Thienylpyridines as a New Fluorescent Reagent. II.[1] Determination of Carboxylic Acid with 5-(4-Pyridyl)-2-thiophenemethanol Using HPLC

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Synopsis. The title compound has been prepared and used as a new derivatization reagent. The reagent reacts with the carboxylic acids in chloroform in the presence of 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide to produce the corresponding fluorescent esters, which can be separated on an octadecyl-silica column. Each 5 fmol of hexadecanoic or octadecanoic acids and about 50 fmol of benzoic and *p*-anisic acids (1 μl injection) (S/N=2) were detected.

Recently, we have reported that a thienylpyridine skeleton is a new fluorophore,[2] and that 5-(4-pyridyl)-2-thiophenecarbaldehyde(**1**) is one of the useful derivatization reagents for aliphatic primary amines in HPLC.[1] The present paper deals with the preparation of a new fluorescent compound, 5-(4-pyridyl)-2-thiophenemethanol(**2**), and with its application to the determination of carboxylic acids. The detection limits of most aromatic carboxylic acids were superior to those of all known reagents,[3,4] and those of aliphatic carboxylic acids were also superior to those of known reagents, except for 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone(**3**).[3] The dynamic linear range was over 4 orders of magnitude.

Experimental

Elemental analyses were performed by the Laboratory for Organic Elemental Microanalysis, Faculty of Pharmaceutical Sciences, Kyoto University. Melting points were uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 FT spectrometer in $CDCl_3$ using TMS as the internal standard. Mass spectra were recorded on a Hitachi M-80B mass spectrometer.

Liquid chromatography experiments were conducted on a HPLC system consisting of two Tosoh CCPE pumps equipped with an MX-8010 mixer, a Rheodyne Model 7410 fixed loop injector, a Shimadzu Shim-pack CLC-ODS column (15 cm×6 mm i.d.; packed with octadecyl-silica gel), and a Shimadzu RF-535 fluorescence monitor. Chromatograms were recorded with a Shimadzu C-R3A chromatopac or with an EPSON PC-286V computer connected with the chromatopac.

Reagent. All chemicals were of reagent grade and were used as received from commercial sources, except for those noted below. Solvents were distilled and water was purified through a Milli-QII system.

Preparation of 2. Aldehyde **1**[1] (2.0 mmol) and a trace amount of Methyl Orange were dissolved in 40 ml of methanol. Sodium cyanotrihydroborate (2.6 mmol) was added, and concd hydrochloric acid–methanol (1 : 5) was added dropwise with stirring to maintain the red color. After 5 h, the methanol was evaporated in vacuo. The residue was taken up in 20 ml of 5% sodium hydroxide solution, and extracted with three 20-ml portions of chloroform. The combined extracts were evaporated under reduced pressure and chromatographed on silica gel (eluent, acetone : ethyl acetate=1 : 1), and recrystallized from chloroform to yield colorless needles **2** in a 60% yield. Mp 139.0—139.5 °C. Found: C, 62.60; H, 4.79; N 7.17%; *m/z*, 191.0404. Calcd for $C_{10}H_9NOS$: C, 62.80; H, 4.74; N, 7.32%; M, 191.0404. ^{1}H NMR (400 MHz, 40 °C) δ=2.5 (1H, br, OH), 4.86 (2H, s, CH_2), 7.01 (1H, d, *J*=3.8 Hz) , 7.36 (1H, d, *J*=3.8 Hz), 7.43 (2H, dd, *J*=4.6 and 1.7 Hz, H-3 and H-5 in pyridine ring), and 8.53 (2H, dd, *J*=4.6 and 1.7 Hz, H-2 and H-6 in pyridine ring). ^{13}C NMR (100 MHz) δ=60.12 (s), 119.77 (d, 2C), 125.27 (d), 126.31 (d), 140.85 (s), 141.61 (s) , 146.79 (s), and 150.25 (d, 2C). Reagent **2** was stable in the crystalline state for a year or longer in daylight at room temperature.

Derivatization Procedure. One fifth ml of chloroform containing 8.0—1.6×10^5 pmol of carboxylic acids and 0.2 ml of chloroform containing 0.8 μmol of 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate(**4**) were added to 0.2 ml of chloroform containing 0.4 μmol of **2** and 1.6 μmol of 4-dimethylaminopyridine(**5**) in a 20-ml screw-capped reaction vial. The reaction vial was tightly closed, heated at 60 °C for 3 h, and then cooled. Each 1 μl of the solution was subjected to HPLC, and determined by monitoring the fluorescence intensities at 360 nm with excitation of 300 nm.

