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Nanomolar Binding of Steroids to Cucurbit[*n*]urils: Selectivity and Applications

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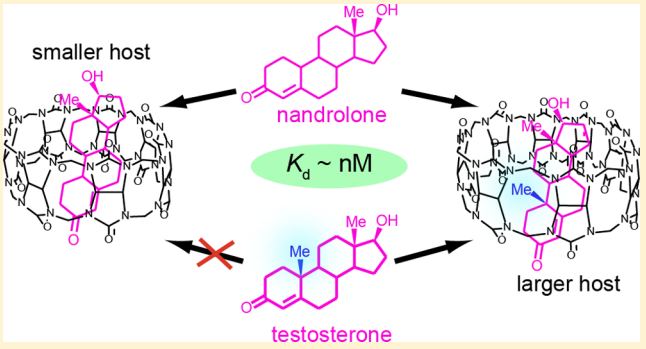
Supporting Information

ABSTRACT: Cucurbit[*n*]urils (CB*n*, *n* = 7, 8) serve as artificial receptors for steroids (21 tested), including the hormones testosterone and estradiol as well as steroidal drugs. Fluorescence displacement titrations and isothermal titration calorimetry (ITC) provided up to nanomolar binding affinities in aqueous solution for these hydrophobic target molecules, exceeding the values of known synthetic receptors. Remarkable binding selectivities, even for homologous steroid pairs, were investigated in detail by NMR, X-ray crystal diffraction, ITC, and quantum chemical calculations. Notably, the CB*n*•steroid complexes are stable in water and buffers, in artificial gastric acid, and even in blood serum. Numerous applications have been demonstrated, which range from the solubility enhancement of the steroids in the presence of the macrocycles (up to 100 times, for drug delivery) and the principal component analysis of the fluorescence responses of different CB*n*•reporter dye combinations (for differential sensing of steroids) to the real-time monitoring of chemical conversions of steroids as substrates (for enzyme assays).



INTRODUCTION

Steroids occur in all biological species, where they carry out important functions (e.g., as membrane components or hormones). Many synthetic drugs, notably anti-inflammatory and contraceptive ones, are steroidal. Biological steroid receptors display a very high specificity and nanomolar affinity to their targets,[1] while natural steroid-transporting proteins, such as albumin, show lower selectivities and millimolar to micromolar affinities.[2] Artificial steroid binders are in demand as chemosensors, solubility enhancers, and drug carriers or stabilizers. Several cyclophanes, cavitands, and cyclodextrins (CDs) are known to bind certain steroids—often cholesterol—with typically millimolar affinities in water.[3−8] One notable exception is the polyanionic CD derivative sugammadex, which has been designed for removal of synthetic steroidal cationic neuromuscular blocking agents.[9] In fact, CDs are approved for use in pharmaceutical steroid formulations,[10−12] but artificial steroid binders with higher affinities and selectivities are required to eventually allow sensing at their typical nanomolar concentrations[13,14] and to reduce unwarranted cargo carrier dissociation of steroid receptor complexes, especially for intravenous or depot injection drug administration.

Because of the absence of conventionally exploited recognition motifs (e.g., hydrogen bonds, charges), the toolbox for steroid binding is limited to the hydrophobic effect and the recognition of their peculiar size or shape. Sufficiently large hosts are capable of tightly immersing the steroidal skeleton and are therefore prime candidates as high-affinity steroid binders. In addition, concave hosts contain high-energy water molecules whose release adds a driving force to host−guest complex formation in aqueous media (the nonclassical hydrophobic effect).[15−19]

Based on simple size-fitting arguments[20,21] and their known high affinities for hydrophobic guests,[20−26] the cucurbit[*n*]uril macrocycles CB7 (*n* = 7) and CB8 (*n* = 8) should serve as promising receptor candidates for steroidal compounds, which we have now tested. In fact, Isaacs and co-workers[27] as well as Macartney et al.[28] have already investigated CB7 and acyclic sulfonated variants of CB structures for *cationic* synthetic steroidal drugs, which encouraged us to conduct a systematic study of 21 representative *naturally occurring* steroids and steroidal drugs (Figure 1). The resulting nanomolar binding allowed us to explore a broad range of highly relevant biological applications.

RESULTS

Our steroid library (Figure 1) included the principal male and female sex hormones, testosterone and estradiol, the main component of oral contraceptives, 17α-ethinyl estradiol, the

