Application of Fluorescent Triazoles to Analytical Chemistry. IV.[1] Development of a New Fluorescent Probe for Basic Drug–α_1-Acid Glycoprotein Binding Measurement

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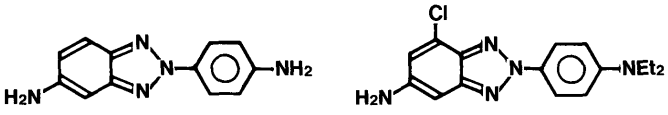
A fluorescent probe applicable to protein binding measurement was developed. Several 2-phenylbenzotriazolyl-5-amine derivatives having an amino group on the 2-phenyl ring were synthesized. Their fluorescence characteristics and the binding affinity to α_1-acid glycoprotein (α_1-AGP) were investigated. They were practically non-fluorescent in aqueous solution, but were highly fluorescent in the presence of α_1-AGP. This fluorescence was due to the binding of these compounds to α_1-AGP. Of all the compounds synthesized, 7-chloro-2-(*p*-diethylaminophenyl)-2*H*-benzotriazolyl-5-amine (CDBA) bound to α_1-AGP most strongly and was used as a fluorescent probe. The binding parameters of CDBA were estimated by the fluorometric titration method to be log $K = 6.18$ and $n = 0.40$. The fluorescence of CDBA in the α_1-AGP solution was markedly quenched in the presence of basic drugs, indicating that these drugs competitively displace CDBA bound to α_1-AGP. The binding constants of the basic drugs were calculated, based on the decrease of fluorescence intensity in the presence of the drug. The binding strength of the drugs tested ranged from 4.7 to 6.8 as log K value.

Keywords fluorescent probe; fluorescence characteristics; protein binding; binding parameter; α_1-acid glycoprotein; competition; basic drug; benzotriazole: 7-chloro-2-(*p*-diethylaminophenyl)-2*H*-benzotriazolyl-5-amine

The fluorescent probe method is one of the most useful means for understanding the interaction between proteins and small molecules. Many fluorescent dyes, for example, 8-anilino-1-naphthalenesulfonate,[2] dansylamino acids[3] and coumarin derivatives[4,5] have been extensively used for the investigation of drug–serum albumin binding. On the other hand, few fluorescent probes are available for studies of the interaction of drugs with α_1-acid glycoprotein (α_1-AGP).[6,7] α_1-AGP has a high affinity for basic drugs and has received considerable attention in recent years, because of its roles in the analysis of the pharmacokinetics of basic drugs.[8] In the previous paper, we found that some benzotriazoles, *e.g.*, 2-(*p*-aminophenyl)-2*H*-benzotriazolyl-5-amine (ABA), might be used as fluorescent probes by applying their character that the compounds, although practically non-fluorescent in aqueous solution, become highly fluorescent in the presence of protein.[1]



ABA CDBA

Chart 1

Based on our previous observations, in order to obtain effective fluorescent probes for α_1-AGP binding study, we synthesized several fluorescent probes by modifying ABA and examined the interaction between these compounds and α_1-AGP. Of these compounds, 7-chloro-2-(*p*-diethylaminophenyl)-2*H*-benzotriazolyl-5-amine (CDBA) was selected as a useful fluorescent probe to estimate the binding constants of basic drugs to α_1-AGP. The binding constants of several basic drugs to α_1-AGP were estimated by the fluorescent probe method.

Experimental

Apparatus A Hitachi 650-60 fluorescence spectrophotometer was used for fluorescence measurement. All melting points were measured with a Yanagimoto micro melting point apparatus.

Reagents and Materials α_1-AGP was purchased from Sigma Chemical Co. Commercially available drugs were used after purification in our laboratories. ABA was synthesized as described in the previous paper.[1] The other chemicals were of reagent grade.

