3-[4-(Bromomethyl)phenyl]-7-(diethylamino)-2*H*-1-benzopyran-2-one (MPAC-Br): A Highly Sensitive Fluorescent Derivatization Reagent for Carboxylic Acids in High-Performance Liquid Chromatography[1)]

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7-Dimethylaminocoumarin derivatives were synthesized and the characteristics of their absorption and emission spectra were investigated. Among them, 3-aryl-7-dimethylaminocoumarins were shown to be promising candidates as the fluorogenic groups for highly sensitive derivatization reagents. Based on the spectroscopic studies, 3-[4-(bromomethyl)phenyl]-7-(diethylamino)-2*H*-1-benzopyran-2-one (MPAC-Br) was designed and synthesized as a fluorescent derivatization reagent for carboxylic acids in HPLC. Saturated carboxylic acids (C_4—C_{18}) were derivatized almost quantitatively into the corresponding fluorescent MPAC-esters by treatment with MPAC-Br. The MPAC-esters of these acids were clearly separated on a reversed-phase HPLC column (Inertsil ODS-2, mobile phase: methanol–water, excitation wavelength 403 nm; emission wavelength 474 nm). The detection limit ($S/N=17$) of lauric acid, as a test compound, was 15 fmol/10 μl.

Key words coumarin; derivatization reagent; HPLC; carboxylic acid; fluorescence detection

Considerable effort has been directed to the development of sensitive and selective derivatization reagents for use in high-performance liquid chromatography (HPLC) with fluorescence detection. Several excellent reagents are currently available for most functional groups, *e.g.*, carboxyl, amino, carbonyl, hydroxyl and thiol groups. However, further research was still needed in order to develop new fluorescent reagents capable of detecting even a trace amount of samples. The improved detection limits of compounds such as biogenic materials obtainable by using derivatization has proved to be one of the major attractions of HPLC in many health and biomedical applications. In the previous papers, we have reported several fluorescent thiol reagents of maleimide type[2)] [*N*-(*p*-(2-benzimidazolyl)phenyl)maleimide (BIPM),[2a)] *N*-(1-anilinonaphthyl-4)maleimide (ANM),[2b)] and *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM)[2b,c)]]. Of these thiol reagents, DACM is widely used in the biological field. The strong fluorescence in a longer wavelength region makes the dialkylaminocoumarin ring one of the candidates of choice for a fluorogenic group. As a part of our continuing search for new fluorescent reagents, we now describe the synthesis of 3-[4-(bromomethyl)phenyl]-7-(diethylamino)-2*H*-1-benzopyran-2-one [**8**: 3-[4-(bromomethyl)phenyl]-7-(diethylamino)coumarin (MPAC-Br)] and the results of fundamental studies on its properties as a new derivatization reagent for HPLC analysis of carboxylic acids.

Results and Discussion

Syntheses and Spectral Properties of 7-Dimethylaminocoumarin Derivatives A series of 7-dimethylaminocoumarin derivatives (**1a**—**d** and **2a**—**d**) was synthesized in order to examine the effect of substituents located at the 3- and 4-position of the coumarin ring on the fluorescence intensity and the wavelength of the fluorescence maximum.

7-Dimethylaminocoumarins (**1a** and **1c**, **d**) were synthesized by the reactions of 2-hydroxy-4-dimethylaminobenzaldehyde[3)] with the corresponding Wittig reagents, and compound **1b** was obtained by the Knoevenagel reaction of the above aldehyde with diethyl malonate. The 4-methylcoumarin compound **2a** was prepared according to the literature method[4)] and its 3-aryl derivatives (**2c** and **2d**) were obtained from **2a** and the corresponding diazonium salts by means of the Meerwein arylation. Compound **2b** was prepared by condensing ethyl cyanoacetate with 2-hydroxy-4-dimethylaminoacetophenone,[5)] followed by hydrolysis, and esterification as described in the experimental section.

The absorption maxima (λ_{max}), the fluorescence maxima ($F\lambda_{max}$), the molar absorptivities, and the fluorescence intensities of **1a**—**d** and **2a**—**d** are listed in Table 1. So far, systematic spectroscopic studies of the compounds synthesized in the present work have afforded the following results.

i) Effect of a 4-Methyl Group: Comparison of the spectra of **1a**—**d** with those of **2a**—**d**, respectively, shows that the 4-methyl group shifts the absorption and the fluorescence maxima to shorter wavelengths, and makes

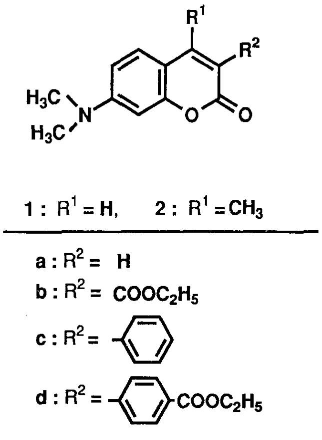

Chart 1

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the absorptivities smaller. But it is difficult to explain the blue shift effect due to the methyl group. As for the fluorescence intensity, the introduction of a methyl group at the 4-position of **1a** and **1b** increases it, while it is diminished in the aryl analogs (**1c** and **1d**).

ii) Effect of a 3-Phenyl Group: Comparison of the spectra of **1a**, **1b**, **2a** and **2b** with those of **1c**, **1d**, **2c** and **2d**, respectively, shows that the introduction of a phenyl group into the 3-position of **1a** and **2a** shifts the absorption maxima to longer wavelengths, but in the cases of **1b** and **2b** there is a shift to shorter wavelengths, that is, a more effective electron-attracting group (in the order of $-COOC_2H_5 > -p-C_6H_4-COOC_2H_5 > -Ph > -H$) at the 3-position tends to shift the absorption maxima to longer wavelengths. As for the fluorescence maxima, the introduction of a 3-phenyl group shifts the fluorescence maxima to longer wavelength in all cases.

iii) Effect of an Ethoxycarbonyl Group: Comparison of the spectra of **1a**, **1c**, **2a** and **2c** with those of **1b**, **1d**, **2b** and **2d**, respectively, shows that the ethoxycarbonyl group shifts both the absorption maxima and the fluorescence maxima to longer wavelengths, and makes the absorptivities larger.