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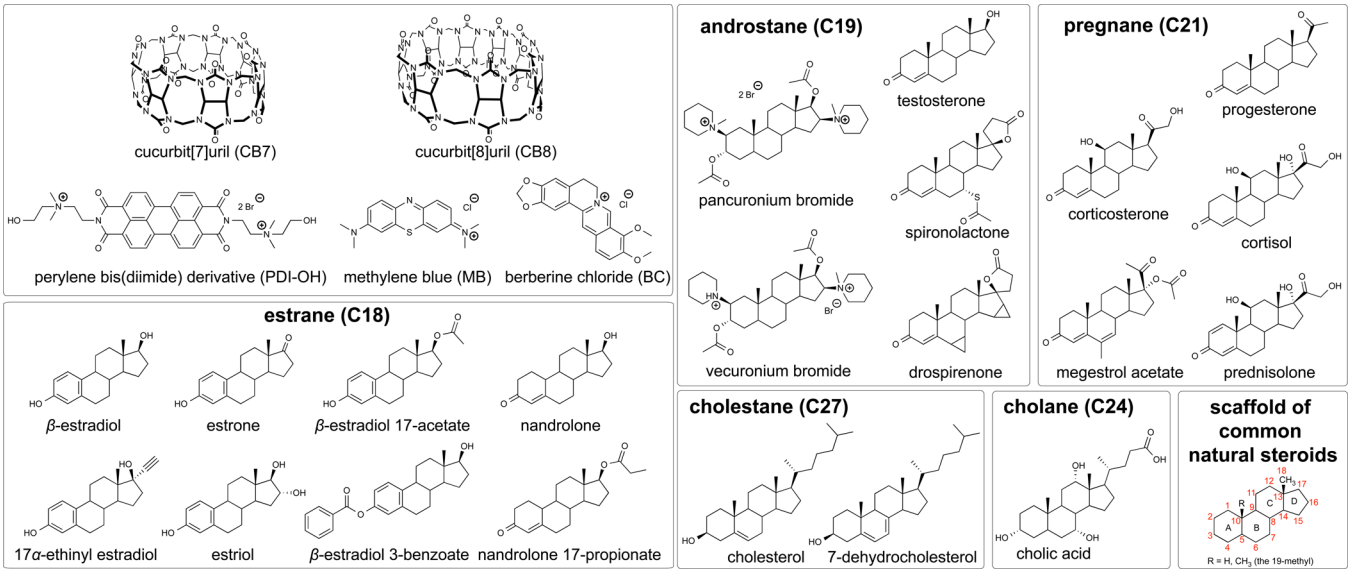

Figure 1. Chemical structures of the cucurbit[*n*]uril macrocycles, the reporter dyes, and the investigated steroids.

membrane component cholesterol, and the muscle relaxants, pancuronium and vecuronium. The anabolic nandrolone (19-nortestosterone) was used as a prototype because its high water solubility (~1 mM)[29] allowed several characterization techniques to be used.

Structural Characterization. Direct evidence for complex formation of nandrolone with CB7 and CB8 was obtained by ^{1}H NMR titration experiments in D_2O, which showed the appearance of additional, mostly upfield shifted, peaks (slow exchange) upon addition of the cucurbit[*n*]uril (CB*n*) macrocycles. For instance, upon addition of CB8 to a solution of nandrolone, a singlet at 0.11 ppm (previously at 0.67 ppm; CH_3 group) and a triplet at 3.10 ppm (previously at 3.55 ppm; CH−O group) emerged, suggesting that the steroid ring D (for nomenclature and atom numbering, see the scaffold of common natural steroids in Figure 1) is immersed in the cavity of the host (Figure S1).[30] In contrast, ring A was found to be mainly located at the CB8 portals (no shift for the CH proton). Similar observations were made for nandrolone binding with CB7 (Figure S2), which shows that nandrolone adopts a similar binding geometry with both homologues. Note that the complete disappearance of the signals associated with free nandrolone after addition of stoichiometric amounts of CB*n* gave a first indication of very strong binding.

^{1}H NMR measurements were feasible for all members of the steroid library (Figure 1) because the presence of the macrocycles caused a sufficiently large solubility enhancement even for the least soluble steroids (see below). The characteristic complexation-induced ^{1}H NMR chemical shifts were observed in all cases (see Supporting Information Figures S1−S19), such that the structural assignments could be conducted analogously to nandrolone. For example, in the case of nandrolone and testosterone, the C and D rings are immersed inside the CB7 cavity, while in the case of drospirenone and progesterone, the B and C rings are complexed.[30]

We were fortunate to grow crystals of the CB7•nandrolone complex and, thus, to obtain atomically resolved structural information about the solid-state inclusion geometry by X-ray diffraction (XRD) experiments (Figure 2).[31] The derived 3D representation of the CB7•nandrolone complex reveals the inclusion of the steroid C and D rings inside the host cavity,

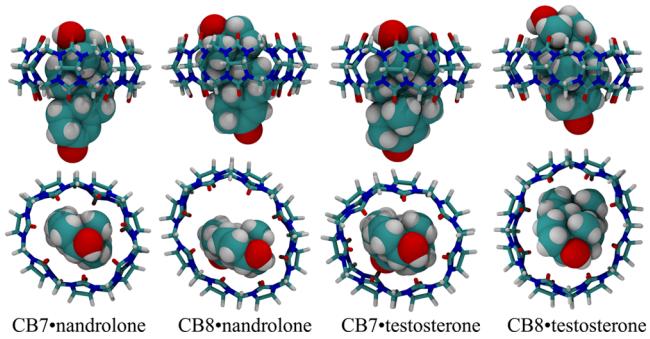

Figure 2. Side and top views of the complex structures of nandrolone and testosterone with CB7 and CB8. Structures were calculated (DFT, wb97xd/6-31G*) except for CB7•nandrolone (experimental XRD structure; see Figure S28 for calculated structure).

consistent with the ^{1}H NMR solution results.[30] Dispersion-corrected quantum chemical calculations (DFT, wb97xd/6-31G*) resulted in a CB7•nandrolone complex geometry (Figure S28) that was virtually identical to the XRD structure. The structure of the CB8•nandrolone complex, for which no crystals could be grown, was therefore calculated with the same method (Figure 2); it showed again the preferential inclusion of the C and D rings, consistent with the ^{1}H NMR results.

