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Effects of Drug Binding on the Esterase Activity of Human Serum Albumin: Inhibition Modes and Binding Sites of Anionic Drugs[1]

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Using the reaction rate of *p*-nitrophenyl acetate (NPA) with human serum albumin (HSA) as an index, the inhibition modes and binding sites of various anionic drugs were investigated in pH 7.4 phosphate buffer at 25°. Eleven anionic drugs including two specific fluorescence probes were chosen on the basis of Sudlow's classification of the drug binding sites, Site I and Site II (Sudlow *et al.*, *Mol. Pharmacol.*, **12**, 1052 (1976)). The types of inhibition caused by these drugs suggested that at least three binding sites were present on HSA. The primarily reactive site (R site) of HSA corresponded to Sudlow's Site II, which corresponds to tyrosine-411 in Brown's HSA sequence. Site I was not involved in the esterase activity of HSA, which required tryptophan-214 (U site). In addition to the R site, HSA had a secondarily reactive site (T site) for NPA, which could also bind the drugs. The binding affinities of the drugs to the R site were estimated and some structural features of the R site were deduced.

Keywords——human serum albumin; protein binding; anionic drug binding sites on human serum albumin; esterase activity of human serum albumin; tyrosine residue of human serum albumin; tryptophan residue of human serum albumin; aromatic carboxylic acids; pyrazolidinedione derivatives; warfarin; fluorescence probes

The characterization of drug binding sites on human serum albumin (HSA), including the estimation of the binding affinities, is pharmacologically and clinically important, particularly from the drug displacement point of view.[3–7] In preceding paper it was reported that N-phenylanthranilic acids and prostaglandins inhibited the reaction of *p*-nitrophenyl acetate (NPA) with HSA, but that warfarin did not affect the reaction,[8] and that in the reactions of various phenyl esters with HSA hydrophobic interaction was involved in the binding of substrates with significant hydrocarbon chain lengths.[9] Means and his co-workers recently reported that the reaction of NPA with HSA was inhibited by small fatty acid anions[10] and by several drugs,[11] and suggested that the tyrosine residue of HSA was involved in the reaction.[12]

Sudlow *et al.*[13] have suggested two specific binding sites on HSA, Site I and Site II, for

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2) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) H.M. Solomon, J.J. Schrogie, and D. Williams, *Biochem. Pharmacol.*, **17**, 143 (1968).
4) R.A. O'Reilly, *Ann. N.Y. Acad. Sci.*, **226**, 293 (1973).
5) L.Z. Benet, "The Effect of Disease States on Drug Pharmacokinetics," ed. by G. Levy, A Ph A Academy of Pharmaceutical Sciences, Washington, D.C., 1976, p. 137.
6) J.T. Slattery and G. Levy, *J. Pharm. Sci.*, **66**, 1060 (1977).
7) M. Otagiri, Y. Otagiri, and J.H. Perrin, *International Journal of Pharmaceutics*, **2**, 283 (1979).
8) K. Ikeda, Y. Kurono, Y. Ozeki, and T. Yotsuyanagi, *Chem. Pharm. Bull.*, **27**, 80 (1979).
9) Y. Kurono, T. Maki, T. Yotsuyanagi, and K. Ikeda, *Chem. Pharm. Bull.*, **27**, 2781 (1979).
10) S.-W. M. Koh and G.E. Means, *Arch. Biochem. Biophys.*, **192**, 73 (1979).
11) N.P. Sollene and G.E. Means, *Mol. Pharmacol.*, **15**, 754 (1979).
12) G.E. Means and H.-L. Wu, *Arch. Biochem. Biophys.*, **194**, 526 (1979).
13) *a*) G. Sudlow, D.J. Birkett, and D.N. Wade, *Mol. Pharmacol.*, **11**, 824 (1975); *b*) *Idem, ibid.*, **12**, 1052 (1976).

anionic drugs on the basis of fluorescence techniques using two specific probes; Site I of HSA showed affinity for bulky heterocyclic molecules with a negative charge (referred to as Site I drugs) and Site II for aromatic carboxylate anions (referred to as Site II drugs).

