Application of Fluorescent Triazoles to Analytical Chemistry. III.[1] Fluorescence Characteristics of 2-Phenylbenzotriazolyl-5-amine Derivatives as Fluorescent Probes

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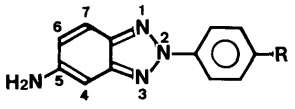
2-Phenylbenzotriazole derivatives with a $COCH_3$ or NH_2 group on the 2-phenyl ring were synthesized and found to be applicable as fluorescent hydrophobic probes. The fluorescence intensities of these compounds, which were intense in non-polar solvents, were dramatically quenched in polar solvents. In particular, 2-(4-acetylphenyl)-2*H*-benzotriazolyl-5-amine (7) showed a much higher ratio of the fluorescence intensity in the organic solvent to that in water (F_o/F_w) than 8-anilino-1-naphthalenesulfonate (ANS): The F_o/F_w values of 7 were 470 for dioxane and 440 for acetone, while those of ANS were 100 for dioxane and 90 for acetone.

We modified compound 7 to obtain water-soluble probes for use in drug–protein binding studies and examined the interaction of these probes with human serum albumin (HSA). All the compounds, which were practically non-fluorescent in the buffer solution, bound to HSA and fluoresced.

Keywords 2-phenylbenzotriazolyl-5-amine; fluorescence characteristics; solvent effect; fluorescent probe; protein binding

Recently, we have reported a new fluorescence derivatization reagent for carboxylic acids in high-performance liquid chromatography.[1] That was the first attempt to use 2-phenylbenzotriazole as an analytical reagent.

In the present work, we investigated the fluorescence characteristics of several 2-phenylbenzotriazoles having various substituents at the 4-position of the 2-phenyl ring with the aim of developing fluorescent probes for protein binding studies as a further application of triazoles to analytical chemistry. It was found that the fluorescence intensities of some derivatives having a $COCH_3$ or NH_2 group on the 2-phenyl ring were drastically quenched in polar solvents. This observation suggests a possible use of these compounds as fluorescent probes for protein binding studies of ligands such as 8-anilino-1-naphthalenesulfonate (ANS)[2] and coumarin derivatives.[3,4]



2-phenylbenzotriazole derivative

Chart 1

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 Human serum albumin (HSA) (Lot. No. 37) and ANS (sodium salt) were purchased from Miles Research Laboratories and Tokyo Kasei Kogyo Co., respectively. All the organic solvents for spectral measurements were purchased from Dojindo Laboratories. The other chemicals were of reagent grade.

Synthesis of Compounds 1—12 Compounds **1**—**12** were prepared by the same method as described previously.[5] The diazonium salt of *p*-substituted aniline was coupled with *m*-phenylenediamine dihydrochloride and the resulting azo compound was converted to the triazole by oxidation with ammoniacal cupric sulfate. Compounds **5** and **9** were obtained by the hydrolysis of **6** and **10**, respectively. Elemental analysis gave the expected values for the desired compounds. All melting points are uncorrected (Table I).

Synthesis of Compounds 13 and 14 Compound **13** was prepared by the same method as described above, except that *N*,*N*-dimethyl-*m*-phenylenediamine dihydrochloride was used instead of *m*-phenylenediamine dihydrochloride. Compound **14** was prepared by the treatment of the 4-CH_2OH derivative in dry dimethyl sulfoxide (DMSO) with *N*,*N'*-dicyclohexylcarbodiimide and H_3PO_4.[6] Elemental analysis gave the expected values for the desired compounds. All melting points are uncorrected (Table I).

Synthesis of Compounds 15—21 Compounds **15**—**18** were prepared by the same method as described above, except that 3,5-diaminobenzoic acid dihydrochloride was used instead of *m*-phenylenediamine dihydrochloride. Compound **19** was prepared by the treatment of compound **7** with propiolactone.[7] Compounds **20** and **21** were prepared by *N*-propylation of compound **7**, followed by the treatment with propiolactone and propanesultone,[8] respectively. Elemental analysis gave the expected values for the desired compounds. All melting points are uncorrected (Table I).

