Cavity depth and width effects on cyclophane–steroid recognition: molecular complexation of cholesterol and progesterone in aqueous solution

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Background: Recent X-ray crystal structures show that steroid-binding proteins contain deep hydrophobic cavities defined by aromatic amino-acid side chains which encapsulate steroid molecules. These cavities resemble the binding site of a synthetic macrotricyclic cyclophane receptor which we recently reported to form complexes with cholesterol in aqueous solution. The binding affinity of the cyclophane–cholesterol complex ($K_a \sim 10^6$ M^{-1}, 295 K) is similar to that measured for the cholesterol complex of steroid-transport proteins such as sterol carrier protein-2 (SCP-2). Here we describe synthesis and binding studies of a related receptor with a cavity that is wider and 2 Å deeper than that of the previous cyclophane, and a comparison of the steroid-binding affinity and selectivity of the two synthetic receptors.

Results: A new tricyclic cyclophane receptor with a 13 Å deep cavity was synthesized to study the effect of increased cavity depth on receptor selectivity for steroids. NMR analysis demonstrated that this receptor provided increased steroidal side-chain encapsulation with a corresponding gain in binding free energy of 0.9 kcal mol^{-1} (in d_4-methanol) as compared to our previously reported 11 Å deep receptor. An unexpected consequence of the increase in cavity depth was a corresponding enlargement of the cavity width, as indicated both by steroid-binding studies and molecular modeling. This enlargement in cavity width increases binding affinity for saturated steroids while decreasing the association strength of unsaturated steroids such as cholesterol. In water, cholesterol binds to the new receptor with $K_a \sim 1.5 \times 10^5$ M^{-1} and exhibits a significant complexation-mediated solubility increase.

Conclusions: Small changes in steroid receptor dimensions have resulted in large differences in steroid selectivity and binding affinity. These results indicate that potentially large gains in steroid-binding free energy may be obtainable from complete hydrophobic encapsulation of the flexible aliphatic steroidal side chain. These results have implications for the design of synthetic receptor mimics of natural steroid binding proteins.

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Introduction

Steroid recognition by protein receptors has several essential and diverse roles in eukaryotic cells [1,2]. Steroid-binding proteins (SBPs) exhibit a wide range of both affinity ($K_a \sim 10^5$–10^{10} M^{-1}) and specificity, depending on their involvement in steroid transport [3], metabolism, or gene regulation [4]. Our approach to studies of steroid recognition has focused on the development of water-soluble synthetic receptors with deep aromatic cavities known as cyclophanes in an effort to mimic the binding properties and understand the mechanisms of recognition of naturally-occurring SBPs [5,6].

High-resolution structural information on complexes of steroids with proteins has only recently become available [7–9]. The X-ray crystal structure analysis of the complexed active site of the tight-binding ($K_a \sim 10^9$ M^{-1}) monoclonal anti-progesterone antibody DB3 [8] indicates that a number of aromatic residues define the steroid-binding pocket. In this progesterone complex, the steroid lies between two cofacial tryptophan residues which form a 'steroid sandwich'. In addition, other aromatic tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) residues form van der Waals contacts with the steroid. The X-ray crystal structure of apo-3α-hydroxysteroid dehydrogenase from rat liver [9] shows a large hydrophobic steroid-binding cavity that is 11 Å deep. The amino acids involved in forming this hydrophobic cavity consist mainly of Tyr, Trp and Phe residues, confirming once more the importance of aromatic residues in steroid complexation.

Structural analysis of SBP–steroid complexes suggests that optimal complexation is promoted by the presence of a hydrophobic cavity, ~11 Å deep, containing aromatic rings between which the steroid can be sandwiched. All these conditions are nicely satisfied in our water-soluble, 11 Å deep receptor **1** (Fig. 1), composed of two ethyne-linked cyclophanes. We recently reported the synthesis and cholesterol-recognition properties of receptor **1** [6]. Here, we describe the synthesis of the expanded, 13 Å deep, butadiyne-linked receptor **2** (Fig. 1) and compare the steroid-binding properties of receptors **1** and **2** in an attempt to gain insight into the mechanisms for selective steroid recognition in biological and synthetic systems.

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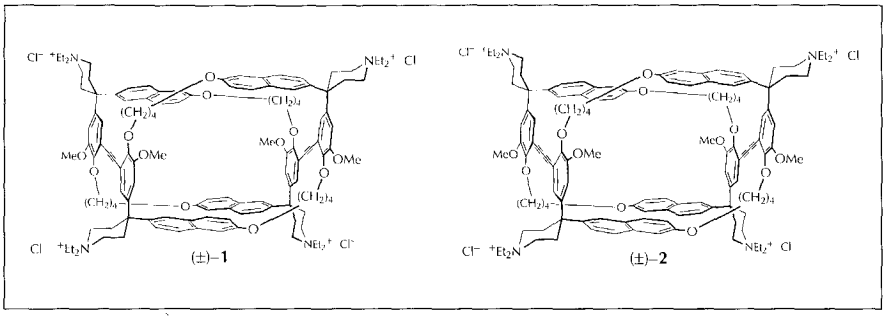

Fig. 1. The synthetic macrotricyclic cyclophane receptors (±)-**1** and (±)-**2**.

