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Monoclonal Antibodies Against Progesterone: Effect of Steroid-carrier Coupling Position on Antibody Specificity

M. Gani,[1]* J. Coley,[1] J. Piron,[1] A. S. Humphreys,[3] J. Arevalo,[2] I. A. Wilson[2] and M. J. Taussig[3]

[1]*Department of Immunology, Unilever Research, Colworth Laboratory, Sharnbrook, Beds. MK44 1LQ, England,* [2]*Department of Molecular Biology, Scripps Research Institute, La Jolla, CA, U.S.A. and* [3]*Laboratory of Structural Studies, Department of Immunology, The AFRC Babraham Institute, Babraham, Cambridge, England*

Monoclonal anti-progesterone antibodies were raised by immunizing mice with progesterone coupled through either the C3, C6 or C11 positions to protein carrier (bovine serum albumin, BSA). The specificity of four antibodies for a range of steroids related to progesterone, some carrying substitutions at various ring positions, was studied by competitive inhibition in an ELISA system. The results demonstrated that the ring coupling position has a determining effect on the cross-reactivity of the antibodies obtained. The patterns of cross-reaction were interpreted in the light of the structure of the combining site of an anti-progesterone antibody (DB3) recently determined by X-ray crystallography, and inferences drawn about the orientation of steroid in the combining sites of the antibodies studied. Specifically, in two antibodies raised against progesterone-11–BSA, the orientation of steroid resembled that of the progesterone–DB3 complex, with positions C11 and C3 exposed and C6 and C20 buried; an antibody raised against progesterone-6–BSA bound steroid in an apparently similar disposition, except that C6 was exposed and C11 buried; finally, in an antibody raised against progesterone-3–BSA, all steroid positions other than C3 were apparently buried in the steroid–antibody complex.

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INTRODUCTION

Steroids are immunogenic haptens when conjugated to protein carriers and as such can produce specific anti-steroid antibody responses. Monoclonal antibodies (mAbs) to progesterone have been raised by conjugation of the 11α-hydroxy steroid to protein by a succinyl linker arm [1–4]. The binding of progesterone to one such mAb, DB3, has recently been analysed at the structural level by X-ray crystallography of the Fab' fragment complexed with steroid [5]. The DB3 structure has provided a molecular basis for understanding the cross-reactivity of the combining site with steroids other than progesterone. Inferences about the nature of the combining sites of other anti-steroid antibodies can now be drawn by analysing their individual patterns of cross-reactivity with structurally related steroid ligands. We report here the binding characteristics of four mAbs raised to progesterone conjugated through different positions on the steroid nucleus (C3, C6 and C11) and assess the structural implications of their differential reactivity by biochemical mapping of their binding pockets. These antibodies are characterized and compared with DB3.

*Correspondence to M. Gani.
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EXPERIMENTAL

Progesterone–protein conjugates

Steroids were obtained from Steraloids Ltd (Croydon, England). Progesterone was coupled to bovine serum albumin (BSA) or alkaline phosphatase (AP) via positions 3, 6 or 11, by mixed anhydride reaction, as described previously [6–8].

Preparation of mAbs

BALB/c mice, 8–12 weeks of age, were immunized intraperitoneally with 50–100 μg of steroid immunogen emulsified with an equal volume of Freund's complete adjuvant. Booster injections with incomplete Freund's adjuvant were given on days 30, 37 and 44. After the fourth immunization, sera were tested for anti-progesterone antibodies by ELISA (below). The

mouse with the highest anti-progesterone activity was selected for fusion and given three further daily injections of immunogen in saline i.p. prior to sacrifice. Fusions were performed using Sp 2/0 cells as described previously [9].

Enzyme linked immunoassay (ELISA)

Polystyrene 96-well microtitre plates were coated overnight at 4°C with rabbit anti-mouse Ig (5 μg/ml in 0.1 M sodium bicarbonate, pH 9.8), followed by washing in phosphate buffered saline (PBS) containing 0.15% Tween 20 and 0.01% sodium azide (PBSTA). 100 μl of each cloned supernatant were added per well, incubated for 1 h at 37°C and the wells washed again. 100 μl of steroid–AP conjugate were added and incubated for 1 h, 37°C. The plates were washed again, 200 μl of substrate solution (1 mg/ml *para*nitrophenyl phosphate in 1 M diethanolamine, pH 9.8, containing 1 mM $MgCl_2$) were added per well and the OD measured at 405 nm after 30 min incubation.

