A new fluorogenic reagent for carboxylic acids, 7-acetylamino-4-mercapto-2,1,3-benzoxadiazole (AABD-SH), derived from an empirical method for predicting fluorescence characteristics

The Analyst

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In our previous studies, we found a relationship between the fluorescence characteristics (the fluorescence intensity, the maximum excitation and emission wavelengths) and Hammett substituent constants (σp) of the substituent groups at the 4- and 7-positions of benzofurazan compounds, which enabled us to predict the fluorescence characteristics of the benzofurazan compounds. This prompted us to design a new fluorogenic derivatization reagent having a benzofurazan skeleton for carboxylic acids along this line of thought. Accordingly, the fluorogenic reagent, 7-acetylamino-4-mercapto-2,1,3-benzoxadiazole (AABD-SH) was synthesized. The reagent itself fluoresced very weakly but fluoresced strongly after the reaction with carboxylic acids. AABD-SH reacted with carboxylic acids within 5 min at room temperature in the presence of triphenylphosphine and 2,2′-dipyridyl disulfide. The derivatives of five carboxylic acids were separated on a reversed phase column and detected fluorimetrically at 524 nm with excitation at 368 nm. The detection limits were 10–20 fmol (signal-to-noise ratio of 3).

Introduction

The sensitive and selective determination of biologically active compounds with carboxylic acid moieties such as fatty acids, bile acids, prostaglandins, and drugs is very important in biological and biomedical sciences. Various fluorescent derivatization reagents for high-performance liquid chromatography (HPLC) have therefore been reported for this purpose.[1–4]

Until now, the following compounds have been reported as fluorescent derivatization reagents for carboxylic acids: (1), fluorescent aryl bromomethanes such as 4-bromomethyl-7-methoxycoumarin (BrMMC),[5] 4-bromomethyl-6,7-dimethoxycoumarin (BrDMC),[6] 3-bromoacetyl-6,7-methylenedioxycoumarin (BrAMDC),[7] 3-bromoacetyl-7-methoxycoumarin,[8] 4-bromomethyl-7-acetoxycoumarin (BrMAC),[9] 3-bromo-methyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (BrDMEQ)[10] and panacyl bromide;[11] (2), fluorescent aryl diazomethanes such as 9-anthryldiazomethane (ADAM)[12] and 1-pyrenyldiazomethane;[13] (3), fluorescent amines and hydrazines such as monodansyl cadaverine,[14] 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionyl carboxylic acid hydrazide (DMEQ-hydrazide),[15] 4-(5,6-dimethoxy-2-benzimidazoyl)benzohydrazide (DMBI-hydrazide),[16] 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI)[17] and 6,7-methylenedioxy-1-methyl-2-oxo-1,2-dihydroquinoxalin-3-yl-propinohydrazide;[18] (4), fluorescent methane sulfonates such as 2-(2,3-naphthalimino)ethyltrifluoromethane sulfonate (NE-OTf)[19] and 2-(2,3-anthracenedicarboximido)ethyltrifluoromethane sulfonate (AE-OTf),[20] and (5), fluorescent benzofurazan amines and hydrazides such as 4-nitro-7-*N*-piperazino-2,1,3-benzoxadiazole (NBD-PZ),[21] 4-(*N*,*N*-dimethylaminosulfonyl)-7-*N*-piperazino-2,1,3-benzoxadiazole (DBD-PZ),[21] 4-(*N*-hydrazinoformylmethyl-*N*-methyl)-amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-CO-Hz)[22] and *N*-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (NBD-CO-Hz).[23]

However, these reagents are not always satisfactory with respect to the detectability of the carboxylic acids, since the fluorescence of the reagent itself and the degradation by-products sometimes interfere with the quantification of the small amount of the analyte. Further, the excitation and emission wavelengths of the derivatives with these reagents are so short that the quantification is often interfered with by biomatrices containing fluorescent substances. Thus, for a useful derivatization reagent for carboxylic acids to be developed it should ideally have the following characteristics: (1), fluorogenic (the reagent itself is not fluorescent); (2), the reagent reacts with carboxylic acids in mild conditions and (3), the formed derivatives fluoresce strongly in the longer wavelength region.

The present work focused on the design of a fluorogenic derivatization reagent for carboxylic acids, since, to our knowledge, there are no ideal fluorogenic reagents for carboxylic acids.