Fluorescence Measurement of HSA Solution HSA was dissolved in 0.1 M phosphate buffer (pH 7.4). HSA solutions of $0.5—10 \times 10^{-5}$ M were used; the concentration was determined by using a value of 0.531 as the extinction coefficient ($E^{0.1\%}_{1\,cm}$) at 279 nm.[9] The triazole solutions were prepared in 0.1 M phosphate buffer (pH 7.4) after being dissolved in a trace amount of 0.1 N NaOH.

Results and Discussion

Fluorescence Characteristics of the 2-Phenylbenzotriazolyl-5-amine Derivatives We synthesized several 2-phenylbenzotriazolyl-5-amine derivatives having various substituents at the 4-position of the 2-phenyl ring and measured their fluorescence in water and five organic solvents. Table II summarizes the fluorescence spectral data. The fluorescence intensity was generally weaker in polar solvents than in non-polar solvents and, in particular, those of **6**, **7** and **9** decreased drastically in water. Among these three compounds, **7** was practically non-fluorescent in polar solvents although it was strongly fluorescent in non-polar solvents. This property of **7** is similar to that of ANS, one of the most widely used fluorescent probes. Hence, compound **7** was taken as a lead compound for further investigation to develop fluorescent probes.

Solvent Effect on the Fluorescence Intensity of Compound 7 and Related Compounds The fluorescence intensity of compound **7** was compared with that of ANS in water, dioxane and acetone. The results are summarized in Table III. We found that the F_o/F_w values, the ratios of the fluorescence intensity in organic solvent (F_o) to that in water (F_w), of **7** were 470 for dioxane and 440 for acetone, while those of ANS were 100 for dioxane and 90 for acetone. Furthermore, the fluorescence intensity of **7** itself was 3.9 times stronger in dioxane and 4.1 times stronger in acetone than that of ANS. Compound **7** was much more sensitive to change of polarity than ANS, indicating that **7**

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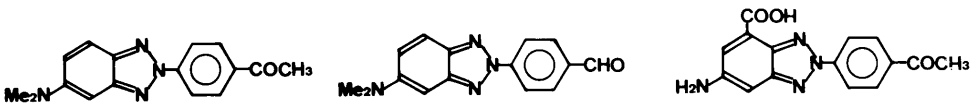
TABLE I. Physicochemical and Analytical Data