In the present study, using the reaction rate of NPA with HSA as an index, we investigated the inhibition modes and the binding sites of various anionic drugs in connection with Sudlow's classification of the binding sites. The types of inhibition caused by these drugs suggested that there might be at least three binding sites on HSA. It was, therefore, necessary to analyze the kinetic results in relation to Sudlow's classification. The primarily reactive Site of HSA corresponded to Sudlow's Site II, which is tyrosine-411 in Brown's HSA sequence.[14a] Site I was not involved in the esterase activity of HSA, which requires tryptophan-214 in the HSA sequence. In addition to the primarily reactive site, HSA appeared to carry a secondarily reactive site for NPA, which was also involved in the drug binding. The binding affinities of drugs to the primarily reactive site of HSA were estimated and some structural features of the site were deduced.

Experimental

Materials——HSA (Fraction V, lot 18c-0515, from Sigma Chem. Co.) was used after purification by the method of Chen.[15] The molecular weight of HSA was assumed to be 69000, and its concentration was determined from the absorption at 278 nm using an extinction coefficient $E_{278}^{0.1\%}$ of 0.531.[16] As Sudlow's Site I drugs, potassium warfarin (WF, from Eisai Co.), oxyphenbutazone (OP, from Japan Ciba-Geigy Co.), phenylbutazone (PB, from Japan Ciba-Geigy Co.) and sulfinpyrazone (SP, from Japan Ciba-Geigy Co.) were chosen. Clofibric acid (CA, synthesized by the method of Jones *et al.*, mp 116—120°; literature[17] mp 118—119°), ibuprofen (IP, from Kakenyaku Kako Co.), flufenamic acid (FA, from Sankyo Co.) and ethacrynic acid (EA, from Japan Merck-Banyu Co.) were used as Sudlow's Site II drugs. Salicylic acid was also employed for comparison. Fluorescence probes, dansylamide (DA) for Site I and dansylsarcosine (DS) for Site II, were obtained commercially. All other chemicals purchased were of reagent grade.

Kinetic Procedures——The reaction of NPA with HSA in the presence and absence of various drugs was followed spectrophotometrically at 400 nm by monitoring the appearance of *p*-nitrophenol. The concentrations of HSA and NPA were always 5.00×10^{-5} M and 1.00×10^{-5} M, respectively, and under these conditions pseudo-first order analysis could be applied, as described in the previous reports.[8,9] The drug concentration was varied widely from zero to 1.00×10^{-3} M; 1/15 M phosphate buffer of pH 7.4 ($\mu=0.2$, adjusted with NaCl) was used, and the temperature was 25°.

The inhibition of the reaction of NPA with HSA by diisopropylfluorophosphate (DFP) was investigated as follows. The reaction of DFP (1.00×10^{-2} M) with HSA (5.00×10^{-5} M) was carried out in pH 7.4 phosphate buffer before the reaction of NPA with the DFP-treated HSA. At the appropriate time interval, 15 μl of 2.01×10^{-3} M NPA in acetonitrile was added to 3 ml of the reaction solution containing the DFP-treated HSA and DFP, and the rate of *p*-nitrophenol appearance was followed at 400 nm.

Fluorescent Measurements——The fluorescence spectra of 1.00×10^{-5} M HSA in the absence and presence of 1.00×10^{-5} M OP and that of 1.00×10^{-5} M OP were measured in a 1 cm quartz cell with a Shimadzu RE-510 spectrofluorophotometer. An excitation wavelength of 300 nm was employed for the measurements, since the single tryptophan residue of HSA was excited at this wavelength.[18]

Results and Discussion

Effects of Site II Drugs on the Reaction Rate of NPA with HSA

Figure 1 shows the effects of CA and IP on the reaction rate of NPA with HSA. In this figure, the ratio (r) of the apparent first-order rate constant (k_{obs}) for the reaction of NPA with HSA in the presence of a drug to that (k_{obs}^0) in its absence was plotted against the value of the

14) *a*) V.M. Rosenor, M. Oratz, and M.A. Rothschild, "Albumin Structure, Function and Uses," ed. by J.R. Brown, Pergamon Press, Oxford, London, 1977, p. 27; *b*) *Idem*, *ibid.*, ed. by R.H. McMenamy, p. 147.
15) R.F. Chen, *J. Biol. Chem.*, **242**, 173 (1967).
16) G.E. Means and M.L. Bender, *Biochemistry*, **14**, 4989 (1975).
17) W.G.M. Jones, J.M. Thorp, and W.S. Waring, Brit. Pat., 860303 (1961).
18) J. Steinhardt, J. Krijn, and J.G. Leidy, *Biochemistry*, **10**, 4005 (1971).

