Application of Fluorescent Triazoles to Analytical Chemistry. II.[1] Fluorescent Derivatization of Carboxylic Acids

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2-(*p*-Aminomethylphenyl)-*N*,*N*-dimethyl-2*H*-benzotriazolyl-5-amine (1) and two related compounds (2 and 3) were synthesized as highly sensitive fluorescence derivatization reagents for carboxylic acids. These reagents reacted with carboxylic acids activated with 2-bromo-1-ethylpyridinium tetrafluoroborate to produce the corresponding fluorescent amides at room temperature. We investigated the fluorescence characteristics and the chromatographic behavior of the benzamide derivatives of the reagents. Compound 1 was selected for use in high-performance liquid chromatography (HPLC). A highly sensitive and convenient fluorescence HPLC method was developed for the determination of carboxylic acids. The detection limit for carboxylic acids was 15 fmol per injection.

Keywords 2-(*p*-aminomethylphenyl)-*N*,*N*-dimethyl-2*H*-benzotriazolyl-5-amine; fluorescence derivatization reagent; fluorescence characteristics; high-performance liquid chromatography; benzotriazole; carboxylic acid; flufenamic acid

Various fluorescence derivatization reagents have been reported for the determination of carboxylic acids by high-performance liquid chromatography (HPLC).[2-9] Such derivatization, however, often requires elevated temperature. 9-Anthryldiazomethane (ADAM), one of the most widely used reagents, reacts with carboxylic acids at room temperature,[5] but interference often makes HPLC separation difficult.

In the present paper, we deal with three benzotriazole derivatives, 2-(*p*-aminomethylphenyl)-*N*,*N*-dimethyl-2*H*-benzotrizolyl-5-amine (**1**), 2-[*p*-(2-aminoethyl)phenyl]-*N*,*N*-dimethyl-2*H*-benzotriazolyl-5-amine (**2**) and 2-(*p*-aminomethylphenyl)-2*H*-benzotriazolyl-5-amine (**3**) as

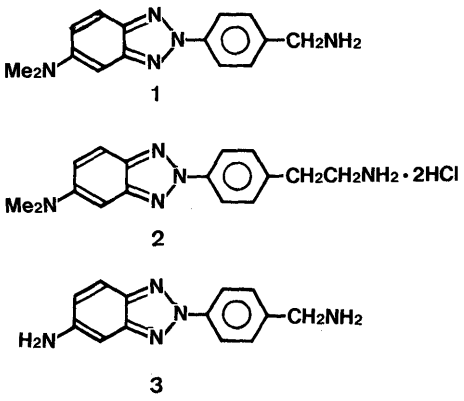


Chart 1

highly sensitive derivatization reagents for carboxylic acids. We investigated the fluorescence characteristics and the chromatographic behavior of the benzamide derivatives of these reagents. The derivatization procedure is in principle based on the method of Lingeman *et al.*[6] 2-Bromo-1-ethylpyridinium tetrafluoroborate (BEPT) is used to activate carboxylic acids and 9-methyl-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-2-one (MDPP) is used as an acid-capturing agent for HBr and HBF_4 formed in the reaction. The reagents reacted with several carboxylic acids in the presence of BEPT and MDPP to produce the corresponding fluorescent amides at room temperature. A highly sensitive and convenient HPLC method was developed for the determination of carboxylic acids using **1** as a reagent.

Experimental

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

HPLC Apparatus: A Shimadzu LC-4A pump equipped with a Shimadzu RF-535 fluorescence HPLC monitor and Rheodyne model 7125 injection valve with a 200 μl sample loop were used. Reversed-phase columns, NOVA PAK $5C_{18}$ (150 × 3.9 mm i.d., Waters Assoc.) and Nucleosil $5C_{18}$ (150 × 4.6 mm i.d., Macherey–Nagel), were used as analytical columns. A Nucleosil $5C_{18}$ (30 × 4.0 mm i.d., Macherey–Nagel) was used as a guard column. A Shimadzu Chromatopac C-R2AX data processor was used for the data treatment.

HPLC Conditions: CH_3CN–H_2O mixtures were used as the mobile phase at the flow rates of 1.0—1.2 ml/min.

Reagents and Materials The chemicals were of reagent grade. CH_3CN (HPLC grade) and Bond Elut (Si) were purchased from E. Merck Co. and Analytichem International, respectively. Commercially available drugs were used after purification in our laboratories.