Synthesis of CDBA An aqueous solution (10 ml) of $NaNO_2$ (700 mg)

TABLE I. Physicochemical and Analytical Data

Compd. No.	Appearance (Recrystn. solvent)	Yield (%)	mp (°C)	Formula	Analysis Calcd (Found)			
					C	H	Cl	N
1	Yellow needles (MeOH–H_2O)	80.8	166—167	$C_{13}H_{13}N_5$	65.25 (65.45)	5.48 5.36	— —	29.27 29.05)
2	Brown plates (MeOH–H_2O)	72.5	211—213	$C_{13}H_{12}ClN_5$	57.04 (57.10)	4.42 4.52	12.95 12.84	25.59 25.35)
3	Brown plates (MeOH–H_2O)	35.4	174—175	$C_{18}H_{14}ClN_5$	64.38 (64.62)	4.20 4.36	10.56 10.60	20.86 20.61)
4	Yellow needles (DMF–H_2O)	65.5	295 (dec.)	$C_{16}H_{17}N_5O$	65.07 (65.36)	5.80 5.78	— —	23.71 23.63)
5	Yellow needles (DMF–H_2O)	45.3	246—247	$C_{16}H_{16}ClN_5O$	58.27 (58.35)	4.89 4.98	10.75 10.75	21.24 21.11)
6	Orange needles (MeOH–H_2O)	49.8	136—137	$C_{16}H_{19}N_5$	68.30 (68.12)	6.81 6.90	— —	24.89 24.57)
7	Orange plates (MeOH–EtOAc)	25.3	187—188	$C_{16}H_{18}ClN_5$	60.85 (61.00)	5.75 5.86	11.23 11.06	22.18 22.02)

DMF, dimethylformamide.

was added dropwise to a solution of *N*,*N*-diethyl-*p*-phenylenediamine dihydrochloride (2.37 g; 10 mmol) in 10% HCl (50 ml) under ice-cooling. After 15 min, ammonium sulfamate (1.5 g) was added. The mixture was stirred for 15 min, then adjusted to pH 5 with sodium acetate. After addition of 5-chloro-*m*-phenylenediamine (1.45 g; 10 mmol), the mixture was further stirred for 2 h. The reaction mixture was adjusted to pH 9 with 1 N NaOH and extracted with EtOAc. The extract was concentrated *in vacuo*. An ammoniacal cupric sulfate solution (10 g of $CuSO_4 \cdot 5H_2O$ dissolved in 60 ml of 14% NH_4OH) was added to a pyridine solution (40 ml) of the residue. The reaction mixture was refluxed for 4 h, then cooled and extracted with EtOAc. The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on a silica-gel column with EtOAc. The eluate was concentrated *in vacuo*.

Elemental analysis and melting point (uncorrected) are shown in Table I.

Synthesis of Other Fluorescent Probes The other compounds were synthesized by the same method as described for the synthesis of CDBA. *m*-Phenylenediamine dihydrochloride was used for compounds **1**, **4** and **6**, instead of 5-chloro-*m*-phenylenediamine. Elemental analysis and melting points are shown in Table I. All melting points are uncorrected.

Fluorometric Titration α_1-AGP solutions were prepared in 0.1 M phosphate buffer of pH 7.4. The concentration of α_1-AGP was determined by using an extinction coefficient ($E^{1\%}_{1\,cm}$) value of 8.93 at 278 nm.[9] ABA and CDBA were prepared in distilled water after being dissolved in a trace amount of 0.1 N HCl.

α_1-AGP solution of an appropriate concentration was titrated by successive additions of a solution of CDBA and the fluorescence intensity was measured at 508 nm with excitation at 418 nm. When the absorbance of the solution at 418 nm was greater than 0.02, a correction for the fluorescence intensity was made by the method of Naik *et al.*[10]

Data Treatment The fraction of bound CDBA, X, was calculated from Eq. 1,

$$X=\frac{F_p-F_o}{F_b-F_o} \tag{1}$$

where F_p and F_o are the fluorescence intensities of a given concentration of CDBA in a solution of low α_1-AGP concentration and in a solution without α_1-AGP, respectively. F_b is the fluorescence intensity of the same concentration of fully bound CDBA in a solution of high α_1-AGP concentration. After the value, X, was found for each point along the titration curve, the results were plotted according to the Scatchard equation.[11]

$$r/D_f=nK_a-rK_a$$

where r is the number of mol of bound CDBA per mol of α_1-AGP, n is the number of binding sites, K_a is the binding constant and D_f is the concentration of free CDBA. The binding constants of drugs were calculated by using the equation of Klotz *et al.*[12]:

$$K_b=\frac{nP_tK_aD_f-K_aD_fD_b-D_b}{B_tK_aD_f-nP_tK_aD_f+K_aD_fD_b+D_b}\cdot\frac{K_aD_f}{D_b} \tag{2}$$