Compd. No.	Appearance (Recrystn. solvent)	Yield (%)	mp (°C)	Formula	Analysis Calcd (Found)		
					C	H	N
1	Colorless needles (MeOH–H_2O)	60.0	187—188	$C_{12}H_{10}N_4$	68.56 (68.57)	4.79 4.75	26.65 26.56)
2	Colorless needles (MeOH–H_2O)	73.0	216—218	$C_{13}H_{12}N_4$	69.62 (69.69	5.39 5.38	24.98 24.89)
3	Colorless needles (MeOH)	85.7	229—230	$C_{13}H_{12}N_4O$	64.99 (65.10	5.03 5.17	23.32 23.22)
4	Colorless needles (MeOH–H_2O)	73.0	176.5—178	$C_{13}H_{12}N_4O$	64.99 (64.83	5.03 5.08	23.32 23.20)
5	Colorless needles (DMF–H_2O)	78.6	over 300	$C_{13}H_{10}N_4O_2$	61.41 (61.13	3.96 4.15	22.04 21.78)
6	Brown plates (DMF–H_2O)	77.0	206—207	$C_{15}H_{14}N_4O_2$	63.82 (63.75	5.00 5.08	19.85 19.83)
7	Yellow needles (DMF–H_2O)	59.0	255—256	$C_{14}H_{12}N_4O$	66.65 (66.61	4.79 4.93	22.21 22.07)
8	Brown needles (THF–H_2O)	56.0	285—295	$C_{13}H_9N_5$	66.37 (66.15	3.86 3.96	29.77 29.53)
9	Colorless needles (MeOH–H_2O)	64.0	201—202	$C_{12}H_{11}N_5$	63.98 (63.87	4.92 4.77	31.09 30.88)
10	Colorless needles (MeOH–H_2O)	83.0	222—224	$C_{14}H_{13}N_5O \cdot 1/2\,H_2O$	60.86 (61.16	5.11 4.91	25.35 25.42)
11	Colorless plates (MeOH–H_2O)	83.0	231—232	$C_{12}H_9ClN_4$	58.90 (58.82	3.71 3.76	22.90 22.86)
12	Orange needles (DMF–H_2O)	69.0	over 300	$C_{12}H_9N_5O_2$	56.47 (56.71	3.55 3.70	27.44 27.35)
13	Yellow needles ($CHCl_3$)	63.0	178.5—180	$C_{16}H_{16}N_4O$	68.55 (68.82	5.75 5.68	19.99 20.00)
14	Yellow plates ($CHCl_3$–MeOH)	28.4	176—178	$C_{15}H_{14}N_4O$	67.65 (67.67	5.30 5.23	21.04 21.03)
15	Colorless needles (DMF–H_2O)	12.5	over 300	$C_{15}H_{12}N_4O_3$	60.81 (60.66	4.08 4.21	18.91 18.65)
16	Colorless needles (MeOH–H_2O)	16.5	231—233	$C_{15}H_{12}N_4O_3$	60.81 (60.67	4.08 4.05	18.91 18.73)
17	Colorless plates (MeOH–H_2O)	58.0	212—214	$C_{15}H_{12}N_4O_3 \cdot 1/4\,H_2O$	59.90 (60.18	4.19 4.17	18.63 18.41)
18	Yellow prisms (DMF–H_2O)	12.0	262—264	$C_{20}H_{14}N_4O_3$	67.03 (66.74	3.94 4.00	15.63 15.54)
19	Yellow needles (MeOH–H_2O)	34.0	215—225	$C_{17}H_{16}N_4O_3$	62.95 (63.13	4.97 5.04	17.27 17.88)
20	Yellow needles (MeOH–H_2O)	42.8	186—189	$C_{20}H_{22}N_4O_3$	65.56 (65.55	6.05 5.97	15.29 15.10)
21	Yellow needles (MeOH–H_2O)	23.3	255—258	$C_{20}H_{23}N_4NaO_4S \cdot 1/4\,H_2O$	53.68 (53.96	5.41 5.38	12.52 12.61)



13 **14** **15**

Chart 2

could be an effective hydrophobic probe. Three derivatives of **7** (**13**, **14** and **15**) also showed similar fluorescence properties to those of **7**: the fluorescence in non-polar solvent was quenched drastically by the addition of a small amount of water, as shown in Fig. 1. These results show that *N*-alkylation at the 5-position and the introduction of a COOH group at the 7-position of the benzotriazole ring do not greatly affect the fluorescent properties and that the quenching by water originates mainly from the presence of the carboxyl group ($COCH_3$ or CHO) on the 2-phenyl ring.

Interaction of the Fluorescent Probe with Protein Synthesis of the Water-Soluble Probes: We thought that compound **7** and related compounds might be useful as fluorescent probes, like ANS. Further attempts were made to obtain some water-soluble probes for the investigation of drug–protein interaction. Keeping the carbonyl group on the 2-phenyl ring, we introduced a COOH group at the 7-position of the benzotriazole ring or an alkylated NH_2 group possessing a COOH or SO_3H group at the 5-position. The same quenching effect of water on the fluorescence as observed for **7** was maintained, while all the probes (**15**—**21**) so obtained (Table IV) were water-soluble.