Table 1. Absorption and Fluorescence Properties of 7-Dimethylaminocoumarin Derivatives (**1a**—**d**, **2a**—**d**) in Ethanol

Compd.	Absorption[a]		Fluorescence[b]		
	λ_{max} (nm)	ε	Ex. (nm)	$F\lambda_{max}$ (nm)	RFI[c]
1a	371	23700	371	453	1.00
1b	412	40600	412	461	0.26
1c	391	31400	392	468	1.35
1d	405	38300	405	484	1.69
2a	366	21800	366	447	1.07
2b	384	25900	385	463	0.51
2c	373	26500	373	462	1.04
2d	380	29800	381	470	0.89

a) Concentration: 2.0×10^{-5} M. *b*) Concentration: 6.0×10^{-6} M. *c*) Relative fluorescence intensity: the fluorescence intensity of **1a** is arbitrarily taken as 1.00.

These results show that 3-aryl-7-dimethylaminocoumarins (**1c** and **1d**) without a 4-methyl substituent strongly fluoresce at significantly longer wavelengths and have larger molar absorptivities, indicating that this fluorogenic group is a promising candidate for use in fluorometric detection in combination with HPLC.

Synthesis of MPAC-Br (8) Based on the spectroscopic studies described above, we have designed and synthesized 3-[4-(bromomethyl)phenyl]-7-(diethylamino)coumarin (**8**, MPAC-Br) which has bromomethyl group as a reacting moiety toward carboxylic acids for the use in HPLC. At first, 3-[4-(bromomethyl)phenyl]-7-dimethylaminocoumarin was synthesized as a derivatization reagent. However, we found that its solubility in organic solvents was very poor, so we next synthesized its *N*-diethyl analog (**8**, MPAC-Br) according to the procedures shown in Chart 2. Methyl 4-[7-(diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzoate (**5**) was prepared by condensing methyl 4-carbomethoxyphenyl acetate[6] (**4**) with 4-diethylamino-2-hydroxybenzaldehyde (**3**) obtained by Vilsmeier formylation of *m*-diethylaminophenol, followed by hydrolysis with 6 N hydrochloric acid to give the carboxylic acid (**6**). The resulting **6** was reduced by the method of Yamada *et al.*[7] to yield the corresponding alcohol (**7**), which was brominated with phosphorus tribromide to give the desired MPAC-Br (**8**), yellow prisms, mp 166—167 °C, in 97% yield. The absorption maximum and the fluorescence maximum of **8** in ethanol appeared at 400 nm ($\varepsilon = 33600$) and 474 nm, respectively. MPAC-Br was stable in the crystalline state for at least a year in daylight at room temperature, and can be stored without appreciable deterioration as an acetonitrile solution in a refrigerator for more than a week.

Derivatization Procedure and Spectroscopic Properties of MPAC-Esters (9a—i) In general, potassium salts and

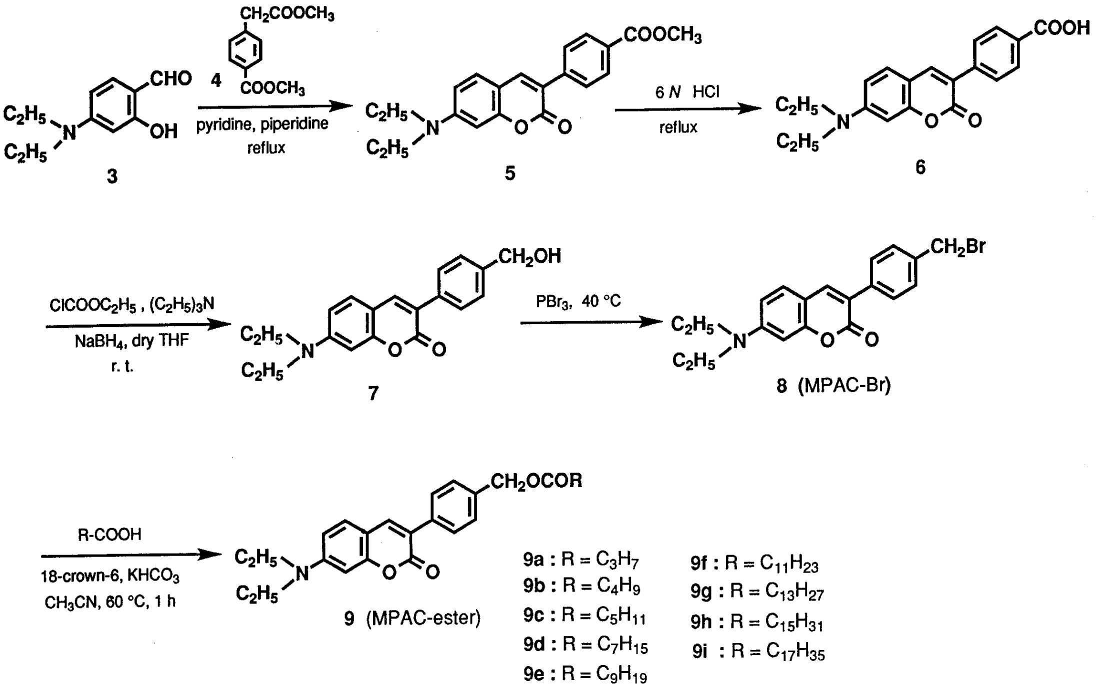


Chart 2

18-crown-6 have been used to facilitate the derivatization (esterification) of fatty acids with reagents having a bromomethyl group.[8] So, we examined their use in the present derivatization system. In order to find the most suitable derivatization conditions, the reaction of lauric acid (C_{12}) with MPAC-Br was examined on a preparative scale by monitoring the disappearance of the MPAC-Br on thin layer chromatography (TLC). The esterification of lauric acid with an equimolar amount of MPAC-Br in acetonitrile (reflux for 1 h) proceeded in 97% yield in the presence of equimolar 18-crown-6 and anhydrous potassium bicarbonate (10 eq). The derivatized products were obtained in good yields in acetonitrile (97%), acetone (95%) or tetrahydrofuran (THF, 96%). Since the reactions proceeded more rapidly in acetonitrile and acetone, acetonitrile was chosen because in acetone a small amount

