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3-Bromomethyl-7-methoxy-1,4-benzoxazin-2-one as a highly sensitive fluorescence derivatization reagent for carboxylic acids in high-performance liquid chromatography

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ABSTRACT

A fluorescence labelling reagent, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (BrMB), was synthesized from 2-amino-5-methoxyphenol and ethyl pyruvate by the Wislecenus reaction followed by bromination. Picomole to nanomole amounts of saturated aliphatic fatty acids were converted into the corresponding fluorogenic esters in the presence of anhydrous potassium carbonate and a crown ether as catalysts. BrMB derivatives of fatty acids were separated on a reversed-phase column and were sensitively detected fluorimetrically at 440 nm with excitation at 345 nm. Quantitative studies revealed that *n*-caproic acid was esterified completely under mild conditions and with sufficient reproducibility. The detection limit of the BrMB derivative of *n*-caproic acid was just below 2 fmol.

INTRODUCTION

Fatty acids are difficult to determine at low concentrations because of their weak UV absorption. High-performance liquid chromatography (HPLC) with fluorescence detection is useful for the determination of trace compounds. Therefore, many fluorogenic reagents have been developed for the determination of carboxylic acids by HPLC, *e.g.*, 4-bromomethyl-7-methoxycoumarin (BrMMC) [1,2], 1-bromoactylpyrene (BAP) [3], 4-bromomethyl-6,7-methylenedioxycoumarin (BrMDC) [4], 4-bromomethyl-6,7-dimethoxycoumarin (BrDMC) [5], 4-bromomethyl-6,7-dimethoxy-1-methyl-2-(1*H*)-quinoxalinone (BrMQ) [6], 9-anthryldiazomethane (ADAM) [7] and others [8–13]. Of these reagents, BrMMC has been widely applied in HPLC assays, but it is poorly soluble in reaction media such as acetonitrile and ethyl acetate used to obtain acyl derivatives, and further the fluorescence intensity of MMC derivatives of carboxylic acids was affected by the solvent composition.

This paper describes the preparation of 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (BrMB), which possesses a new 1,4-benzoxazin-2-one moiety, a highly fluorescent compound, and the applicability of the reagent to the determination of femtomole levels of carboxylic acids by HLC with fluorescence detection.

EXPERIMENTAL

Materials

All chemicals for synthesis were of guaranteed-reagent grade and all organic solvents for chromatographic purposes were of special grade for HPLC, obtained from Wako (Osaka, Japan). Water was purified with a Milli-Q water purification unit (Millipore, Bedford, MA, USA). Linear C_3–C_{20} saturated fatty acids and 18-crown-6 were pur-

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chased from Sigma (St. Louis, MO, USA) and phenyltrimethylammonium tribromide from Aldrich (Milwaukee, WI, USA).

Instruments

A Hitachi (Tokyo, Japan) Model 650-40 spectrofluorimeter photometer was used for the measurement of fluorescence spectra. The HPLC system consisted of a Model LC-5A solvent-delivery pump (Shimadzu, Kyoto, Japan) equipped with a Rheodyne (Cotati, CA, USA) Model 7125 sample injector and a Shimadzu Model RF-550 fluorescence HPLC monitor, which was linked to a Shimadzu Model C-R6A chromatographic integrator.

Synthesis of reagents

The following methods were used for analysis of the products of the syntheses. Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured using a Model JNM-GX270 spectrometer (JEOL, Tokyo, Japan) at 270 MHz and chemical shift values were expressed in ppm downfield from tetramethylsilane as a internal standard. A Perkin-Elmer (Norwalk, CT, USA) Model 1750 infrared spectrometer was used for infrared spectra determinations. Mass spectra were measured with a Model DX-300 mass spectrometer (JEOL) in the electron impact mode.