Antibody specificity screen

Initial ELISA screening was carried out using progesterone–AP coupled in the same position as the immunogen (i.e. antibodies raised against progesterone-3–BSA were screened against progesterone-3–AP, etc). Supernatants of positive hybrids were then screened with all progesterone–AP conjugates, i.e. in which progesterone was coupled via positions 3, 6 or 11.

Specificity for different steroids was determined by competition assay [10]. In brief, the ELISA method was performed in the presence of free steroid at various dilutions, competing with a fixed amount of antibody giving 60–70% maximal binding in the absence of steroid. Cross-reaction with other steroids is defined as $100X/Y\%$, where X is the concentration of homologous steroid (progesterone) and Y the concentration of heterologous steroid required to produce 50% inhibition.

RESULTS

Specificity of mAbs for progesterone conjugated to AP at different positions

A panel of anti-progesterone mAbs, raised against three different immunogens, was obtained and screened against progesterone–AP in which the steroid was coupled at the 3, 6 or 11 position. Four mAbs of interest were selected, showing the specificity patterns summarized in Table 1, with titration curves in Fig. 1. Antibody 3562 was apparently specific for the conjugate in which the enzyme and BSA were coupled through the same position, but was clearly of low affinity. Two antibodies had dual reactivity, namely 3568 which reacted well with 3- and 6-conjugated AP (preferentially with the 6-conjugate), and 4159 which reacted equally well with 3- and 11-conjugated AP. Finally, antibody 2533 was reactive with all three progesterone–AP conjugates, though discriminating clearly among them. With the exception of 4159, the mAbs showed a preference for the steroid linked through the same position as in the conjugate used for immunization. For comparison, the specificity of mAb DB3 (raised against progesterone-11–BSA) is also included in Table 1; DB3 reacted equally well in ELISA with 11- and 3-conjugated progesterone–BSA, but was almost completely unreactive with the 6-conjugate.

Specificity of selected mAbs for different steroids

A series of progesterone-related steroids was used to inhibit the binding of the four mAbs against progesterone–AP conjugates. Cross-reactivity (relative to progesterone) was quantified from the amount of each steroid required to produce 50% inhibition. The results are shown in Table 2.

Anti-progesterone-11α-hemisuccinyl–BSA mAbs 4159 and 2533. The mAbs 4159 and 2533 bound strongly to the 11α-hemisuccinyl (HS) and 3-carboxymethyloxime (CMO) derivatives of progesterone (# 2 and # 4 in Table 2), with a small preference for the former, but had little or no binding affinity for 6β-HS–progesterone (# 3) (though binding of 2533 for the latter could be shown by using the weaker binding progesterone-6–AP as the competitor). They cross-reacted well with both 5α- and 5β-pregnane-3,20-diones (# 11, # 12). The mAb 2533 did not react with androsterone (# 13) and discriminated among the hydroxyprogesterones, reacting strongly with 11α-hydroxyprogesterone (# 6), but very weakly with 17α-hydroxy- or 6β-hydroxyprogesterone (# 7, # 8), 5β-pregnanolone (# 9) or pregnanediol (# 10). For the most part, this pattern of reactivity is similar to DB3 [1, 4], with the exception of 5β-pregnanolone. In contrast, 4159 had a much wider reactivity and bound most derivatives tested; interestingly, it bound the 6β-hydroxy (# 8) but not the 6β-HS (# 3) derivative of progesterone.

