sequences were used for recording

all spectra. The spectra were processed using the Bruker TopSpin 3.6.3 software package.

Computational investigation. Search for energetically favourable complex structures was performed using the conformer-rotamer ensemble sampling tool (CREST) based on the GFN methods[77,78] by applying the iterative meta-dynamic sampling for non-covalently bound complexes, clusters or aggregates (NCI-iMTD mode). In these computations the analytical linearised Poisson–Boltzmann (ALPB) solvation model was also used to account for the implicit influence of water. The identified best complex structures were further refined at the SMD(water)–r^{2}SCAN-3c level of theory[79,80] using the Orca 6.0 program package[81–83] and the obtained minima were verified by frequency computations.

Author contributions

Conceptualization: AU, JP; data curation: AU, KP, KKG, SK, MŠ; formal analysis: AU, KP, KKG, SK, MŠ, JP; funding acquisition: JP; investigation: AU, KP, KKG, SK, GT, MŠ, JP; methodology: MŠ, JP; project administration: JP; resources: JP, MŠ; supervision: AU, JP; validation: AU, GT, MŠ, JP; visualization: AU, KP, KKG, SK, MŠ; writing – original draft: AU, JP; writing – review & editing: AU, KP, MŠ, JP.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d6nj02208a.

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