The macrotricyclic cyclophane receptors **1** and **2** form complexes with cholesterol in aqueous solution. The binding affinity for cholesterol is in the range of 10^5–10^6 M^{-1}, which is comparable to that of steroid transport proteins such as serum albumin ($K_a \sim 10^5$–10^6 M^{-1}) [2] or sterol carrier protein-2 (SCP-2) ($K_a \sim 10^6$ M^{-1}) [3].

We designed and prepared receptor **2** for comparison with receptor **1**, to try to understand some of the rules underlying specific steroid recognition. We chose to explore the effect of increasing cyclophane-cavity depth on steroid side-chain selectivity. The complexation of the sparingly water-soluble steroids cholesterol, progesterone and testosterone was studied by solid–liquid extraction experiments in water. Binding of the steroids shown in Fig. 2 was studied in d_4-methanol using a more sensitive ^{1}H NMR titration method.

Results and discussion

Chemical synthesis

Fig. 3 illustrates the synthesis of receptor **2** from the phenol shown as compound **3** [5]. Iodination of compound **3** with iodine monochloride in the presence of triethylamine proceeded smoothly and selectively to afford *ortho*-iodophenol **4** (55 %), which was then cyclized to the diiodo cyclophane **5** in 45 % yield. Diisobutylaluminum hydride (DIBAL-H) was used in the reduction of the tertiary amide functions to give compound **6** (82 %), and Heck coupling with trimethylsilylacetylene yielded the bis(silylethynylated) macrocycle **7** (92 %). Deprotection with potassium carbonate in methanol provided the diethynyl cyclophane **8** (94 %) which, upon Glaser–Hay macrocyclization, furnished as a single macrotricyclic product (see below) the racemic D_2-symmetric (±)-**9** in a remarkable 42 % yield. Subsequent quaternization with ethyl iodide followed by ion exchange (DOWEX Cl^-) afforded in 88 % yield the water-soluble macrotricyclic receptor (±)-**2**. By a similar route, in which the tertiary amide functions in **5** were not reduced prior to the Glaser–Hay coupling, the tricyclic tetrakis(tertiary amide) **10** was obtained, which proved to be very useful in the assignment of the receptor symmetry.

Receptor symmetry, properties, and dimensions

Glaser–Hay macrocyclization provided only the chiral, D_2-symmetric macrotricyclic products (±)-**9** (R_f 0.17, SiO_2, CH_2Cl_2/Et_3N 20:1) and (±)-**10** (R_f 0.30, SiO_2,

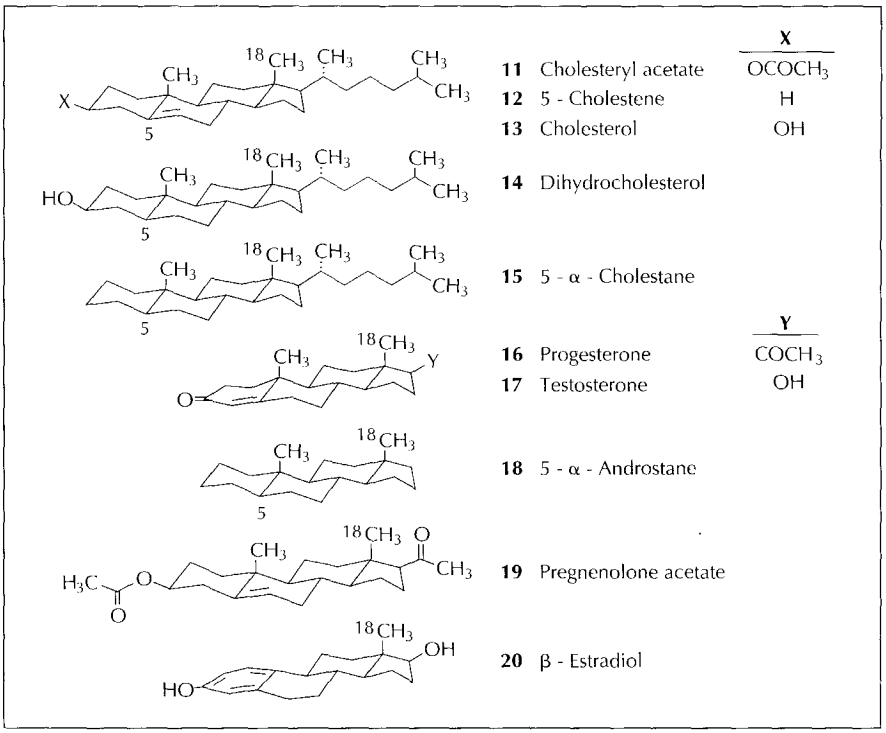

Fig. 2. Steroids investigated in complexation studies with receptor (±)-**2**.