Table 2. Physical Properties of MPAC-Esters **9a**—**j**

Compd.	Yield (%)	mp (°C)	IR (Nujol) cm^{-1}	MS m/z (M$^+$)	Formula	Analysis (%) Calcd			Found			Absorption[a] ε (401 nm)	RFI[b] (474 nm) (Ex. 403 nm)
						C	H	N	C	H	N		
9a	96	70—71	1740, 1705	393	$C_{24}H_{27}NO_4$	73.26	6.92	3.56	73.28	6.99	3.57	36600	0.96
9b	99	92—93	1740, 1710	407	$C_{25}H_{29}NO_4$	73.68	7.17	3.44	73.60	7.22	3.39	36900	0.98
9c	98	81—82	1720, 1710	421	$C_{26}H_{31}NO_4$	74.08	7.41	3.32	73.92	7.42	3.25	36900	1.00
9d	94	63.5—64.5	1720	449	$C_{28}H_{35}NO_4$	74.80	7.85	3.12	74.81	7.91	3.12	36800	0.99
9e	94	67.5—69	1730, 1700	477	$C_{30}H_{39}NO_4$	75.44	8.23	2.93	75.40	8.29	2.91	37100	1.00
9f	97	58—59.5	1720	505	$C_{32}H_{43}NO_4$	76.00	8.57	2.77	76.09	8.54	2.69	36700	1.00
9g	97	46—47.5	1740, 1710	533	$C_{34}H_{47}NO_4$	76.51	8.88	2.62	76.39	8.89	2.68	37200	1.00
9h	97	64.5—65.5	1750, 1710	561	$C_{36}H_{51}NO_4$	76.96	9.15	2.49	77.02	8.98	2.52	37000	0.99
9i	97	64.5—66	1740, 1705	589	$C_{38}H_{55}NO_4$	77.37	9.40	2.37	77.32	9.37	2.46	36700	0.98
9j	98	91—92	1745, 1700	365	$C_{22}H_{23}NO_4$	72.31	6.34	3.83	72.25	6.42	3.87	36300	0.97

a) Concentration in ethanol: 2.0×10^{-5} M. *b*) Relative fluorescence intensity: the fluorescence intensity of **9f** is arbitrarily taken as 1.00. Concentration in ethanol: 6.0×10^{-6} M.

Table 3. ^{1}H-NMR Spectral Data for MPAC-Esters **9a**—**j**

Compound	^{1}H-NMR (CDCl$_3$, 400 MHz) δ
9a	0.93 (3H, t, $J=7$ Hz, CH_3), 1.22 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.35 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.14 (2H, s, CH_2OCO), 6.54 (1H, s, ArH), 6.60 (1H, d, $J=9$ Hz, ArH), 7.31 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9b	0.92 (3H, t, $J=7$ Hz, CH_3), 1.23 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.3—1.5 (2H, m, C–$CH_2$–C), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.37 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.14 (2H, s, CH_2OCO), 6.54 (1H, s, ArH), 6.60 (1H, d, $J=9$ Hz, ArH), 7.32 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9c	0.89 (3H, t, $J=7$ Hz, CH_3), 1.23 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.2—1.4 (4H, m, C–$(CH_2)_2$–C), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.36 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.14 (2H, s, CH_2OCO), 6.54 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.32 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9d	0.88 (3H, t, $J=7$ Hz, CH_3), 1.23 (6H, t, $J=7$ Hz), $N(CH_2C\underline{H}_3)_2$), 1.2—1.4 (8H, m, C–$(CH_2)_4$–C, 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.36 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.13 (2H, s, CH_2OCO), 6.53 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.31 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9e	0.87 (3H, t, $J=7$ Hz, CH_3), 1.23 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.2—1.4 (12H, m, C–$(CH_2)_6$–C), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.36 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.13 (2H, s, CH_2OCO), 6.54 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.31 (1H, d, $J=9$ Hz, ArH), 7.39 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9f	0.87 (3H, t, $J=7$ Hz, CH_3), 1.23 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.2—1.4 (16H, m, C–$(CH_2)_8$–C), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.36 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.13 (2H, s, CH_2OCO), 6.53 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.31 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9g	0.87 (3H, t, $J=7$ Hz, CH_3), 1.23 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.2—1.4 (20H, m, C–$(CH_2)_{10}$–C), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.36 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.13 (2H, s, CH_2OCO), 6.54 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.32 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9h	0.88 (3H, t, $J=7$ Hz, CH_3), 1.23 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.2—1.4 (24H, m, C–$(CH_2)_{12}$–C), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.36 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.13 (2H, s, CH_2OCO), 6.54 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.31 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9i	0.88 (3H, t, $J=7$ Hz, CH_3), 1.22 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 1.2—1.4 (28H, m, C–$(CH_2)_{14}$–C), 1.6—1.8 (2H, m, $COCH_2C\underline{H}_2$), 2.35 (2H, t, $J=7$ Hz, $COCH_2$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.13 (2H, s, CH_2OCO), 6.53 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.31 (1H, d, $J=9$ Hz, ArH), 7.39 (2H, d, $J=8$ Hz, ArH), 7.69 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)
9j	1.23 (6H, t, $J=7$ Hz, $N(CH_2C\underline{H}_3)_2$), 2.11 (3H, s, $COCH_3$), 3.43 (4H, q, $J=7$ Hz, $N(C\underline{H}_2CH_3)_2$), 5.13 (2H, s, CH_2OCO), 6.53 (1H, d, $J=2$ Hz, ArH), 6.60 (1H, dd, $J=9$, 2 Hz, ArH), 7.31 (1H, d, $J=9$ Hz, ArH), 7.40 (2H, d, $J=8$ Hz, ArH), 7.70 (1H, s, ArH), 7.70 (2H, d, $J=8$ Hz, ArH)