Binding of the Fluorescent Probes with Protein: All the water-soluble probes (**15**—**21**), which were practically non-fluorescent in the buffer solution, fluoresced in the presence of HSA. Figure 2 shows the fluorescence spectra of **15** in the

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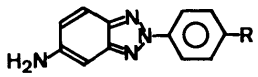
TABLE II. Fluorescence Characteristics of Triazole Derivatives



Compd. No.	R	RFI (%)[a] [Ex (nm)/Em (nm)]					
		Water	Ethanol	DMSO	Dioxane	Chloroform	Cyclohexane
1	H	43.0 (352/496)	52.4 (367/473)	65.2 (383/477)	62.6 (366/439)	37.6 (358/436)	75.6 (357/408)
2	CH_3	53.0 (352/494)	59.0 (365/472)	74.0 (381/473)	69.4 (366/436)	45.6 (357/431)	94.6 (355/406)
3	CH_2OH	50.4 (354/501)	62.2 (366/475)	73.6 (382/478)	74.0 (368/441)	46.4 (358/438)	72.0 (357/410)
4	OCH_3	66.0 (353/492)	68.6 (364/467)	81.8 (378/467)	86.4 (366/434)	62.6 (358/429)	121.2 (356/405)
5	COOH	41.2 (360/515)	64.6 (370/483)	53.8 (399/522)	88.2 (380/463)	34.0 (370/465)	11.2 (382/429)
6	COOEt	5.0 (360/520)	53.0 (386/513)	52.2 (400/527)	94.0 (381/464)	61.6 (371/462)	98.2 (368/422)
7	$COCH_3$	—	—	15.2 (400/549)	94.4 (385/471)	62.2 (374/479)	54.2 (375/423)
8	CN	20.4 (369/534)	57.6 (396/515)	46.6 (402/530)	89.0 (386/469)	57.4 (376/469)	79.0 (373/428)
9	NH_2	1.0 (360/493)	60.8 (371/463)	118.8 (385/456)	156.2 (371/431)	110.8 (364/430)	156.2 (360/407)
10	NHAc	69.6 (358/500)	85.8 (369/474)	96.8 (384/473)	109.2 (369/436)	74.4 (362/435)	20.0 (365/408)
11	Cl	47.8 (355/503)	59.4 (371/481)	71.4 (390/488)	71.0 (371/447)	44.4 (362/441)	88.2 (361/413)
12	NO_2	—	—	—	2.4 (334/368)	—	2.4 (383/429)

a) In comparing the fluorescence intensities of triazole derivatives (2×10^{-7} M), that of quinine sulfate (2×10^{-7} M in 0.1 N H_2SO_4) was taken as 40 (at 350/450 nm). RFI: relative fluorescence intensity.

TABLE III. Fluorescence Characteristics of Compound 7 and ANS

Compound	RFI (%)[a] [Ex (nm)/Em (nm)]		
	Water[b]	Dioxane	Acetone
7	1 (360/505)	470 (388/473)	440 (393/510)
ANS	1.2 (350/515)	120 (380/466)	108 (377/465)

a) In comparing the fluorescence intensities of the compounds (2×10^{-7} M), that of quinine sulfate (2×10^{-7} M, 0.1 N H_2SO_4) was taken as 200 (at 350/450 nm). *b*) Contained 0.01% dioxane.

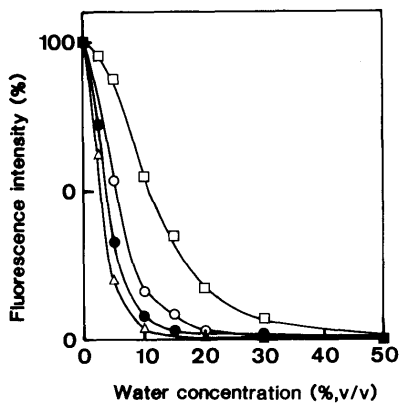
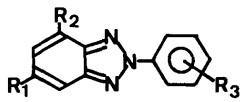


Fig. 1. Effect of Water Concentration on the Fluorescence Intensity of Triazole Derivatives in Dioxane–Water Mixtures

●, compound **7**; ○, compound **13**; △, compound **14**; □, compound **15**.