Structural analyses through a combination of NMR and DFT calculations also exposed some distinct differences. For example, while the binding geometries of nandrolone were qualitatively similar for CB7 and CB8 as hosts, those for the slightly larger testosterone differed markedly between CB7 and CB8 (Figure 2, Figure S28, and Table 1). The larger CB8 cavity offers sufficient space for the steroid to adopt a slightly tilted coconformation, and in this case, the depth of immersion depends on the presence of the 19-methyl group (=R in general steroid structure in Figure 1), which is located at carbon 10 and connects rings A and B. Without this methyl group, the A ring remains excluded from the cavity, while with it, the B ring becomes centrally immersed, allowing both the A and C rings to be immersed as well.[30] The presence of the 19-methyl group emerged therefore as a critical element for steroid binding, a

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Table 1. Thermodynamic Data for the CB7 and CB8 Complexes with Selected Steroids[a]

host	steroid [PC/%][b]	K (10^6 M^{-1})	ΔH (kJ mol^{-1})	$-T\Delta S$ (kJ mol^{-1})
CB7	nandrolone [70]	11.2 [3.7][c]	−52.2 [−40.9][c]	12.0 [3.4][c]
	testosterone [73]	<0.001		
	prednisolone	<0.001		
	cholic acid	<0.001		
	vecuronium[d]	0.47 [0.22][e]		
	pancuronium[d]	0.59 [0.13][e]		
CB8	nandrolone [56]	21	−37.6	−4.2
	testosterone[f] [59]	110	−40.5	−5.4
	prednisolone	1.4	−29.9	−5.1
	cholic acid	0.19	−46.2	16.0
	vecuronium	[6200][g]	−41.2[h]	14.7[i]
	pancuronium	[202][g]	−34.7[h]	12.7[i]

[a]Binding constants and enthalpies as well as entropies of binding were obtained by ITC experiments performed at 25 °C in water; typical errors, determined by repeating the CB8 titration with nandrolone six times, are 15% in K, 2.0 kJ mol^{-1} in ΔH, and 2.5 kJ mol^{-1} in $T\Delta S$. [b]Packing coefficient in square brackets; the PC value from the experimental CB7•nandrolone XRD structure is 69%. [c]Measurement in square brackets performed in 10 mM sodium phosphate buffer. [d]A 2:1 host−guest binding stoichiometry with identical affinities for both binding sites has been found by Macartney et al.[28] The tabulated values refer to CB7 binding to one binding site. The ΔH and $T\Delta S$ could not be accurately determined. [e]Reported in ref 28. [f]ITC measurement performed by a competitive titration with displacement of methyl viologen. [g]Value in square brackets obtained by competitive fluorescence titrations with CB8•$(BC)_2$. [h]Binding constants above 5×10^7 M^{-1} could not be reliably obtained by direct ITC titrations. Nonetheless, the corresponding ΔH values could be measured accurately.[33] [i]Calculated from the K (and derived ΔG) value and the ΔH value given in this table.

conclusion that was confirmed by the trends in affinity constants (see below).

Binding Constants by Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) experiments with nandrolone as guest and CB7 and CB8 as hosts were carried out in aqueous solution. The data (Figure 3) verified the

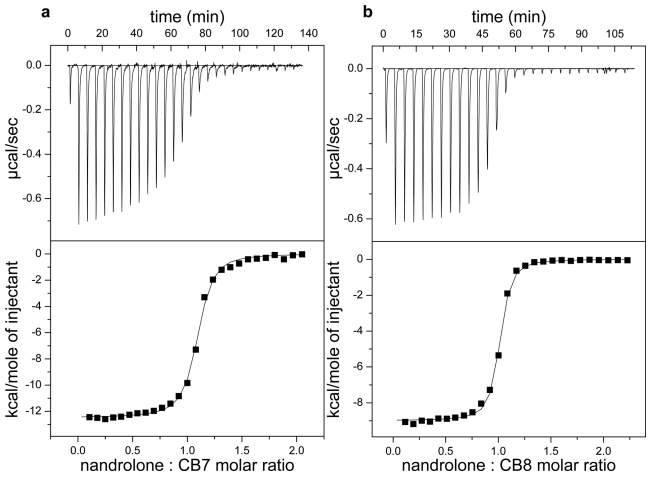

Figure 3. ITC isotherms for the titration of nandrolone (200 μM) into (a) CB7 (23 μM) and (b) CB8 (18 μM) in aqueous solution at 25 °C.