Anti-progesterone-6β-HS–BSA mAb 3568. Antibody 3568 bound progesterone conjugated to HS or CMO groups at the 6- or 3-positions, respectively, but did not bind 11α-HS–progesterone (# 2). The binding

Table 1. Summary of specificity patterns of selected anti-progesterone mAbs

Clone	Immunogen	Conjugate Prog-3–AP	Prog-6–AP	Prog-11–AP
2533	Prog-11-HS–BSA	+ +	+	+ + +
4159	Prog-11-HS–BSA	+ +	—	+ +
3568	Prog-6-HS–BSA	+ +	+ + +	—
3562	Prog-3-HS–BSA	+	—	—
DB3[a]	Prog-11-HS–BSA	+ +	—	+ +

The table shows the extent of cross-reactivity of the five monoclonal anti-progesterone antibodies under study with three progesterone–AP (Prog–AP) conjugates. The positions of coupling of progesterone to BSA in the immunogens are indicated (HS = hemisuccinyl). DB3 specificity measured by RIA against progesterone-conjugated BSA. $^{+++}$, maximum $OD_{280\,nm}$ (optical density) reading in ELISA > 1; $^{++}$, $OD_{280\,nm}$ max. 0.6–1; $^{+}$, OD max. < 0.6.

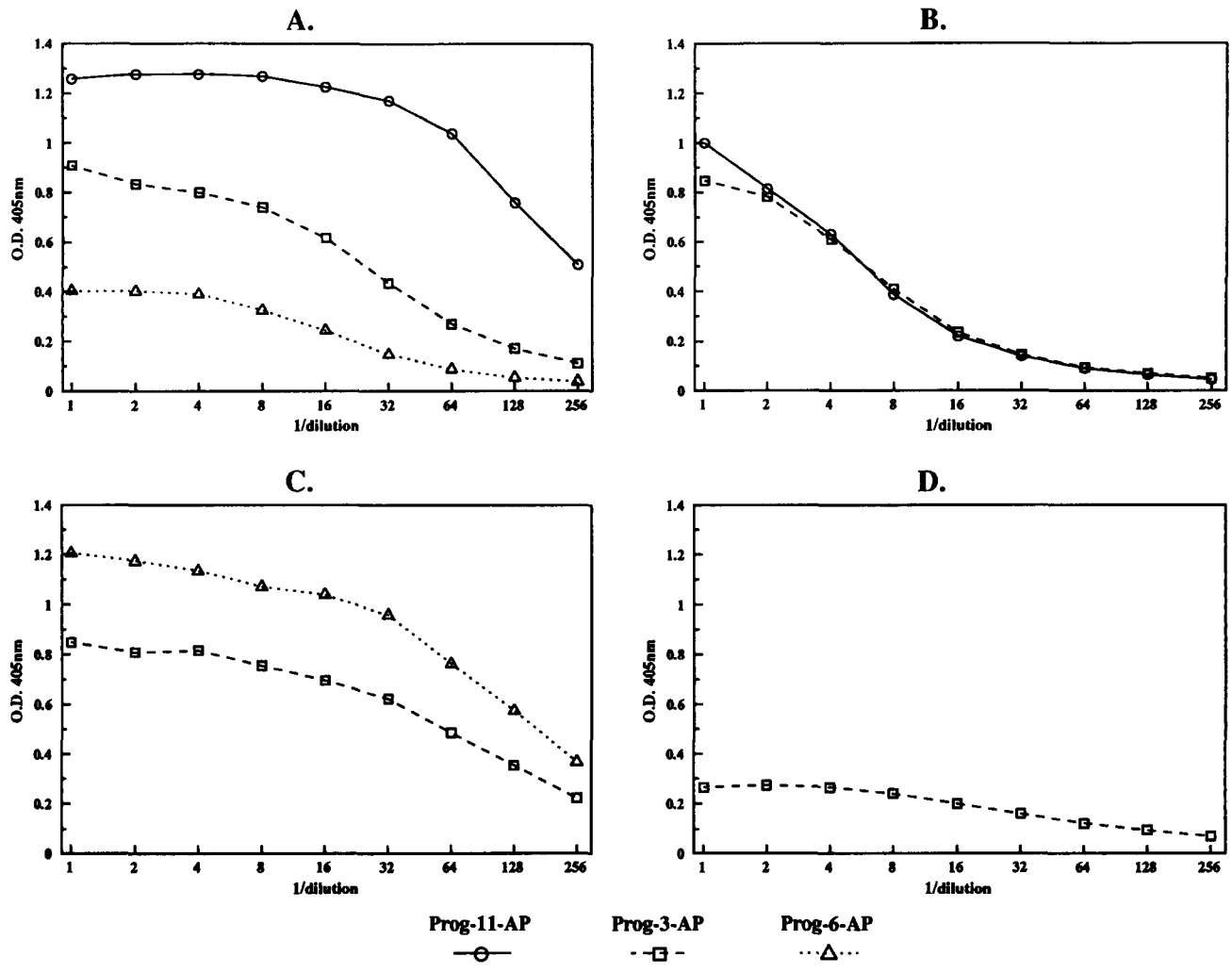

Fig. 1. ELISA titration curves for monoclonal anti-progesterone antibodies 2533 (A), 4159 (B), 3568 (C) and 3562 (D) against three different progesterone–AP (Prog–AP) conjugates, progesterone-11–AP, progesterone-3–AP and progesterone-6–AP. O.D., optical density. 1/dilution, inverse of dilution of supernatant containing monoclonal anti-progesterone.

of the 6- and 3-conjugated progesterones was over 10-fold stronger than for progesterone itself (# 1), suggesting a stabilizing contribution from the attached side-chain. 3568 showed a clear-cut pattern of cross-reactivity with 5α- and 5β-pregnanediones (# 11, # 12), pregnanolone (# 9) and 6-hydroxy-progesterone