TABLE IV. Excitation and Emission Maxima of Water-Soluble Triazole Derivatives in the HSA Solution



Compd. No.	R_1	R_2	R_3	Ex (nm)/Em (nm)
15	NH_2	COOH	*p*-$COCH_3$	398/517
16	NH_2	COOH	*m*-$COCH_3$	382/486
17	NH_2	COOH	*o*-$COCH_3$	375/460
18	NH_2	H	*p*-COC_6H_5	398/520
19	$NHCH_2CH_2COOH$	H	*p*-$COCH_3$	400/520
20	$N<^{CH_2CH_2CH_3}_{CH_2CH_2COOH}$	H	*p*-$COCH_3$	400/505
21	$N<^{CH_2CH_2CH_3}_{CH_2CH_2CH_2SO_3Na}$	H	*p*-$COCH_3$	400/505

buffer solution of the presence and absence of HSA. We tried to determine the binding sites of these probes on the HSA molecule. The inhibition of binding of the probes to HSA by site I and II drugs as classified according to Sudlow *et al.*[10,11] was examined. But the binding sites could not be identified because both site drugs decreased the fluorescence intensity of the probes and the Scatchard plots[12] did not show competitive inhibition. It is thus somewhat difficult to apply these probes to evaluate drug–protein binding at least for site I and II drugs. A search for some different applications of these probes to protein binding studies is in progress.

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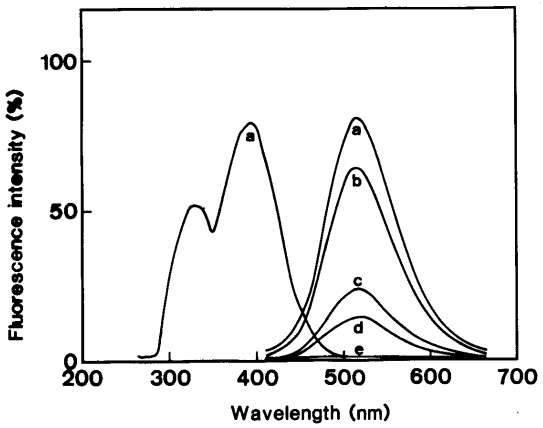

Fig. 2. Excitation and Emission Spectra of Compound **15** (3×10^{-6} M) in HSA Solution

a, in the presence of 1×10^{-4} M HSA; b, in the presence of 5×10^{-5} M HSA; c, in the presence of 1×10^{-5} M HSA; d, in the presence of 5×10^{-6} M HSA; e, in the absence of HSA.

Compound **9** also fluoresced in the presence of not only HSA but also α_1-acid glycoprotein. An application of **9** and related compounds to a protein binding study will be described in the following paper.

References and Notes

1) Part II: S. Narita and T. Kitagawa, *Chem. Pharm. Bull.*, **37**, 831 (1989).
2) J. K. H. Ma, H. W. Jun and L. A. Luzzi, *J. Pharm. Sci.*, **62**, 2038 (1973).
3) S. Goya, A. Takadate, H. Fujino, M. Otagiri and K. Uekama, *Chem. Pharm. Bull.*, **30**, 1363 (1982).
4) A. Takadate, Y. Ohkubo, M. Irikura, S. Goya, M. Otagiri and K. Uekama, *Chem. Pharm. Bull.*, **33**, 1522 (1985).
5) S. Narita, T. Kitagawa and E. Hirai, *Chem. Pharm. Bull.*, **33**, 4928 (1985).
6) K. E. Pfitzner and J. G. Moffatt, *J. Am. Chem. Soc.*, **85**, 3027 (1963).
7) C. D. Hurd and S. Hayao, *J. Am. Chem. Soc.*, **74**, 5889 (1952).
8) D. Horiguchi, M. Saito, T. Imamura and K. Kina, *Anal. Chim. Acta*, **151**, 457 (1983).
9) G. E. Means and M. L. Bender, *Biochemistry*, **14**, 4989 (1975).
10) G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.*, **11**, 824 (1975).
11) G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.*, **12**, 1052 (1976).
12) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

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