Structure-Function Relationships in the Complexation of Steroids by a Synthetic Receptor

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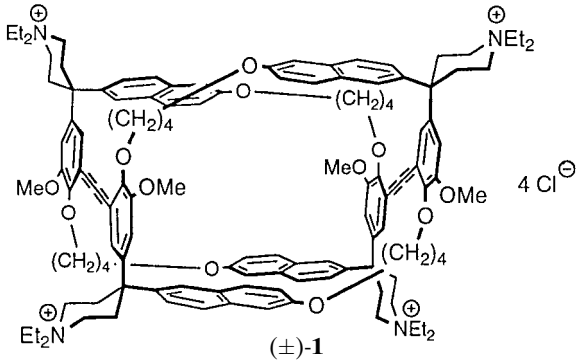
The complexation between the double-decker cyclophane (±)-**1** and a series of 30 steroids was investigated in CD_3OD by ^1H-NMR titrations. The geometries of the complexes, in which the substrates are axially included in the 13-Å deep and 9 Å × 12 Å wide receptor cavity, were estimated based on the complexation-induced changes in chemical shift (CIS) of the steroidal Me group resonances. Computer modeling provided additional support for the geometries deduced from the experimental data. The log *P* (octanol/H_2O) values of the steroids were determined experimentally by HPLC or calculated using the program CLOGP. Although steroids with a high log *P* form some of the most stable complexes with (±)-**1**, a general correlation between the thermodynamic driving force for association $-\Delta G^0$ and the partition coefficient was not observed. It can, therefore, be concluded that inclusion complexation is not only driven by the preference of the steroid to transfer from the polar solvent into the lipophilic binding cavity but also by specific host-guest interactions. A series of structure-function relationships was revealed. *i*) Steroids with an isoprenoidal side chain at C(17) form some of the most stable complexes ($-\Delta G^0$ up to 4.8 kcal mol^{-1}), with side-chain encapsulation contributing as much as 1.2 kcal mol^{-1} to the association strength. In these complexes, the receptor is slipping in a dynamic process over both the tetracyclic core and the lipophilic side chain. *ii*) Pregnane derivatives, which lack the isoprenoidal side chain, are tightly encapsulated with their tetracyclic core. Upon introduction of double bonds, the core flattens, and binding affinity drops substantially. *iii*) The presentation of steroidal OH groups to the receptor cavity is accompanied by energetically unfavorable functional-group desolvation, which strongly reduces the host-guest binding affinity. In contrast, inclusion of steroidal carboxylate or keto groups into the cavity does not substantially change complexation strength as compared to the unsubstituted derivatives. *iv*) Addition of extra Me groups to the steroidal A ring does not have a large effect on the association strength; however, complex geometries may change significantly. *v*) Receptor (±)-**1** shows a remarkably high affinity towards progesterone ($-\Delta G^0 = 4.7$ kcal mol^{-1}) despite the low log *P* value (3.87) of this steroid. Small changes in the progesterone structure lead to large reductions in complex stability, which clearly demonstrates that the double-decker cyclophane is a selective molecular receptor.

1. Introduction. – Steroids are ubiquitous in eukaryotic organisms where they exert a broad variety of essential functions [1]. A detailed understanding – at the molecular level – of their biological action, transport, and metabolism is of considerable interest to fields such as chemical and molecular biology or medicinal chemistry [2][3]. In particular, the mechanism of action of steroid-binding proteins belonging to the receptor superfamily, which further contains thyroid hormone, vitamin D, and retinoic acid receptors, has attracted much attention, and details of steroid hormone-mediated signal-induction and transcription processes are rapidly being revealed [4][5].

Molecular recognition is at the heart of biological steroid function, and in recent years, X-ray crystal-structure analyses of enzyme and antibody complexes with a

variety of steroids have been accomplished [6–8]. They provided significant insight into the principles governing biological steroid complexation and revealed some general recognition features. The tetracyclic core of the steroid is bound by lipophilic amino acid residues – often aromatic side chains – which form a large hydrophobic pocket. Steroid-binding cavities are, in most cases, 11–17 Å deep. Polar functional groups at the steroidal A and D rings usually undergo H-bonding to hydrophilic amino-acid side chains lined at the extremities of the pocket. Interestingly, the steroid-binding cavities in proteins resemble in size and shape the binding sites of synthetic double-decker cyclophane receptors such as (±)-**1**, which we developed in recent years for the complexation and dissolution of cholesterol in aqueous solution [9][10] (for other water-soluble synthetic steroid receptors, see [6][11–14]).



(±)-**1**

Here, we report extensive ^{1}H-NMR investigations on structure-function relationships in the complexation of steroids by the previously reported [9b,c] double-decker cyclophane receptor (±)-**1** in CD_3OD. Computer-modeling studies indicated that (±)-**1** possesses a cylindrical, highly preorganized 13-Å deep and 9 Å × 12 Å wide lipophilic cavity (*Fig. 1*). Initial ^{1}H-NMR binding studies had shown that steroids form stable 1 : 1

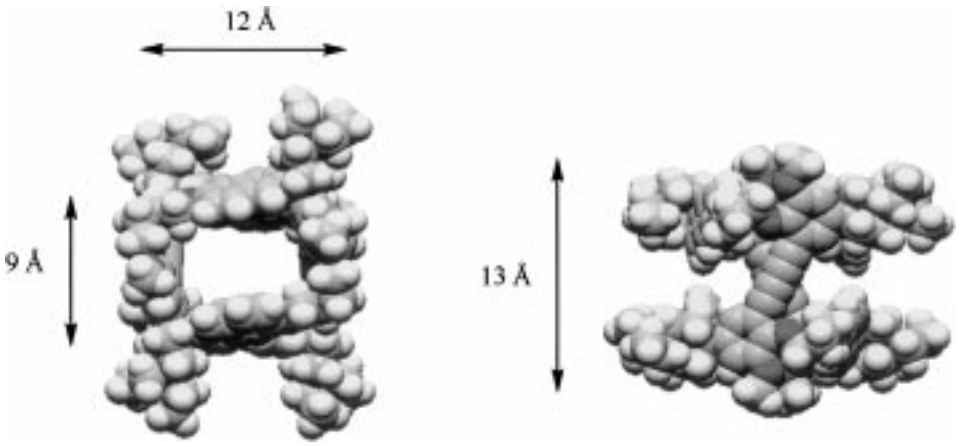


Fig. 1. *View of the double-decker cyclophane* (±)-**1** *onto the cavity entrance* (left) *and onto the buta-1,3-diynediyl bridges* (right) [9b,c]

complexes with (±)-**1** in CD_3OD, in which the tetracyclic skeleton (rings A–D) is axially included in the deep cavity. Additional encapsulation of the isoprenoidal side chain at C(17) in cholestane derivatives was found to make a substantial contribution to the binding free enthalpy $-\Delta G^0$ [9b,c]. The present work analyzes systematically how structural changes in the tetracyclic core and the side chain, as well as substituent effects, affect the complexation of steroids in a deep apolar cavity.