of impurity was detected in TLC. As for the base catalysts, potassium bicarbonate and potassium carbonate gave good results, so weakly basic potassium bicarbonate was selected. The derivatization of a series of saturated (C_4—C_{18}) fatty acids with MPAC-Br was next carried out, giving much the same results as in the case of the test compound (C_{12}) (Chart 2 and Table 2). The structures of MPAC-esters (**9a**—**i**) were determined on the basis of the spectral and analytical data (Table 2 and Table 3). All of the MPAC-derivatives (**9a**—**i**) had absorption maxima and fluorescence maxima at 401 nm and 474 nm, respectively, with much the same values of molar absorptivity and fluorescence intensity in ethanol (Table 2).

Table 4 shows the spectroscopic data for the MPAC-ester of myristic acid (**9g**) in several solvent systems often used in reversed-phase chromatography. Both the absorption maxima and the fluorescence maxima shift slightly toward the blue as the solvent polarity decreases. It is known that the emission maxima of some coumarin compounds shift toward the blue as the solvent polarity decreases, and the results obtained above are in agreement with those reported previously.[2d)] Since the variation of shift value in the spectra of MPAC-ester (**9g**) in the solvents used in this work is small, it is possible to separate and detect various carboxylic acids by using a gradient elution technique with essentially the same detector sensitivity. In addition, these results suggest that aqueous methanol is suitable as a mobile phase in reversed-phase chromatography of MPAC-esters of fatty acids with gradient elution.

Optimal Conditions for HPLC Analysis of Fatty Acids In order to investigate the optimal conditions for HPLC analysis, reaction temperature, the amount ratio of reagent to sample, and the amount of catalyst were varied, using the derivatization procedure described in the experimental section. The derivatization yield of the test compound (lauric acid) with MPAC-Br was estimated by comparing the fluorescence intensity of the product (**9f**) at 474 nm with that of an internal standard **9g** at regular time intervals. The derivatization reaction of lauric acid apparently occurred at moderately low temperatures, though higher temperatures allowed the fluorescence to develop more rapidly. At 60 °C and above, the peak area for lauric acid was almost maximal (97%) after heating for 10 min. Thus, heating at 60 °C was employed in subsequent work. Furthermore, the derivatization of lauric acid was examined with various concentrations of MPAC-Br and 18-crown-6, by monitoring the fluorescent peak of **9f** on the chromatogram. The results indicated that about 10- to 20-fold and 10-fold molar excess of reagent and catalyst, respectively, were suitable (Fig. 1). Consequently, the derivatization of carboxylic acids was carried out under the following conditions: heating at 60 °C for 30 min in acetonitrile with 10-fold molar excess of each of MPAC-Br and 18-crown-6, in the presence of a large excess of potassium bicarbonate.

Table 4. Absorption and Fluorescence Properties of **9g** in Various Solvent Systems

Solvent	Absorption[a)]		Fluorescence[b)]		
	λ_{max} (nm)	ε	Ex. (nm)	$F\lambda_{max}$ (nm)	RFI[c)]
C_2H_5OH	401	37200	403	474	1.00
CH_3OH	402	36900	403	475	0.98
$CH_3OH:H_2O$ (90:10)	406	36700	407	480	1.01
CH_3CN	398	36800	400	473	1.02

a) Concentration: 2.0×10^{-5} M. *b)* Concentration: 6.0×10^{-6} M. *c)* Relative fluorescence intensity: the fluorescence intensity in ethanol is arbitrarily taken as 1.00.

Regarding the calibration curve, a linear relationship between the ratio of the peak areas of lauric acid MPAC-ester (**9f**) to that of the internal standard (**9g**) and the amount of lauric acid was observed in the range of 30 fmol—100 pmol/injection volume (10 μl) of the acid (linear correlation coefficient: 0.999), and the detection limit in this case was 15 fmol/10 μl ($S/N=17$). The reproducibility of this procedure was good, with the coefficient of variation for 500 fmol of lauric acid not exceeding 2.7% ($n=8$).

In order to investigate the usefulness of the present method, the simultaneous determination of saturated fatty

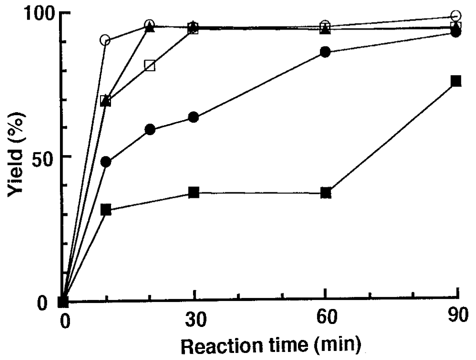


Fig. 1. Fluorescent Derivatization with Various Amount Ratios of MPAC-Br (**8**) to Lauric Acid

Derivatization reaction and detection were carried out according to the procedure as described in the experimental section. [MPAC-Br]/[lauric acid]=—■—, 1; —●—, 2; —□—, 5; —▲—, 10; —○—, 20.