where K_a and K_b are the binding constants for CDBA and drug, and D_f and D_b are the concentrations of free and bound CDBA, respectively. P_t and B_t are the total concentrations of α_1-AGP and drug, respectively, and n is the number of binding sites. Rearrangement of Eq. 2 gives

$$K_bP_t(K_aD_f+1)\cdot r^2+K_aD_f(K_bB_t-nK_bP_t+K_aD_f+1)\cdot r-nK_a^2D_f^2=0 \tag{3}$$

where r is D_b/P_t. Hence, Eq. 4 can be derived from Eq. 3:

$$r=\frac{-\beta+\sqrt{\beta^2-4\alpha\gamma}}{2\alpha} \tag{4}$$

where α is $K_bP_t(K_aD_f+1)$, β is $K_aD_f(K_bB_t-nK_bP_t+K_aD_f+1)$ and γ is $-nK_a^2D_f^2$. The inhibition data (r *versus* D_f) were fitted to Eq. 4 by a nonlinear least-squares method based on a Gauss–Newton algorithm. The program was run on a FACOM M340R digital computer.

Results and Discussion

Interaction between ABA and α_1-AGP As reported in the previous paper,[1] the fluorescence intensity of ABA depends upon the polarity of the solvent; for example, ABA is practically non-fluorescent in water but is highly fluorescent in dimethylsulfoxide, dioxane, chloroform and cyclohexane. Such character suggests that when ABA binds to protein and is placed in the hydrophobic environment of the protein molecule, it becomes fluorescent in an aqueous medium. ABA seems to be a useful fluorescent probe for protein binding measurement.

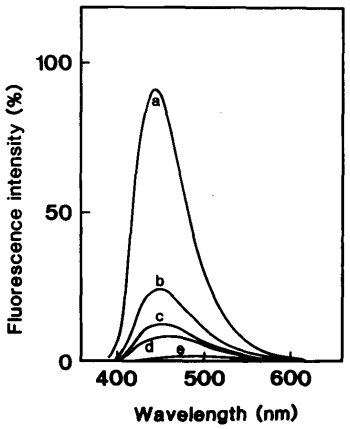


Fig. 1. Fluorescence Spectra of ABA (1×10^{-5} M) in the α_1-AGP Solution

a, in the presence of 5×10^{-5} M α_1-AGP; b, in the presence of 1×10^{-5} M α_1-AGP; c, in the presence of 5×10^{-6} M α_1-AGP; d, in the presence of 3×10^{-6} M α_1-AGP; e, in the absence of α_1-AGP.

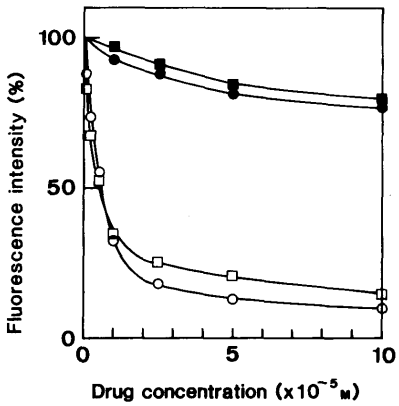


Fig. 2. Effect of Various Drugs on the Fluorescence Intensity of ABA in the α_1-AGP Solution

○, chlorpromazine; □, quinidine; ■, flufenamic acid; ●, ethacrynic acid.

α_1-AGP has a high binding affinity for basic compounds.[13,14] Therefore, the basic compound ABA was expected to bind to α_1-AGP. The possible use of ABA as a fluorescent probe was investigated. As shown in Fig. 1, ABA was practically non-fluorescent in the buffer solution, but fluoresced in the presence of α_1-AGP, and its fluorescence intensity increased with α_1-AGP concentration. The fluorescence of ABA, however, decreased when the drugs were added to the α_1-AGP solution. Figure 2 shows the inhibition of the ABA binding to α_1-AGP with increasing concentrations of basic or acidic drugs. Basic drugs, chlorpromazine and quinidine, markedly quenched the fluorescence with increasing concentration, while acidic drugs, flufenamic acid and ethacrynic acid, slightly decreased the fluorescence. These observations indicate that basic ABA binds to α_1-AGP to give the fluorescence and that bound ABA dissociates owing to displacement by the basic drugs. Thus, ABA should be useful as a fluorescent probe if the basic drug inhibits the ABA binding to α_1-AGP at the same

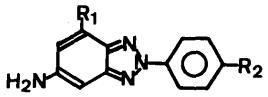
binding site on the protein.