Results and Discussion

Preparation and Properties of 2. Reagent **2** was prepared in a 60% yield by the reduction of **1** with cyanotrihydroborate ion under the conditions described for the reduction of acetophenone;[5] its structure was proved by its ^{1}H and ^{13}C NMR spectra, MS, and elemental analysis. The alcohol **2** a stable crystalline substance, behaved as a weak base with pK_a 4.64 in 40% aqueous methanol. A nonprotonated form of **2** exhibited more excellent fluorescent properties than a protonated one. Its new carboxylic esters(**6**) also strongly fluorescenced and the pH dependence of the spectra was similar to that of 4-(2-thienyl)pyridine.[6]

Determination of Carboxylic Acids. Figure 1 shows typical chromatograms for the linear saturated fatty acid esters of **2**. The C2—C18 fatty acid esters of **2** were separated on a reversed-phase column (Shim-pack CLC-ODS) with methanol. Methanol gave a complete separation for the C12—C18 long-chain fatty acid esters, though the peaks for the esters of C2—C7 short-chain fatty acids almost overlapped those for the derivatization reagent **2** and/or an adjacent peak. When a gradient elution using 30—0%(v/v) aqueous methanol was applied, however, the esters of short-chain fatty acids were completely separated.

Three condensing reagents, i.e., **4**, dicyclohexylcarbodiimide(**7**), and diethyl phosphorocyanidate(**8**), were examined for the esterification of linear fatty acids with **2** in chloroform, since no esterification occurred in the absence of a condensing reagent; reagents **4** and **7** accelerated the esterification, but reagent **8** did not.[7] Figure 2 shows the effect of the concentration of **4** and **7** on the peak area of the esters of C2—C7 acids. The

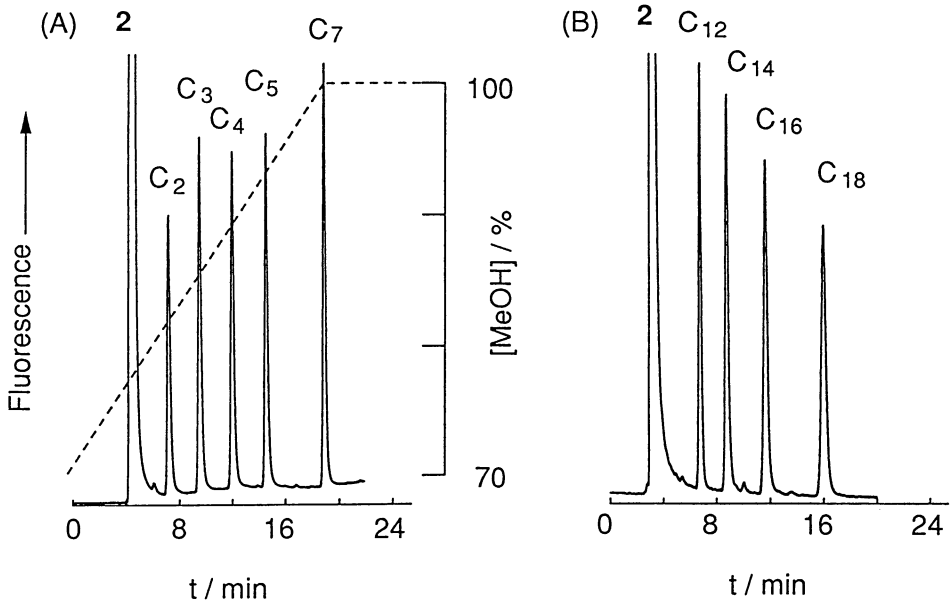

Fig. 1. Chromatograms for the esters of C2—C18 linear saturated fatty acids.
The esters are denoted by the numbers of carbon atoms of fatty acid, C*n*. Column, 150×6 mm i.d. Shim-pack CLC-ODS; eluent, (A) 30—0% (v/v) aqueous methanol and (B) methanol, 1.0 ml min^{-1}; sample size, 1 μl (70 pmol); precolumn reaction, a portion (0.2 ml) of a standard mixture of the acids (each 0.2 mmol dm^{-3}) was derivatized according to the procedure described in the experimental section.

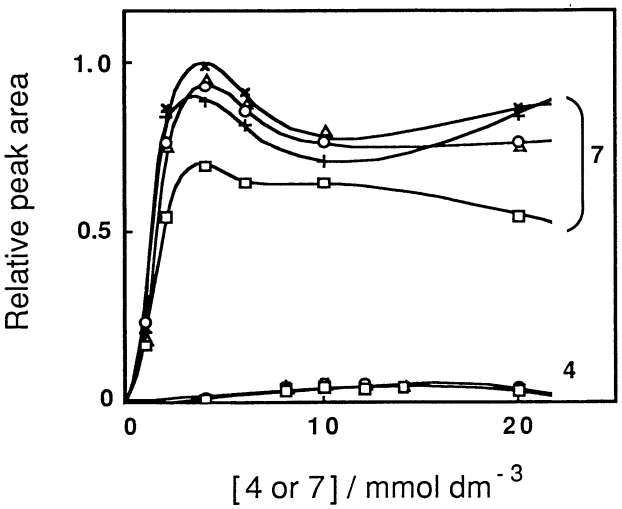

Fig. 2. Effect of the concentration of a condensing reagent, **4** or **7**, on the peak area of the esters of C2—C7 acids: (—□—) C2, (—○—) C3, (—△—) C4, (—×—) C5, and (—+—) C7 acids.
The conditions are as described in Fig. 1, except for a condensing reagent. The values of the vertical axis are represented as the ratio of the peak area of the esters to the maximum area of the C5 acid ester.

