Testosterone Receptor Binding Mimic Constructed Using Molecular Imprinting

S. H. Cheong, S. McNiven, A. Rachkov, R. Levi, K. Yano, and I. Karube*

Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153, Japan

Received July 11, 1996; Revised Manuscript Received December 23, 1996[⊗]

ABSTRACT: The molecular imprinting technique was used in the synthesis of polymers having a high affinity for testosterone. These polymers thus function as receptor binding mimics for the drug. Various polymerization conditions were examined in order to determine their influence on the binding strength and selectivity of the binding mimics. Using a series of similar steroids, we were able to identify features of the molecules which affect their affinity for the polymer matrix. The efficacy of covalent and noncovalent imprinting methods was also compared. As determined by HPLC, the most selective (noncovalently imprinted) polymer bound testosterone over 4 times more strongly than did a nonimprinted polymer and at least 3 times more selectively than steroids of similar structure.

Introduction

Molecular imprinting[1,2] is now a well established technique for the construction of polymers having a high affinity for a target molecule. The general procedure for creating these MIPs involves (i) the assembly of polymerizable "functional monomers" around a "template" molecule in a solution containing a high ratio of cross-linker, (ii) polymerization of the mixture, and (iii) removal of the template to afford the imprinted polymer. The resulting molecularly imprinted polymers (MIPs) are macroporous matrices possessing microcavities with a three-dimensional structure complementary in both shape and chemical functionality to that of the template about which they were formed. The high degree of cross-linking enables the microcavities to maintain their shape after removal of the template, and thus the functional groups are held in an optimal configuration for rebinding the template, allowing the receptor to "recognize" the original substrate. Thus they have been developed for the binding of drugs,[3−7] herbicides,[8−10] and other biologically important molecules such as amino acids and their derivatives,[11] peptides,[12] proteins,[13,14] nucleotides,[15] and nucleotide bases.[16,17] The advantages that MIPs possess over biopolymers are cost, ease of preparation, and stability over a great range of temperatures. Recent work[4] by Mosbach's group compared the selectivities and cross-reactivties of MIPs and antibodies for theophylline and diazepam and found them to be equal, confirming the utility and, in this case, superiority of this method.

In spite of their biological and clinical importance there are only a few instances[18−21] where steroids have been used as substrates in molecular imprinting. The increasing illegal use of anabolic steroids in sport also necessitates a rapid, sensitive, and inexpensive means of detecting the drug and its metabolites. Moreover, the recent successes of hormone therapy for the aging and the imminent release of a male contraceptive make the anabolic-androgenic steroids[22] worthy of greater attention.

The steroids are a family of large, structurally similar organic molecules. They possess a rigid, stereochemically complex hydrocarbon skeleton with a variety of substituents at the extremities. They are thus ideally suited for a study aimed at identifying those factors which affect the binding of these substrates to our artificial receptors. In spite of the great need for such work, we are aware of few extensive studies on the optimization of polymerization conditions for a given template,[23,24] and those studies were concerned with quite a different problem: the enantioresolution of racemic phenylalanine anilide. Thus we were prompted to carry out our own study in order to determine the optimal conditions for our particular substrate. Moreover, by examining the behavior of a number of probes with differing shapes and functionalities, we expected to gain a lot more information than we would using a single compound.

We chose a series of compounds such that we could examine the effects of shape, distribution of functionality, and one-point versus two-point binding. The structures of the various steroids are shown in Figure 1. By examining the behavior of these probes in receptors created under varying polymerization conditions (different functional monomers, solvents, template/monomer ratios, *etc.*), we expected not only to optimize the selectivity of our receptor but also to be able to identify those parameters which are important in determining the ability of MIPs to recognize template molecules.

This work concerns the optimization of a testosterone-selective MIP with the aim of eventually exploiting such systems in the construction of steroid sensors.

Experimental Section

General Information. Infrared spectra were recorded on a Horiba FT-200 spectrometer using KBr disks. UV−visible spectra were recorded on a Hitachi U-2000 instrument. NMR spectra were recorded using a JEOL JNM-A500 spectrometer. Microanalyses were performed by the Graduate School of Science, The University of Tokyo. All reagents were of the highest grade available. Polymerizable components were purified[32] prior to use.

Testosterone Methacrylate (6). Testosterone (3.16 g, 10.9 mmol), methacryloyl chloride (4.00 mL, 40.9 mmol), and triethylamine (10 mL, 82 mmol) were dissolved in dry (molecular sieves) $CHCl_3$ (100 mL) and stirred at room temperature under nitrogen for 18 h. The solution was washed with dilute HCl (50 mL), 1 M $NaHCO_3$ (50 mL), and saturated NaCl solution (50 mL) and dried on anhydrous $MgSO_4$. The solution was reduced in volume and chromatographed on silica gel using 1% MeOH in CH_2Cl_2 as eluent. Fractions containing the desired material were combined, and the solvent was evaporated. The ester was precipitated from methanol by the

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.