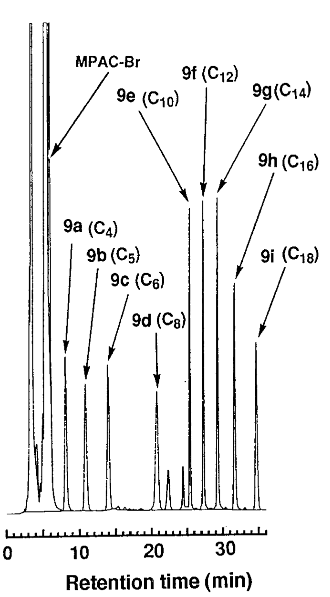


Fig. 2. Chromatogram of MPAC-Esters of Saturated Fatty Acids (**9a**—**i**)

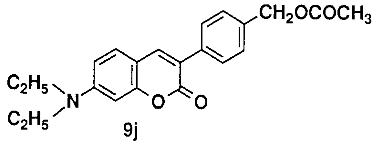
Each peak area corresponds to 2 pmol of fatty acid.

acids (C_4—C_{18}) was examined. As shown in Fig. 2 the peaks of the MPAC-derivatives (**9a**—**i**) of nine fatty acids were clearly separated within 35 min by gradient elution with methanol–water as the mobile phase and were identified by comparison with authentic samples prepared previously.

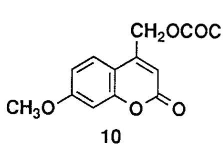
Comparison of the Spectroscopic Properties of MPAC-Ester with that of the 7-Methoxy Analogue and MMC-Ester A number of derivatization reagents have been developed for carboxylic acid analysis by HPLC with fluorescence detection.[8,9] Of these reagents, 4-(bromomethyl)-7-methoxycoumarin (Br-MMC) has been most widely used for the detection of carboxylic acids.[8a] To confirm the applicability of MPAC-Br for carboxylic acid analysis, we compared the spectroscopic properties of acetic acid MPAC-ester (**9j**) with that of MMC-ester[10] (**10**) (Table 5). It was shown that both the absorption maximum and the fluorescence maximum of **9j** appear at about 80 nm longer wavelength than those of **10**. The fluorescence intensity and the molar absorptivity of MPAC-ester (**9j**) were 15 and 2.5 times as strong as those of MMC-ester (**10**), respectively.

It is known that an electron-donating group at the 7-position of the coumarin ring increases the fluorescence intensity.[11] To assess the auxochromic effect of the diethylamino group in the 3-arylcoumarin system, the spectral properties of **9j** and **11** were compared. As shown in Table 5, the fluorescence excitation and the emission maxima of **9j** in ethanol were considerably red-shifted and its fluorescence intensity and molar absorptivity were 1.8 and 1.5 times as strong as those of **11**, respectively. It is considered that the 7-diethylamino group is of importance as the auxochrome in this coumarin system, as described in a previous paper.[12]

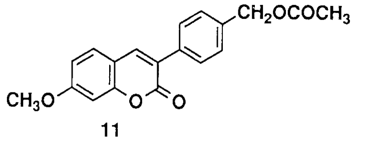
Table 5. Absorption and Fluorescence Properties of MPAC-Ester (**9j**), MMC-Ester (**10**) and 7-Methoxycoumarin Derivative (**11**) in Ethanol



9j



10



11

Compd.	Absorption[a]		Fluorescence[b]		
	λ_{max} (nm)	ε	Ex. (nm)	Fλ_{max} (nm)	RFI[c]
9j	401	36300	403	474	14.7
10	322	14400	323	393	1.0
11	341	24100	342	424	8.0

a) Concentration: 2.0×10^{-5} M. *b*) Concentration: 6.0×10^{-6} M. *c*) Relative fluorescence intensity: the fluorescence intensity of **10** is arbitrarily taken as 1.0.

In comparison with the properties of fluorogenic reagents reported previously,[9a–f] the newly developed MPAC-Br requires a slightly elevated temperature for derivatization in nonaqueous media. However, the reagent is superior in respect of stability, quantitative yield in derivatization, and high sensitivity, allowing detection of carboxylic acids at a femtomol level by HPLC. In addition, MPAC-Br affords esters having much longer wavelengths of absorption maximum (401 nm) in comparison with those of the above-mentioned reagents (259—388 nm).[9] These results suggest that MPAC-Br is suitable as a fluorescence derivatization reagent for the HPLC determination of biologically important carboxylic acids.

Experimental

Apparatus All melting points were determined on a Yamato melting point apparatus (model MP-21) and are uncorrected. Infrared spectra (IR) were recorded on JASCO A-102 and JASCO FT/IR-300 spectrometers. Nuclear magnetic resonance spectra (NMR) were taken on JEOL FX-90Q and JEOL JNM-EX 400 spectrometers. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane (TMS, 0.0 ppm) as an internal standard. The abbreviations used are as follows: br, broad; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. Mass spectra (MS) were determined with JEOL JMS-QH-100 and Shimadzu GC MS-9100-MK gas chromatograph-mass spectrometers with a direct inlet system. Absorption and fluorescence spectra were measured with a Hitachi 288 dual-wavelength spectrophotometer and a Hitachi F-4100 fluorescence spectrophotometer, respectively. The HPLC system consisted of a Hitachi L-6200 pump, a Rheodyne Model 7125 injector valve, a Hitachi F-1040 fluorescence spectrophotometer, a Hitachi D-2500 chromato-integrator and a Gasukuro Kogyo/Model-545 degassing unit. The column was Inertsil ODS-2 (150 × 4.6 mm i.d.; particle size, 5 μm; Gasukuro Kogyo, Tokyo).