3-Methyl-7-methoxy-1,4-benzoxazin-2-one (**1**). This synthesis was performed using the Wislecenus reaction [14]. To an aqueous solution (60 ml) of 5-methoxy-2-nitrophenol (0.03 mol) was added sodium hydrosulphite (36 g) and the mixture was refluxed for 30 min under an inert gas. 2-Amino-5-methoxyphenol was separated as amber-coloured prisms after cooling on ice. Ethyl pyruvate (0.03 mol) was added to a solution of 2-amino-5-methoxyphenol (0.03 mol) in ethanol (45 ml) and acetic acid (11 ml). The mixture was kept in the dark with stirring overnight at ambient temperature. The orange-yellow crystals formed were separated by filtration and recrystallized from ethanol, 2.38 g, yield 41.6%, m.p. 126°C. Elemental analysis: calculated for $C_{10}H_9NO_3$, C 62.82, H 4.75, N 7.33; found, C 62.91, H 4.85, N 7.30%. NMR (C^2HCl_3), ppm: 2.52 (3H, s, CH_3); 3.87 (3H, s, OCH_3); 6.75 (1H, d, J=2.6 Hz); 6.90 (H, dd, J=2.6 and 8.8 Hz); 7.60 (1H, d, J=8.8 Hz). Mass spectrum: m/z 192 (M^+), 106 (base peak). IR (KBr): 3040–2956, 1730, 1616, 1566, 1442, 1356, 1256, 1164, 1096, 1024 cm^{-1}.

3-Bromomethyl-7-methoxy-1,4-benzoxazin-2-one (**2**). To an ice-cold solution of **1** (1.09 g) in tetrahydrofuran (20 ml) was added phenyltrimethyl ammonium tribromide (0.01 mol) and the mixture was stirred gently overnight in a refrigerator. The crude mixture was diluted with 50 ml of diethyl ether and washed with 25 ml of 0.1 *M* $NaHSO_3$, followed by 25 ml of 0.1 *M* $NaHCO_3$ and distilled water. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was purified by silica gel (50 g) column chromatography and eluted with *n*-hexane–dichloromethane (1:1). The dried material was recrystallized from *n*-hexane–dichloromethane (1:1). BrMB was obtained as faint yellow prisms, 1.66 g, yield 62.0%, m.p. 146°C. NMR (C^2HCl_3), ppm: 3.90 (3H, s, OCH_3); 4.54 (2H, s, CH_2); 6.78 (1H, d, J=2.6 Hz); 6.94 (1H, dd, J=2.6 and 8.8 Hz); 7.67 (1H, d, J=8.8 Hz). Elemental analysis: calculated for $C_{10}H_8NO_3Br$, C 44.47, H 2.99, N 5.19, Br 29.59; found, C 44.42, H 2.99, N 5.18, Br 29.41%. Mass spectrum: m/z 271 (M^+), 190 (base peak). IR (KBr): 3072–2948, 1730, 1620, 1544, 1364, 1272, 1214, 1198, 1124, 1022 cm^{-1}.

Preparation of n-caproic acid derivative (3) as fluorescence reference. The authentic BrMB derivative of *n*-caproic acid was synthesized on a semi-micro preparative scale in order to evaluate its reactivity. To a solution of *n*-caproic acid (1.2 mmol) in 5 ml of acetonitrile were added BrMB (0.6 mmol) and triethylamine (1.2 mmol). The resulting solution was allowed to stand at 40°C for 1 h, then evaporated to dryness *in vacuo*. The residue was purified on a silica gel column with a mixture of *n*-hexane and dichloromethane (1:1) as eluent. The main fraction was evaporated to dryness *in vacuo* and the residue was purified by repeated recrystallization from diethyl ether to give faint yellow needles (**3**), yield 42.0%, m.p. 48°C. NMR (C^2HCl_3), ppm: 0.92 (3H, t, J=6.3 Hz, CH_3); 1.2–1.9 [6H, m, $(CH_2)_3$]; 2.47 (2H, t, J=7.5 Hz, $OCOCH_2$); 3.89 (3H, s, OCH_3); 5.26 (2H, s, CH_2OCO); 6.78 (1H, d, J=2.6 Hz); 6.92 (1H, dd, J=2.6 and 8.8 Hz); 7.65 (1H, d, J=8.8 Hz). Elemental analysis: calculated for $C_{16}H_{19}NO_5$, C 62.94, H 6.27, N 4.59; found, C 62.99, H 6.23, N 4.55%. Mass spectrum: m/z 305 (M^+), 207 (base peak). IR (KBr): 2932, 1726, 1620, 1566, 1420, 1340, 1284, 1172, 1158, 1026 cm^{-1}.