In our previous paper,[24] we investigated the effects of the substituent group at the 4- and 7-positions on the fluorescence characteristics of the benzofurazan (2,1,3-benzoxadiazole) compounds and found the relationship between the fluorescence characteristics of these compounds and the Hammett substituent constants (σp) of the substituent groups at 4- and 7-positions. Namely, taking the sum of the σp at the 4- and 7-positions of benzofurazan compounds as the abscissa and the difference of the σp as the vertical axis on this plane coordinate, the points corresponding to the fluorescent compounds were concentrated in two areas, whereas in contrast those corresponding to the non-fluorescent compounds scattered out of these two areas. Using this relationship, we could predict the fluorescence characteristics from the chemical structures of 4,7-disubstituted benzofurazan compounds and could thus design better fluorogenic derivatization reagents.

In this article, we report the development of a fluorogenic derivatization reagent having a benzofurazan skeleton for

carboxylic acids based on the relationship thus obtained and examined the reactivity of the reagent as well as the detectability of the analytes.

Experimental

Materials

NBD-Cl (4-chloro-7-nitro-2,1,3-benzoxadiazole), potassium *o*-ethyldithiocarbonate, triphenylphosphine (TPP), 2,2′-dipyridyl disulfide (DPDS), *n*-caprylic acid, *n*-capric acid, lauric acid, myristic acid and palmitic acid were of guaranteed grade (Tokyo Kasei Co., Tokyo, Japan). Silica gel 60 was obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q reagent system (Millipore, Bedford, MA, USA). All other reagents were of analytical- or guaranteed-reagent grade and used without further purification.

Apparatus

Melting points were measured on a micro melting-point apparatus (Yanagimoto, Tokyo, Japan) and uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a JEOL GSX-400 spectrometer (Tokyo, Japan) with tetramethylsilane as an internal standard (abbreviations used: s = singlet, d = doublet, t = triplet, q = quadruplet). Mass spectra were measured on a Hitachi M-1200H mass spectrometer (atmospheric pressure chemical ionization (APCI) system) (Tokyo, Japan). Fluorescence spectra were measured on a Hitachi F-4010 fluorescence spectrometer (Tokyo, Japan).

Synthesis of 7-acetylamino-4-mercapto-2,1,3-benzoxadiazole (AABD-SH)

4-Ethyldithiocarbonyl-7-nitro-2,1,3-benzoxadiazole (NO_2/$SCSOC_2H_5$). NBD-Cl (500 mg) was dissolved in 30 ml of acetonitrile. To the solution, potassium *o*-ethyldithiocarbonate (400 mg) was added slowly. The mixture was stirred for 30 min at room temperature and evaporated to dryness. The residue was chromatographed on silica gel [dichloromethane–hexane (1 + 2)] to afford 660 mg of the product as yellow crystals, mp 31 °C. ^{1}H NMR ($CDCl_3$): δ 8.52 (1 H, d, J = 8.0 Hz), 7.84 (1 H, d, J = 8.0 Hz), 4.63 (2 H, q), 1.29 (3 H, t). APCI-MS: m/z = 285 (M^-).

7-Amino-4-ethyldithiocarbonyl-2,1,3-benzoxadiazole (NH_2/$SCSOC_2H_5$). NO_2/$SCSOC_2H_5$ (200 mg) was dissolved in a mixture of 15 ml of methanol, 2 ml of dichloromethane and 5 ml of concentrated hydrochloric acid. After addition of 200 mg of iron powder, the mixture was vigorously stirred for 30 min. The reaction mixture was concentrated under reduced pressure and 50 ml of water was added. The solution was extracted with 100 ml of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was chromatographed on silica gel [dichloromethane–hexane (1 + 1)] to afford 70 mg of red powder, mp 139–140 °C. ^{1}H NMR ($CDCl_3$): δ 7.44 (1 H, d, J = 8.0 Hz), 6.36 (1 H, d, J = 8.0 Hz), 4.94 (2H, s, broad), 4.58 (2 H, q), 1.27 (3 H, t). APCI-MS: m/z = 255 (M^-).