7-(Dimethylamino)-2*H*-1-benzopyran-2-one (1a) A mixture of 2-hydroxy-4-dimethylaminobenzaldehyde[3] (540 mg, 3.27 mmol) and carbethoxymethylenetriphenylphosphorane[13] (1.22 g, 3.51 mmol) was heated at 170—180 °C under a nitrogen atmosphere for 1 h. The resultant mixture was purified by column chromatography on silica gel using AcOEt–hexane (1 : 2, v/v) as the eluent to give **1a** (532 mg, 86%). Yellow needles (from AcOEt), mp 162—163 °C (lit.[14] mp 161—161.5 °C).

Ethyl 7-(Dimethylamino)-2-oxo-2*H*-benzopyran-3-carboxylate (1b) The title compound was prepared from 2-hydroxy-4-dimethylaminobenzaldehyde,[3] ethyl malonate, and piperidine in pyridine according to the reported method.[15] Yellow needles (from EtOH), mp 169—170 °C (lit.[16] mp 170 °C).

7-(Dimethylamino)-3-phenyl-2*H*-1-benzopyran-2-one (1c) A mixture of the 2-hydroxy-4-dimethylaminobenzaldehyde[3] (165 mg, 1.0 mmol) and carbethoxybenzylidenetriphenylphosphorane[17] (551 mg, 1.3 mmol) in mesitylene (15 ml) was refluxed for 39 h. The reaction mixture was directly subjected to column chromatography on silica gel using AcOEt–hexane (1 : 2, v/v) as the eluent to give **1c** (119 mg, 45%). Yellow prisms (from AcOEt), mp 219—220 °C. IR (Nujol): 1690 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 90 MHz) δ: 3.04 (6H, s, $N(CH_3)_2$), 6.5—6.7 (2H, m, ArH), 7.3—7.5 (4H, m, ArH), 7.6—7.8 (3H, m, ArH). MS *m*/*z*: 265 (M^+). *Anal.* Calcd for $C_{17}H_{15}NO_2$: C, 76.96; H, 5.70; N, 5.28. Found: C, 77.02; H, 5.88; N, 5.39.

Ethyl 4-[(7-Dimethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzoate (1d) i) A solution of ethyl 4-chloromethylbenzoate (11.6 g, 58 mmol) and triphenylphosphine (23.0 g, 88 mmol) in xylene (75 ml) was refluxed for 15 h, and the precipitated 4-carbethoxybenzyltriphenyl phosphonium chloride was collected and washed with benzene and ether. Yield 21.1 g (79%), mp 240—241.5 °C. Next, a suspension of the resulting phosphonium chloride (230 mg, 0.5 mmol) and KH (40 mg, 1.0 mmol) in toluene (8 ml) was refluxed for 1 h. Then ethyl chloroformate (0.1 ml, 75.0 mmol) was added dropwise at room temperature until the red color of the solution disappeared. The precipitate was filtered off and the mother liquor was evaporated to dryness. The residue was chromatographed on silica gel using hexane–AcOEt (2 : 1, v/v) as the eluent to give carbethoxy-4-carbethoxybenzylidenetriphenylphosphorane (142 mg, 57%). Colorless needles (from AcOEt–hexane), mp 176—177 °C. IR (Nujol): 1700 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 90 MHz) δ: 0.81 (3H, t, J=7 Hz, CH$_2$C$\underline{H}_3$), 1.32 (3H, t, J=7 Hz, CH$_2$C$\underline{H}_3$), 3.88 (2H, q, J=7 Hz, C$\underline{H}_2$CH$_3$), 4.27 (2H, q, J=7 Hz, C$\underline{H}_2$CH$_3$), 7.0—7.7 (19H, m, ArH). MS m/z: 496 (M$^+$). *Anal.* Calcd for $C_{31}H_{29}O_4P$: C,74.99; H, 5.89; P, 6.24. Found: C, 74.87; H, 5.92; P, 6.24. This compound was also obtained in almost the same yield by stirring at room temperature in the presence of $[(CH_3)_3Si]_2NLi$ in place of KH as a base.

ii) A mixture of 2-hydroxy-4-dimethylaminobenzaldehyde,[3] (825 mg, 5.0 mmol) and carbethoxy-4-carbethoxybenzylidenetriphenylphosphorane (2.92 g, 5.9 mmol) was heated at 220 °C under a nitrogen atmosphere for 1 h. The resultant mixture was purified by column chromatography on silica gel using AcOEt–hexane (1 : 1, v/v) as the eluent to give **1d** (900 mg, 53%). Yellow needles (from CHCl$_3$–hexane), mp 204—205 °C. IR (Nujol): 1690 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 90 MHz) δ: 1.41 (3H, t, J=7 Hz, CH$_2$C$\underline{H}_3$), 3.09 (6H, s, N(CH$_3$)$_2$), 6.5—6.7 (2H, m, ArH), 7.36 (1H, d, J=8 Hz, ArH), 7.78 (2H, d, J=8 Hz, ArH), 8.08 (2H, d, J=8 Hz, ArH). MS m/z: 337 (M$^+$). *Anal.* Calcd for $C_{20}H_{19}NO_4$: C, 71.20; H, 5.68; N, 4.15. Found: C, 71.24; H, 5.87; N, 4.37.