largest peaks were obtained at 4 mmol dm^{-3} of the reagent **4** solution for all the acids, where no side reaction occurred, although above this concentration other small peaks were also observed; a 4 mmol dm^{-3} **4** solution was used in subsequent work. The maximum and constant peak area were attained at temperatures higher than 60 °C and heating times longer than 3 h; heating for 3 h at 60 °C was employed in subsequent work.

Table 1. Linear Ranges and Detection Limits of Saturated Fatty Acids[a)]

Saturated fatty acid	Linear range /fmol	Detection limit (S/N=2)/fmol
Acetic acid	5.8×10^{3}—120×10^{3}	5800
Propionic acid	170—84×10^{3}	170
Butyric acid	420—84×10^{3}	420
Valeric acid	29—58×10^{3}	15
Heptanoic acid	27—53×10^{3}	13
Dodecanoic acid	110—110×10^{3}	110
Tetradecanoic acid	11—110×10^{3}	11
Hexadecanoic acid	12—120×10^{3}	5
Octadecanoic acid	12—120×10^{3}	5

a) Conditions as described in Fig. 1.

The derivatization of C18 acid with **2** went to completion under the present conditions, since both the retention time and the peak area of HPLC for a sample prepared from 2.0×10^{-5} mol dm^{-3} C18 acid solution were consistent with those of a 2.0×10^{-5}mol dm^{-3} 5-(4-pyridyl)-2-thenyl octadecanoate[6)] solution. Table 1 shows the linear ranges and detection limits (S/N=2) obtained from the calibration curves for C2—C18 acids. The linear-correlation coefficients were more than 0.9991 for all the acids within the tabulated ranges of the concentration. These limits were superior to those of the known reagents, except for **3**.[3)] The lower detection limit of C2 acid was higher than that estimated from the IFS value,[6)] because a small peak for a trace amount of the C2 acid ester and a large peak for a large excess of reagent **2** overlapped. The coefficients of

Table 2. Retention Time, Peak Area, and pK_a of Aromatic Carboxylic Acids[a]

Aromatic carboxylic acid	Retention time/min	Relative peak area[b]	pK_a
o-Bromobenzoic acid	6.7	0.10	2.85[c]
m-Bromobenzoic acid	10.0	0.19	3.81[c]
p-Bromobenzoic acid	9.5	0.28	4.00[c]
o-Anisic acid	5.1	0.65	4.09[c]
m-Anisic acid	6.7	0.62	4.09[c]
p-Anisic acid	7.7	1.43	4.49[c]
Benzoic acid	6.9	1.00	4.21[c] 5.79[d]
p-Toluic acid	8.9	1.29	6.12[d]

a) Conditions as stated in Fig. 1, except for the eluent (95% methanol) and concentration of the acid standard solution (each 1.5 mmol dm^{-3}). b) Expressed as a ratio of the peak area of the analyte to that of benzoic acid. c) Ref. 8. d) Ref. 9.

variation for 1.8×10^{-7} mol dm^{-3} C5 and 1.6×10^{-7} mol dm^{-3} C7 acid solutions (n=7) were 2.1 and 1.9%, respectively.

Some aromatic carboxylic acids were made to react with **2** under the derivatization conditions described above in order to produce fluorescent derivatives which could be separated by HPLC. Table 2 shows the retention time and peak area with the pK_a values of the corresponding acids. The samples prepared from 1.5 mmol dm^{-3} acid solutions were analyzed on an ODS column using 85% methanol as the moving phase. The peak area of the esters increased with increasing pK_a: the esterification of aliphatic, benzoic, and *o*-bromobenzoic acids, of which the pK_a values were 4.7—4.9, 4.21, and 2.85,[8] occurred in 100, 20, and 3% conversions, respectively. The linear ranges for benzoic and *p*-anisic acids were 98 fmol—490 pmol and 110 fmol—550 pmol; the linear-correlation coefficients were 0.9997 and 0.9998, respectively, and the detection limits (S/N=2) were 50 and 40 fmol. The coefficients of variation for 2.5 mmol dm^{-3} benzoic and 2.8 mmol dm^{-3} *p*-anisic acid solutions (n=7) were 2.3 and 2.1%. No description has been found in the literature concerning the limits and ranges for the determination of aromatic carboxylic acids, except that by Lingeman et al.,[4] who reported that the limit and range for benzoic acid were 100 fmol (S/N=3) and 0.1—1.0 μg (0.8—8.0 nmol), respectively. The present method was therefore superior to that reported by Lingeman et al.

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