Synthesis of 1—3 **1**: The diazonium salt of *N*-(*p*-aminobenzyl)-acetamide (6.6 g, 40 mmol) was coupled with *N*,*N*-dimethyl-*m*-phenylenediamine dihydrochloride (8.4 g, 40 mmol), followed by oxidation with an ammoniacal cupric sulfate. The resulting acetylated derivative of **1** was hydrolyzed with 10% HCl in EtOH. The crude product was recrystallized from MeOH to give **1** (6.8 g, yield, 63.2%) as yellow needles, mp 127—128 °C. IR (Nujol): 3300, 3380 cm^{-1}. *Anal.* Calcd for $C_{15}H_{17}N_5$: C, 67.39; H, 6.41; N, 26.20. Found: C, 67.15; H, 6.37; N, 26.15.

2: The trifluoroacetylated derivative of **2** was synthesized as described above by using *N*-(*p*-aminophenethyl)-2,2,2-trifluoroacetamide hydrochloride (8.1 g, 40 mmol) instead of *N*-(*p*-aminobenzyl)acetamide. The resulting compound was hydrolyzed with 5 N NaOH in EtOH. The crude product was obtained from the ethanolic HCl solution and recrystallized from MeOH to give **2** (7.5 g, 52.8%) as colorless plates, mp 254—258 °C. IR (Nujol): 2450, 2550 cm^{-1}. *Anal.* Calcd for $C_{16}H_{21}Cl_2N_5$: C, 54.24; H, 5.97; Cl, 20.01; N, 19.77. Found: C, 54.15; H, 6.08; Cl, 19.76; N, 19.62.

3: **3** was synthesized as described above using *m*-phenylenediamine dihydrochloride (5.4 g, 40 mmol) instead of *N*,*N*-dimethyl-*m*-phenylenediamine dihydrochloride. The resulting compound was recrystallized from MeOH to give **3** (5.1 g, 53.4%) as yellow plates, mp 206—207 °C. IR (Nujol): 3150, 3300, 3350, 3430 cm^{-1}. *Anal.* Calcd for $C_{13}H_{13}N_5$: C, 65.25; H, 5.48; N, 29.27. Found: C, 65.27; H, 5.51; N, 29.18.

Preparation of the Fluorescent Amides (1a—e, 2a and 3a) A carboxylic acid (1 mmol) was dissolved in CH_3CN (10 ml). After addition of BEPT (1.1 mmol), MDPP (2.4 mmol) and the derivatization reagent (**1**, **2** or **3**, 1 mmol), the reaction mixture was stirred for 30 min at room temperature. The crude product was chromatographed on a silica gel column with $CHCl_3$. After evaporation of the eluate, the residue was recrystallized from $CHCl_3$–MeOH.

1a: 114 mg (30.7%) of **1a** was obtained as orange needles, mp 166—167 °C. IR (Nujol): 1627, 3280 cm^{-1}. *Anal.* Calcd for $C_{22}H_{21}N_5O$: C, 71.14; H, 5.70; N, 18.85. Found: C, 71.09; H, 5.73; N, 18.83.

1b: 251 mg (81.1%) of **1b** was obtained as yellow needles, mp 196—197 °C. IR (Nujol): 1630, 3300 cm^{-1}. *Anal.* Calcd for $C_{17}H_{19}N_5O$: C, 66.00; H, 6.19; N, 22.64. Found: C, 66.19; H, 6.22; N, 22.70.

1c: 200 mg (44.4%) of **1c** was obtained as colorless needles, mp 136—137 °C. IR (Nujol): 1630, 3300 cm^{-1}. *Anal.* Calcd for $C_{27}H_{39}N_5O$: C, 72.12; H, 8.74; N, 15.58. Found: C, 72.01; H, 8.72; N, 15.52.

1d: 190 mg (41.7%) of **1d** was obtained as green needles, mp 143—145 °C. IR (Nujol): 1643, 3300 cm^{-1}. *Anal.* Calcd for $C_{28}H_{33}N_5O$: C, 73.82; H, 7.30; N, 15.37. Found: C, 73.98; H, 7.31; N, 15.43.

1e: 145 mg (27.3%) of **1e** was obtained as green needles, mp 175—176 °C. IR (Nujol): 1628, 3360 cm^{-1}. *Anal.* Calcd for $C_{29}H_{25}F_3N_6O$: C, 65.65; H, 4.75; F, 10.74; N, 15.84. Found: C, 65.63; H, 4.75; F, 10.56; N, 15.83.