7-(Dimethylamino)-4-methyl-2*H*-1-benzopyran-2-one (2a) This compound was prepared from *m*-dimethylaminophenol and ethyl acetate according to the reported method.[4]

Ethyl 7-(Dimethylamino)-4-methyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2b) i) A mixture of 2-hydroxy-4-dimethylaminoacetophenone[5] (1.08 g, 6 mmol), ethyl cyanoacetate (1 ml, 9 mmol) and sodium ethoxide (54 mg, 0.8 mmol) in ethanol was refluxed for 4 h. After removal of the solvent, the residue was chromatographed on silica gel using CHCl$_3$ as the eluent to give 3-cyano-7-(dimethylamino)-4-methyl-2*H*-1-benzopyran-2-one (207 mg, 15%). Yellow needles (from AcOEt), mp 251—252 °C. IR (Nujol): 2225, 1710 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 90 MHz) δ: 2.63 (3H, s, CH$_3$), 3.13 (6H, s, N(CH$_3$)$_2$), 6.45 (1H, d, J=2 Hz, ArH), 6.67 (1H, dd, J=9, 2 Hz, ArH), 7.48 (1H, d, J=9 Hz, ArH). MS m/z: 228 (M$^+$). *Anal.* Calcd for $C_{13}H_{12}N_2O_2$: C, 68.41; H, 5.30; N, 12.27. Found: C, 68.31; H, 5.38; N, 12.44.

ii) A mixture of the cyano compound obtained above (207 mg, 0.9 mmol), 6% sodium hydroxide solution (9 ml) and pyridine (7 mg) was heated at 40 °C for 7 h. The resulting yellow solution was acidified with 2 N hydrochloric acid. The precipitated product was collected by filtration and recrystallized from ethanol to give 7-(dimethylamino)-4-methyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (159 mg, 72%). Reddish-yellow needles (from AcOEt–hexane), mp > 300 °C (dec.). IR (Nujol): 1725 cm^{-1}. MS m/z: 247 (M$^+$).

iii) *N,N'*-Dicyclohexylcarbodiimide (418 mg, 2.00 mmol), ethanol (0.1 ml, 2.00 mmol) and 4-dimethylaminopyridine (10 mg, 0.08 mmol) were added to a suspension of the resulting carboxylic acid (100 mg, 0.40 mmol) in THF (12 ml), and the reaction mixture was stirred at room temperature for 5 h. The precipitate was filtered off and the mother liquor was evaporated to dryness. The residue was chromatographed on silica gel using CHCl$_3$–hexane–acetone (10 : 8 : 1, v/v) as the eluent to give **2b** (5 mg, 5%). Reddish-yellow needles, mp 129—130 °C. IR (Nujol): 1700 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 90 MHz) δ: 1.39 (3H, t, J=7 Hz, OCH$_2$C$\underline{H}_3$), 2.41 (3H, s, CH$_3$), 3.06 (6H, s, N(CH$_3$)$_2$), 4.40 (2H, q, J=7 Hz, OC$\underline{H}_2$CH$_3$), 6.45 (1H, d, J=3 Hz, ArH), 6.62 (1H, dd, J=9, 3 Hz, ArH), 7.46 (1H, d, J=9 Hz, ArH). MS m/z: 275 (M$^+$). *Anal.* Calcd for $C_{15}H_{17}NO_4$: C, 65.44; H, 6.22; N, 5.09. Found: C, 65.49; H, 6.35; N, 5.09.

7-(Dimethylamino)-4-methyl-3-phenyl-2*H*-1-benzopyran-2-one (2c) The title compound was prepared from 7-dimethylamino-4-methyl-2*H*-1-benzopyran-2-one (**2a**) and benzenediazonium chloride according to the reported method.[18] Yellow needles (from EtOH), mp 169—170 °C (lit.[18] mp 126 °C). IR (Nujol): 1705 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 90 MHz) δ: 2.24 (3H, s, CH$_3$), 3.07 (6H, s, N(CH$_3$)$_2$), 6.5—6.7 (2H, m, ArH), 7.2—7.6 (6H, m, ArH). MS m/z: 279 (M$^+$). *Anal.* Calcd for $C_{18}H_{17}NO_2$: C, 77.39; H, 6.13; N, 5.01. Found: C, 77.36; H, 6.23; N, 4.98.

Ethyl 4-[7-(Dimethylamino)-4-methyl-2-oxo-2*H*-1-benzopyran-3-yl]benzoate (2d) Compound **2d** was obtained from 4-carbethoxybenzenediazonium chloride in place of benzenediazonium chloride by the same method as used for the preparation of **2c**. The product **2d** was purified by column chromatography (benzene—AcOEt, 20 : 1, v/v). Yield, 22%. Reddish-yellow needles (from AcOEt), mp 153—154 °C. IR (Nujol): 1700 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 90 MHz) δ: 1.40 (3H, t, J=7 Hz, OCH$_2$C$\underline{H}_3$), 2.24 (3H, s, CH$_3$), 3.07 (6H, s, N(CH$_3$)$_2$), 4.40 (2H, q, J=7 Hz, OC$\underline{H}_2$CH$_3$), 6.5—6.8 (2H, m, ArH), 7.39 (2H, d, J=9 Hz, ArH), 7.53 (1H, s, ArH), 8.11 (2H, d, J=9 Hz, ArH). MS m/z: 351 (M$^+$). *Anal.* Calcd for $C_{21}H_{21}NO_4$: C, 71.78; H, 6.02; N, 3.99. Found: C, 71.70; H, 6.05; N, 4.14.

Methyl 4-[7-(Diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzoate (5) 4-(Diethylamino)-2-hydroxybenzaldehyde (**3**) was prepared by the same method as used for the preparation of its 4-dimethylamino analogue,[3] but with *m*-diethylaminophenol in place of *m*-dimethylaminophenol. A mixture of **3** (8.69 g, 45 mmol), methyl 4-carbomethoxyphenyl acetate (**4**,[6] 15.6 g, 75 mmol) and piperidine (2 ml) in pyridine (68 ml) was heated at 100 °C for 15 h. The pyridine and piperidine were evaporated off, and the residue was recrystallized from AcOEt–hexane to give **5** (6.57 g). An additional crop (5.44 g) was obtained from the mother liquor by purification with silica gel column chromatography (CHCl$_3$–hexane–acetone, 20 : 18 : 1, v/v). The total yield of **5** was 76%. Reddish-yellow prisms, mp 179—180.5 °C. IR (Nujol): 1700 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 400 MHz) δ: 1.23 (6H, t, J=7 Hz, N(CH$_2$C$\underline{H}_3$)$_2$), 3.44 (4H, q, J=7 Hz, N(C$\underline{H}_2$CH$_3$)$_2$), 3.93 (3H, s, OCH$_3$), 6.54 (1H, d, J=2 Hz, ArH), 6.61 (1H, dd, J=9, 2 Hz, ArH), 7.34 (1H, d, J=9 Hz, ArH), 7.78 (1H, s, ArH), 7.80 (2H, d, J=8 Hz, ArH), 8.07 (2H, d, J=8 Hz, ArH). MS m/z: 351 (M$^+$). *Anal.* Calcd for $C_{21}H_{21}NO_4$: C,71.78; H, 6.02; N, 3.99. Found: C, 71.65; H, 6.12; N, 4.06.