AABD-SH. NH_2/$SCSOC_2H_5$ (50 mg) was dissolved in a mixture of 1 ml of pyridine and 1 ml of acetic anhydride. The mixture was stirred at 70 °C for 30 min and evaporated to dryness. The residue was dissolved in 5 ml of ethanol followed by the addition of 1 ml of 10% NaOH aqueous solution. The mixture was stirred at 60 °C for 10 min. The solution was acidified with hydrochloric acid and concentrated under reduced pressure. The resultant solution was extracted with 50 ml of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was chromatographed on silica gel [dichloromethane–methanol (95 + 5)] to afford 25 mg of yellow powder, mp 214 °C. ^{1}H NMR ($CDCl_3$): δ 8.17 (1 H, d, J = 8.0 Hz), 7.91 (1 H, s), 7.23 (1 H, d, J = 8.0 Hz), 4.06 (1 H, s), 2.27 (3 H, s). APCI-MS: m/z = 209 (M^-).

AABD-$SCOCH_3$. AABD-SH (5 mg) was dissolved in a mixture of 1 ml of dichloromethane and 1 ml of acetic anhydride. The mixture was stirred at 40 °C for 10 min and evaporated to dryness. The residue was chromatographed on silica gel [ethyl acetate–methanol (95 + 5)] to afford 5 mg of pale yellow powder, mp 165 °C. ^{1}H NMR ($CDCl_3$): δ 8.25 (1 H, d, J = 8.0 Hz), 8.10 (1 H, s), 7.45 (1 H, d, J = 8.0 Hz), 2.43 (3 H, s), 2.26 (3 H, s). APCI-MS: m/z = 250 ($M - H^-$).

Derivatization of carboxylic acids with AABD-SH

To a vial (500 μl volume) were added 20 μl of AABD-SH in dichloromethane (20 mM), 20 μl of mixed carboxylic acids (100 μM each of *n*-caprylic acid, *n*-capric acid, lauric acid, myristic acid and palmitic acid) in acetonitrile, 20 μl of TPP (20 mM) and 20 μl of DPDS (20 mM) in acetonitrile. The vial was capped and allowed to stand for 15 min at room temperature. Then 20 μl of acetonitrile was added and an aliquot (1.0 μl) of the reaction mixture was subjected to HPLC.

Time course of the reaction of AABD-SH with *n*-capric acid

n-Capric acid (100 μM) in acetonitrile was derivatized with AABD-SH (20 mM) in dichloromethane, TPP (20 mM) and DPDS (20 mM) in acetonitrile according to the procedure described above and an aliquot (1.0 μl) of the reaction mixture was subjected to HPLC after appropriate reaction periods.

Fluorescence spectra

The solutions of AABD-SH (1.0 μM) and AABD-$SCOCH_3$ (1.0 μM) in acetonitrile were used for the measurement of the fluorescence spectra. For the measurement of the fluorescence intensity, the maximum excitation and emission wavelengths in various solvents, the solution of AABD-$SCOCH_3$ (2.0 mM) in acetonitrile was diluted to a concentration of 2.0 μM with the solvent.

High-performance liquid chromatography

The high-performance liquid chromatograph consisted of a Hitachi L-6300 intelligent pump, a Hitachi L-1080 fluorescence detector and a Hitachi D-2500 integrator. The separation of the derivatives was studied on an analytical column, TSK gel ODS-80TsQA (250 × 4.6 mm id, 5 μm) (TOSOH, Tokyo, Japan) using isocratic elution from 0 to 15 min (acetonitrile, 80% in water, v/v), linear gradient elution from 15 to 35 min (acetonitrile, 80 to 100% in water, v/v), and isocratic elution from 35 min (acetonitrile, 100%) at a flow rate of 1.0 ml min^{-1}. The column temperature was ambient. The eluate was monitored with fluorescence detection (excitation at 368 nm, emission at 524 nm).

Results and discussion

Design and synthesis of the fluorogenic derivatization reagent for carboxylic acids

Fig. 1 shows the relationship between the fluorescence intensity of 4,7-disubstituted benzofurazan compounds and Hammett substituent constant (σp) at the 4- and 7-positions, obtained previously.[24] In this figure, the abscissa and the ordinate were the sum of σp at the 4- and 7-positions and the difference of σp at the 4- and 7-positions, respectively. The seventy 4,7-disubstituted benzofurazan compounds were classified into three groups according to their relative fluorescence intensity (RFI; fluorescence intensity of 4-amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole was arbitrarily taken as 1.0) [RFI = 0–1, having no or weak fluorescence (○); RFI = 1–5, having moderate fluorescence (▲); RFI > 5, having strong fluorescence (■)]. The fluorescent compounds, represented as closed squares and closed triangles, were concentrated in two areas (A and B), in contrast, non-fluorescent compounds scattered out of these areas. These results showed that the compounds in these two areas are strongly fluorescent and the compounds out of these areas are non-fluorescent or weakly fluorescent, suggesting that the compounds which are out of these areas before reaction and move into these areas after reaction are suitable as fluorogenic derivatization reagents.