2a: 183 mg (47.4%) of **2a** was obtained as brown needles, mp 155—156 °C. IR (Nujol): 1630, 1645, 3370 cm^{-1}. *Anal.* Calcd for $C_{23}H_{23}N_5O$: C, 71.67; H, 6.01; N, 18.17. Found: C, 71.50; H, 6.12; N, 18.12.

3a: 158 mg (46.0%) of **3a** was obtained as orange needles, mp 255—257 °C. IR (Nujol): 1635, 3270, 3360, 3470 cm^{-1}. *Anal.* Calcd for $C_{20}H_{17}N_5O$: C, 69.96; H, 4.99; N, 20.40. Found: C, 69.60; H, 5.01; N, 20.17.

Derivatization Procedure Solutions (50 μl each) of BEPT (3.7 mM), MDPP (2.7 mM) and **1** (3.7 mM) were added successively to 0.5 ml of the test solution of carboxylic acid in CH_3CN (*ca.* 2—50 ng per each 0.5 ml), in a stoppered 10-ml vial. After mixing for 10 s, the mixture was allowed to stand for 30 min at room temperature. Then, 0.5 ml of the reaction mixture was applied to a Bond Elut column and eluted with 1.5 ml of CH_3CN. The eluate was diluted with CH_3CN to make 2.5 ml. The resulting solution (20 μl) was injected into the chromatograph.

Results and Discussion

Evaluation of Fluorescence Derivatization Reagents The fluorescence characteristics of three amides, **1a**—**3a**, were investigated. As shown in Table I, the fluorescence intensities at the maximum wavelength were almost the same. The maximum emission wavelengths of **1a** and **2a** were longer than that of **3a** by 30 nm. The effect of H_2O concentration on the fluorescence intensity was examined. In aqueous CH_3CN, the intensity of **3a** was affected a little by H_2O concentration. Although the intensities of **1a** and **2a** remained almost constant in the low concentration range of H_2O, they were greatly affected at over 50% H_2O.

The chromatographic behavior of the three amides were also examined (Table I). The sensitivity of **3a** evaluated from HPLC analysis was 2.5 times higher than those of **1a** and **2a**. The retention times of **1a** and **2a** were much longer than that of **3a**, indicating that **1** and **2** were preferable to **3** for the separation of the biological samples. We selected **1** as a reagent for further investigation; the retention time of **2a** was longer than that of **1a**, but **2** is sparingly soluble in CH_3CN (this reagent was isolated as an HCl salt).

Fluorescence Characteristics of Amides of 1 The fluorescence characteristics of the isolated amides of **1** (**1a**—**e**) were measured. Figure 1 shows the fluorescence spectra of **1e** derived from flufenamic acid. As shown in Table II, the substituents of the amide moiety did not affect the fluorescence characteristics, the intensity or the wavelength.

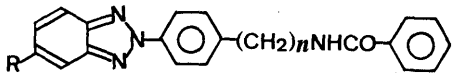
Derivatization Conditions Derivatization conditions were examined using flufenamic acid (50 ng/0.5 ml) as a test compound.

Various concentrations (0.04—5 mM) of **1** were used together with BEPT and MDPP. A constant peak area was obtained at concentrations greater than 2.8 mM for 1, 2.7 mM for BEPT and 1 mM for MDPP.

This reaction proceeded rapidly at room temperature (*ca.* 22 °C). The maximum and constant peak areas were attained at 10—45 min; standing for 30 min at room temperature was employed in this work.

Pretreatment Conditions To remove excess of reagent, a simple pretreatment procedure with a silica column such as a Bond Elut column was established. When the reaction mixture was passed through the column, **1** was strongly retained on the column and only the amide produced was eluted with CH_3CN. Figure 2 shows chromatograms obtained by a typical derivatization procedure. No interfering peaks were detected at the retention time of flufenamic acid.