For this investigation, CD_3OD rather than D_2O was chosen as the solvent. The stability of the complexes formed in D_2O is difficult to assay accurately by ^{1}H-NMR titrations due to severe signal broadening resulting from host-guest exchange processes on the NMR time scale. Also, the solubility of many of the steroidal substrates is quite limited in D_2O. A determination of thermodynamic binding data in D_2O by solid-liquid extraction experiments lacks the accuracy for meaningful comparative studies; furthermore, such assays do not provide any structural information on the complexes formed. In contrast, sharp ^{1}H-NMR resonances are measured for both binding partners at fast host-guest exchange in CD_3OD, and all steroids and their inclusion complexes are well-soluble. Although apolar complexation in CD_3OD is weaker than in D_2O (for example, we measured for the 1:1 complex between (±)-**1** and cholesterol (**2**) in H_2O: $K_a = 1.5 \times 10^5$ l mol^{-1}, $-\Delta G^0_{295K} = 6.5$ kcal mol^{-1}; in CD_3OD: $K_a = 900$ l mol^{-1}, $-\Delta G^0_{298K} = 4.1$ kcal mol^{-1} [9b,c]), extensive studies on apolar molecular recognition with cyclophane receptors have shown that relative binding strengths follow the same trend in both solvents [15]. Therefore, we believe that the principles for the molecular recognition of steroids revealed by this study in CD_3OD also apply to the complexation in H_2O and to biological processes.

2. Results and Discussion. – 2.1. *Results from Previous Work*. The steroids **2**–**11** that had been investigated as substrates in an initial complexation study are shown in *Fig. 2*, and the results of ^{1}H-NMR binding titrations at constant guest concentration in CD_3OD at 298 K are summarized in *Table 1* [9b,c]. Receptor (±)-**1** forms axial 1:1 inclusion complexes with steroidal substrates, driven by solvophobic forces and apolar host-guest interactions. Aliphatic steroids bind more strongly than flat aromatic steroids, which have a poor geometrical complementarity for the wide receptor cavity. A substantial contribution to the binding free enthalpy results from encapsulation of the terpenoidal side chain at C(17): the complex of 5α-cholestane (**8**) is 1.2 kcal mol^{-1} more stable than the complex of 5α-androstane (**10**), which lacks this side chain. Encapsulation of the steroidal side chain was evidenced experimentally by large upfield complexation-induced shifts (CIS) of the ^{1}H-NMR signals assigned to its Me groups. Depending on their nature and polarity, functional groups attached to the steroidal core profoundly affect the association strength: whereas progesterone (**6**) with a MeCO group at C(17) forms one of the most stable complexes ($-\Delta G^0 = 4.7$ kcal mol^{-1}), testosterone (**7**) with an OH group at C(17) is only weakly bound ($-\Delta G^0 = 3.1$ kcal mol^{-1}). These remarkable side-chain and substituent effects initiated the studies reported in the following.

2.2. *New Complexation Studies*. According to the nature of their tetracyclic cores, the steroidal substrates **12**–**41** provided by *Schering AG* can be subdivided into three groups: derivatives of androst-5-ene **12**–**21**, which can be viewed as cholesterol-like (*Fig. 3,a*), derivatives of androst-4-en-3-one **22**–**33**, which are testosterone- or

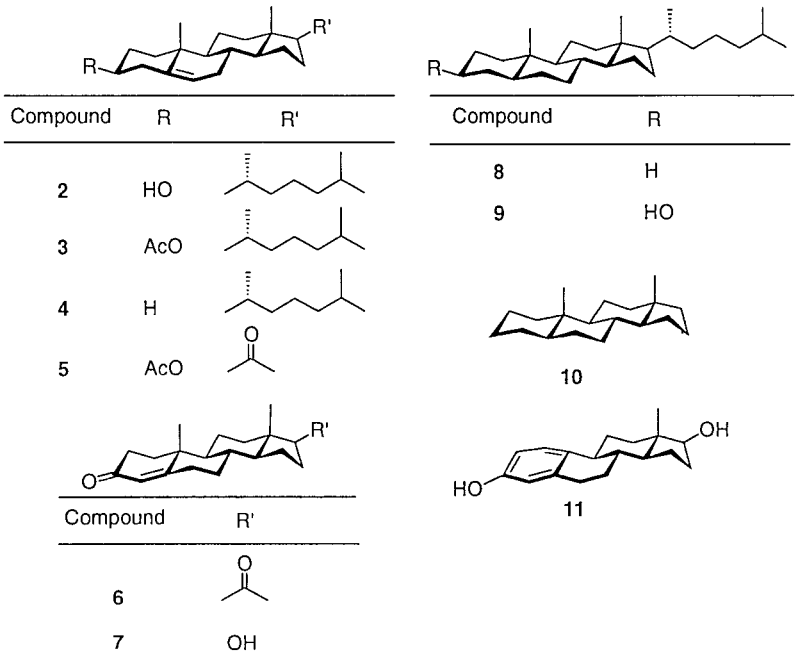


Fig. 2. *The steroids* **2**–**11** *investigated in initial binding studies with receptor* (±)-**1** [9b,c]

Table 1. *Association Constants* K_a *and Binding Free Enthalpies* $-\Delta G^0$ *from 500-MHz* ^{1}*H-NMR Titrations* (CD_3OD, 298 K) *for 1:1 Inclusion Complexes between* (±)-**1** *and Steroids* **2**–**11** [9b,c]. Also given are the calculated CIS for the steroidal Me(18) group at saturation binding $\Delta\delta_{sat}$ and, in brackets, the maximum observed CIS $\Delta\delta_{max\ obs}$. log *P* Values for the partitioning between octanol and H_2O are also shown.