S0024-9297(96)01014-5 CCC: $14.00

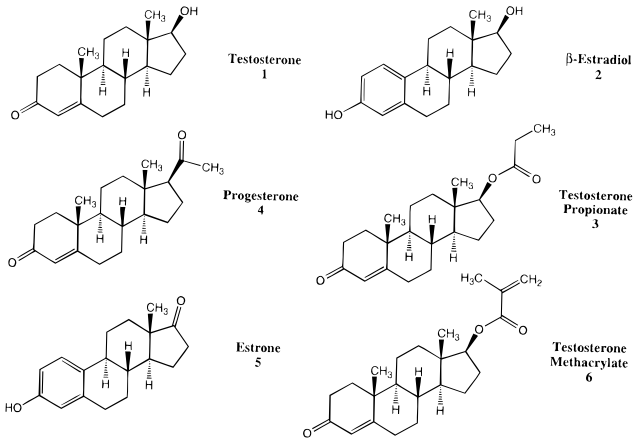

Figure 1. Steroids used in these studies.

addition of water, recrystallized similarly, and washed with cold methanol. The yield of tiny, off-white needles was 1.37 g, 35%. IR (KBr): 2900, 1720, 1680, 1640, 1480, 1300, 1190, 1020, 1000, 950, 850 cm^{-1}. 500 MHz ^{1}H-NMR ($CDCl_3$): δ (ppm) 6.10 (d, 1H, J = 45 Hz, *trans*-H_2C=CMe), 5.74 (d, 1H, HC=C), 5.54 (s, 1H, J = 45 Hz, *cis*-H_2C=CMe), 4.67, 4.66, 4.65, 4.64 (dd, 1H, H-17, J = 10 Hz, HC−O), 2.2−2.5 (m, 5H), 2.0−2.1 (m, 1H), 1.95 (s, 3H, H_3C−C=), 1.8−1.9 (m, 2H), 1.65−1.75 (m, 2H), 1.5−1.6 (m, 3H), 1.3−1.15 (m, 2H), 0.99 (s, 3H, 10-CH_3), 1.15−0.85 (m, 3H), 0.88 (s, 3H, 13-CH_3). 126 MHz ^{13}C-NMR ($CDCl_3$): δ (ppm)[25] 199.46 (C=O, C3), 170.92 (methacryl quaternary), 167.38 (quaternary, C5), 136.69 (methacryl CO_2), 125.13 (CH, C4), 123.98 (CH, C17), 82.68 (methacryl CH_2), 53.71 (CH, C9), 50.24 (CH, C12), 42.75 (quaternary, C13), 38.63 (quaternary, C10), 36.69 (CH_2, C14), 35.72 (quaternary, C1), 35.43 (CH, C8), 33.95 (CH_2, C2), 32.75 (CH_2, C6), 31.51 (CH_2, C7), 27.59 (CH_2, C16), 23.58 (CH_2, C15), 20.55 (CH_2, C11), 18.35 (methacryl CH_3−C=), 17.42 (CH_3, C19), 12.14 (CH_3, C18). Anal. Calcd for $C_{23}H_{32}O_3$: C, 77.49; H, 9.05. Found: C, 77.30; H, 9.14.

Polymerization. The procedure for the synthesis of the standard polymer P2 is as follows. The template (1 mmol) was dissolved in $CHCl_3$ (7.50 mL) in a 50 mL glass flask, and 2,2′-azobis(2,4-dimethylvaleronitrile) [V-65] (50 mg), ethylene glycol dimethacrylate [EGDM] (5.00 mL), and the functional monomer methacrylic acid [MA] (8 mmol) were added. The samples were then degassed by ultrasonication under vacuum and purged with nitrogen. The flasks were then sealed and the mixtures heated at 40 °C for at least 16 h. The bulk polymers were then ground and washed with boiling ethanol until the template could no longer be detected (λ_{max} 238 nm) in the supernatant. Nonimprinted polymers were produced under exactly the same conditions with the omission of the template. The polymerization conditions for all other materials are shown in Table 1.

The covalently imprinted polymer was prepared in a similar fashion using **6** as the template. Hydrolyses were carried out in refluxing methanolic 1 M NaOH (MeOH:H_2O, 1:1) and the polymers reprotonated with either acetic acid or MeOH:HCl (10:1).