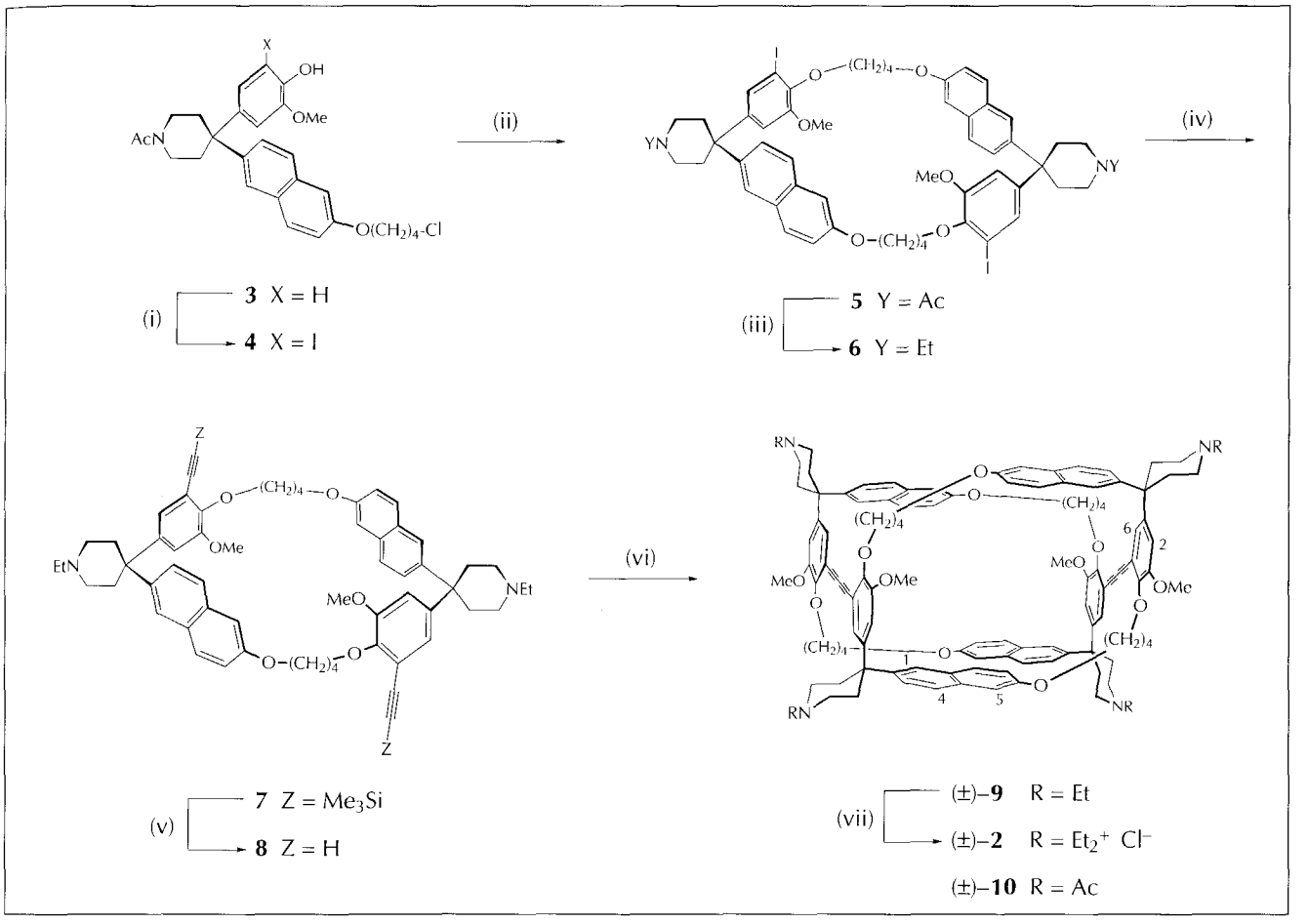

Fig. 3. The synthesis of receptor (±)-**2**. Conditions used were: (i) ICl, triethylamine, CH_2Cl_2, 0 °C, 1.5 h, 55 %. (ii) Cs_2CO_3, CH_3CN, reflux, 3 d, 45 %. (iii) DIBAL-H, CH_2Cl_2, 0 °C, 1 h, 82 %. (iv) Ethynyltrimethylsilane, ($PdCl_2(PPh_3)_2$), diethylamine, CuI (cat.), 100 °C, pressure bottle, 18 h, 92 %. (v) K_2CO_3, MeOH/THF (1:1), 20 °C, 3 h, 94 %. (vi) CH_2Cl_2, CuCl, *N,N,N',N'*-tetramethylethylenediamine, air, 20 °C, 16 h, 42%. (vii) Ethyl iodide, $CHCl_3$, 20 °C, 4 d, followed by Dowex (Cl^-), 88 %.

CH_2Cl_2/CH_3OH 20:1) as established by thin-layer chromatography and by the presence of only one set of resonances in the corresponding ^{13}C and ^1H NMR spectra. None of the alternative, achiral, C_{2h}-symmetrical isomer was isolated. D_2-symmetry of the macrotricycles was established unambiguously by the enantiomeric resolution of (±)-**10** on a chiral stationary HPLC phase (S,S-Whelk-O1 column, Regis Technologies Inc., methanol/dichloromethane 1:20). On-line polarimetric detection showed that (–)-**10** had a shorter retention time than (+)-**10**. Molecular Dynamics (MD) simulations with MacroModel [10] indicated that D_2-symmetric (±)-**9** was 4 kcal mol^{-1} more stable than the corresponding C_{2h}-isomer. This calculated difference in thermodynamic stability could provide an explanation for the exclusive isolation of the racemic product.