Analytical derivatization of carboxylic acids

In a 4-ml amber-coloured screw-capped vial were placed 1 ml of acetonitrile solution of carboxylic acids, 0.05 ml of 40 m*M* BrMB in acetonitrile, 0.05 ml of 40 m*M* 18-crown-6 in acetonitrile and 10 mg of potassium carbonate. After being mixed, the reaction solution was allowed to stand at 40°C for 10 min. A 10-μl aliquot of the reaction mixture was injected into the HPLC system. The BrMB derivatives of carboxylic acids were separated by HPLC and quantified by fluorescence detection.

Chromatographic conditions

Optimum separation of BrMB derivatives of C_6–C_{13} straight-chain fatty acids could be achieved by using a reversed-phase ODS YMC Pack A-312 column (150 × 6 mm I.D.; particle size 5 μm) (YMC, Kyoto, Japan) and acetonitrile–water (80:20) as mobile phase. The mobile phase was degassed by brief sonication before use and then pumped isocratically at 1.5 ml/min at ambient temperature. The excitation and emission wavelengths were adjusted to 345 and 440 nm, respectively.

RESULTS AND DISCUSSION

Synthesis of 7-methoxy-1,4-benzoxazin-2-one as a fluorescent probe

Many fluorescent labelling reagents are known for the sensitive chromatographic determination of carboxylic acids [1–13]. ADAM [7] and BrMMC [1,2] have been used extensively for trace determinations of prostanoids in biological specimens. However, the former reagent is unstable and the fluorescence intensity of the latter derivatives is affected by solvents. Various 1,4-benzoxazin-2-ones have been reported [14–17], which were synthesized by condensation of α-keto esters with *o*-aminophenols, but no mention was made of their fluorescence properties except for 6,7-methylenedioxy [18], 6,7-dimethoxy [18] and 7-amino derivatives [19]. Our attention became directed toward substituted 1,4-benzoxazin-2-ones, especially with electron-donating substituents, such as amino, alkylamino, hydroxy and alkyloxy groups, in the 7-position as new fluorescent probes for the sensitive determination of trace drugs by HPLC. Of these compounds, we synthesized 7-methoxy-1,4-benzoxazin-2-one with a reactive functional group at the 3-position, because the 7-methoxy derivative of 3-methyl-1,4-benzoxazin-2-one has the strongest fluorescence intensity, as shown in Table I. The order of the relative fluorescence intensities is essentially the same as the order of the Hammett substituent constants, except for the amino derivatives.

The synthetic route is shown in Fig. 1. 2-Amino-5-methoxyphenol was previously obtained [20] by catalytic hydrogenation (PtO_2–H_2) of 5-methoxy-2-nitro-phenol in hydrochloric acid–ethanol. In this work, 2-amino-5-methoxyphenol was obtained by reduction using sodium hydrosulphite. The aminophenol reacted with ethyl pyruvate under mild conditions and gave the condensed-ring 3-methyl-7-methoxy-1,4-benzoxazin-2-one. This

TABLE I

FLUORESCENCE SPECTRAL PROPERTIES OF 3-METHYL-1,4-BENZOXAZIN-2-ONE ANALOGUES

Substituent		I_F[a]	Fluorescence[b]	
6-position	7-position		λ_{ex} (max.) (nm)	λ_{em} (max.) (nm)
H	H	4.5	355	430
H	CH_3	13	336	428
H	OH	450	400	472
H	OCH_3	520	342	436
$-OCH_2O-$		480	370	480
H	NH_2	42	380	510
H	$N(CH_3)_2$	120	410	535

[a] I_F = relative fluorescence intensity [4-methyl-7-methoxycoumarin (MMC) = 100].
[b] Fluorescence spectra were measured in ethanol solution.