High-Performance Liquid Chromatography Studies. Particles were wet sieved in EtOH, and the 25−45 μm fraction was packed into 100 × 4.6 (i.d.) mm HPLC columns. Analyses were performed using a Gilson system equipped with a UV−vis detector (set at 225 nm for these compounds) with MeCN as eluent at a rate of 2 mL/min. A 10 μL aliquot of a 0.2 mM solution of the steroid was injected, and the retention times were compared using acetone as a void marker. Quoted retention times were obtained from at least three successive experiments. The *capacity factor* was calculated as $k' = (t - t_v)/t_v$, where t is the retention time of the analyte and t_v is the retention time of the void marker, in this case, acetone. The *separation factor* $\alpha = k'_x/k'_y$ is the ratio of the retention times of components x and y on a single polymer. Thus α_{imp} and α_{non} are indices of the separation of components x and y on the imprinted and nonimprinted polymers, respectively. We define the *imprinting factor* as $I = k'_{imp}/k'_{non}$, where k'_{imp} and k'_{non} are the capacity factors of the same compound on the imprinted and nonimprinted polymers. Thus k' is a measure of the affinity of the analyte for the polymer, while I is a measure of the effect of the imprinting process. Consequently, comparison of I values for the various steroids gives a measure of how well the inherent selectivity of the polymer improves upon imprinting, and we define a *selectivity factor* $S = I_x/I_y$. A measure of the polymer's ability to actually separate the components is the *resolution* R_s, given by $2(t_x - t_y)/(w_x + w_y)$, where w is the (projected) width of the peak at the baseline.[26]

Table 1. Performance Indices for Initial Methacrylic Acid Polymers

polymer	template/monomer ratio	steroid	k'_{imp}	α_{imp}	k'_{non}	α_{non}	I	S^a
P1	1:4	1	1.39	1	0.795	1	1.75	1
		2	1.13	1.24	0.928	0.857	1.21	1.44
		3	0.398	3.50	0.331	2.40	1.20	1.46
		4	0.530	2.63	0.464	1.71	1.14	1.53
		5	0.464	3.00	0.398	2.00	1.17	1.50
P2	1:8	1	5.10	1	1.26	1	4.05	1
		2	1.52	3.35	1.19	1.06	1.28	3.16
		3	0.597	8.55	0.464	2.72	1.28	3.16
		4	0.795	6.42	0.597	2.11	1.33	3.04
		5	0.530	9.63	0.464	2.72	1.14	3.55
P3	1:12	1	6.56	1	1.59	1	4.12	1
		2	1.92	3.42	1.26	1.26	1.52	2.71
		3	0.729	9.00	0.464	3.43	1.57	2.63
		4	1.06	6.19	0.663	2.40	1.60	2.58
		5	0.597	11.0	0.398	4.00	1.50	2.75

[a] By definition $\alpha = 1$, $S = 1$ for testosterone.

Results and Discussion

Noncovalent Imprinting. We chose the noncovalent imprinting method, pioneered and extensively developed by Mosbach and co-workers, for the bulk of this work, as is it the more convenient and bases the binding of our templates on H-bonding, which is a dominant interaction in biological systems.

Template/Monomer Ratio. Polymers based on methacrylic acid are widely used, so the first parameter we investigated was that of the ratio of template to functional monomer. According to the work of Mosbach and Sellergren, each template requires 3 or 4 equiv of functional monomer in order to produce a sufficiently selective polymer. On the basis of NMR studies, they concluded that an L-phenylalanine anilide template associates with a maximum of three MA monomers in solution prior to polymerization.[27] They then proposed a model involving a 2:1 complex. A later study by Sellergren[23] established that a polymer comprising approximately 25 mol % MA resulted in optimal separation of racemic phenylalanine anilide. Nonetheless, many different molecules having different functional groups[28] and different degrees of functionalization[13,29] have been used as templates, with different ratios of functional monomer to template. Thus, in order to find the optimum conditions for our particular template, we synthesized polymers P1, P2, and P3 (Table 1) containing 4, 8, and 12 mol of methacrylic acid, *i.e.*, 2, 4, and 6 equiv of functional monomer per H-bonding functionality on the template at a constant (1:37) template:cross-linker ratio.