Interaction between CDBA and α_1-AGP The binding affinity of ABA was relatively weak for use as a probe in drug–protein binding studies. Some similar compounds to ABA were synthesized to obtain a probe with stronger affinity to α_1-AGP than ABA. Various basic substituents at the 4-position of the 2-phenyl ring and a chlorine atom at the 7-position of the benzotriazole ring were introduced. All the compounds exhibited similar fluorescing and quenching phenomena in the α_1-AGP solution. Table II summarizes the fluorescence maximum wavelength in the α_1-AGP solution and the binding affinity. As a measure of the binding affinity, log *nK* values were calculated. Introduction of a chlorine atom and a basic substituent into the compounds resulted in higher affinity and longer wavelength. Compound **7** (CDBA) with the highest binding strength was selected. This compound has higher solubility in the acidic solution than the other analogs and its fluorescence at the longer wavelength region is advantageous for measurement with little interference from the protein. Figure 3 shows the fluorescence spectra of CDBA in the presence of α_1-AGP. The addition of α_1-AGP resulted in a large increase in the fluorescence intensity of CDBA.

CDBA was bound to human serum albumin (HSA) and fluoresced (excitation, 400 nm; emission, 485 nm). The binding parameter, however, could not be determined, since the fluorescence property of CDBA bound to HSA was different at high and low concentrations of HSA; the fluorescence intensity of CDBA in the presence of 1×10^{-5} M HSA was higher than that in the presence of 1×10^{-4} M HSA.

Determination of the Binding Parameters of CDBA to α_1-AGP The fluorometric titration of α_1-AGP with CDBA was performed according to the method of Naik *et al.*[10] The CDBA solution was successively added to the α_1-AGP solution and the fluorescence intensity was measured. Figure 4 shows the titration curves which were made by plotting the fluorescence intensity against the CDBA concentration. Two titrations of the α_1-AGP solutions, 5×10^{-5} and 1×10^{-4} M, resulted in straight lines (line a). The two curves were the same as each other. This shows that all CDBA added was bound to α_1-AGP at both protein concentrations. When a low concentration of α_1-AGP, 3×10^{-6} M, was titrated, the titration curve was not linear (curve b), suggesting that CDBA was only partially bound to α_1-AGP. The plateau region of curve b indicates saturation of the α_1-AGP binding site. In the presence of drugs (imipramine and chlorpromazine), the titration plot was a curve (c and d) situated under the curve b. In this case, the

TABLE II. Excitation and Emission Maxima of Triazole Derivatives in the α_1-AGP Solution and the Binding Affinities



Compd. No.	R_1	R_2	Ex (nm)	Em (nm)	log *nK*
1	H	NHMe	385	465	4.54
2	Cl	NHMe	396	482	4.85
3	Cl	NHC_6H_5	400	505	5.60
4	H	Mor[a]	380	480	4.70
5	Cl	Mor[a]	396	502	5.38
6	H	NEt_2	395	475	5.02
7	Cl	NEt_2	418	508	5.77
ABA	H	NH_2	375	455	4.17

a) Mor = 

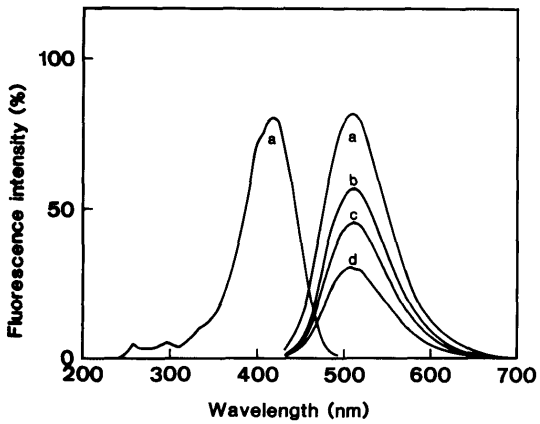


Fig. 3. Excitation and Emission Spectra of CDBA (1×10^{-5} M) in the α_1-AGP Solution

a, in the presence of 5×10^{-5} M α_1-AGP; b, in the presence of 5×10^{-6} M α_1-AGP; c, in the presence of 3×10^{-6} M α_1-AGP; d, in the presence of 1×10^{-6} M α_1-AGP.