Table 2. Cross-reactivities of anti-progesterone mAbs

mAb No.	Posn Immun.	Posn Conj.	Steroids and cross-reactivity # 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	# 10	# 11	# 12	# 13
3562	3	3	100	0.0	0.0	633	13	0.0	0.0	0.0	0.0	0.0	66	0.0	0.0
3568	6	3	100	0.0	600	517	46	0.0	0.0	150	65	0.0	78	50	4.0
3568	6	6	100	0.0	1650	1320	71	0.0	0.0	132	73	0.0	94	71	0.0
2533	11	3	100	100	0.0	13	1.0	100	1.0	3.0	1.0	1.0	44	17	1.0
2533	11	6	100	103	9.0	22	0.6	100	0.5	2.0	1.0	0.0	32	12	0.3
2533	11	11	100	600	0.0	100	0.0	380	0.0	5.0	0.0	0.0	46	33	0.0
4159	11	3	100	100	4.0	32	4.1	100	5.9	16	4.7	4.0	22	71	6.0
4159	11	11	100	100	0.0	42	30	91	34	42	33	31	40	81	33
DB3	11	11	100	200	0.0	10	10	100	0.4		25	0.0	100	50	0.1

Steroids: # 1, progesterone; # 2, progesterone-11α-HS; # 3, progesterone-6β-HS; # 4, progesterone-3-CMO, # 5, pregnenolone; # 6, 11α-hydroxyprogesterone; # 7, 17α-hydroxyprogesterone; # 8, 6β-hydroxyprogesterone; # 9, 5β-pregnan-3α-ol-20-one; # 10, 5β-pregnan-3α,20α-diol; # 11, 5α-pregnan-3,20-dione; # 12, 5β-pregnan-3,20-dione; # 13, 5α-androstan-3α-ol-17-one. ND, not done.

The table shows the extent of cross-reactivity of five anti-progesterone mAbs with 13 steroids related to progesterone. The 'Posn Immun.'column indicates the position at which progesterone was coupled to BSA in the immunizing conjugate. The 'Posn Conj.' column indicates the progesterone–AP (AP) reagent used in the ELISA (i.e. progesterone-3–AP, progesterone-6–AP or progesterone-11–AP). Cross reactions were determined by competitive ELISA with the free steroids and calculated as described in Experimental.