Steroid	K_a [a]) [l mol^{-1}]	$-\Delta G^0$ [kcal mol^{-1}]	$\Delta\delta_{sat}$ ($\Delta\delta_{max\ obs}$) Me(18) [ppm]	log *P*	log *P* (CLOGP) [e])
Cholesterol (**2**)	900	4.1	– 0.97 (– 0.64)	–	9.43
Cholesteryl acetate (**3**)	2300	4.6	– 1.33 (– 0.95)	–	10.37
5-Cholestene (**4**)	2300	4.6	– 1.20 (– 0.85)	–	11.55
Pregnenolone acetate (**5**)	2100	4.5	– 1.68 (– 1.33)	–	4.81
Progesterone (**6**)	2600	4.7	– 1.63 (– 1.30)	3.87 [b])	3.85
Testosterone (**7**)	200	3.1	– 1.13 (– 0.34)	3.45 [c])	3.35
5α-Cholestane (**8**)	2700	4.7	– 1.10 (– 0.81)	–	12.09
Dihydrocholesterol (**9**)	1200	4.2	– 0.93 (– 0.67)	–	10.00
5α-Androstane (**10**)	370	3.5	– 1.16 (– 0.51)	–	8.13
17β-Estradiol (**11**)	170	3.0	– 1.10 (– 0.29)	3.30 [d])	3.78

[a]) Titrations at constant steroid concentration. Reproducibility of K_a values: ± 10%. [b]) From [16]. [c]) From [17]. [d]) From [18]. [e]) Calculated with the program CLOGP [19].

progesterone-like (*Fig. 3,b*), and derivatives of androsta-1,4-dien-3-one **34**–**39** (*Fig. 3,c*). Two compounds, 5β-cholestan-3-one (**40**) and 5β-cholest-1-en-3-one (**41**), do not fit into any of these categories (*Fig. 3,d*).

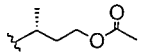
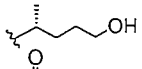
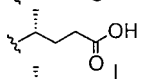
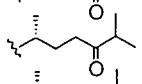
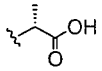
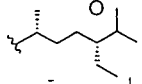
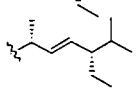
Binding studies were performed by means of 500-MHz ^{1}H-NMR titrations in CD_3OD at 298–300 K with the steroid concentration being held constant at 0.25 mM.

Table 2. *Association Constants* K_a *and Binding Free Enthalpies* $-\Delta G^0$ *from 500-MHz* ^{1}*H-NMR Titrations* (CD_3OD, 298–300 K) *for 1:1 Inclusion Complexes between (±)-***1** *and Steroids* **12**–**41**. Also given are the calculated CIS for the steroidal Me(18) group at saturation binding $\Delta\delta_{sat}$ and, in brackets, the maximum observed CIS $\Delta\delta_{max\ obs}$. log *P* Values for the partitioning between octanol and H_2O are also shown.

Steroid [a])	K_a [b]) [l mol^{-1}]	$-\Delta G^0$ [kcal mol^{-1}]	$\Delta\delta_{sat}$ ($\Delta\delta_{max\ obs}$) Me(18) [ppm]	log *P* [c])	log *P* (CLOGP) [d])	log *P** [e])
23-Acetoxy-24-norchol-5-en-3β-ol (ZK134266, **12**)	1200	4.2	−1.15 (−0.94)	>7	6.39	6.39
3β-Hydroxychol-5-enoic-24-acid (ZK115757, **13**)	1000	4.1	−1.51 (−1.26)	5.67 [f])	6.02	6.00
24-Oxocholesterol (ZK134306, **14**)	1000	4.1	−1.13 (−0.90)	7.02	6.82	6.84
β-Sitosterol (ZK46000, **15**)	540	3.7	−1.21 (−0.82)	≫7	10.39	10.56
Stigmasterol (ZK 17736, **16**)	320	3.4	−1.35 (−0.75)	≫7	9.84	9.99
Chol-5-ene-3β,24-diol (ZK134264, **17**)	280	3.3	−1.45 (−0.78)	5.97	6.02	6.00
Pregnenolone (ZK5553, **18**)	240	3.3	−1.34 (−0.66)	4.28	3.91	3.80
3β-Hydroxybisnorchol-5-enoic-22-acid (ZK115758, **19**)	180	3.1	−1.21 (−0.52)	4.40 [g])	4.87	4.80
Dehydroepiandrosterone (ZK5062, **20**)	130	2.9	−1.06 (−0.37)	3.15	2.95	2.80
Androst-5-ene-3β,17β-diol (ZK4802, **21**)	[h])	[h])	(−0.06)	3.00	3.41	3.28
(20*S*)-20-Acetoxymethylpregn-4-en-3-one (ZK138155, **22**)	3100	4.8	−1.67 (−1.53)	6.05	5.80	5.77
4-Methylcholest-4-en-3-one (ZK789832, **23**)	3000	4.7	−1.46 (−1.33)	≫7	9.92	10.07
Cholest-4-en-3-one (ZK136274, **24**)	2100	4.5	−1.32 (−1.16)	≫7	9.40	9.53
Ergosta-4,22-dien-3-one (ZK32036, **25**)	2100	4.5	−1.39 (−1.23)	≫7	9.25	9.37
25-Hydroxycholest-4-en-3-one (ZK134308, **26**)	1900	4.5	−1.50 (−1.33)	7.52	7.19	7.23
2α-Methylcholest-4-en-3-one (ZK134286, **27**)	1700	4.4	−1.04 (−0.90)	≫7	9.92	10.07
16α,17-Epoxyprogesterone (ZK5197, **28**)	1000	4.1	−1.50 (−1.20)	3.35	3.18	3.04
1α-Methylcholest-4-en-3-one (ZK226154, **29**)	900	4.1	−1.40 (−1.05)	≫7	9.92	10.07
(20*S*)-20-Hydroxymethylpregn-4-en-3-one (ZK17235, **30**)	700	3.9	−1.76 (−1.30)	4.67	4.90	4.84
16-Dehydroprogesterone (ZK47520, **31**)	700	3.9	−1.42 (−1.04)	3.70	3.70	3.58
2,2-Dimethylprogesterone (ZK35063, **32**)	400	3.5	−1.31 (−0.81)	4.87	4.88	4.81
20-Hydroxy-20-methylpregn-4-en-3-one (ZK133861, **33**)	320	3.4	−1.26 (−0.70)	4.70	4.68	4.61
1-Methylcholesta-1,4-dien-3-one (ZK226155, **34**)	2400	4.6	−1.08 (−0.97)	≫7	9.70	9.84
Cholesta-1,4-dien-3-one (ZK92151, **35**)	2200	4.6	−1.02 (−0.90)	≫7	9.18	9.30
4-Methylcholesta-1,4-dien-3-one (ZK229281, **36**)	1900	4.5	−1.07 (−0.94)	≫7	9.70	9.84
2-Methylcholesta-1,4-dien-3-one (ZK226153, **37**)	1800	4.4	−0.97 (−0.84)	≫7	9.70	9.84
1-Dehydroprogesterone (ZK34525, **38**)	700	3.9	−1.19 (−0.68)	3.17	3.63	3.51
(20*S*)-20-Hydroxymethylpregna-1,4-dien-3-one (ZK18364, **39**)	350	3.5	−1.29 (−0.77)	4.01	4.68	4.61
5α-Cholestan-3-one (ZK46023, **40**)	3000	4.7	−1.53 (−1.39)	≫7	9.54	9.68
5α-Cholest-1-en-3-one (ZK136275, **41**)	2500	4.6	−1.27 (−1.14)	≫7	9.40	9.53