TABLE I. Fluorescence Characteristics, Retention Times and Detection Limits of Triazole Derivatives (**1a**—**3a**)



Compd. No.	R	*n*	Fluorescence characteristics[a]			Retention times and detection limits[b]	
			Ex. (nm)	Em. (nm)	RFI (%)	Retention time (min)	Detection limit (fmol/inj.)
1a	NMe_2	1	390	488	100	10.6	15
2a	NMe_2	2	384	486	102	12.2	15
3a	NH_2	1	366	456	105	4.2	6

a) Observed in CH_3CN solution (2×10^{-7} M). *b*) HPLC conditions: column, Nucleosil $5C_{18}$ (150 × 4.6 mm i.d.); guard column, Nucleosil $5C_{18}$ (30 × 4.0 mm i.d.); mobile phase, $CH_3CN : H_2O = 4 : 3$; flow rate, 1.0 ml/min.

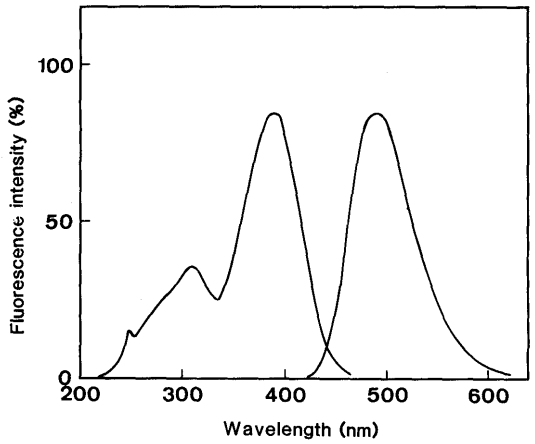
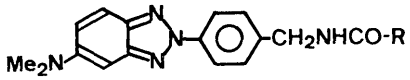
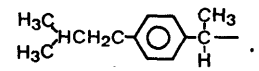


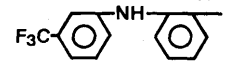
Fig. 1. Excitation and Emission Spectra of Compound **1e** in CH_3CN (2×10^{-7} M)

TABLE II. Fluorescence Characteristics of Amide Derivatives of **1** (2×10^{-7} M)



Compd. No.	R	Ex. (nm)	Em. (nm)	RFI (%)
1a	C_6H_5–	390	488	100
1b	CH_3–	385	486	102
1c	$CH_3(CH_2)_{10}$–	388	488	98
1d	*a*)	388	487	105
1e	*b*)	387	489	104

a) R = 

b) R = 

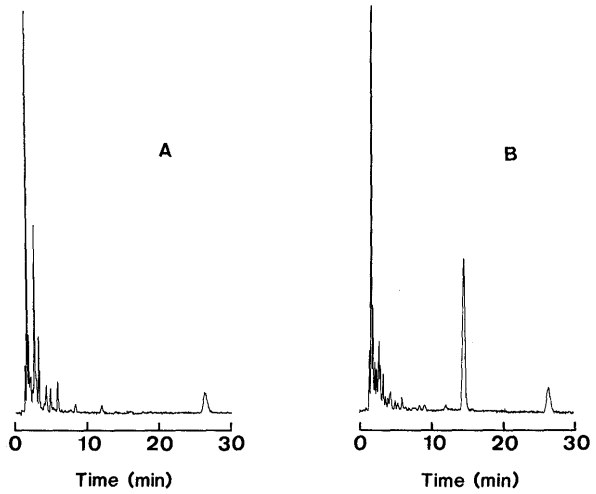


Fig. 2. Chromatograms of Flufenamic Acid after Derivatization with **1**

A, blank; B, flufenamic acid (150 pg/injection). HPLC conditions: column, NOVA PAK $5C_{18}$ (150 × 3.9 mm i.d.); guard column, Nucleosil $5C_{18}$ (30 × 4.0 mm i.d.); mobile phase, $CH_3CN : H_2O = 2 : 1$; flow rate, 1.0 ml/min.

Evaluation of the Derivatization Procedure The overall recoveries of flufenamic acid and ibuprofen throughout the assay were estimated. Flufenamic acid and ibuprofen were recovered as **1e** and **1d** in yields of 84% and 86%, respectively.

The derivatization procedure was applied to the determination of several carboxylic acids; 15 drugs containing a carboxyl group and 5 fatty acids. All the compounds showed a linear relationship between the peak area and the concentration in the range of *ca.* 2—50 ng/0.5 ml. The detection limit was 15 fmol per injection.

The new fluorescence derivatization reagent, **1**, is highly sensitive and reacts with a wide variety of carboxylic acids at room temperature in high yield. This reagent should be useful for the determination of carboxylic acids by HPLC.

References and Notes

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