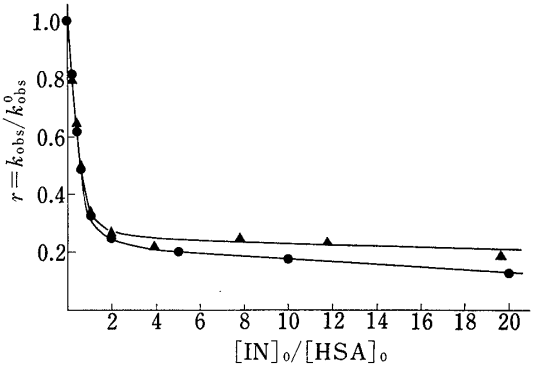

Fig. 1. Effects of CA and IP on the Reaction Rate of NPA with HSA

●, CA; ▲, IP.
The concentration of HSA was 5.00×10^{-5} M.

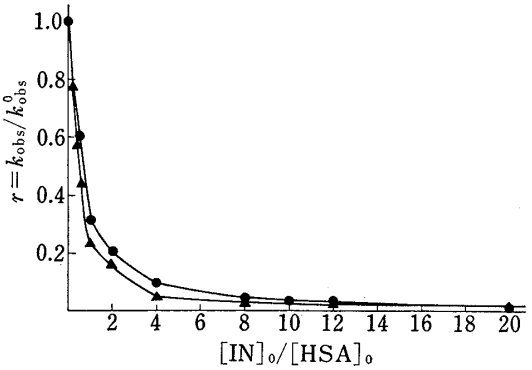

Fig. 2. Effects of FA and EA on the Reaction Rate of NPA with HSA

●, FA; ▲, EA.
The concentration of HSA was 5.00×10^{-5} M.

total drug concentration ($[IN]_0$) divided by the initial concentration of HSA ($[HSA]_0$). The inhibitions by CA and IP were very strong and the r values decreased linearly up to approximately a 1:1 ratio of drug to HSA. With further increase in the drug concentration, the r value no longer decreased, and appeared to reach a plateau value of approximately 0.2. This reactivity at excess drug concentrations could not be explained by simple competitive inhibition, because in the case of simple competitive inhibition the r value in the presence of a large excess of drug relative to HSA would correspond to the ratio (r_{sp}) of the spontaneous hydrolysis rate constant (k_0) of NPA to k^0_{obs} and the value of r_{sp} was about 0.003 under these experimental conditions. Two possibilities can be considered for the remaining reactivity. One possibility is non-competitive inhibition or mixed-type inhibition by the drug, *i.e.*, the complex (IN·HSA) formed between the drug and HSA still has reactivity with NPA, though it is less than that of the native (uncomplexed) HSA. The other is that HSA has multiple reactive sites for NPA and that the drug is bound only to the primarily reactive site of HSA. Examination of the bindings of other drugs and the reaction of DFP with HSA may cast further light on these possibilities.

The effects of FA and EA on the reaction rate of NPA with HSA are illustrated in Figure 2. At relatively low drug concentrations, up to a ratio of about 2 on the abscissa, the inhibition curves due to FA and EA were similar to those of CA and IP in Figure 1. The r values, however, declined with further increase of drug concentration and asymptotically reached about 0.02 to 0.04. The remaining reactivities, $r=0.02$ to 0.04, in Figure 2 were significantly different from those, $r\fallingdotseq0.2$, in Figure 1.

Chignell[19] suggested that HSA had three strong binding sites for FA. On the other hand, it was reported that CA was strongly bound to one high-affinity binding site of HSA.[20] The difference between these binding properties of FA and CA to HSA may lead to the different remaining reactivities observed for CA and IP and for FA and EA. Since CA and IP bind only to the primarily reactive site of HSA, the remaining reactivities of about 20% might correspond to a secondarily reactive site of HSA, ruling out mixed type inhibitions by CA and IP. The multiple bindings of FA and EA to both sites of HSA might account for the small remaining reactivities of only about 2 to 4%.