[a]) Internal ZK number from *Schering*. [b]) Reproducibility of K_a values: ±10%. [c]) Measured at pH 7. [d]) Calculated with the program CLOGP [19]. [e]) $\log P^* = 1.0436 \times CLOGP - 0.2784$. [f]) Measured at pH 2; at pH 7: 2.07. [g]) Measured at pH 2; at pH 7: 0.23. [h]) Very weak binding, $-\Delta G^0 < 1.4$ kcal mol^{-1}.

a)

Compound	R	Compound	R
12		17	
13		18	
14		19	
15		21	
16			

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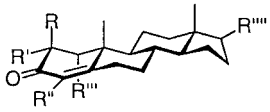
b)



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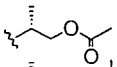
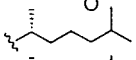
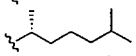
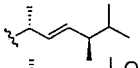
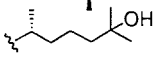
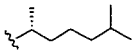
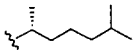
22	H	H	H	H	
23	H	H	Me	H	
24	H	H	H	H	
25	H	H	H	H	
26	H	H	H	H	
27	H	Me	H	H	
29	H	H	H	Me	
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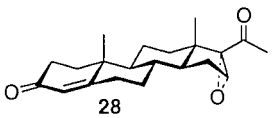

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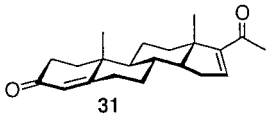

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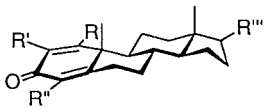

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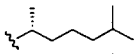
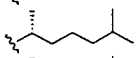
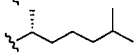
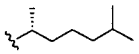
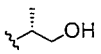
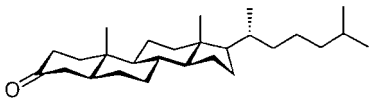
34	Me	H	H	
35	H	H	H	
36	H	H	Me	
37	H	Me	H	
38	H	H	H	
39	H	H	H	

d)



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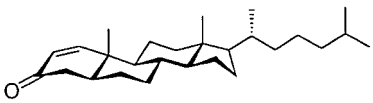

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Fig. 3. *Steroids investigated in the new binding studies:* a) *with an androst-5-ene core* (**12**–**21**), b) *with an androst-4-en-3-one core* (**22**–**33**), c) *with an androsta-1,4-dien-3-one core* (**34**–**39**), *and* d) *others* (**40**, **41**)

The upfield-shifted resonance of the steroidal Me(18) group was monitored in all titrations, and the binding free enthalpies for the exclusively formed 1:1 host-guest complexes were evaluated by nonlinear least-squares curve-fitting analysis. Only one set of resonances was observed for host and guest during the titrations. The absence of any significant differential CIS for the resonances in the two diastereoisomeric complexes, which are formed between racemic receptor and enantiomerically pure steroid, suggests that these complexes have similar geometries and stabilities. Therefore, the association constants given in *Tables 1* and *2* are average values for both complexes. Association strength varies from $-\Delta G^0 = 4.8$ kcal mol^{-1} for **22** to < 1.4 kcal mol^{-1} for **21**. The library of compounds selected for this study is biased by the previous experience with a related double-decker receptor featuring ethynediyl instead of buta-1,3-diynediyl bridges between the two cyclophane moieties [9]. ^{1}H-NMR Binding studies in CD_3OD showed almost no affinity of this receptor for corticoids and bile acids featuring multiple polar groups on the tetracyclic core. Thus, these substrates, as well as others with multiple polar functional groups on the core, were not considered here.

The association constant K_a reflects the relative stability of two states, the one of the solvated free binding partners, and the other of the solvated host-guest complex. To analyze whether the transfer of steroidal substrates from the polar solvent into the

lipophilic host cavity is mainly driven by their enhanced affinity for the less polar environment, we determined their log *P* values [20] for the partitioning between octanol and H_2O according to the HPLC method defined by the *OECD* guideline [21]. Alternatively, the partitioning coefficients were calculated using the program CLOGP [19]. Experimental and calculated values are in good agreement; therefore, it is reasonable to use the calculated values for the range of $\log P \gg 7$, in which experimental values cannot be determined (*Table 2*). The good correlation between measured and calculated values allowed an additional data extrapolation from the linear relationship $\log P^* = 1.0436 \times CLOGP - 0.2784$ ($R^2 = 0.9475$) (*Table 2*) [20].

Although log *P* values refer to the partitioning between octanol and H_2O, we use them here to estimate, in a first approximation, the driving force for the transfer of the steroids from CD_3OD (instead of H_2O) into the hydrophobic interior of the receptor (instead of octanol). A strong correlation of $-\Delta G^0$ values for the various complexes of (±)-**1** with the log *P* values for the different steroids would indicate that the different affinity of the apolar substrates for the two phases – polar solvent and lipophilic receptor cavity – largely determines the driving force for complexation. The absence of such correlation, on the other hand, would demonstrate that specific molecular-recognition processes, in addition to specific functional-group solvation effects [13][15][22], control the driving force for complexation. Such a large role of specific host-guest interactions in determining the thermodynamic quantities is clearly evidenced in the comparison between the complexes formed by progesterone (**6**) and testosterone (**7**). Both steroids have similar log *P* values (3.87 and 3.45, resp.), yet the inclusion complex of the former is by 1.6 kcal mol^{-1} more stable than the one formed by the latter (*Table 1*).

2.3. *Effects of the Steroidal Side Chain at C(17) on Complex Stability.* A large stabilizing contribution of an isoprenoidal side chain at C(17) had initially been revealed in the comparison between the complexes formed by 5α-cholestane (**8**; $-\Delta G^0 = 4.7$ kcal mol^{-1}) and 5α-androstane (**10**; $-\Delta G^0 = 3.5$ kcal mol^{-1}) [9b,c]. A similar stabilizing effect was also observed in this new study. Compounds **23**, **24**, **27**, **34**–**37**, **40**, and **41** possess the same side chain as cholestane, and these lipophilic substrates ($\log P > 9$) all form complexes with a large binding free enthalpy $-\Delta G^0$ between 4.4 and 4.7 kcal mol^{-1} (*Table 2*). The driving force is not reduced when the isoprenoidal side chain bears a terminal OH group, as in **26** ($-\Delta G^0 = 4.5$ kcal mol^{-1}), since in the inclusion complex this functional group is located outside the cavity. However, if the side chain is shortened, attached polar groups (OH, COOH) encounter energetically unfavorable desolvation upon inclusion complexation, and, correspondingly, the binding free enthalpy becomes reduced. The complex may actually adopt a less favorable conformation (with respect to host-guest interactions) to avoid the costly desolvation of the functional group. Such effects are responsible for the significantly lower $-\Delta G^0$ values measured for **13** (4.1 kcal mol^{-1}), **17** (3.3 kcal mol^{-1}), **30** (3.9 kcal mol^{-1}), **33** (3.4 kcal mol^{-1}), and **39** (3.5 kcal mol^{-1}). It is noticeable that the log *P* values (between 4 and 6) for these substrates are much reduced as compared to those for the steroids bearing an intact isoprenoidal side chain. The poorest binding is observed when the isoprenoidal side chain is completely replaced by a β-OH group at C(17): androst-5-ene-3β,17β-diol (**21**) was found to bind hardly at all ($-\Delta G^0 < 1.4$ kcal mol^{-1}).