At first, as the candidate for the reactive group for carboxylic acids, NH_2, OH and SH groups were considered. Among them, the SH group was the best with regard to the reactivity to carboxylic acids, and thus the SH group was selected as the reactive group at the 4-position. Then, a substituent group at the 7-position was selected to be an acetylamino group to afford 7-acetylamino-4-mercapto-2,1,3-benzoxadiazole (AABD-SH) considering the relationship above described: AABD-SH [①] was located out of the fluorescent area in Fig. 1 and moves into the fluorescent area in Fig. 1 after reaction with carboxylic acids [②]. Thus, AABD-SH seemed to be appropriate as the fluorogenic reagent for carboxylic acids (Fig. 2).

AABD-SH was synthesized by the reaction of NBD-Cl with potassium *o*-ethyldithiocarbonate, followed by reduction of the nitro group with Fe–HCl, acetylation of the amino group and hydrolysis to afford a yellow powder. The structure of AABD-SH was confirmed by NMR and mass spectrometry.

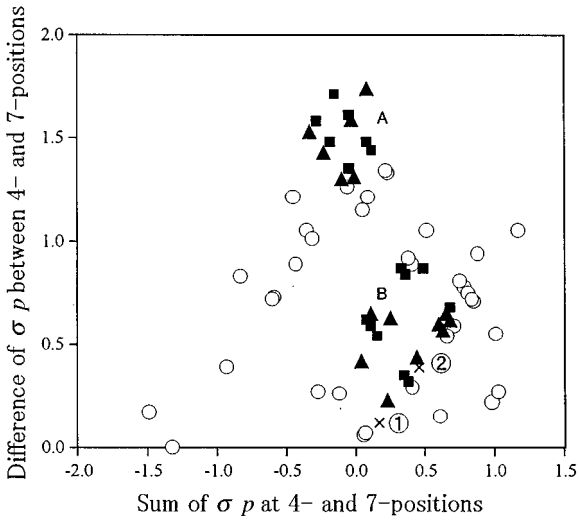

Fig. 1 The design of fluorogenic reagents for carboxylic acids using the relationship between the fluorescence intensity of 4,7-disubstituted benzofurazan compounds and the Hammett substituent constants (σp) at 4- and 7-positions. ① and ② denote AABD-SH and AABD-$SCOCH_3$, respectively. RFI = 0–1, no or weak fluorescence (○); RFI = 1–5, moderate fluorescence (▲); RFI > 5, strong fluorescence (■).

The fluorescence characteristics of AABD-SH and AABD-$SCOCH_3$

The fluorescence spectra of AABD-SH and AABD-$SCOCH_3$ in acetonitrile are shown in Fig. 3. As expected, AABD-$SCOCH_3$ fluoresced strongly (maximum excitation wavelength (λ_{ex})/maximum emission wavelength (λ_{em}) = 368 nm/511 nm in acetonitrile). On the contrary, AABD-SH, the reagent itself, fluoresced very weakly and the fluorescence intensity was more than thirty times lower compared with AABD-$SCOCH_3$. Also, the λ_{em} was different from that of AABD-$SCOCH_3$; ($\lambda_{ex}/\lambda_{em}$ = 367 nm/486 nm in acetonitrile). These results indicate that AABD-SH is suitable as a fluorogenic reagent for carboxylic acids.

The λ_{ex}, λ_{em} and the relative fluorescence intensities of AABD-$SCOCH_3$ in the various solvents are summarized in Table 1. Those in acetonitrile–water of various ratios are shown in Table 2. The fluorescence intensity of AABD-$SCOCH_3$

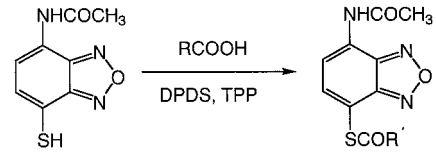

Fig. 2 Chemical structure of AABD-SH and the reaction with carboxylic acids in the presence of TPP and DPDS.