To further confirm the presence of the secondarily reactive site rather than mixed type inhibitions by CA and IP, the effect of DFP on the reaction rate of NPA with HSA was investigated. Figure 3 shows the effect of the reaction period of DFP with HSA on the apparent first-order rate constant (k_{obs}) for the reaction of NPA with the DFP-treated HSA. The rate

19) C.F. Chignell, *Mol. Pharmacol.*, **5**, 455 (1969).
20) A.A. Spector, E.C. Santos, J.D. Ashbrook, and J.E. Fletcher, *Ann. N.Y. Acad. Sci.*, **226**, 247 (1973).

constant decreased with increase of the reaction period of DFP with HSA, and finally reached a plateau value. The abrupt decrease in the rate constant is considered to be due to reversible DFP·HSA complex formation. The subsequent slower decrease in the rate constant is caused by the irreversible formation of diisopropylphosphoryl-HSA, in which the hydroxyl group of tyrosine of HSA is reacted with DFP, as found by Sanger[21] and Means and Wu.[12] It is interesting that the remaining reactivity of the DFP-treated HSA was about 20% of the reactivity of untreated HSA, and the inhibition by DFP was almost equal to those by CA and IP (Figure 1). Thus, this inhibition by DFP also suggests that the remaining reactivities in Figure 1 were due to the secondarily reactive site of HSA, and not to mixed type inhibition by CA and IP.

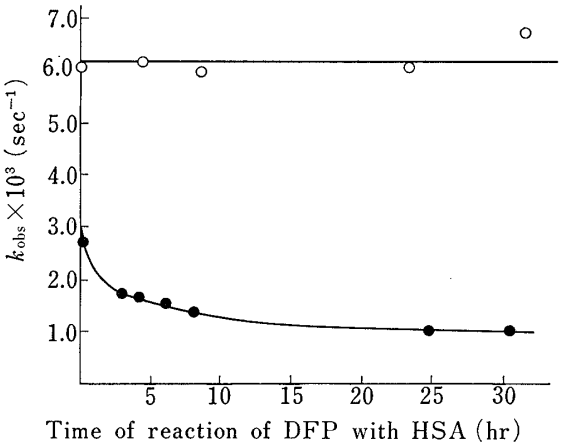

Fig. 3. Effect of the Reaction Period of DFP with HSA on the Apparent First-order Rate Constant for the Reaction of NPA with DFP-Treated HSA

●, $[DFP]_0=1.00\times10^{-2}$ M; ○, $[DFP]_0=0.0$.
The reactions of NPA with DFP-treated HSA and with untreated HSA were carried out in pH 7.4 phosphate buffer containing 0.5% (v/v) acetonitrile.

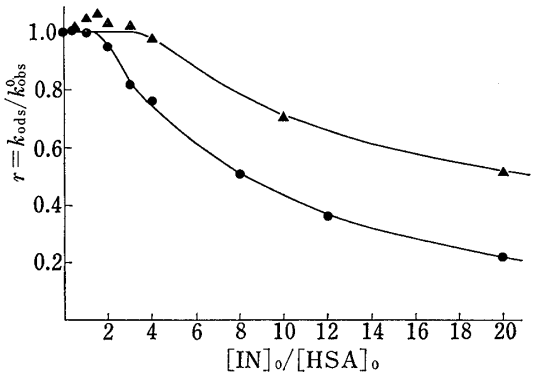

Fig. 4. Effects of WF and OP on the Reaction Rate of NPA with HSA

●, WF; ▲, OP.
The concentration of HSA was 5.00×10^{-5} M.

It should be emphasized that the secondarily reactive site of HSA has not been found by Means and his co-workers in similar inhibition studies using DFP[12] and CA and IP.[11] The remaining reactivities in their studies were always about 5% of the initial reactivity in the absence of the inhibitors. When the effects of these inhibitors on the rate were examined by us under their experimental conditions, results similar to theirs were obtained. Therefore, the discrepancy regarding the remaining reactivities observed in their studies and in Figures 1 and 3 of this study might have arisen from the differences in the buffer constituents employed and pH. They used 0.06 M triethanolamine–HCl buffer of 0.02 M ionic strength at pH 8.0. On the other hand, 1/15 M phosphate buffer of 0.2 M ionic strength at pH 7.4 was employed in this study. The conformation of HSA in the organic buffer of low ionic strength may differ from that in the inorganic buffer of high ionic strength[14b] which has been commonly used for studies on the interaction between drugs and HSA in the neutral pH region. Sudlow *et al.* also used 0.1 M phosphate buffer, pH 7.4, containing 0.9% NaCl.[13] The secondarily reactive site of HSA in the inorganic buffer may become more exposed on the albumin surface, so that the site may take a more favorable position for reaction with NPA and interaction with the drugs.