The binding contribution of the side chain at C(17) is altered not only by variation of its length and attachment of polar functional groups, but also by additional alkyl substituents. Whereas one additional Me group as in **25** (4.5 kcal mol^{-1}) does not change the complexation driving force, $-\Delta G^0$ drops upon attachment of an additional Et group in **15** (3.7 kcal mol^{-1}) or **16** (3.4 kcal mol^{-1}). Since the log *P* values of the latter substrates are very high, this drop must be explained by unfavorable steric interactions between the bulky side chain and the receptor in the complex.

Experimental evidence for side-chain incorporation into the cavity was obtained by ^{1}H-NMR spectroscopy. *Fig. 4* shows the CIS measured for the steroidal Me groups in the complexes of the cholestane derivatives **2**, **35**, and **41**, and the pregnane derivative **22**. Also depicted are the energy-minimized inclusion geometries of the steroids obtained by simulations within MacroModel [23] using the AMBER* force field [24]. The modeling revealed full axial incorporation of the B,C,D rings of the steroid as well as of large parts of the side chain at C(17). The functional groups at C(3) in the A ring, as well as the termini of the side chain at C(17), protrude from the cavity into the solution. This binding picture is corroborated by the upfield CIS measured for the Me groups. The upfield shifts are particularly pronounced for the Me(18), Me(19), and Me(21) groups, whereas those of the terminal Me groups of the side chain are significantly smaller. Interestingly, the CIS for the Me groups on or near the core are much larger at saturation binding of the pregnane derivative **22** than of the cholestane derivatives **2**, **35**, and **41** (*Fig. 4*). Apparently, the B,C,D rings of the former are more tightly encapsulated than those of the latter. A larger conformational homogeneity of the inclusion complex with **22** is possibly enforced by the preference of its two terminal polar groups to protrude into the solvent for solvation, whereas, in the complexes of the cholestane derivatives, the receptor can slip over the lipophilic tetracyclic frame and the isoprenoidal side chain, adopting several energetically favorable conformations in a dynamic equilibrium.

2.4. *Effects of Double Bonds in the Tetracyclic Steroidal Core.* The tighter encapsulation of the tetracyclic core of pregnane derivatives, as compared to cholestane derivatives (*Sect. 2.3*), is also reflected in the way the association with (±)-**1** is affected by structural changes in the core, such as a flattening due to the introduction of double bonds. The introduction of a double bond into the A or B ring of cholestane derivatives hardly affects the binding affinity, and differences in $\Delta(\Delta G^0)$ amount to only up to 0.2 kcal mol^{-1}. This is nicely seen in comparisons of the binding affinity of the following pairs 5α-cholestane (**8**)/5-cholestene (**4**), dihydrocholesterol (**9**)/cholesterol (**2**), or 5α-cholestan-3-one (**40**)/5α-cholest-1-en-3-one (**41**). Even the addition of a second double bond to cholesta-1,4-dien-3-one (**35**), which fully flattens the A ring, does not affect the association strength. The receptor presumably slips away from the flattened rings of these substrates, which show poor complementarity to the wide cavity, and positions itself more towards the isoprenoidal side chain.

In contrast, the introduction of additional double bonds strongly affects the binding of pregnane derivatives (lacking the hydrophobic side chain at C(17)). Thus, progesterone (**6**, $-\Delta G^0 = 4.7$ kcal mol^{-1}) binds by 0.8 kcal mol^{-1} better than 1-dehydroprogesterone (**38**) or 16-dehydroprogesterone (**31**). Note that all three derivatives feature very similar log *P* values. In the absence of the isoprenoidal side chain, the receptor has no opportunity to slip away from the flattened, poorly complementary A ring and the

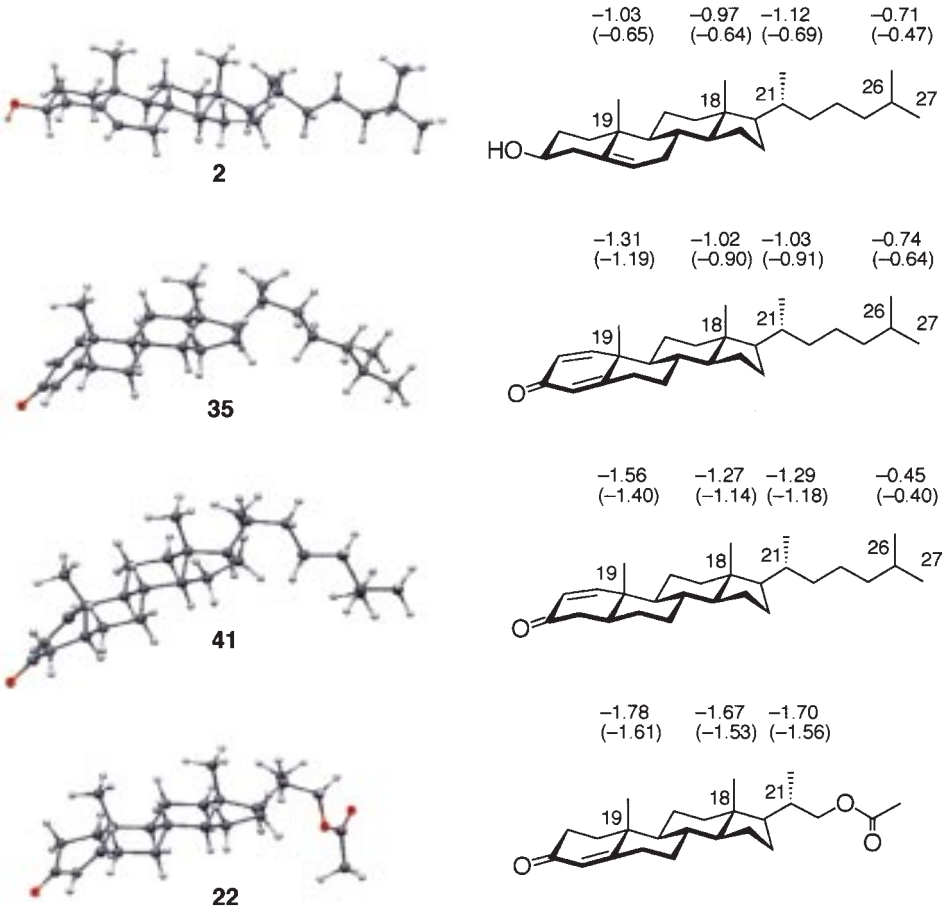
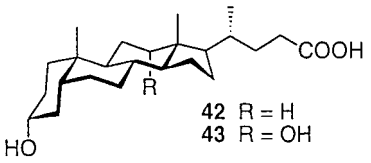