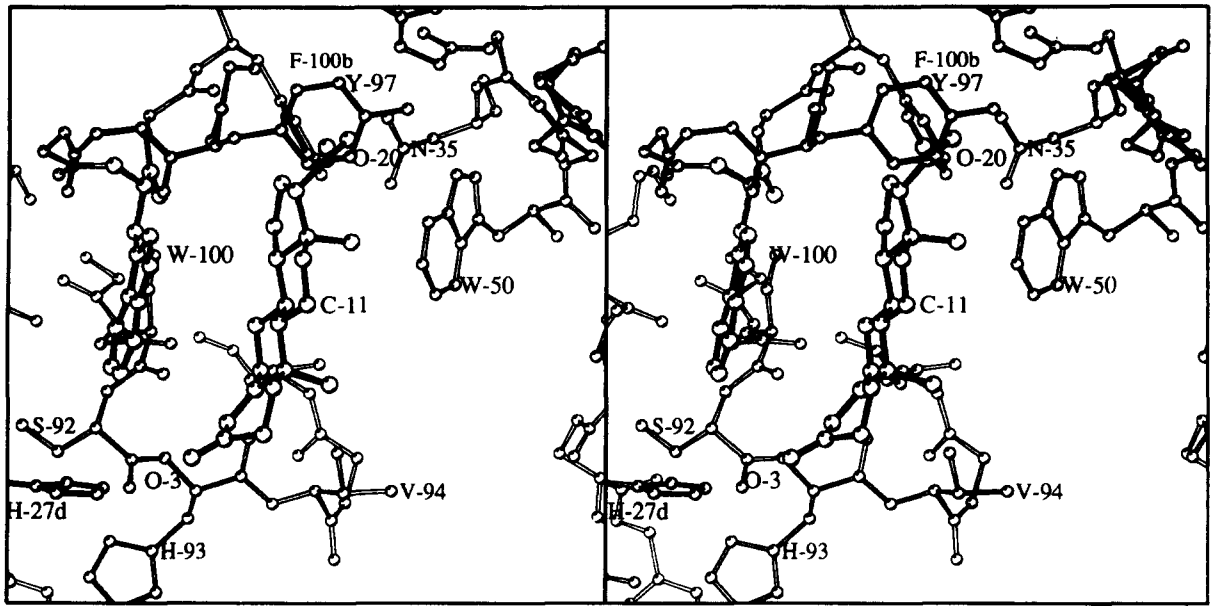

Fig. 2. View of the combining site of monoclonal anti-progesterone antibody DB3 complexed with progesterone, taken from the 2.7 A resolution crystallographic structure [5]. Steroid (with reference positions numbered) and the antibody contact residues in the site are shown. Steroid atom O-3 forms a hydrogen-bond with H-27d (light chain), while steroid atom O-20 forms a hydrogen bond with N-35 (heavy chain) (for details, see Ref. [5]).

(# 8). It failed to bind progesterone hydroxylated at positions 11, 17 or 20 (# 6, # 7, # 10). With the exception of the interposition of 11- and 6-hydroxyprogesterones, this cross-reactivity pattern is very similar to 2533 and DB3 [1, 4].

Anti-progesterone-3CMO–BSA mAb 3562. The mAb 3562 appeared to be specific for only one of the three conjugated progesterones, namely progesterone-3CMO (# 4), the homologue of the immunogen used to raise this mAb; it bound this derivative more strongly than progesterone (# 1). The only other cross-reactions detected were with 5α-pregnan-3,20-dione (# 12) and pregnenolone (# 5).

DISCUSSION

Antibodies to steroids can be raised by immunizing with steroid–protein conjugates. The specificities of the antibodies obtained depend partly on the nature of the steroid link to the protein carrier, in particular the position on the steroid nucleus at which the linker is attached, since this determines the orientation of the steroid in the antibody combining site. For a ligand such as a steroid, the binding site is likely to consist of a hydrophobic cavity or pocket in which most of the ligand surface becomes buried. When the unconjugated steroid interacts with the antibody, the atom or group through which it was attached to the carrier protein is orientated to the outside of the site and exposed to solvent; otherwise, the majority of the steroid is buried and interacts with the side chains of the antibody hypervariable loops [5]. Shape complementarity between the steroid and the antibody in combination with the specific antibody–ligand interactions determine what other steroids can be accommodated in addition to the original immunogen (i.e. cross-reactivity). In general, steroids differing at positions where direct contact is made with antibody side-chains can be expected to have a lower binding affinity than the immunizing steroid, whereas differences at positions exposed to the outside of the antibody site should have relatively little effect. Thus, given the rigidity of the steroid nucleus, steroids with substitutions at defined positions can be used as probes to analyse the topography of steroid combining sites.