The primarily reactive site of HSA was named R site, and considered to correspond to tyrosine-411[21,12] in Brown's sequence of HSA.[14a] Thus the R site is identical with Sudlow's Site II. The secondarily reactive site was designated as T site, but its position has not

21) F. Sanger, *Proc. Chem. Soc.*, **1963**, P. 76.

been identified yet. The drugs binding only to the R site, as shown in Figure 1, were referred to as R type drugs and those binding to both the R and T sites (Figure 2) were referred to as R—T type drugs.

Effects of Site I Drugs on the Reaction Rate of NPA with HSA

Figure 4 shows the effects of WF and OP on the reaction rate of NPA with HSA. WF and OP did not initially inhibit the reaction up to a drug-to-HSA ratio of about 1 to 3, but decreased the rate at higher ratios. There have been many studies on the interactions of WF with HSA using various methods.[3,4,5,13,22,23] It has been reported that HSA has two classes of binding sites for WF, that is, high and low affinity sites.[22] The high-affinity binding site of HSA for WF was the single tryptophan residue,[23] Trp-214 in Brown's sequence of HSA.[14a]

To investigate whether the binding of OP was also related to the tryptophan residue of HSA at a low OP-to-HSA ratio, the fluorescence spectra of HSA were measured (Figure 5). An excitation wavelength of 300 nm was chosen, since the single tryptophan residue of HSA was excited at this wavelength.[18] The intensity of the emission spectrum of HSA decreased in the presence of OP at an OP-to-HSA ratio of 1. The reduced emission spectrum indicates that the binding of OP occurred near the single tryptophan residue of HSA. Therefore, in the initial plateau region observed in Figure 4 the drug was bound mainly to the tryptophan residue, and with further increase of drug concentration, the drug was bound gradually to the R site of HSA. Drugs showing this type of inhibition were defined as U—R type drugs, and the drug binding site of HSA not involved in the esterase activity was denoted as U site. Thus, the U site was identical with Sudlow's Site I and Trp-214 in Brown's HSA sequence.[14a]

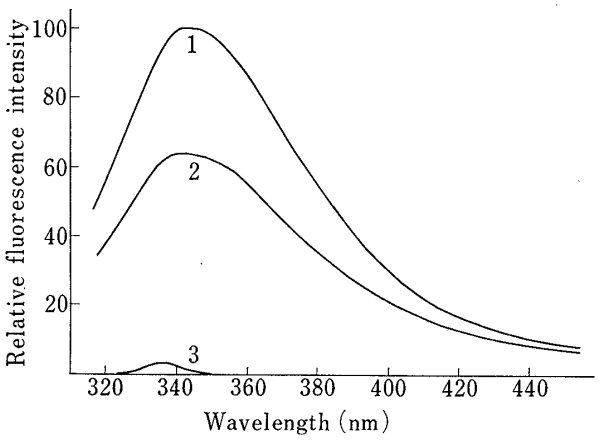

Fig. 5. Fluorescence Emission Spectra excited at 300 nm

1: 1.00×10^{-5} M HSA.
2: 1.00×10^{-5} M HSA in the presence of 1.00×10^{-5} M OP.
3: 1.00×10^{-5} M OP.

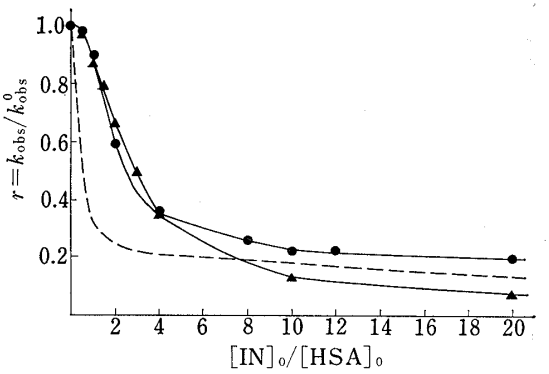

Fig. 6. Effects of PB and SP on the Reaction Rate of NPA with HSA

●, PB; ▲, SP.
The dashed curve shows the result for CA for comparison. The concentration of HSA was 5.00×10^{-5} M.