Fig. 4. Left: *Calculated* (MacroModel, AMBER*) *lowest-energy conformations of steroids* **2**, **22**, **35**, and **41** *complexed by (±)-***1**. The receptor is omitted for clarity. Right: *Calculated upfield CIS at saturation binding* ($\Delta\delta_{sat}$) *and, in parenthesis, the maximum observed CIS* ($\Delta\delta_{max\ obs}$) *for the steroidal Me group* ^{1}*H-NMR resonances*

attractive *van der Waals* contacts in the formed complex are reduced. A reduction in binding strength is also observed upon increasing the degree of unsaturation in (20*S*)-20-hydroxymethylpregnene derivatives (**30** *vs.* **39**).

2.5. *Hydroxy Substituents.* Partial or full incorporation of a steroidal OH group into the apolar receptor cavity of (±)-**1** leads to an energetically unfavorable desolvation of this polar group, which, in return, reduces the association strength. Previous studies with a steroid-binding macromonocyclic cyclophane in D_2O/CD_3OD 1:1 [13] had shown that lithocholic acid (**42**), which does not bear polar groups on rings B and C, forms a complex that is by 2 kcal mol^{-1} more stable than the complex of deoxycholic acid (**43**), which possesses one α-OH group at C(12) of ring C. To minimize the energetically unfavorable desolvation of this OH group, deoxycholic acid prefers



adopting a different orientation in the complex (as compared to lithocholic acid), which positions the polar group more outside of the cavity [25].

In the present study, the measured reductions in binding strength, due to unfavorable complexation-induced desolvation of OH groups, are less pronounced, since solvation effects are generally reduced in pure CD_3OD as compared to D_2O/CD_3OD 1:1 [15], and since these groups are positioned more at the periphery of the substrates, as compared to the central position of the OH group in deoxycholic acid (**43**). Nevertheless, a significant destabilization of the inclusion complexes of OH-substituted steroids was measured, as was already mentioned in *Sect. 2.3.* Androst-5-ene-3β,17β-diol (**21**), with both OH groups directly attached to the tetracyclic skeleton, is hardly bound at all by (±)-**1**, whereas compounds such as testosterone (**7**) and chol-5-ene-3β,24-diol (**17**) form weak complexes ($-\Delta G^0 = 3.1$ to 3.3 kcal mol^{-1}). Also for reasons of unfavorable complexation-induced desolvation of an OH group, the complexes of cholesterol (**2**) or dihydrocholesterol (**9**) are by 0.5 kcal mol^{-1} less stable than the corresponding complexes of 5-cholestene (**4**) and 5α-cholestane (**8**), respectively (*Table 1*). It should, however, also be mentioned that, with increasing number of OH groups, the log *P* value of the steroid decreases, reflecting enhanced stabilization of the substrates by the polar solvent.

2.6. *Carboxy and Carbonyl Derivatives.* This study clearly shows that the solvation requirements of carboxylate and keto groups are lower than those of OH groups, and that, as a result, it is less costly to transfer these groups into an apolar environment depleted of H-bond donor sites. Several examples document the more favorable complexation of acetates as compared to the corresponding OH derivatives: cholesteryl acetate (**3**) binds better than cholesterol (**2**) ($\Delta(\Delta G^0) = 0.5$ kcal mol^{-1}), (20*S*)-20-acetoxymethylpregn-4-en-3-one (**22**) better than (20*S*)-20-hydroxymethyl-pregn-4-en-3-one (**30**) (($\Delta(\Delta G^0) = 0.9$ kcal mol^{-1}), and pregnenolone acetate (**5**) better than pregnenolone (**18**) ($\Delta(\Delta G^0) = 1.2$ kcal mol^{-1}). Part of the more favorable association of the acetates can be attributed to their higher lipophilicity, since the log *P* values increase upon passing from the OH derivatives to the corresponding acetates. An additional stabilization of the acetate inclusion complexes by CH$\cdots\pi$ interactions between the acetyl Me groups and the aromatic rings of the receptor could also be effective [9c][26]. We did not, however, gain experimental evidence in form of particularly large upfield CIS of the acetyl protons for such interactions, since their ^{1}H-NMR resonances are masked by the signals of the receptor.

An enhanced binding is also generally observed upon changing from OH derivatives to the corresponding ketones. Thus, the complex of 5α-cholestan-3-one (**40**) is more stable than that of dihydrocholesterol (**9**) ($\Delta(\Delta G^0) = 0.5$ kcal mol^{-1}). A particularly large increase in stability ($\Delta(\Delta G^0) => 1.3$ kcal mol^{-1}) is measured upon moving from diol **21** to hydroxy ketone **20**. Remarkably, the log *P* values for both **20** and **21** are nearly the same, and the large energetic difference must, therefore, result

from specific functional-group-solvation requirements. In the inclusion complex of **20**, the receptor can position itself more towards the B-D rings in order to avoid encapsulation and unfavorable desolvation of the OH group at C(3). In the corresponding complex of **21**, this alternative does not exist, and partial desolvation of the OH groups can hardly be avoided upon inclusion complexation.

The two carboxylic acids **13** ($-\Delta G^0 = 4.1$ kcal mol^{-1}) and **19** ($-\Delta G^0 = 3.1$ kcal mol^{-1}) most probably are bound in the non-dissociated form in CD_3OD, and *Coulombic* interactions between the carboxylate of the guest and the quaternary ammonium centers of the receptor should, therefore, not contribute to the association strength. The reduction in binding strength upon moving from **13** to **19** is readily explained by the increasing proximity of the COOH residue to the steroidal core. To avoid interference with the COOH group and its high solvation requirement, the receptor encapsulates preferentially the steroidal core rather than the side chain. This is reflected by the large upfield CIS measured for the Me(18) ^{1}H-NMR resonance of bound **13** (*Table 2*).