The retention factors k'_{imp} of all substrates increased with the MA content, as did α_{imp}. Sellergren[23] found similar behavior but in that case the retention factor was greatest at *ca.* 50% MA while the separation was best at about half that amount. For practical purposes, all we require is baseline separation of the analytes, and

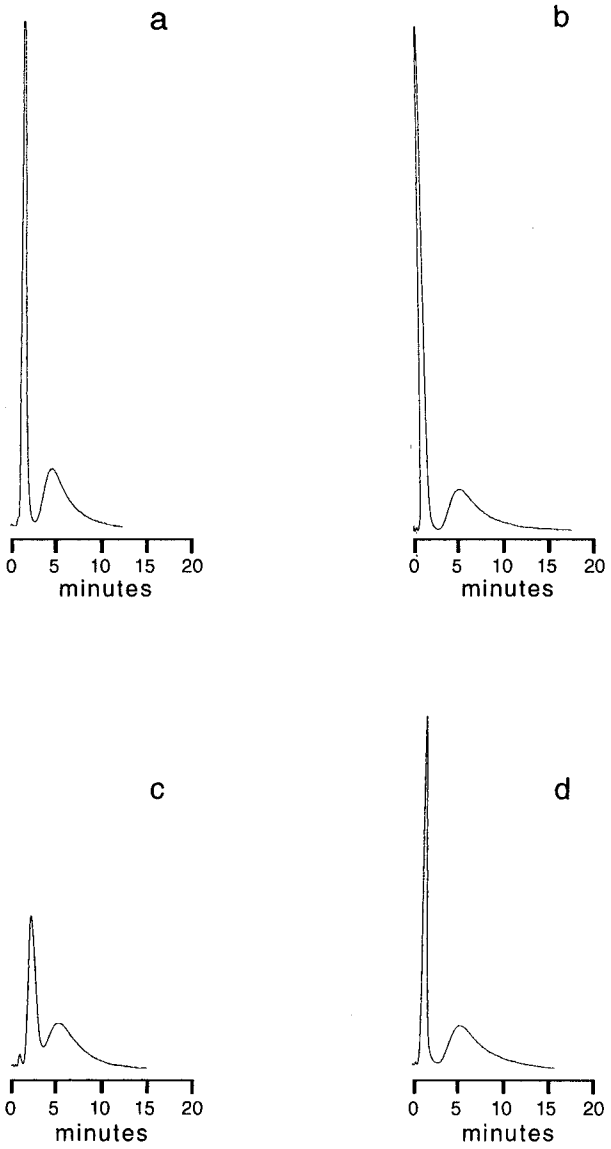

Figure 2. Chromatograms obtained using P2. Mixtures of testosterone and (a) progesterone, $R_s = 1.16$, (b) testosterone propionate, $R_s = 1.26$, (c) β-estradiol, $R_s = 0.80$, and (d) estrone, $R_s = 1.30$.

examination of the separation factors alone is usually enough to judge the utility of an imprinted polymer. To this end, values of approximately $\alpha_{imp} = 6$ are sufficient for the physical separation of testosterone from the other compounds (Figure 2). According to this criterion it would appear that P3 is the best polymer, although R_{imp} and α_{imp} are only marginally greater than for P2. However, R_{non} and α_{non} also increase, indicating that nonspecific interactions also become stronger as the proportion of methacrylic acid increases. Inspection of the *I* values shows that, in spite of this, the effect of imprinting increases with the ratio of methacrylic acid to template for *all* substrates. This indicates that the imprinted polymers are forming binding cavities which more closely complement the shape and functionality of molecules of this general structure—large hydrophobic blocks with polar functionalities at diagonal corners. Although the imprinting factors for P3 are greater than those for P2, comparison of the *S* values shows that the *selectivity* for testosterone is much greater for P2 than P3. Since we are interested not only in the ability of the substrate to *separate* the various steroids but in its ability to selectively *recognize* them, P2 was taken as the standard against which the other polymers were compared.

Effect of Functional Monomer. While acrylic and methacrylic acids are routinely used as functional monomers in MIPs, other functionalities may, in fact, be superior. In particular, "chelating" functional monomers which are able to bind to the template at multiple points should result in considerable increases in the thermodynamic stability[30] of the template-functional monomer complex and yield much more highly selective substrates. We replaced methacrylic acid with DAM (2-(diethylamino)ethyl methacrylate), a strongly basic functional monomer, and synthesized P4. The properties of this polymer were vastly inferior to those of P2, so no further optimization was performed.