The modeling revealed that connecting the two macrocyclic moieties by butadiyne bridges increased the depth of the cavity from ≈ 11 Å (in ethyne-linked receptor **1**) to ≈ 13 Å (in receptor **2**), allowing encapsulation of the aliphatic side chain of cholesterol to a larger extent (Fig. 4). In addition, MacroModel-minimized, low-energy structures of receptor **2** showed the butadiyne linkages in a twisted orientation, allowing the naphthyl moieties to orient in a cofacial fashion more readily, resulting in a substantial enlargement in width of the cyclophane cavity of receptor **2** (Fig. 4).

Receptor **2** is highly soluble in water (solubility: 4 mg ml^{-1}; 1.7 mM; critical aggregation concentration (CAC): 1.7 mM in D_2O), although the additional hydrophobic surface area from the longer butadiyne linker decreases the solubility and CAC somewhat in comparison to the ethyne-bridged receptor **1** (solubility: 6 mg ml^{-1}; 3.0 mM; CAC: 2.5 mM in D_2O).

Aqueous solution complexation studies

Receptor **2** solubilizes hydrophobic steroids in water [11–13]. The complexation strength in water was evaluated using solid–liquid extraction [14], and the results, combined with those of previously-reported studies with receptor **1** [6], are summarized in Table 1.

Extraction of solid cholesterol with a 1 mM aqueous solution of receptor **2** provided a 0.42 ± 0.07 mM solution of cholesterol; thus, complexation increased the

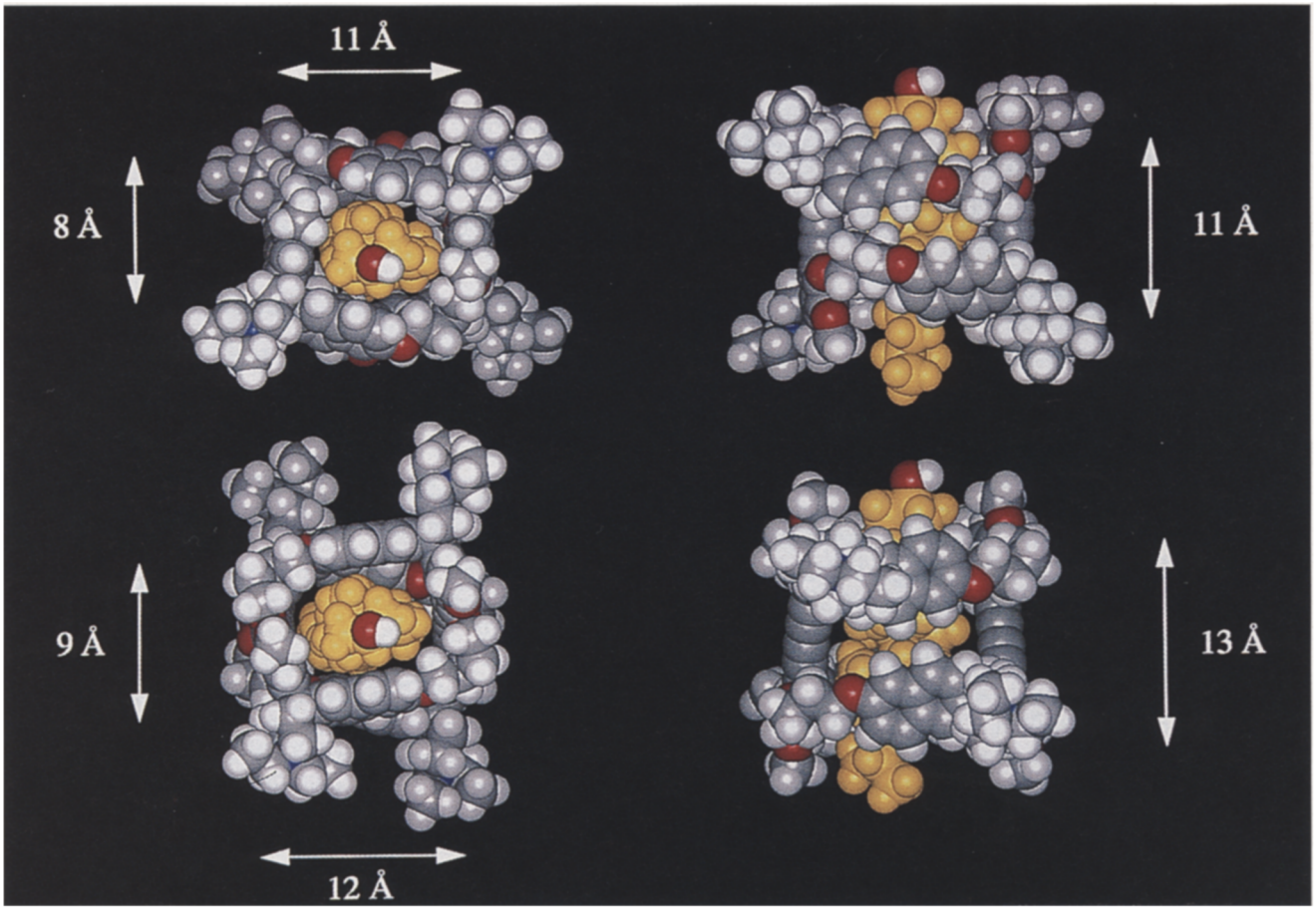

Fig. 4. Comparison of two views of MacroModel-minimized structures of **1** (top) and **2** (bottom) complexed to cholesterol (yellow). The views on the left show the greater width of the cavity of **2** (≈ 12 Å x 9 Å) as compared to **1** (≈ 11 Å x 8 Å). Dimensions were calculated at the cavity entrance; the larger distance is between the cofacial phenyl rings, the smaller between the naphthalene hydrogen atoms H–C(8) (for numbering, see Fig. 3). The views on the right show the greater depth of the receptor cavity in **2** (≈ 13 Å) as compared to **1** (≈ 11 Å).