postulated 1:1 binding ratio.[32] The ITC experiments afforded binding constants of 1.1×10^7 M^{-1} with CB7 and 2.1×10^7 M^{-1} with CB8 (Tables 1 and 2). Such high absolute values for binding of a natural anabolic steroid by synthetic receptors in water are unmatched. For exemplary comparison, β-CD has a maximum binding affinity toward steroids of 10^5 M^{-1}, reported for taurochenodeoxycholate, a negatively charged bile acid. Cyclodextrins designed for natural steroid binding, that is, with appended hydrophobic binding units, do not exceed affinity values of 5.7×10^6 M^{-1}, which were reached for lithocholic acid.[3]

Table 2. Binding Constants of Steroids with CB8[a]

steroid[b]	K (10^6 M^{-1})	steroid[b]	K (10^6 M^{-1})
vecuronium	6200[c]	pancuronium	200[c]
drospirenone	115[c]	testosterone	110
progesterone	93 [81][d]	nandrolone	[21][e]
megestrol ac.	16	corticosterone	13
β-estradiol 17-ac.	6.5	nandrolone 17-prop.	4.9
cortisol	4.0	estrone	2.5
β-estradiol	1.9	17α-ethinyl estradiol	1.8
β-estradiol 3-bz.	1.8	prednisolone	1.0
spironolactone	1.0	estriol	0.8
cholic acid	0.2 [0.2][c]	7-dehydrocholesterol	<0.1[f]
		cholesterol	<0.1[f]

[a]Obtained by competitive fluorescence titration experiments with BC as reporter dye; 20% error. [b]Abbreviations: ac., acetate; bz., benzoate; prop., propionate. [c]Aqueous steroid stock solution. [d]PDI−OH as reporter dye. [e]Internal standard; determined by ITC (see Table 1). [f]No significant fluorescence change.

ITC experiments could be performed for some additional (Table 1), but not all, steroids because a favorable combination of sufficient intrinsic water solubility and affinity was required. Besides nandrolone, this requirement was met for testosterone, prednisolone, cholic acid, vecuronium, and pancuronium. Note that the presence of the additional 19-methyl group in testosterone, prednisolone, and cholic acid suppressed the binding of these steroids to CB7, while the binding with CB8 followed the order of their hydrophobicity, that is, testosterone > nandrolone > prednisolone > cholic acid. Vecuronium and pancuronium benefit from their cationic nature, which matches the cation receptor properties of CBs;[23,34,35] this leads to the largest binding constants with CB8. Conversely, CB7 cannot slide onto the steroidal skeleton of vecuronium and pancuronium for steric reasons; here, 2 equiv of CB7 were reported to bind to the two piperidine substituents.[28]

Thermodynamic Parameters by ITC. The formation of the CBn•nandrolone complexes is enthalpically driven, with

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higher values for CB7 than for CB8 (Table 1). This is typical for the formation of CB*n* inclusion complexes with hydrophobic guests and originates from the high-energy water release from the host cavity.[15] It is interesting to note that the formation of the CB8 complexes with nandrolone, testosterone, and prednisolone are also entropically favored, which is in accordance with the "classical" hydrophobic contribution of the steroid.[19] Conversely, the complexation of nandrolone by the smaller CB7 is strongly entropically disfavored, which points to a binding mode tighter than that for CB8, in accordance with the calculated packing coefficients (PC values in Table 1); for CB7, these are significantly larger than the optimal value of ∼55%.[20,21,36]

Importantly, the enthalpic and entropic contributions varied strongly in the presence of salt, for example, $\Delta\Delta H$ = 11 kJ mol^{-1} and $-T\Delta\Delta S$ = −9 kJ mol^{-1} for CB7•nandrolone in 10 mM phosphate buffer versus neat water (Table 1), while the corresponding free energy of binding was much less affected ($\Delta\Delta G$ = 3 kJ mol^{-1}, corresponding to the observed factor of 3 difference in K values). An enthalpy−entropy compensation applies, which leads to comparably small salt effects on steroid binding to CB*n*.

Binding Constants by Dye Displacement Titrations. The binding of nandrolone to CB7 and CB8 could be independently determined by optical displacement titrations, which are based on the competitive displacement of a chromophoric or fluorescent dye from a macrocycle by an analyte.[37−40] Several reporter dyes suitable for displacement titrations with CB7 are known.[20,39,41] In this study, we selected berberine chloride (BC) and methylene blue (MB) as pH-insensitive dyes for CB7 as host. Their affinities for CB7 are $K_{CB7•BC}$ = 1.6 × 10^6 M^{-1} for BC[42] and $K_{CB7•MB}$ = 1.3 × 10^7 M^{-1} for MB.[43] The displacement of CB7•BC[44,45] by addition of nandrolone yielded affinities in neat water of 4.8 × 10^7 M^{-1} (cuvette-based measurement) and 3.7 × 10^7 M^{-1} (microplate-based measurement) by fitting the fluorescence titration data; the resulting K values are in good agreement with the affinities for CB7•nandrolone obtained by ITC (1.1 × 10^7 M^{-1}), considering the variation in methods.