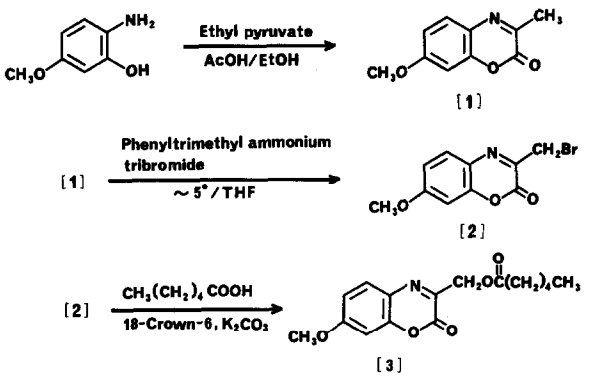

Fig. 1. Synthesis scheme for the fluorescent derivatization reagent BrMB (**2**), and derivatization of *n*-caproic acid with BrMB. AcOH = Acetic acid; EtOH = ethanol; THF = tetrahydrofuran.

TABLE II

FLUORESCENCE SPECTRAL PROPERTIES OF BrMB ESTER OF *n*-CAPROIC ACID IN VARIOUS SOLVENTS

Solvent	I_F[a]	Fluorescence	
		λ_{ex} (max.) (nm)	λ_{em} (max.) (nm)
n-Hexane	4.7	338	426
Benzene	11	346	429
Dichloromethane	29	374	427
Acetone	25	343	427
Ethyl acetate	15	337	423
Acetonitrile	38	341	429
Ethanol	51	342	436
Methanol	69	340	440
Water	100	346	451

[a] I_F = relative fluorescence intensity (water = 100).

compound was easily converted into the bromomethyl derivative (BrMB) by bromination of the methyl group at the 3-position on treatment with phenyltrimethylammonium tribromide.

The reagent, BrMB, proved to be stable at room temperature for at least 6 months with protection from humidity and light, and the acetonitrile solution was also stable for at least 1 week when stored in a refrigerator.

Fluorescence properties of BrMB derivatives

The excitation and emission spectra of the fluorescent ester **3** in methanol–water (80:20) were measured. The solution is similar to the mobile phase used for the chromatographic separation of BrMB derivatives of fatty acids. Under this condition, the excitation and emission maxima were obtained at 345 and 440 nm, respectively.

The fluorescence intensity of BrMB derivatives was affected by the polarity and pH of the solvent. The effects of the solvent on the fluorescence were therefore examined before the analytical technique was developed.

Table II lists the excitation and emission maxima and the relative fluorescence intensities of the purified BrMB ester of *n*-caproic acid in various media. Strong fluorescence was observed in methanol and acetonitrile.

The effects of water concentration and pH on the fluorescence intensity of **3** were investigated. The fluorescence intensity was almost maximum and constant at water concentrations of 20–40% in aqueous methanol and 20–60% in aqueous acetonitrile. The most intense fluorescence was constant between pH 3 and 6.

These results suggest that aqueous acetonitrile is suitable as a mobile phase in the reversed-phase HPLC of BrMB derivatives of fatty acids.

Derivatization of carboxylic acids with BrMB

The derivatization reaction of carboxylic acids with BrMB afforded the ester in the presence of a phase-transfer catalyst in acetonitrile. The fluorescence intensity–time profile of the reaction of *n*-caproic acid with BrMB was tested in the presence of 18-crown-6 and potassium carbonate in acetonitrile at 25 and 40°C. Under these derivatization conditions, the rate of formation of the fluorescent ester was rapid, the reaction being completed within 10 min at 40°C, and a slow decrease in the fluorescence intensity was observed. However, when the reaction mixture was stored in an amber-coloured glass vial at 4°C, the fluorescent derivatives were stable for at least 1 day. This property of BrMB is better than that of BrMMC and BrMDC, where complete esterification of *n*-caproic acid took at least 30 min at the same temperature [4].