solubility of cholesterol by a factor of 90. From these data, the binding free energy for the receptor **2**–cholesterol complex of 1:1 stoichiometry in water was calculated as $\Delta G^0 = -7.1 \pm 0.4$ kcal mol^{-1}. Thus, the affinity of butadiyne-linked receptor **2** towards cholesterol is significantly lower than that of ethyne-linked receptor **1** ($\Delta(\Delta G^0)_{1-2} = 1.1$ kcal mol^{-1}).

Surprisingly, extraction of testosterone (**17**) with a 1 mM solution of receptor **2** provided a 1.09 ± 0.11 mM solution of testosterone in water. This result has two possible interpretations: (i) one equivalent of testosterone is extracted into solution upon treatment with receptor **2** because of very strong 1:1 complex formation ($\Delta G^0 << -7.0$ kcal mol^{-1}) or (ii) more than one molecule of testosterone is binding in the deep butadiyne-linked cavity. Since the molecular-modeling studies suggested a wider binding site for receptor **2** than for receptor **1**, we strongly favor the latter explanation. With their flat enone-type A-rings, two testosterone molecules should

Table 1. Association constants (K_a) and binding free energies (ΔG^0) for 1:1 steroid complexes of receptors **1** and **2** as determined at 295 K by solid–liquid extraction in H_2O.[a]

Steroid	Receptor	K_a [M^{-1}]	ΔG^0 [kcal mol^{-1}]	Maximum aqueous solubility [μM]
Cholesterol (**13**)	**2**	1.5 x 10^5	– 7.1	4.7[b]
Cholesterol (**13**)	**1**	1.1 x 10^6	– 8.2	4.7[b]
Progesterone (**16**)	**2**	1.5 x 10^5[c]	– 7.1[c]	29[d]
Testosterone (**17**)	**1**	6.8 x 10^4	– 6.5	83[d]

[a]Reproducibility of $\Delta G^0 \pm 0.4$ kcal mol^{-1}. [b]See [22]. [c]A 1:2 receptor–steroid stoichiometry cannot be fully ruled out; the given K_a and ΔG^0 values are only meaningful if a 1:1 complex forms exclusively. [d]See [23].

be able to arrange themselves in a cofacial π-stacking alignment in the receptor cavity. To form a 1:2 complex, the two substrates could penetrate the binding site from different cavity sides. Previous studies had shown that [2.2] and [2.4]paracyclophanes, which contain two cofacially-aligned benzene rings, can be incorporated into the cavity of a receptor structurally related to the two macrocyclic moieties that are linked together to form the binding sites in receptors **1** and **2** [15].

Extraction of solid progesterone (**16**) with a 1 mM solution of receptor **2** provided a 0.83 ± 0.04 mM solution of this steroid hormone, allowing calculation of $\Delta G^0 = -7.1 \pm 0.4$ kcal mol^{-1} for formation of a 1:1 complex. However, it cannot be ruled out that progesterone, like testosterone, also forms a complex with 1:2 receptor–substrate stoichiometry, although the bulkier acetyl side chain could possibly provide steric hindrance to such an association mode. A 1:2 stoichiometry of receptor–substrate association is not possible for cholesterol: Corey–Pauling–Koltum (CPK) models and computer modeling clearly show that the bulkier saturated A-ring and the aliphatic side chain prevent two cholesterol molecules from penetrating the binding site of either receptor **1** or receptor **2**.

Job plots of the complexes of receptor **2** with testosterone, progesterone and cholesterol in d_4-methanol clearly indicate that only 1:1 complexation takes place in this solvent [16]. In water, the much stronger hydrophobic effect presumably forces the flat A-rings of two testosterone molecules (and possibly also of two progesterone molecules) into the wide cavity of receptor **2**, thus increasing the amount of this steroid dissolved in water.

Since the binding site in receptor **1** is significantly narrower than in receptor **2**, we believe that receptor **1** forms a testosterone inclusion complex with exclusive 1:1 stoichiometry also in water [6].

Comparison of the properties of receptors 1 and 2 by complexation studies in d_4-methanol

The binding of the steroids shown in Fig. 2 to receptor **2** was studied by ^{1}H NMR titrations in d_4-methanol (Table 2). Similarly-accurate binding assays were not possible in D_2O due to low steroid solubilities and exchange kinetics on the NMR time scale. Furthermore, as discussed above, the stoichiometry of complexes of steroids with flat unsaturated A-rings and no aliphatic side chains is not well defined in water whereas the complexes in d_4-methanol have 1:1 stoichiometry, independent of the steroid structure.