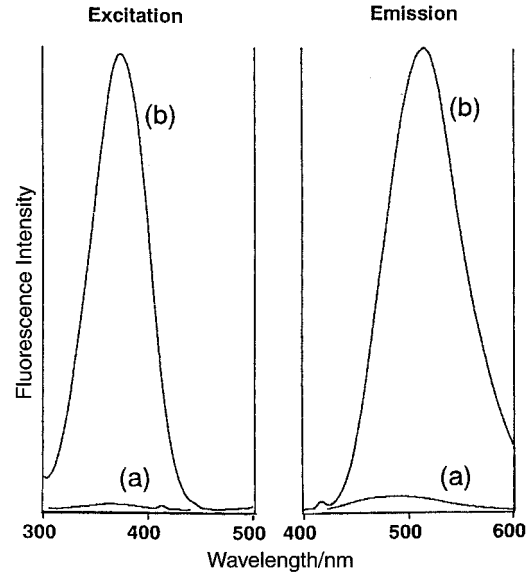

Fig. 3 Fluorescence spectra of AABD-SH (1.0 μM) (a) and AABD-$SCOCH_3$ (1.0 μM) (b) in acetonitrile. The excitation and emission spectra of AABD-SH were recorded at an emission of 470 nm and at an excitation of 367 nm, respectively. The excitation and emission spectra of AABD-$SCOCH_3$ were recorded at an emission of 510 nm and at an excitation of 369 nm, respectively.

Table 1 Maximum excitation and emission wavelengths and relative fluorescence intensities of AABD-$SCOCH_3$ in various solvents

Solvent	λ_{ex}/nm	λ_{em}/nm	RFI[a]
Benzene	371	497	100
Dichloromethane	368	504	87
Acetonitrile	368	511	62
Propan-2-ol	366	517	26
Ethanol	366	517	17
Methanol	366	522	10

[a] RFI: the fluorescence intensity in benzene was taken as 100.

decreased with the increase of solvent polarity, especially in the protic solvents; λ_{em} shifted bathochromically. These phenomena were similar to other derivatization reagents with a benzofurazan structure.[25–27]

The λ_{em} of AABD-$SCOCH_3$ was longer than the derivatives with a coumarin skeleton (410–470 nm),[5–8] BrDMEQ[10] and DMEQ-hydrazide[15] (435 nm), ADAM (412 nm)[12] NE-OTf (394 nm),[19] AE-OTf (456 nm)[20] and HPCI (455 nm)[17] and panacyl bromide (470 nm),[11] giving superiority to AABD-SH with regard to the avoidance of the interference from biomatrices.

Time course study of the reaction of *n*-capric acid with AABD-SH

n-Capric acid was selected as a representive carboxylic acid. The time course study of the derivatization of *n*-capric acid with AABD-SH was performed in the presence of TPP and DPDS at room temperature. As shown in Fig. 4, the peak area reached a plateau after 5 min at room temperature, meaning that the thiol group reacted rapidly with carboxylic acids under these conditions. The derivatization of carboxylic acids with conventional reagents requires heating or longer reaction periods [BrMMC (60 °C, 15 min),[5] BrDMC (70 °C, 30 min),[6] BrDMEQ (80 °C, 20 min),[10] ADAM (60 min),[12] HCPI hydrazide (30 min)[17]] suggesting its superiority to other reagents with regard to the reactivity as the derivatization reagent for carboxylic acids.

Table 2 Maximum excitation and emission wavelengths and relative fluorescence intensities of AABD-$SCOCH_3$ in various ratios of water–acetonitrile

Acetonitrile (%)	λ_{ex}/nm	λ_{em}/nm	RFI[a]
100	368	511	100
90	367	522	38
80	369	522	23
70	367	523	18
60	366	524	14
50	365	523	11
40	363	525	1
30	361	523	6
20	361	523	5
10	360	524	5

[a] RFI: the fluorescence intensity in acetonitrile was taken as 100.