Effect of Porogen. The inert solvent used in the polymerization mixture may play a major role in determining the properties (surface area, internal pore volume, *etc.*) of the resulting polymer. Moreover, since polar solvents are more able to solvate polar molecules, this leads to the disruption of H-bonds between, in this case, the template and the functional monomer. In Whitcombe's[19] case, addition of as little as 3% of a H-bonding solvent to a hexane medium was sufficient to prevent the binding of cholesterol to an MIP. This is analogous to the situation observed in "small molecule" host−guest chemistry. The general procedure is to chose the least polar solvent in which the reagents dissolve, in order to maximize the interactions between the template and the functional monomer(s). We prepared P5 using THF instead of $CHCl_3$ and found the polymer showed reasonable selectivity for the template but by no means as much as did P2. In contrast, Sellergren[23] found that substituting benzene for MeCN during the polymerization resulted in a much higher retention factor but a decreased separation. Interestingly, a 1:1 mixture gave the desired results, indicating that it may be possible to optimize the solvent mixture in every case. Our results nonetheless confirm the tendency of more polar porogens to destabilize hydrogen-bonded complexes and inhibit the binding of substrates to receptors.

Polymerization Temperature. To ensure strong, selective binding of the substrate, it is important that the template molecule preorganize the functional monomer(s) in a stable configuration prior to polymerization. Since this preorganization takes place in solution, it is necessarily a dynamic process. The labile functional monomers are in constant flux with the template, and thus a number of binding modes coexist at any time. This results in a heterogeneity or, by analogy with antibodies, a "polyclonality" of binding sites. One way to increase the strength of the template−functional monomer interactions is to decrease the kinetic energy of the system. Thus we examined the effect of the polymerization temperature on the performance of the polymers. P6 was polymerized at 60 °C under otherwise identical conditions, and the contrast with P2 is immediately obvious. Even though P6 gives reasonable separation factors, the retention factors are quite low, indicating that the substrates have a low affinity for the polymer. The higher temperature is expected to drive the equilibrium away from the template-functional monomer complex toward the unassociated species, resulting in a decrease in the number of strongly binding cavities. In a similar investigation Sellergren[23] compared materials polymerized at 40 and 60 °C (subsequently annealing both at 90 °C and then 120 °C)

Table 2. Polymerization Conditions and Selected Data for Imprinted Polymers

		k'_{imp} (steroid)					α_{imp}[b] (steroid)			
polymer	feature[a]	1	2	3	4	5	2	3	4	5
P4	DAM as monomer	0.464	2.78	0.266	0.331	1.92	0.167	1.74	1.40	0.241
P5	THF as porogen	1.99	1.06	0.530	0.729	0.331	1.87	3.75	2.73	6.01
P6	polymerized at 60 °C	1.52	0.862	0.331	0.464	0.266	1.77	4.60	3.28	5.73
P7	Polymerized at room temp using UV	2.39	1.19	0.464	0.597	0.398	2.00	5.14	4.00	6.00
P8	PGDM as cross-linker	0.729	1.06	0.199	0.266	0.398	0.687	3.66	2.74	1.83
P9	covalently imprinted, hydrolyzed 6 h	1.26	1.26	0.398	0.597	0.530	1.00	3.17	2.11	2.38

[a] Standard polymerization conditions are given in the Experimental Section. Only deviations from this procedure are listed. DAM = 2-(diethylamino)ethyl methacrylate, PGDM = propylene glycol dimethacrylate. [b] By definition $\alpha = 1$ for testosterone.

and found that while higher retention factors and better resolution were observed at the lower temperature; the separation factors were almost unchanged. Conversely, we polymerized P7 with UV irradiation at room temperature. Retention and separation factors are slightly higher than those for P6, as has been noted previously. Shea and Sellergren[24] compared materials obtained by either thermal polymerization at 60 °C or photopolymerization at 15 °C and found the latter to be superior stationary phases for the resolution of racemic phenylalanine anilide. However, the retention and separation factors of P7 are still far below those obtained with P2. We attribute this to a lesser degree of polymerization occurring under UV irradiation than at 40 °C. In fact, the performance of Shea's photopolymerized materials was shown to improve after high-temperature treatment of the initially formed polymer. It may be that there is a tradeoff between the extent of polymerization and stabilization of the template−functional monomer complex, and optimal conditions may be found for each combination of template and monomer. Nonetheless, the deleterious effect of high temperatures during the polymerization has been demonstrated.