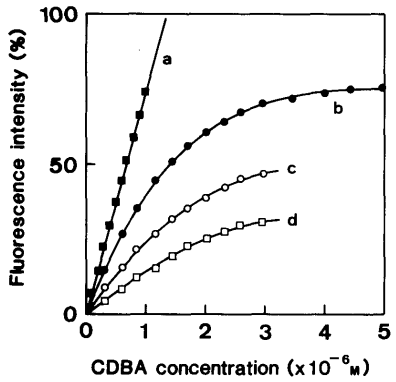


Fig. 4. Fluorometric Titration Curves of α_1-AGP with CDBA

a, at high α_1-AGP concentration (1×10^{-4} M and 5×10^{-5} M); b, at low α_1-AGP concentration (3×10^{-6} M); c, at low α_1-AGP concentration in the presence of imipramine (7.4×10^{-6} M); d, at low α_1-AGP concentration in the presence of chlorpromazine (3.6×10^{-6} M).

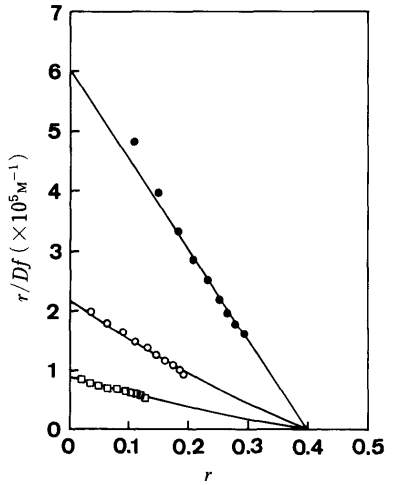


Fig. 5. Scatchard Plots for the Binding of CDBA to α_1-AGP

●, in the absence of drug; ○, in the presence of imipramine (7.4×10^{-6} M); □, in the presence of chlorpromazine (3.6×10^{-6} M). The solid lines are theoretical curves.

TABLE III. Binding Constants of Basic Drugs

Drug	log K_b	Literature
Chlorpromazine	6.46	6.54,[13] 5.53,[16] 5.04[17]
Amitriptyline	5.58	5.53,[15] 5.30[17]
Trimipramine	5.61	
Imipramine	5.54	5.39,[13] 5.34,[17] 4.96[18]
Desipramine	4.86	
Prochlorperazine	6.24	
Levomepromazine	6.01	4.95[17]
Promethazine	4.94	
Quinidine	6.18	
Diltiazem	5.40	
Pindolol	5.33	
Nicardipine	6.78	
Lidocaine	5.12	5.24[19]
Propranolol	5.85	6.05,[13] 5.59,[19] 5.00[16]

fluorescence intensity was lowered by the competition of the added drug with CDBA. Figure 5 shows a Scatchard plot of the titration data in Fig. 4. The plot resulted in a linear relationship, indicating one class of binding site on α_1-AGP. The binding constant, K_a, and the number of binding sites, n, were calculated to be $1.50 \times 10^6\ \text{M}^{-1}$ and 0.40, respectively.

Determination of the Binding Constant of Basic Drug The Scatchard plots for the binding data of imipramine and chlorpromazine gave straight lines. As shown in Fig. 5, the slopes of the two straight lines were lower than that observed in the absence of the drugs. On the other hand, the intercepts of the lines in the presence of the drugs were identical with that in the absence of the drugs. The facts indicate that competition occurred between CDBA and each of the drugs at the same binding site on α_1-AGP. The binding constants of imipramine and chlorpromazine estimated by using the equation of Klotz *et al.*[12] are summarized together with the results for other basic drugs in Table III. The binding constants, K_b, obtained by this method agree approximately with the values from the literature.

Conclusion

A new fluorescent probe, CDBA, for protein binding measurement was developed. The probe binds to α_1-AGP with the binding strength of log $K=6.18$. Its binding is competitive with respect to that of many basic drugs at the same binding site on the protein. The binding constants of basic drugs could be measured by the fluorescent probe method. The log K values of the drugs tested ranged from 4.7 to 6.8.

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