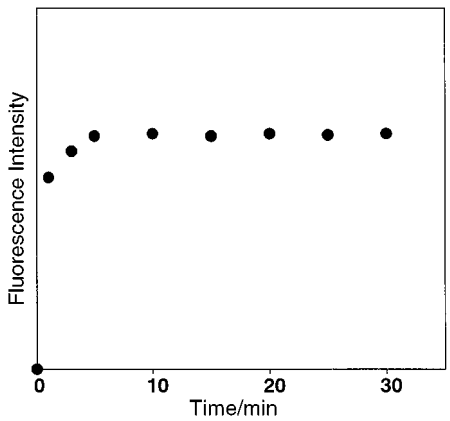

Fig. 4 Time course for the derivatization reaction of *n*-capric acid with AABD-SH. The closed circles (●) denote the fluorescence intensity determined on a reversed phase HPLC. The experimental details are described in the text.

Separation of the derivatives of carboxylic acids with AABD-SH on a reversed phase column

The chromatogram of derivatives thus obtained is shown in Fig. 5. As expected, the interfering peaks from the reagent and the degradation products were extremely small, since the reagent, AABD-SH, itself fluoresces very weakly compared with the derivatives. The peaks of the derivatives were clearly separated from each other and the interfering peaks. The calibration curves for carboxylic acids were linear over the range from 0.1 to 10 pmol per injection (r = 0.99). The relative standard deviations of the peak area for five replicate measurement of carboxylic acids at 10 pmol per injection were 2.5–4.0%. The detection limits attained were 10–20 fmol per injection at a signal-to-noise ratio of 3, suggesting that AABD-SH was more sensitive than BrMMC (10 pmol),[5] BrMDC (500 fmol),[6] panacyl bromide (250 fmol),[11] ADAM (100 fmol),[12] dansyl cadaverine (100 fmol),[14] equivalent to BrMAC (10 fmol),[11] HCPI (7–57 fmol)[17] and slightly less sensitive than DMEQ-hydrazide (3–5 fmol),[15] NE-OTf (4 fmol),[19] AE-OTf (0.5–3 fmol),[20] DBD-PZ (3–5 fmol),[21] DBD-CO-HZ (3–9 fmol)[22] and NBD-CO-Hz (2–4 fmol).[23] However, these reagents themselves fluoresce strongly, thus the fluorescence of the excess reagent and the degradation products interfere with the quantification of trace amounts of intended derivatives. These facts indicate that AABD-SH, the new fluorogenic reagent, is superior as a derivatization reagent with regard to the detectability of carboxylic acids.

In conclusion, considering the relationship between the fluorescence intensity of the 4,7-disubstituted benzofurazan compounds and Hammett substituent constants at the 4- and 7-positions, a new fluorogenic reagent for carboxylic acids, AABD-SH, was designed. AABD-SH was superior to other reagents with regard to its sensitivity and reactivity, and in the absence of interfering peaks in the chromatograms. Thus, AABD-SH seems to be useful for the derivatization of a small amount of biologically important carboxylic acids. These results showed that our approach, employing the relationship between the fluorescence characteristics and the chemical

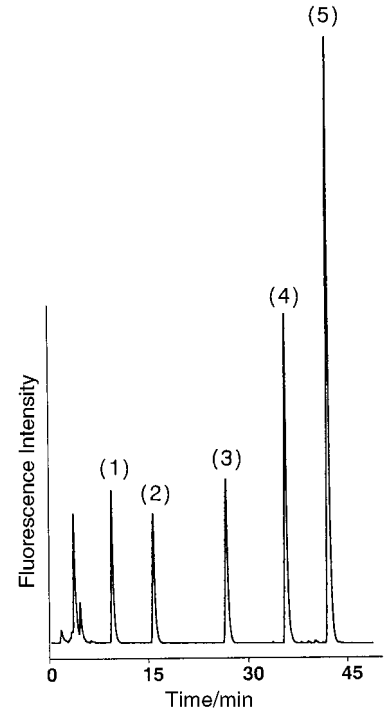

Fig. 5 Chromatogram of carboxylic acids derivatized with AABD-SH: (1) *n*-caprylic acid, 10 pmol; (2) *n*-capric acid, 10 pmol; (3) lauric acid, 10 pmol; (4) myristic acid, 10 pmol; (5) palmitic acid, 10 pmol. The derivatization conditions and HPLC conditions are described in the text.

structure, was effective in the development of new fluorogenic reagents. Further theoretical study is in progress[28] for the precise prediction of the fluorescence intensity of 4,7-disubstituted benzofurazan compounds from the chemical structure.

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