Nature of the Cross-Linker. Early studies by Wulff[2a] on the optimization of polymer selectivity have resulted in EGDM being almost universally employed as the cross-linking agent. Later work by Shea explored the use of bis(meth)acrylamides[24,31] and Mosbach[13] showed that polymers using trimethylolpropane trimethacrylate (TRIM) were capable of higher loadings than EGDM supports. Nonetheless, EGDM is still the usual choice, at least in the initial stages of polymer optimization. We examined the effect of using propylene glycol dimethacrylate (PGDM), differing by only a single methylene group, as a cross-linker and found it gave a polymer with characteristics quite different from P2. Not only did the imprinted polymer retain β-estradiol considerably more strongly than the template but also the order in which the various steroids were eluted changed. Although separation factors of 2.7 (progesterone) and 3.6 (testosterone propionate) were achieved, it is surprising that such a small change in the structure of the cross-linker produces such a striking result. By way of comparison, Wulff[2a] achieved separation factors of 2.5−3.0 using an 80−90% butanediol dimethacrylate-cross-linked polymer for the resolution of phenyl-D,L-mannoside. Using MA polymers cross-linked with a mixture of EGDM and alkyl bis(methacrylamide)s (38:38:19), Sellergren and Shea[24] obtained separation factors of 2.4 (ethylene) and 1.6 (butane) for the resolution of racemic phenylalanine anilide. The rigid 1,3-diaminobenzene bis(methacrylamide) performed equally as well as EGDM. The difference is therefore most likely due to the added flexibility and rotational freedom conferred by the extra methylene group. It should be noted that the cross-linker constitutes approximately 80% of our MIPs.

Effect of Steroid Structure. In terms of affinity for the various polymers, testosterone is the most strongly retained compound on the imprinted polymers but β-estradiol is also quite well retained.

β-Estradiol has a phenolic A-ring and lacks the C18 methyl group, making the A and B rings fairly planar (structures minimized using CSC Chem3D Plus 3.1.2). Testosterone has a chair, chair structure with a rather sterically demanding C18 methyl group. More importantly, the phenol of β-estradiol and the α,β-unsaturated ketone of testosterone point in different directions. In spite of these differences in structure and the orientation of their H-bonding functionalities they bind very strongly to the nonimprinted polymer. These are the only steroids possessing a hydroxyl group at the 17-position and it therefore appears that this single factor is responsible for the affinity of these compounds for both the imprinted and nonimprinted polymers. It is clear that the rather acidic (and therefore potentially strongly H-bonding) phenol group is not responsible for the strong binding of β-estradiol, as estrone, which possesses the phenol but has a ketone instead of the 17-hydroxyl group, is eluted quite rapidly.

Examination of the retention factors k'_{imp} for P2 indicates that the next most important influence on the binding strength of the substrate is the hydrocarbon skeleton. Progesterone and testosterone propionate differ from testosterone only in the substituent at the 17-position yet have significantly smaller retention factors. That such minor differences have such profound effects on the binding is testament to the ability of MIPs to accurately recognize the template molecules. Estrone, which has neither the tetosterone skeleton nor the 17-hydroxyl group is only weakly retained on the column. It is interesting that this order of elution is generally preserved for all the imprinted polymers examined in this study. That β-estradiol is more strongly retained than both testosterone propionate and progesterone indicates that the interaction between the 17-hydroxyl group and the carboxylic acid group of MA is much stronger than that of the 3-ketone. This can also be seen upon consideration of the k'_{non} values (Table 1).

The affinity of the various steroids for the polymers should be analyzed in terms of the *complementarity* of receptor and substrate. The strength with which the various compounds bind within the microcavities is a direct reflection of how much they resemble testosterone in terms of shape and the disposition of their functional groups. Each steroid examined has functionalities at the 3- and 17-positions and thus should be capable of interacting in some way with the prearranged functional monomers. The high affinity of the polymer for β-estradiol indicates that the C- and D-rings fit extremely well into the testosterone imprint and the poor fit at the 3-position is of lesser importance. Testosterone propionate and progesterone differ from testosterone

only in the substituent at the 17-position and thus are able to bind to the A-, B-, and C-ring portions of the testosterone imprint. Estrone, which has no functionality in common with testosterone, binds only weakly to the cavities by virtue of a vague resemblance to the template. This illustrates the concept of two-, one-, and zero-point binding of the steroids to the substrate.