These principles have been demonstrated at the atomic level in the recent 2.7 A X-ray structure of the complex of an anti-progesterone monoclonal antibody Fab' fragment (DB3) with progesterone [5]. The DB3 antibody was raised against progesterone coupled to BSA via a succinyl linker at the steroid C11-position (progesterone-11α-HS–BSA). In the complex, progesterone is bound in a sandwich between two heavy chain (V_H) tryptophan residues (Trp50H, Trp100H) and further oriented by hydrogen bonds to the ketones at positions C3 and C20. The steroid is 85% buried in the site and its conformation is consistent with that of free progesterone deposited in the Cambridge Structural Data Bank [11, 12]. The bound steroid orientation is such that the C11- and C3-positions are exposed to solvent, while the C6- and C17-positions are buried in the pocket (Fig. 2). This mode of binding explains the strong cross-reactivity of the DB3 antibody with

progesterone derivatized at the C3 and C11 positions (11α-hydroxyprogesterone, progesterone-11α-HS, progesterone-3-CMO) and steroids differing from progesterone only in saturation of the A ring (5α- and 5β-pregnan-3,20-dione, pregnanolone), and the much weaker binding of steroids with substitution (or reduction) at the 6, 17 or 20 positions (progesterone-6β-HS, 17α-hydroxy-progesterone, pregnanediol, androsterone) (Table 2).

The crystallographic structure of the progesterone–DB3 complex can be used to aid the interpretation of the cross-reactivity patterns of other anti-steroid antibodies and to draw conclusions about the steroid orientation in binding sites. Antibody 2533, also raised against progesterone-11α-HS–BSA, exhibits a cross-reactivity pattern closely resembling that of DB3, in which alteration to the A ring and at positions C11 and C3 are well tolerated, while differences at the C6, C17 and C20 positions lead to a sharp loss of binding affinity (Table 2). Thus, we can suggest that the orientation of steroid in the combining site of 2533 is similar to that for DB3 in Fig. 2. This does not necessarily apply to all antibodies raised against this conjugate, as mAb 4159 shows a much wider cross-reactivity. mAb 4159 displays some intriguing cross-reactions not seen for either DB3 or 2533, e.g. with 17α-hydroxyprogesterone, androsterone, pregnanediol and 6β-hydroxyprogesterone. On the one hand, the finding that 4159 bound to 11- and 3-conjugated progesterones, but not to the 6-conjugate, suggests that steroid orientation is similar to that in the DB3 site; however, the ability to bind steroids with significant alterations at the 17-end and with 6β-hydroxyprogesterone is at variance with this model. The fit of steroid in the 4159 binding site appears to differ from DB3 or 2533. There are various possible explanations for this difference: the antibody may be able to accommodate steroids in more than one orientation, in which spaces in the progesterone–4159 complex can be filled by small substituents (such as hydroxyl groups), or the 4159 pocket may adjust to fit various ligands by an induced fit mechanism, as has been reported for peptides [13, 14], protein [15] and DNA [16] complex structures.

A second antibody with a structurally explicable pattern of cross-reactivity is 3568, raised against progesterone-6β-HS–BSA. Its cross-reactions closely resemble those of DB3 and 2533, with the notable exception of steroids substituted at the C6 or C11 positions, for which binding is reversed from the DB3 pattern. This is consistent with the nature of the conjugates (through positions 6 vs 11) used to raise the antibodies. Thus, the orientation of progesterone in the 3568 site apparently resembles that of DB3, but with position C6 exposed at the top and C11 buried. This means that, compared with the DB3 complex, the steroid in the 3568 complex should be inverted along its long axis; the C18 and C19 methyl groups (β-face of the steroid) will then point towards residue 100 H rather than 50 H (assuming they form contacts with the steroid similar to those of DB3) and the interaction of the 20–21 carboxymethyl side arm will also differ. Sequence comparison between 3568 and DB3 will be of considerable interest in elucidating further the nature of the interaction between progesterone and the 3568 antibody.

Finally, antibody 3562 raised against progesterone-3-CMO–BSA was particularly restricted in its specificity, binding progesterone derivatized at the 3-position (progesterone-3-CMO), but not derivatives at the 6, 11, 17 or 20 positions. Moreover, 3562 (but not DB3, 3568 or 2533) was able to distinguish 5α- and 5β-pregnane-3,20-dione enantiomers; 3562 binds to the 5α-form in which the A-ring conformation is coplanar with the B, C and D rings, but not to the 5β-form in which the A-ring conformation is considerably out of the plane [17]. An interpretation consistent with this high degree of specificity is that progesterone is even more buried in the 3562 site than in DB3, with only the C3-ketone exposed.

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