The BC dye and the perylene bis(diimide) derivative PDI−OH dye are suitable reporter dyes for CB8.[46] Their effective binding constants were taken as 2.0 × 10^7 and 1.0 × 10^5 M^{-1}, respectively, as determined by competitive titrations with nandrolone, a steroid whose binding constant was accurately known from ITC (Table 1). This indirect referencing (see Supporting Information) was required because of the different complexation stoichiometries for BC (2:1)[20,47,48] and PDI−OH (1:1)[49] with CB8. Figure 4 shows several dye displacement titrations for nandrolone and cholic acid binding to CB8. The values extracted for cholic acid were 1.9 × 10^5 and 1.6 × 10^5 M^{-1} with BC and PDI−OH as reporter dyes, respectively, in good agreement with the constant of 1.9 × 10^5 M^{-1} obtained by ITC (Table 1).

The emission-based method has the advantage that it operates—in contrast to NMR and ITC titrations—at very low (micromolar) concentrations, which also allows poorly water-soluble analytes to be tested with high sensitivity.[50] Indeed, fluorescence displacement titrations could be applied to all 21 steroids (Table 2), although weak binders caused no displacement in the dilute solutions, such that only upper limits of the binding constants could be provided for some guests (such as for cholesterol with CB8, Table 2). It was found that CB8 shows a promiscuous, high-affinity binding character for

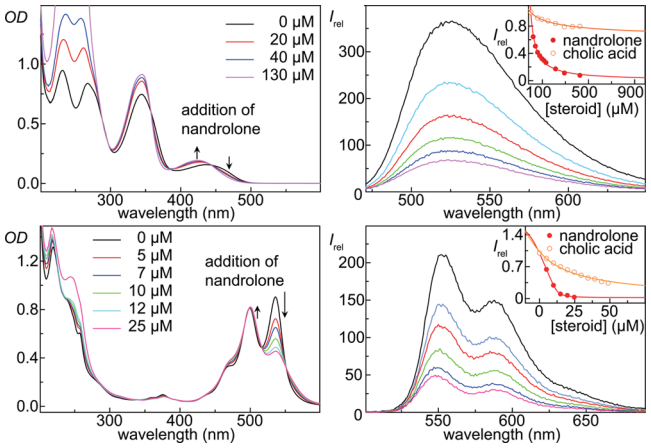

Figure 4. UV−vis (left) and fluorescence (right) spectra of 45 μM BC (λ_{exc} = 445 nm) with 20 μM CB8 (top) and 20 μM PDI−OH (λ_{exc} = 420 nm) with 15 μM CB8 (bottom) aqueous solutions upon addition of nandrolone or cholic acid. Insets: Fluorescence emission intensities at 520 nm (top) and 550 nm (bottom) upon addition of different concentrations of cholic acid.

all steroids (Tables 1 and 2), while CB7 is selective for vecuronium, pancuronium, and nandrolone since only those were found to bind with high affinities (Table 1).

The comprehensive selectivity data from the fluorescence screening experiments confirmed the conclusions that had emerged already from the structural case studies (see above), for example, in regard to the strong binding of steroids with a 19-methyl group to CB8 versus the steric hindrance of the same group when CB7 was offered as host (cf. K values of testosterone and nandrolone), but showed also that a positioning of an OH group inside the CB cavities is unfavorable (see values for corticosterone and progesterone), presumably because the required desolvation of this polar group reduces the driving force for immersion. The combined absolute affinities also exposed trends in hydrophobicity. Thus, we observed a binding of testosterone to CB8 2−3 times stronger than that of nandrolone (Tables 1 and 2), in line with their log$P$ values (3.3 for testosterone versus 2.6 for nandrolone).[51]

Solubility Enhancement. NMR spectroscopy was used to probe the solubility enhancement of the steroids in the presence of the macrocycles (Table 3 and Table S2), providing important pharmaceutical benchmarks.[52−55] Solubility enhancements served also as tangible evidence for binding, even where spectroscopic titrations pointed to weaker affinities, such as for testosterone with CB7.

Stability of the Complexes in Biological Fluids. Potential applications of CB*n*•steroid complexes require the stability of CB*n*•steroid complexes not only in water but also in complex biological fluids. This is a frequent obstacle for applications of macrocycles because their affinities may drop steeply in the presence of additives.[56,57] Specifically, for the larger CB*n* hosts, the affinities decrease frequently significantly in the presence of salts (cations).[58,59]

Stability in gastric acid (for oral administration) and blood serum (for intravenous administration or steroid detection in blood samples) is of particular importance. Consequently, we investigated the stability of the CB7•nandrolone and the CB8 complexes of nandrolone, testosterone, and progesterone in an artificial gastric acid fluid (150 mM DCl, 150 mM NaCl, 20 mM KCl; 0.32 M total ionic strength) by ^{1}H NMR