The butadiyne-linked receptor **2** is similar to ethyne-linked receptor **1** in forming stable complexes with apolar steroids in methanol. Selectivity differences are evident, however, due to the fact that the cavity of receptor **2** is wider as well as deeper (Table 2), as discussed below.

i) In d_4-methanol, ethyne-linked receptor **1** is the more effective receptor for cholesterol (**13**) and derivatives **11** and **12** by 0.2–0.4 kcal mol^{-1}. The presence of a double bond at C(5) in steroids such as cholesterol seems to lower the affinity for receptor **2**. A comparison of the binding of the two tricyclic receptors **1** and **2** to the fully aliphatic substrate 5-α-cholestane ($\Delta(\Delta G^0)_{2-1} = 0.7$ kcal mol^{-1}) and other steroids indicates that unsaturation of the guest at C(5) is favorable by 0.8 kcal mol^{-1} ($\Delta(\Delta G^0_1)_{12-15}$) for complexation by receptor **1** but unfavorable by 0.1 kcal mol^{-1} ($\Delta(\Delta G^0_2)_{15-12}$ and $\Delta(\Delta G^0_2)_{14-13}$) for complexation by receptor **2**. These effects can be explained by differences in cavity width as revealed by molecular modeling (Fig. 4). The narrower cavity in receptor **1** provides better van der Waals contacts to flatter unsaturated steroids, whereas the wider

Table 2. Association constants (K_a) and binding free energies (ΔG^0) from ^{1}H NMR titrations for 1:1 steroid complexes formed by receptors **1** and **2** in CD_3OD at 298 K.[a]

	Steroid	Receptor 2			Receptor 1	
		K_a[b] (M^{-1})	ΔG^0 (kcal mol^{-1})	$\Delta\delta_{max\ obs}$ ($\Delta\delta_{sat}$) CH_3 (18)	K_a[b,c] (M^{-1})	ΔG^0[c] (kcal mol^{-1})
15	5-α-Cholestane	2700	−4.7	0.81 (1.10)	870	−4.0
16	Progesterone	2600	−4.7	1.30 (1.63)		
11	Cholesteryl acetate	2300	−4.6	0.95 (1.33)	4800	−5.0
12	5-Cholestene	2300	−4.6	0.85 (1.20)	3200	−4.8
19	Pregnenolone acetate	2100	−4.5	1.33 (1.68)		
14	Dihydrocholesterol	1200	−4.2	0.67 (0.93)		
13	Cholesterol	900	−4.1	0.64 (0.97)	1500	−4.3
18	5-α-Androstane	370	−3.5	0.51 (1.16)	500	−3.7
17	Testosterone	200	−3.1	0.34 (1.13)	2100	−4.5
20	β-Estradiol	170	−3.0	0.29 (1.10)	390	−3.5

[a]Also shown are the maximum observed complexation-induced changes in chemical shift ($\Delta\delta_{max\ obs}$), as well as the shifts at saturation binding ($\Delta\delta_{sat}$) of the protons of the CH_3(18) methyl group, which were evaluated in the titrations with receptor **2**. For the $\Delta\delta_{max\ obs}$ ($\Delta\delta_{sat}$) values in the complexes with receptor **1**, see [6]. [b]Reproducibility of K_a values: ± 10 %. [c]Taken from [6].

cavity in butadiyne-linked receptor **2** has a better complementarity to fully aliphatic substrates. In the modeling studies, the energy-minimized structure of the complex of receptor **1** and cholesterol shows 12 short C···C contacts below 3.7 Å, whereas only 7 such contacts were observed in the complex with receptor **2**. In contrast, 5-α-cholestane has seven such short C···C contacts in the complex with receptor **2** and six such contacts when bound to receptor **1**.

ii) Side-chain complexation is promoted by the deeper cavity of receptor **2**. Comparison of the binding of 5-α-cholestane (**15**) with 5-α-androstane (**18**) ($\Delta(\Delta G^0_2)_{15-18}$ = 1.2 kcal mol^{-1}) indicates that the side chain makes a 0.9 kcal mol^{-1} higher contribution to the free energy of complexation by receptor **2** than by receptor **1** ($\Delta(\Delta G^0_1)_{15-18}$ = 0.3 kcal mol^{-1}). Evidence for significantly greater encapsulation of the steroidal side chain by receptor **2** was also obtained in the ^{1}H NMR spectra of complexes of both receptors **2** and **1** in CD_3OD (Fig. 5). When measured at equivalent degrees of complexation with both receptors, 5-α-cholestane (**15**) exhibits diagnostic upfield shifts of the resonances assigned to the methyl groups at the steroid nucleus and the side chain. The weaker binding of receptor **1** (ΔG^0 = −4.0 kcal mol^{-1}) induces larger upfield shifts in the resonances of methyl groups at the steroid nucleus, whereas the stronger binding of receptor **2** (ΔG^0 = −4.7 kcal mol^{-1}) induces larger upfield shifts in the signals assigned to the methyl groups of the steroidal side chain, indicating much more efficient encapsulation of this hydrophobic moiety.

iii) One striking difference in the binding selectivity (1.4 kcal mol^{-1}) of butadiyne-linked receptor **2** and ethyne-linked receptor **1** is apparent upon comparing their complexation behavior towards testosterone (**17**). Whereas receptor **1** binds testosterone strongly (ΔG^0 = −4.5 kcal mol^{-1}) in d_4-methanol, receptor **2**, with its wider cavity, forms a much weaker 1:1 complex (ΔG^0 = −3.1 kcal mol^{-1}). In addition, comparison of the affinities of progesterone (**16**) and testosterone (**17**) to receptor **2** indicates that the free energy difference between binding a hydroxyl (testosterone) and a methyl ketone (progesterone) substituent is 1.6 kcal mol^{-1}. Presumably this difference reflects favorable CH···π interactions [17] between the acetyl group and the aromatic receptor binding site. Also, the costs for partial desolvation of an OH-group (in **17**) upon incorporation of the steroid D ring into the deep receptor cavity may be higher than those for the partial desolvation of a CH_3CO group (in **16**). Favorable CH···π interactions may also be responsible for the increased binding affinity of receptor **1** for cholesteryl acetate (**11**) over the more hydrophobic 5-cholestene (**12**) ($\Delta(\Delta G^0_1)_{11-12}$ = 0.2 kcal mol^{-1}).