Figure 6 shows the effects of PB and SP on the reaction rate of NPA with HSA. For comparison, the results with CA shown in Figure 1 are also indicated by a dashed curve in Figure 6. The initial curves for PB and SP also showed slight shoulders compared with those for R or R—T type drugs and differed slightly from those for WF and OP in Figure 4. These differences in the initial inhibition curves can probably be accounted for as follows. PB and SP were bound to both the U and R sites of HSA, and the binding affinities to the U site were slightly larger than those to the R site. Consequently, PB and SP were also classified as U—R

22) R.F. Mais, S. Keresztes-Nagy, J.F. Zaroslinsky, and Y.T. Oester, *J. Pharm. Sci.*, **63**, 1423 (1974).
23) C.F. Chignell, *Mol. Pharmacol.*, **6**, 1 (1970).

type drugs. Furthermore, SP might also be bound weakly to the T site since the remaining reactivity at high concentrations of SP was slightly less than in the cases of PB and CA.

It should be noted that U—R type inhibition does not necessarily imply that the U site is the tryptophan residue of HSA. If a drug is bound both to a high affinity site(s) other than Trp-214, which is not involved in the esterase activity, and to the R site, inhibition similar to the U—R type inhibition would be observed. Although the drug binding site(s) not involved in the esterase activity is not identified by means of this kinetic method alone, informations obtained on the existence of the site(s) and the binding affinity of the drug would provide a significant insight for further examination.

Binding Affinities of Drug to the R Site of HSA

The drugs examined showed three inhibition types, R, R—T and U—R types. Since it was too difficult to determine the binding constants between each drug and the individual sites of HSA, estimation of the binding affinity to the R site of HSA only was attempted. The determination of the individual binding constants is now in progress using an analog computer. The concentration of the drug giving the r value of 0.5 in the inhibition curve, IN_{50}, was used for estimation of the binding affinity. The value of IN_{50} may be a measure of the drug binding affinity to the R site of HSA, since it was assumed for the results shown in Figures 1, 2, 4, and 6 that the bindings of the drug to the individual sites on HSA were independent and that the drug was bound to the each site with the individual binding constant.

Table I lists the IN_{50} values for all of the drugs examined. The R and R—T type drugs belonging to the Site II drugs had IN_{50} values of about 2.5×10^{-5} M to 3.5×10^{-5} M. These values suggest that the bindings of the drugs to the R site of HSA were very strong and were stoichiometric, since the HSA concentration of 5.00×10^{-5} M was always used. Although SA showed R—T type inhibition, the value of IN_{50} was larger than those for the Site II drugs. Thus, an appropriate distance between the apolar and bulky group and the anionic group in the drug molecule may be necessary for very strong binding to the R site of HSA.

Table I. The IN_{50} Values for the Drugs examined

R type drugs	IN_{50} (M)	R-T type drugs	IN_{50} (M)	U-R type drugs	IN_{50} (M)
CA	2.80×10^{-5}	FA	3.53×10^{-5}	WF	4.06×10^{-4}
IP	3.07×10^{-5}	EA	2.45×10^{-5}	OP	1.10×10^{-3}
DS	3.45×10^{-5}	SA	8.60×10^{-5}	PB	1.25×10^{-4}
				SP	1.48×10^{-4}
				DA	*a*

a) DA did not inhibit the reaction of NPA with HSA at concentrations of DA up to saturation.

The IN_{50} values for Sudlow's Site I drugs were larger than those for the Site II drugs. Furthermore, it is of interest to compare the values of IN_{50} for OP, PB, and SP because of the similarity of their molecular structures. The almost equal values of IN_{50} for PB and SP suggest that the diphenyl moiety at the 1- and 2-position of the pyrazole ring rather than the lengthy group at the 4-position interacts with the hydrophobic region at the R site of HSA. Accordingly, the R site of HSA may consist of a cationic region due to arginine and lysine at each end and a hydrophobic region due to tyrosine and threonine.[14a,18] The hydroxyl group of OP might contribute unfavorably to the binding of the diphenyl portion to the R site of HSA, resulting in the larger value of IN_{50} than those for PB and SP.

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