Covalent Imprinting. The covalent imprinting method involves the copolymerization of functional monomers and a template molecule conjugated to a polymerizable group. After polymerization the template is "split off" from the polymer to expose the binding sites. While noncovalent imprinting suffers from the disadvantage that only a small percentage[23,28] of the binding sites can be reoccupied after removal of the template, the main disadvantage of covalent imprinting is that, at least for esters[20,32,33] and amides,[34−36] only a small percentage of the templates can be removed from the microcavities. Furthermore, the EGDM cross-linker is also hydrolyzed under the splitting conditions and this results in a more swellable and less selective polymer.[24] Nevertheless, some success has been realized using this technique, particularly because a very high percentage of the available site can be reoccupied. We copolymerized **6** and 4 equiv of MA in the presence of excess EGDM, assuming that each molecule of **6** would require the usual 4 equiv of MA to bind the 3-keto group while the carboxylate released upon hydrolysis would provide a well-positioned binding group for the 17-hydroxyl functionality. Prior to hydrolysis, the esterified polymer P8 behaves very similarly to the nonimprinted P1—very short retention times, low separation factors, and a small preference for β-estradiol over testosterone (see tables). Hydrolysis of the polymer for 1 h and subsequent reprotonation gave a polymer with only slightly improved properties. We supposed that the hydrolysis had not proceeded to a sufficient extent so the treatment was continued for a further 5 h. The amount of testosterone released from the polymer was measured by UV spectroscopy and found to be much less than that after the first hydrolysis. Upon acidification, polymer P9 showed only slightly improved separation and gave identical retention times for testosterone and β-estradiol. Similar polymers covalently imprinted with steroidal 3- or 17-methacrylates[20] also showed resistance to hydrolysis using NaOH/MeOH and NaOMe/MeOH and were eventually hydrolyzed with $LiAlH_4$/THF. This, however, reduced the residual functional group to an alcohol and was therefore unsuitable for our studies. Thus we hydrolyzed the polymer for another 6 h, acidified it, and this time noted a decrease in performance relative to P9. While there is some separation of the steroids on our covalently imprinted polymers, the noncovalent imprinting method is vastly superior for these substrates. In contrast, using noncovalent imprinting, Whitcombe[19] was unable to produce a polymer which bound cholesterol, even with 10% MA in the polymerization mixture. An interesting observation made during these experiments was that after treatment with NaOH/MeOH the polymers, containing sodium carboxylate functionalities, bound estrone and, moreover, β-estradiol extremely strongly. Very long retention times were observed, and the chromatograms showed such broad peaks that they were evident only as a slow increase in the height of the baseline. Presumably, this is attributable to the phenol functionality, the acidic proton being bound extremely strongly to the carboxylate anion of the substrate. This information should be useful in the design of MIPs for phenol-containing substrates.

Conclusion

A thorough investigation of the factors influencing the selectivity of MIPs for a closely related series of steroidal compounds has exemplified a number of basic tenets for the design of artificial receptors produced by molecular imprinting methods. Analysis of the retention factors reveals that, in this case, the 17-hydroxyl groups are dominant in determining the affinity of the substrate for the receptor. Our optimized testosterone receptor bound testosterone more than 4 times more effectively than a nonimprinted polymer and afforded separation factors of between 3.3 and 9.6 for a series of structurally similar steroids.

The excellent selectivity among closely related molecules emphasizes the ability of the molecular imprinting method to create receptors with both shape and functional group complementarity. The ability to prepare receptors with a predetermined selectivity has great implications in the fields of enantioseparation, catalysis, immunoassay, and the construction of biomimetic sensors. Current work is directed toward constructing steroid sensors using this technology.

Acknowledgment. This project was supported by the Japanese Ministry of Education, Science, and Culture (Large-scale Research Projects) under the New Program System, Grants-in-Aid for Scientific Research. S.H.C., S.M., A.R., and R.L. are grateful recipients of JSPS Fellowships. We extend our sincere thanks to Yohei Yokobayashi for many hours of stimulating discussion.

References and Notes

(1) For the latest reviews, see: (a) Mosbach, K.; Ramstrom, O. *Bio/Technology* **1996**, *14*, 163. (b) Steinke, J.; Sherrington, D.; Dunkin, I. *Adv. Polym. Sci.* **1995**, *123*, 80. (c) Wulff, G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1812.

(2) For earlier reviews and more specific issues see: (a) Wulff, G. *Polymeric Reagents and Catalysts*; ACS Symposium Series 308; American Chemical Society: Washington, DC, 1986; p 186. (b) Wulff, G. In *Bioorganic Chemistry in Healthcare and Technology*; Pandit, U. K., Alderwiereldt, F. C., Eds.; Plenum Press: New York, 1991; p 55. (c) Wulff, G. *Trends Biotechnol.* **1993**, *11*, 85. (d) Andersson, L. I.; Ekberg, B.; Mosbach, K. In *Molecular Interactions in Biseparations*; Ngo, T. T., Ed.; Plenum Press: New York, 1993; p 383. (e) Mosbach, K. *Trends Biochem. Sci.* **1994**, *19*, 9. (f) Shea, K. J. *Trends Polym. Sci.* **1994**, *2*, 166. (g) Nicholls, I. A.; Andersson, L. I.; Mosbach, K.; Ekberg, B. *Trends Biotechnol.* **1995**, *13*, 47.

(3) Kempe, M.; Mosbach, K. *J. Chromatogr., Sect. A* **1994**, *664*, 276.

(4) Vlatakis, G.; Andersson, L. I.; Muller, R.; Mosbach, K. *Nature* **1993**, *361*, 645.