iv) Both receptors **1** and **2** are specific for aliphatic steroids, with flat aromatic hormones such as β-estradiol being even more weakly bound by the wider receptor **2** (ΔG^0= −3.0 kcal mol^{-1}) than by receptor **1** (ΔG^0 = −3.5 kcal mol^{-1}).

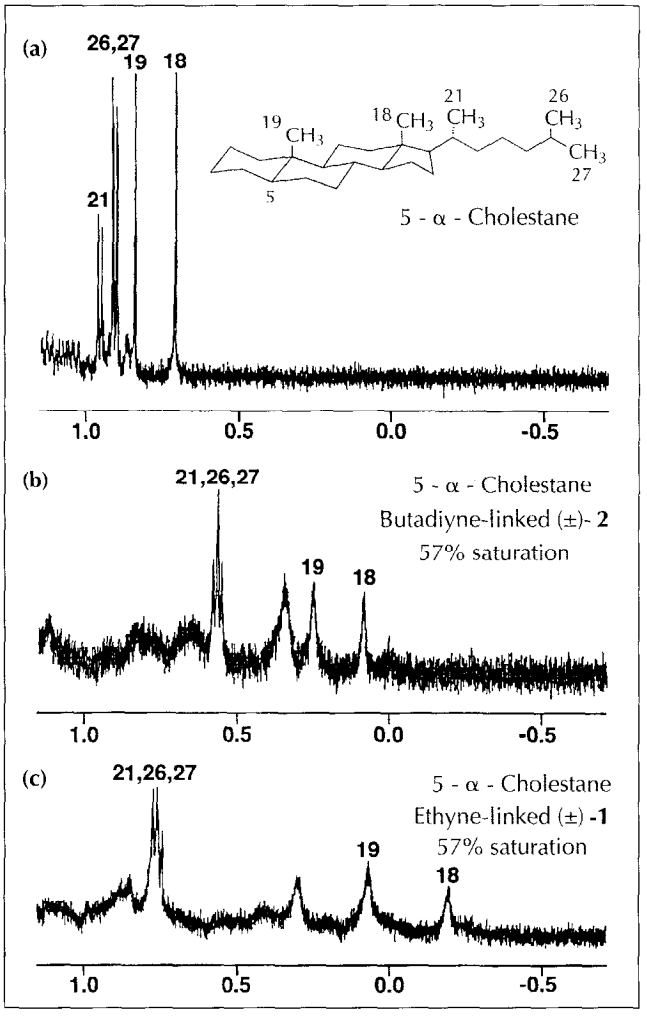

Fig. 5. Receptor **2** shows significantly greater side-chain encapsulation of 5-α-cholestane than receptor **1**. The ^{1}H NMR spectral regions (500 MHz) show the resonances of the methyl groups on the side chain (CH_3(21), CH_3(26), CH_3(27)), the C–D ring junction (CH_3(18)), and the A–B ring junction (CH_3(19)) of free and complexed 5-α-cholestane (**15**) in CD_3OD at 298 K. **(a)** Free 5-α-cholestane at 0.25 mM. **(b)** 5-α-Cholestane (0.25 mM) and receptor **2** (0.625 mM); 57 % degree of complexation. **(c)** 5-α-Cholestane (0.25 mM) and receptor **1** (1.75 mM); 57 % degree of complexation. The stronger-binding cyclophane, receptor **2** (ΔG^0= − 4.7 kcal mol^{-1}), induces the largest upfield shifts of the resonances of the side-chain methyl groups, whereas the weaker-binding cyclophane, receptor **1** (ΔG^0= − 4.0 kcal mol^{-1}), induces larger upfield shifts of the methyl resonances at the steroid nucleus.

Significance

The development of highly specific, tight-binding steroid receptors could provide new strategies for interfering with biologically important steroids *in vivo* and potentially lead to a new class of pharmacological agents. Receptors specific for cholesterol may offer an alternative pharmacological strategy for the dissolution of cholesterol deposits such as those in atherosclerotic plaques [18,19]. Our efforts to design and prepare such specific cholesterol receptors led to the synthesis of two water-soluble macrotricyclic

cyclophanes (receptors 1 and 2) which dissolve cholesterol in aqueous solution. Observed steroid-binding selectivities can be explained by the differences in cavity width and depth between the otherwise structurally closely-related receptors. Whereas receptor 1, composed of two monocycles linked by two ethyne-tethers and possessing a shallower cavity, is more specific for flatter steroids with a double bond at C(5) (such as cholesterol), the deeper and wider cyclophane, receptor 2, constructed with longer butadiyne-linkers, prefers fully-saturated steroids with acetyl or aliphatic side chains. Comparison of the binding performance of the two receptors has provided novel insights into the underlying rules for biotic and abiotic steroid recognition, and this information should be useful in the future for the design of even more efficient and selective binders for specific steroids.