Separation of BrMB derivatives of fatty acids

Fig. 2 shows a typical chromatogram of a reaction mixture of fatty acids with BrMB obtained by reversed-phase HPLC using aqueous acetonitrile as

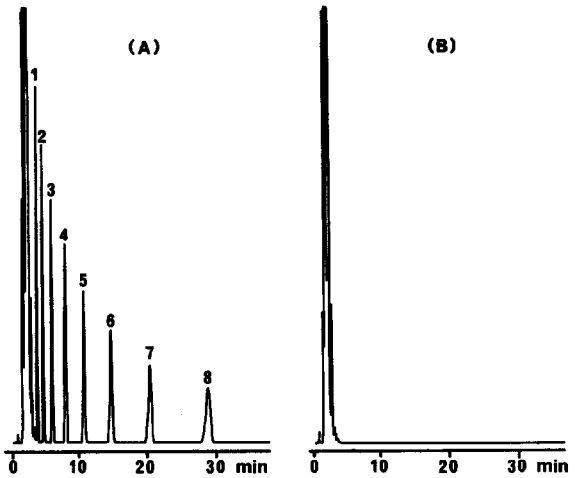

Fig. 2. Chromatographic separation of BrMB derivatives of fatty acids. (A) Reaction mixture of C_6–C_{13} fatty acids. Peaks: 1 = hexanoic acid (C_6), 2 = heptanoic acid (C_7), 3 = octanoic acid (C_8), 4 = nonanoic acid (C_9), 5 = decanoic acid (C_{10}), 6 = undecanoic acid (C_{11}), 7 = dodecanoic acid (C_{12}), 8 = tridecanoic acid (C_{13}). (B) Blank of reaction mixture without fatty acid. HPLC conditions are given in the text. Amount injected: 5 pmol each.

the eluent. A good separation can be achieved with the YMC-Pack A-312 column and acetonitrile–water (80:20) as the mobile phase. The eight fatty acids derivatives were resolved within 30 min.

The capacity factors (k') increased with increasing number of methylene groups in the carboxylic acid; the plot of log k' *versus* carbon number gave approximately a straight line, as illustrated in Fig. 3.

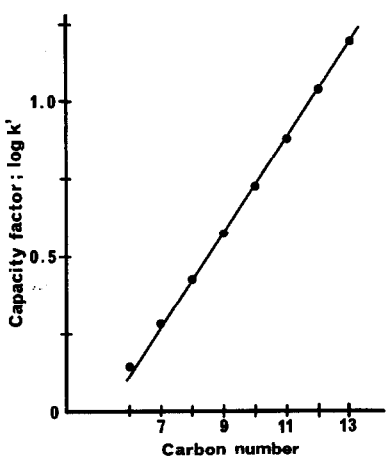

Fig. 3. Relationship between carbon chain number of fatty acids and capacity factor (log k') of BrMB derivatives in reversed-phase HPLC.

TABLE III

REPRODUCIBILITY OF THE PROPOSED METHOD

Fatty acid	Relative standard deviation (%)[a]		
	1.8 pmol	9.0 pmol	45 pmol
C_6	1.86	2.43	0.75
C_7	2.30	2.22	0.79
C_8	2.25	3.15	1.08
C_9	2.11	2.07	0.97
C_{10}	2.00	1.88	0.93
C_{11}	1.60	1.92	0.86
C_{12}	2.56	2.07	0.95
C_{13}	2.76	1.40	0.65

[a] $n = 5$.

Linearity of response, precision and detection limit

Calibration graphs of peak areas *versus* concentrations of fatty acids labelled with BrMB were plotted. A good linear detector response (linear regression coefficient = 0.999) was observed for all the fatty acids in the range 0.7– 45 pmol injected on-column (number of fatty acid concentrations tested = 7, $n = 3$ at each concentration). The detection limits of the fatty acids are in the range 2–10 fmol at a signal-to-noise ratio of 3.

Multiple derivatization ($n = 5$) of fatty acids followed by HPLC gave peak areas with relative standard deviations of 0.65–3.15% in the range 1.8–45 pmol of fatty acids analysed, as shown in Table III.

It is expected that this method will be suitable for the derivatization and separation of carboxylic acids with satisfactory reliability and its high sensitivity may provide much more precise information on biologically important various organic acids. This methods is now being applied to trace determinations of some non-steroidal anti-inflammatory drugs in biological specimens for pharmacokinetic studies.

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