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Table 3. Aqueous Solubilities and Solubility Enhancement Factors of Naturally Occurring Steroids in D_2O in the Presence of CB8 (<100 μM) and CB7 (4 mM), Respectively, As Determined by ^1H NMR

	solubility (μM)	enhancement factor	
steroid	no host	CB8	CB7
drospirenone	36[a]	2	≥4
testosterone	114[b]	2	≥5
progesterone	33[c]	8	21
nandrolone	810[d]	<2	5
megestrol acetate	5[e]	72	74
corticosterone	314	2	<2
cortisol	696[f]	<2	<2
estrone	48[g]	2	11
β-estradiol	9[h]	7	120
17α-ethinyl estradiol	30[i]	<2	<2
β-estradiol 3-benzoate	1[j]	33	36
prednisolone	483[k]	<2	2
spironolactone	48[l]	5	<2
estriol	75[m]	2	7
cholic acid	324[n]	<2	2
cholesterol	0.2[o]	120	255

[a]Alternative values: 6 μM in ref 60. [b]83 μM,[61] 104 μM,[62] 161 μM.[29] [c]29 μM,[61] 35 μM,[62] 25 μM.[29] [d]1003 μM.[29] [e]Ref 60. [f]786 μM,[61] 897 μM.[29] [g]Ref 63. [h]18 μM,[61] 48 μM,[63] 11 μM.[29] [i]34 μM,[61] 16 μM,[63] 34 μM.[29] [j]Ref 61. [k]596 μM,[61] 481 μM.[29] [l]0.1 μM.[60] [m]45 μM.[63] [n]120 μM,[64] 235 μM.[65] [o]Ref 62.

spectroscopy (see Figure S26). Similar to the findings in neat D_2O, upfield shifts of the methyl protons were observed in the presence of CB7, indicating that the CB7•steroid complexes are stable in gastric fluid. For the less soluble CB8, a dye displacement assay was used to assess steroid binding in the presence of 1 M potassium phosphate buffer or blood serum. In both media, steroid addition to the CB8•reporter dye complex caused dye displacement, indicating that steroid binding to the macrocycle occurred (see Figure S27). Consequently, CB7 and CB8 retain their high propensity to complex steroids also in the presence of saline buffers and complex biological fluids.

Differentiation of Steroids by Principal Component Analysis. The detection of steroids by fluorescence displacement titrations opens the door for numerous sensing applications. For example, the dye displacement measurement format can be transferred from quartz cuvettes to disposable microtiter plates to allow rapid and economic screening applications (see Figure 5). Furthermore, the method can be used to measure binding constants, and when the binding constant of an analyte is known, its concentration can be determined. Moreover, each steroid gives a distinct response to different reporter pairs (composed of different dyes and/or hosts; see above), which can be employed for differential sensing, that is, to identify the nature of an analyte through the fluorescence response pattern.[66,67] To demonstrate, the emission intensity response changes were measured in microplate format for the repetitive addition of a representative set of steroids to several CB*n*•reporter dye combinations (i.e., CB7•BC, CB7•MB, CB8•PDI−OH, and CB8•$(BC)_2$). Mathematical treatment by principal component analysis (PCA)[68] resulted in a three-dimensional score plot that is shown in Figure 5. This demonstrates that 10 steroids of the 12 tested can be uniquely identified by their differential response to different reporter pairs. Only the progesterone/testosterone

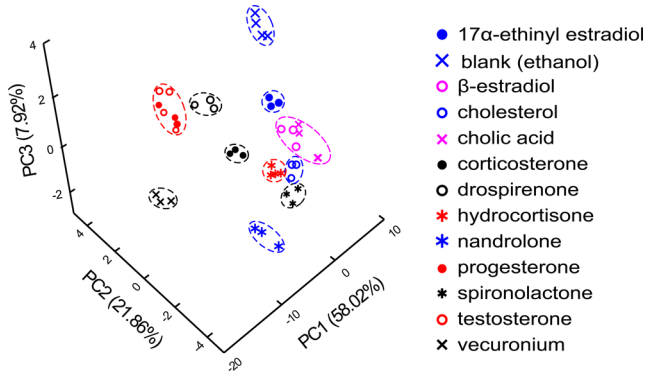

Figure 5. Score plot of the first three principal components obtained by PCA of 12 steroidal analytes (+blank). The percent of total variance is given in brackets for each principal component. The data used were obtained as the fluorescence response observed by addition of varying concentrations of the steroidal analytes to CB7•BC, CB7•MB, CB8•PDI−OH, and CB8•$(BC)_2$ reporter pairs in a microplate format (see Supporting Information).

and β-estradiol/cholic acid pairs showed overlapping confidence intervals, which could be potentially remedied by expanding the sensor array. We are unaware of any other artificial sensor array with comparable performance in steroid recognition.