2.7. *Additional Me Groups on the Tetracyclic Steroidal Core.* The binding affinity of the steroid is, in most cases, not much changed, when an additional Me group is added to different positions on the A ring. This is shown by comparing the complex formed by cholest-3-en-3-one (**24**; $-\Delta G^0 = 4.5$ kcal mol^{-1}) to those formed by the analogous steroids **23** ($-\Delta G^0 = 4.7$ kcal mol^{-1}), **27** ($-\Delta G^0 = 4.4$ kcal mol^{-1}), and **29** ($-\Delta G^0 = 4.1$ kcal mol^{-1}) bearing an additional Me group. Also, cholesta-1,4-dien-3-one (**35**) and the additionally methylated **34**, **36**, and **37** form complexes of similar association strength.

A more detailed ^{1}H-NMR analysis, however, reveals that the geometries of the inclusion complexes might differ substantially, depending on position and orientation of the additional Me group. This is illustrated in *Fig. 5* for the inclusion complexes of 1α-methylcholest-4-en-3-one (**29**) and 1-methylcholesta-1,4-dien-3-one (**34**). Compound **29** features a simply unsaturated A ring with an axial Me group at C(1), whereas compound **34** possesses a doubly unsaturated, flattened A ring that is bent out of the mean plane of the B-D rings and, in addition, bears a Me group at C(1) in plane with the A ring (*Fig. 5*). The analysis of the CIS clearly shows that compound **29** is preferentially enclosed with its tetracyclic core, while the side chain at C(17) protrudes out of the cavity into the solution. The largest upfield CIS are observed for the Me groups on the core ($\Delta\delta_{sat}$ up to 1.40 ppm), whereas the resonance of the terminal Me group of the side chain is only weakly shifted ($\Delta\delta_{sat} = 0.17$ ppm). In contrast, steroid **34** is preferentially encapsulated with its isoprenoidal side chain, and the A ring protrudes out of the cavity. In this complex, the Me groups on the tetracyclic core encounter only moderate upfield shifts ($\Delta\delta_{sat}$ between 0.53 and 1.08 ppm), whereas the terminal Me groups of the side chain are remarkably shielded ($\Delta\delta_{sat} = 0.92$ ppm) by the aromatic rings lining the receptor cavity. The strong preference for inclusion of the side chain of **34** might be due to the flattened A ring and its particularly bent orientation with respect to the B-C ring skeleton. However, computational energy minimizations of the complex also suggest that the Me group at C(1) may extend the width of the A ring too much for a good fit into the binding site.

2.8. *The Case of Progesterone.* Despite its low log *P* value (3.87) and the lack of an extended side chain at C(17), progesterone forms one of the most stable complexes

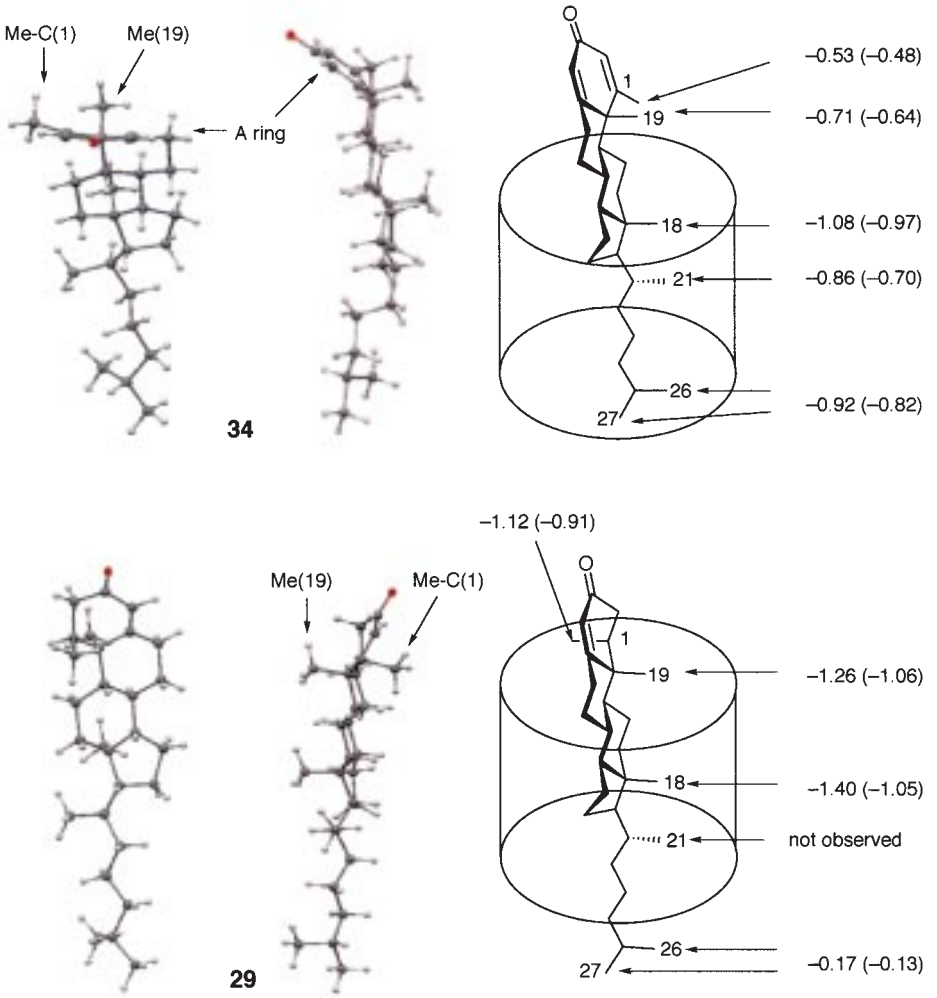


Fig. 5. Left: *Calculated* (MacroModel, AMBER*) *lowest-energy conformations of* **29** *and* **34** *complexed to (±)-***1** *in views onto and parallel to the tetracyclic steroidal core.* The receptor is omitted for clarity. Right: *Schematic drawing of the inclusion geometries which are preferred according to the modeling and the* ^{1}*H-NMR studies.* Calculated upfield CIS at saturation binding ($\Delta\delta_{sat}$) and, in parenthesis, the maximum observed CIS ($\Delta\delta_{max\,obs}$) for the steroidal Me group ^{1}H-NMR resonance are given.