(5) Andersson, L. A.; Müller, R.; Vlatakis, G.; Mosbach, K. *PNAS* **1995**, *92*, 4788.

(6) Andersson, L. I.; Muller, R.; Mosbach, K. *Macromol. Rapid Commun.* **1996**, *17*, 65.

(7) Andersson, L. *Anal. Chem.* **1996**, *68*, 111.

(8) Matsui, J.; Doblhoff-Dier, O.; Takeuchi, T. *Chem. Lett.* **1995**, 489.

(9) Matsui, J.; Miyoshi, Y.; Takeuchi, T. *Chem. Lett.* **1995**, 1007.

(10) Kugimiya, A.; Matsui, J.; Takeuchi, T.; Yano, K.; Muguruma, H.; Elgersma, A.; Karube, I. *Anal. Lett.* **1995**, *28*, 2317.

(11) Andersson, L. A.; O'Shannessy, D. J.; Mosbach, K. *J. Cromatogr.* **1990**, *513*, 167.

(12) Ramstrom, O.; Nicholls, I. A.; Mosbach, K. *Tetrahedron: Asymmetry* **1994**, *5*, 649.

(13) Kempe, M.; Mosbach, K. *J. Chromatogr., Sect. A* **1995**, *691*, 317.

(14) Kempe, M.; Glad, M.; Mosbach, K. *J. Mol. Recognit.* **1995**, *8*, 35.

(15) Piletsky, S. A.; Fedoryak, D. M.; Kukhar, V. P. *Dokl. Akad. Nauk. Ukr. SSR, Ser. B* **1990**, *4*, 53; *Chem. Abstr.* **1991**, *114*, 165457q.
(16) Shea, K. J.; Spivak, D. A.; Sellergren, B. *J. Am. Chem. Soc.* **1993**, *115*, 3368.
(17) Mathew, J.; Buchardt, O. *Bioconjugate Chem.* **1995**, *6*, 524.
(18) Ramstrom, O.; Ye, L.; Mosbach, K. *Chem. Biol.* **1996**, *3*, 471.
(19) Whitcombe, M. J.; Rodrigez, M. E.; Villar, P.; Vulfson, E. N. *J. Am. Chem. Soc.* **1995**, *117*, 7105.
(20) Byström, S. E.; Börje, A.; Akermark, B. *J. Am. Chem. Soc.* **1993**, *115*, 2081.
(21) Piletsky, S. A.; Parhometz, Y. P.; Lavryk, N. V.; Panasyuk, T. L.; El'skaya, A. V. *Sens. Actuators B* **1994**, *18*−*19*, 629.
(22) Hoberman, J. M.; Yesalis, C. E. *Sci. Am.* **1995**, 76.
(23) Sellergren, B. *Makromol. Chem.* **1989**, *190*, 2703.
(24) Sellergren, B.; Shea, K. J. *J. Chromatogr.* **1993**, *635*, 31.
(25) Blunt, J. W.; Stothers, J. B. *Org. Magn. Reson.* **1977**, *9*, 439.
(26) Snyder, L. R.; Kirkland, J. J. *Introduction to Modern Liquid Chromatography*; Wiley Interscience: New York, 1974.
(27) Sellergren, B.; Lepisto, M.; Mosbach, K. *J. Am. Chem. Soc.* **1988**, *110*, 5853.
(28) Kempe, M.; Mosbach, K. *Anal. Lett.* **1991**, *24*, 1137.
(29) Moradian, A.; Mosbach, K. *J. Mol. Recognit.* **1989**, *2*, 167.
(30) Nicholls, I. A. *Chem. Lett.* **1995**, 1035.
(31) Shea, K. J.; Stoddard, G. J.; Shavelle, D. M.; Wakui, F.; Choate, R. M. *Macromolecules* **1990**, *23*, 4497.
(32) Shea, K. J.; Thompson, E. A.; Pandey, S. D.; Beauchamp, P. S. *J. Am. Chem. Soc.* **1980**, *102*, 3149.
(33) Damen, J.; Neckers, D. C. *J. Am. Chem. Soc.* **1980**, *102*, 3265.
(34) Wulff, G.; Sarhan, A. In *Chemical Approaches to Understanding Enzyme Catalysis: Biomimetic Chemistry and Transition-State Analogs*; Green, B. S., Ashani Y., Chipman D., Eds.; Elsevier: Amsterdam, 1982; p 106.
(35) Sarhan, A.; Wulff, G. *Makromol. Chem.* **1982**, *183*, 1603.
(36) Sarhan, A.; Wulff, G. *Makromol. Chem.* **1982**, *183*, 85.

MA961014L