Real-Time Monitoring of Enzymatic Reactions with Steroidal Substrates. The striking selectivity for binding of steroids with closely related structures immediately suggested that supramolecular tandem enzyme assays could be set up.[39] As an extension of (static) indicator displacement assays,[38] this strategy enables the time-resolved detection of reaction-induced concentration changes of analyte mixtures by a continuous re-equilibration of host−dye and host−analyte binding during an enzymatic reaction.[69]

With respect to steroids, a CB*n*-based supramolecular tandem assay could be utilized to monitor their production, functionalization, or destruction by enzymatic processes in real time. This is particularly useful because most steroids are poorly water-soluble and non- or only poorly chromophoric (absorbance in the UV; no emission) such that direct spectroscopic reaction monitoring is challenging. As a proof of principle, the steroid binding data display that nandrolone 17-propionate has a lower affinity for CB8 than nandrolone (Table 2). Consequently, enzymatic conversion of nandrolone 17-propionate to nandrolone by pig liver esterase (PLE) converts a weak competitor of the reporter dye BC for CB8 into a strong competitor. This leads to a concomitant displacement of the fluorescent dye from CB8 and a readily detectable fluorescence switch-off response (Figure 6).

■ DISCUSSION

The conventional design of steroid receptors is facing distinct challenges. On one hand, the cavity of most artificial receptors, such as β-CD, is too small to encapsulate the steroidal skeleton. Therefore, pendant, noncyclic alkyl substituents were utilized as the receptor recognition site for the binding of cholestanes, that is, steroids with an elongated alkyl tail. On the other hand, most steroidal hormones and drugs belong to the classes of estranes, androstanes, and pregnanes, all of which do not possess suitable long alkyl substituents, such that the steroidal core needs to serve as the recognition site itself. However, sufficiently large hosts are rare and, as is the case of γ-CD, are often

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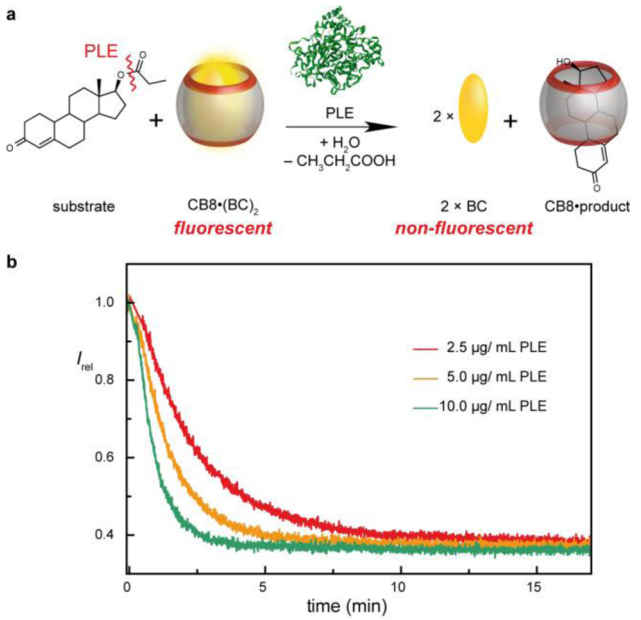

Figure 6. (a) Schematic equilibrium established between the host and guests in the course of an enzymatic conversion. (b) Fluorescence kinetic traces monitoring the enzymatic conversion of 5 μM nandrolone 17-propionate to nandrolone by different concentrations of PLE in the presence of 4.5 μM BC and 2 μM CB8 in 10 mM sodium phosphate buffer, pH 7.4; λ_{exc} = 340 nm and λ_{em} = 520 nm.

conformationally flexible, such that host cavity collapse competes with steroid binding. In addition to steric fitting arguments ("lock-and-key" principle), the exploitation of the particularly strong nonclassical hydrophobic effect in confined, barrel-shaped host cavities has recently emerged as an additional design principle for analyte binding in water.[15] This effect is caused by the geometric shielding of the cavity water molecules from hydrogen bonding with their neighbors and bulk water molecules. Binding of analytes to such a confined, hydrophobic host cavity leads to the release of these high-energy water molecules. This release is strongly enthalpically favored because new water−water hydrogen bonds are being formed.[15,70]

Steroidal compounds, with a diameter of ca. 6 Å for each ring A−D and a total length of 12.5 Å, should fit virtually perfectly and thus bind strongly with the 1 nm wide CB8 macrocycle (7.2 Å portal diameter and 7.7 Å height), while CB7 may be too narrow, with its 5.4 Å diameter, despite its identical height. Coincidentally, the calculated PC of the CB8•nandrolone complex matches with 56% essentially the ideal "55% solution" proposed by Rebek.[20,36] Indeed, CB8 was found to be a *general* receptor for steroids, mimicking the features of albumin proteins, albeit some steroidal analytes show a distinctly lower affinity for CB8. Most of these cases can be rationalized by a guest desolvation effect, that is, a decrease in hydrophobic driving force upon increasing the hydrophilicity of the steroidal skeleton through hydroxylation (compare corticosterone with cortisol). Nevertheless, CB8 shows generally a higher affinity than the natural steroid receptor albumin, which can enable the use of CB8 as a general solubilization agent or drug delivery vehicle for steroidal compounds.