Materials and methods

Critical aggregation concentration (CAC) evaluation of receptor 2

The CAC of receptor **2** was determined by the absence of significant ^{1}H-NMR chemical shift changes upon dilution of a D_2O solution from 1.7 mM to 0.5 mM at 295 K. For more details, see [14].

Empirical molecular modeling and molecular dynamics

The MacroModel V4.0 AMBER* force field [10] was used for the molecular dynamics (MD) simulations of compound **9**, modified to include butadiyne (–C≡C–C≡C–) parameters for the evaluation of the total energies of the isomers. A crystal structure of 1,4-diphenylbutadiyne [20] provided values for bond lengths and angles, whereas stretching and bending force constants were adapted from AMBER*-supplied sp^2-ethyne-sp^2 parameters. Equilibration of the D_2- and C_{2h}-isomers of compound **9** for 500 ps (AMBER*, 300 K, $CHCl_3$) was followed by minimization of the lowest-potential-energy structure obtained, and repeated 200 ps simulations on low-energy minimized structures were carried out until convergence of total energies was reached.

*HPLC enantiomeric resolution of macrotricycle **6***

The resolution was conducted by Dr Christopher Welch at Regis Technologies Inc., 8210 Austin Ave., P.O. Box 519, Morton Grove, IL 60053, USA. The stationary phase used to resolve compounds (±)–**10** was a (*S*, *S*) Whelk-O1 column with methanol/dichloromethane 1:20 as the eluant. The flow rate was 1 ml min^{-1} with UV detection at 254 nm. By on-line polarimetric detection, it was shown that the (–)-enantiomer was eluted first (retention time (t_r) 8.12 min), followed by the (+)-enantiomer (t_r 10.25 min) (Jon Brice, research group of Professor W.H. Pirkle at the University of Illinois, Urbana-Champaign, in conjunction with Regis Technologies Inc.).

*Analytical characterization of receptor **2***

M_p 260 °C (dec.). ^{1}H NMR (500 MHz, $(CD_3)_2SO$, 400 K, see Fig. 3 for atom numbering) δ 1.19–1.27 (*bm*, 24 H, $N^+CH_2CH_3$), 1.89–1.99 (2 x *bm*, 16 H, CH_2CH_2), 2.60–2.89 (3 x *bm*, 16 H, ArAr'CCH_2), 3.07–3.48 (4 x *bm*, 32 H, ArAr'$CCH_2CH_2N^+$, $N^+CH_2CH_3$), 3.80–3.87 (*bm*, 12 H, OCH_3), 4.08–4.24 (4 x *m*, 16 H, phOCH_2, napOCH_2), 6.81–6.86 (*m*, 4 H, ph H–C(6)), 7.05–7.14 (*bm*, 12 H, ph H–C(2), nap H–C(3), nap H–C(7)), 7.19 (*bs*, 4 H, nap H–C(5)), 7.56 (*m*, 4 H, nap H–C(4)), 7.62–7.78 (*bm*, 8 H, nap H–C(1), nap H–C(8)); Anal. calc'd for $C_{128}H_{148}N_4O_{12}Cl_4 \cdot 7\ H_2O$ (2202.55): C 69.74, H 7.50, N 2.54; found: C 69.89, H 7.81, N 2.91.

Full experimental protocols for the syntheses of receptor **2**, macrotricycle **10**, and the previously-communicated receptor **1** [6] will be provided in a full paper elsewhere. All new compounds reported here were fully characterized by electron-impact or fast-atom-bombardment mass spectrometry, ^{13}C- and ^{1}H-NMR, infrared spectroscopy, and elemental analysis or high-resolution mass spectra.

Solid–liquid extraction binding assay

Solutions of receptor **2** (1 mM) in H_2O were sonicated for 45 min in the presence of excess solid steroid and allowed to equilibrate at 295 ± 2 K for 2 h. Centrifugation of the suspension was followed by filtration of the supernatant liquid through a 0.45 mm cellulose acetate filter. Removal of water under reduced pressure was followed by addition of CD_3OD, which allowed determination of percent steroid extracted by ^{1}H-NMR integration. For the calculation of K_a, see [14]. The association constants are average values of two diastereomeric complexes which are of similar stability (see below), and data shown in Table 1 are averaged over triplicate runs. Reproducibility of ΔG^0 values: ± 0.4 kcal mol^{-1}. The solubilization of progesterone by receptor **1** was not studied.

^{1}H-NMR titration binding assay

Association constants were determined by non-linear least-squares curve fitting of 500 MHz ^{1}H NMR titrations (298 K) using the program *Associate 1.6* [21]. In these titrations, the steroid concentration was held constant (usually 0.25 mM) and the receptor concentration varied (usually between 0.2 mM and 2 mM) to provide between 10 and 90 % of saturation binding. The complexation-induced change in chemical shift of the steroidal CH_3(18) methyl resonance was monitored and evaluated in all titrations. Association constants shown are an average value of the two diastereomeric complexes. This approximation is justified by the absence of any significant differential complexation-induced shifts for the resonances of the diastereomeric complexes, including CH_3(18), indicating similar complex geometries and stabilities. Reproducibility of K_a values: ± 10 %.

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