($-\Delta G^0 = 4.7$ kcal mol^{-1}). With the exception of pregnenolone acetate (**5**) and epoxyprogesterone (**28**), there are no other androstane or pregnane derivatives in the entire study binding with $-\Delta G^0 > 4$ kcal mol^{-1}. Computer simulations indicate that progesterone has a particularly good fit to the receptor cavity. The deep inclusion of its tetracyclic core is supported by the large upfield CIS of the Me(18) resonance ($\Delta\delta_{sat} =$ 1.63 ppm; *Table 1*). According to the simulations, progesterone has eleven short (≤ 3.7 Å) C$\cdots$C and three short C$\cdots$O contacts with the receptor; the latter involving the carbonyl O-atom at C(20). By comparison, 5α-cholestane (**8**), which binds with the

same complexation free enthalpy as progesterone, but has a much larger hydrophobic surface, also undergoes only twelve short C···C contacts with the receptor. Progesterone, with an Ac side chain at C(17), and cholest-4-en-3-one (**24**; $-\Delta G^0 =$ 4.5 kcal mol^{-1}), with the same tetracyclic core but with an isoprenoidal side chain at C(17), show nearly identical binding. Their log *P* values, however, differ greatly (3.87 for **5** and *ca.* 9.40 for **24**), conclusively showing that binding by receptor (±)-**1** is not a simple partitioning between two phases of different polarity (CD_3OD and the interior of the cyclophane cavity), but that specific host-guest interactions control the association strength. Accordingly, minor changes in the progesterone structure already lead to large reductions in binding strength: the epoxy derivative **28** binds weaker by 0.6 kcal mol^{-1}, compounds **31** and **38**, each with an additional double bond, weaker by 0.8 kcal mol^{-1}, and the methylated derivative **32** ($\log P = 4.87$) weaker by 1.2 kcal mol^{-1}. The specific binding of progesterone clearly shows that inclusion complexation by (±)-**1** is a true molecular-recognition event.

3. Conclusions. – The inclusion complexation between the double-decker cyclophane (±)-**1** with a deep cavity and a series of 30 steroids was investigated in CD_3OD by ^{1}H-NMR titrations. The high accuracy of the data, together with the large number of complexes investigated, allowed the identification of various, meaningful structure-function relationships, which are not easily obtained in studies with biological receptors. The evaluation of the measured CIS of the steroidal Me group ^{1}H-NMR resonances provided a good view of the geometries of the complexed formed. The experimental structures were further corroborated by computer modeling, and the rigidity of both receptor and steroidal substrates ensured a high confidence level of the calculated structures. To evaluate whether axial inclusion of the steroids by the host is driven by specific host-guest interactions, or whether the driving force for complexation is mainly provided by the greater preference of the steroid for the more lipophilic interior of the cavity as compared to the CD_3OD solution, log *P* (octanol/water) values of the steroids were determined experimentally or calculated using the CLOGP program. Although steroids with a high log *P* value tend to form more stable complexes than those with low log *P* values, there exists no general correlation between the thermodynamic driving force for complexation $-\Delta G^0$ and the partition coefficient. In fact, progesterone, with one of the lowest log *P* values, forms one of the most stable complexes. Thus, we can conclude that (±)-**1** not only provides a lipophilic phase for the steroid, but that it also acts as a specific receptor.

The following structure-function relationships were identified: *i*) Steroids with an isoprenoidal side chain at C(17) form some of the most stable complexes ($-\Delta G^0$ up to 4.8 kcal mol^{-1}), with side-chain inclusion contributing up to 1.2 kcal mol^{-1} in binding free enthalpy. There is competition between inclusion of the tetracyclic core and the side chain, and the measured CIS indicate that the receptor is slipping over the two fragments in a dynamic process. If double bonds are introduced into the A and B rings, which become flattened and less complementary in shape to the wide binding site, the receptor shows a tendency to slip away from these rings onto the isoprenoidal side chain, and the overall binding affinity is hardly changed. *ii*) Pregnane derivatives, which lack the isoprenoidal side chain, are tightly encapsulated with their tetracyclic core. If the core is flattened by introduction of double bonds, binding affinity drops

substantially. *iii*) The incorporation of steroidal OH groups into the receptor cavity is accompanied by energetically unfavorable functional-group desolvation, and can lead to very weak binding, as was observed for **21**. On the other hand, the solvation requirements of carboxylates and keto groups are much lower, and the incorporation of these functional groups into the lipophilic host cavity does not reduce the steroid binding affinity. *iv*) Additional Me groups on the tetracyclic steroidal core do not alter the association strength; however the analysis of measured CIS shows that the geometries of the complexes can change significantly. *v*) Progesterone has a particularly high complementarity to the receptor cavity and forms one of the most stable complexes. Even small structural changes in progesterone derivatives reduce this complementarity and strongly affect the stability of the formed complexes. These are clear characteristics of a selective receptor. This work confirms once more that investigations with small artificial receptors can ideally complement studies with large biological protein receptors in providing fundamental insight into molecular-recognition phenomena.

Experimental Part

General. The steroids **12**–**41** provided by *Schering AG* were used in the binding studies without further purification. Receptor (±)-**1** was synthesized according to the published procedure [9c]. For use in multiple-binding studies, (±)-**1** was recycled from solutions containing the steroid complexes by chromatography on *Sephadex* (MeOH) and recrystallization from Et_2O/MeOH 50:1.

Computer Modeling. The simulations were done with MacroModel V. 5.5 [23] using the AMBER* force field [24]. For the determination of the complex geometries, the steroids were introduced into the previously [9b,c] generated structure of (±)-**1**. After initial minimizations of the complexes (GB/SA solvation model for H_2O [27]), the generated conformations were subjected to a 10 000-step Monte-Carlo simulation. For the evaluation of the short intermolecular C···C contacts in the complexes, the program Cerius V. 3.5 of *BIOSYM Technologies, Inc.* (San Diego, 1997) was used.

^{1}H-NMR Binding Titrations in CD_3OD. NMR Spectra were measured on a *Bruker AMX 500* instrument. Association constants were determined by nonlinear least-squares curve fitting of 500-MHz ^{1}H-NMR titration data (298 K) using the program Associate V. 1.6 [28]. In the titrations, the steroid concentration was kept constant (0.25 mM), and the receptor concentration varied (between 0.25 and 4.0 mM) to provide between 10 and 90% saturation binding. The complexation-induced change in chemical shift (CIS) of the steroidal Me(18) resonance was monitored and evaluated in all titrations; for some steroids, additional Me resonances were also followed. The reproducibility of the K_a values is ±10%.

Partition Coefficients log P (octanol/H_2O). They were obtained by the HPLC method according to *OECD* guidelines [21]. HPLC was performed on an anal. column packed with a commercially available reverse phase (*Kromasil 100, C_8*) with MeOH/phosphate buffer 3:1 as the eluent. The log *P* determinations were made at pH 7 and, for carboxylic acids **13** and **19**, also at pH 2. log *P* Values between 0 and 6 are in the measuring range of the method. For steroids with log $P > 6$, an extrapolation was done or a limit is given. All calculated log *P* (CLOGP) values were obtained with the program CLOGP [19] based on the method by *Hansch* and *Leo* [20].

We thank Dr. *Monica Sebova* and Dipl.-Chem. *Anne Sophie Droz* for conducting the ^{1}H-NMR binding titrations.

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Received August 4, 1999