Conversely, the PC value of the small(est) steroid nandrolone with the smaller-diameter CB7 homologue (70%) is far beyond the optimal 55 ± 9% PC range and coincides with maximally observed PC values in other systems.[36] Therefore, even subtle structural modifications such as an additional methyl group (testosterone versus nandrolone) or an additional ester group (nandrolone 17-propionate versus nandrolone) lead to a drastic decrease in the binding affinity for CB7. Thus, CB7, as opposed to CB8, serves as a high-affinity *and* highly selective receptor for certain steroids.

It is fortunate that the highly abundant cholestanes and cholic acid are significantly more weakly bound by CB8 (and CB7) than other steroids. In fact, cholestanes and cholic acid primarily function to improve the physical features of membranes and as surfactants in the digestive tract but not as hormones/drugs and are thus much less attractive sensing targets. Conversely, β-CD binds cholestane steroids the strongest, which impedes its use as a sensor for hormone/drug-like steroids and can cause biocompatibility issues.[71,72] Specifically, cholesterol complexation by β-CD can cause hemolysis by membrane cholesterol depletion in vivo, which accounts for the acute toxicity of this macrocycle. The complementary orthogonal selectivity of CB8 and β-CD is of significant importance for the design of new steroid receptors and also illustrates that different steroid binding mechanisms operate for both hosts, that is, inclusion of the steroidal scaffold (for CB8 and CB7) versus inclusion of the alkyl side chains (for β-CD and derivatives).

The poor solubility of steroids in aqueous media, associated with a low bioavailability, is a major hurdle to be overcome in their use as orally administrable steroidal drugs.[73] The use of cyclodextrins as a chemical additive can significantly enhance the solubility of steroids in water; however, the rather low affinity for many steroids limits the scope.[3] With the biocompatible and high-affinity binders CB7 and CB8 in hand, much higher solubility enhancement factors (e.g., up to 120-fold increase in the solubility of β-estradiol with CB7, cf. Table 3) may be achieved with a lower amount of additive. Since CB*n* can be easily prepared in a kilogram scale[74] and since exploratory toxicity studies with CB7 and CB8 are promising,[52,53,75−77] pharmaceutical formulations, in particular, as oral drug carriers, may come into reach.[28,41,52,54,78−83]

Artificial receptors hold additional promise for drug sequestering to turn off the activity of a drug after its action is not required anymore. For instance, injection of the clinically used sugammadex facilitates displacement of the muscle relaxants vecuronium from the receptor protein after surgery[84] in order to ease the waking time of the patient. However, its rather low affinity for vecuronium ($K_d \sim \mu$M) requires the use of larger doses, which increases side effects and costs. Isaacs et al. showed that acyclic cucurbiturils can strongly bind steroidal neuromuscular blocking agents[27] and function as broad-spectrum reversal agents in vivo.[85] Surprisingly, even the parent CB8 has a remarkably higher (>1000) affinity for vecuronium ($K_d \leq$ nM) than sugammadex.

In addition to potential in vivo applications, CB*n* additives may also prove to be useful for in vitro uses: on one hand, steroid solubilization by CB*n* can provide water-soluble steroid formulations needed in certain biochemical studies and cell culture experiments. In fact, we are currently developing a bacterial assay in which CB8•$(BC)_2$ can be used to follow the depletion of testosterone from a bacterial culture broth that degrades it. On the other hand, immobilized CB*n* filter materials could be devised for the removal of persistent steroids and steroidal drug impurities from blood serum or wastewater.[86] More sophisticated sensing applications can be developed when CB*n* hosts are combined with suitable reporter

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dyes. Sensitive sensing strategies for steroids are in demand to detect steroids or monitor their concentration. When the differential fluorescence response of different CB*n*•reporter dye complexes upon addition of steroids is recorded and appropriately analyzed, the steroid recognition by CBs can be exploited to differentiate steroids, as shown in Figure 5. Moreover, in the presence of the CB•dye complexes, real-time changes in steroid concentrations due to chemical or enzymatic conversions can be readily followed by supramolecular tandem assay formats.[39,41,87,88] We have demonstrated this for the PLE-catalyzed hydrolysis of a nandrolone ester (Figure 6).

CONCLUSIONS

Cucurbit[*n*]urils have extraordinarily high binding affinities for selected steroids (CB8: estranes, androstanes, pregnanes; CB7: nandrolone) and steroidal drugs (CB7 and CB8), both in aqueous solutions and buffers, as well as in biological media such as gastric acid and blood serum. As a consequence, effective steroid solubilization can be achieved through the host−guest complexation with CB*n*, which may be useful to increase their bioavailability or to remove persistent steroid impurities. Furthermore, the detection of steroids at low micromolar concentrations is possible through the use of emission-based binding assays that were shown to be applicable for parallelization in microplate reader format. Because cucurbit[*n*]urils are commercially available and biocompatible, we envision significant developments for their use as high-affinity binders for steroids.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b07655.

Experimental details and additional figures and tables (PDF)

X-ray data for CB7•nandrolone (CIF)

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Notes

The authors declare no competing financial interest.

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