4-[7-(Diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzoic Acid (6) A suspension of **5** (7.1 g, 20 mmol) in 6 N HCl aqueous solution (240 ml) was refluxed for 19 h. Most of the solvent was evaporated *in vacuo*, and the residue was neutralized with saturated NaHCO$_3$ aqueous solution. The resulting precipitates were collected by filtration and recrystallized from acetone to give **6** (6.0 g, 89%). Reddish-yellow prisms, mp 282—283.5 °C. IR (Nujol): 1700, 1680 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.14 (6H, t, J=7 Hz, N(CH$_2$C$\underline{H}_3$)$_2$), 3.2—3.5 (1H, br s, OH), 3.45 (4H, q, J=7 Hz, N(C$\underline{H}_2$CH$_3$)$_2$), 6.56 (1H, d, J=2 Hz, ArH), 6.74 (1H, dd, J=9, 2 Hz, ArH), 7.52 (1H, d, J=9 Hz, ArH), 7.86 (2H, d, J=8 Hz, ArH), 7.98 (2H, d, J=8 Hz, ArH), 8.19 (1H, s, ArH). MS m/z: 337 (M$^+$). *Anal.* Calcd for $C_{20}H_{19}NO_4$: C, 71.20; H, 5.68; N, 4.15. Found: C, 71.39; H, 5.66; N, 4.20.

7-(Diethylamino)-3-[4-(hydroxymethyl)phenyl]-2*H*-1-benzopyran-2-one (7) Ethyl chloroformate (2.2 ml, 23 mmol) and triethylamine (3.2 ml, 23 mmol) were added to a solution of carboxylic acid (**6**, 1.69 g, 5 mmol) in THF (200 ml) at room temperature, and the whole was stirred for 1 h at the same temperature. Next, a solution of NaBH$_4$ (2.27 g, 60 mmol) in H$_2$O (4.8 ml) was added to the above solution during 30 min, and the mixture was stirred for an additional 1 h. After acidification of the reaction mixture with acetic acid, the THF was evaporated *in vacuo* and the residue was treated with CHCl$_3$ and H$_2$O. The organic layer was washed with H$_2$O, dried over MgSO$_4$, and evaporated to dryness. The residue was chromatographed on silica gel using CHCl$_3$ as the eluent to give **7** (1.32 g, 81%). Yellow needles (from AcOEt–hexane), mp 153—154°C. IR (Nujol): 3425, 1680 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 400 MHz) δ: 1.23 (6H, t, J=7 Hz, N(CH$_2$C$\underline{H}_3$)$_2$), 1.6—1.8 (1H, br s, OH), 3.43 (4H, q, J=7 Hz, N(C$\underline{H}_2$CH$_3$)$_2$), 4.73 (2H, s, C$\underline{H}_2$OH), 6.54 (1H, d, J=2 Hz, ArH), 6.60 (1H, dd, J=9, 2 Hz, ArH), 7.32 (1H, d, J=9 Hz, ArH), 7.41 (2H, d, J=8 Hz, ArH), 7.70 (2H, d, J=8 Hz, ArH), 7.70 (1H, s, ArH). MS m/z: 323 (M$^+$). *Anal.* Calcd for $C_{20}H_{21}NO_3$: C, 74.28; H, 6.55; N, 4.33. Found: C, 74.40; H, 6.56; N, 4.34.

3-[4-(Bromomethyl)phenyl]-7-(diethylamino)-2*H*-1-benzopyran-2-one (8, MPAC-Br) A mixture of the alcohol (**7**, 3.23 g, 10 mmol) and PBr$_3$ (86 ml, 900 mmol) was stirred at 40—50 °C for 3 d. The reaction mixture was poured into ice-water, and the resulting precipitates were collected by filtration. The precipitates were dissolved in CHCl$_3$ and the organic solution was washed with saturated aqueous NaHCO$_3$, and H$_2$O, dried over MgSO$_4$, and evaporated. The residue was recrystallized from AcOEt–hexane to give MPAC-Br (**8**, 3.73 g, 97%). Yellow needles, mp 166—167 °C. IR (Nujol): 1685 cm^{-1}. ^{1}H-NMR (CDCl$_3$, 400 MHz) δ: 1.22 (6H, t, J=7 Hz, N(CH$_2$C$\underline{H}_3$)$_2$), 3.43 (4H, q, J=7 Hz, N(C$\underline{H}_2$CH$_3$)$_2$), 4.53 (2H, s, –CH$_2$Br), 6.53 (1H, d, J=2 Hz, ArH), 6.60 (1H, dd, J=9, 2 Hz, ArH), 7.32 (1H, d, J=9 Hz, ArH), 7.43 (2H, d, J=8 Hz, ArH), 7.69 (2H, d, J=8 Hz, ArH), 7.71 (1H, s, ArH). MS m/z: 385, 387 (M$^+$). *Anal.* Calcd for $C_{20}H_{20}NO_2Br$: C, 62.19; H, 5.22; N, 3.63; Br, 20.68. Found: C, 62.27; H, 5.27; N, 3.61; Br, 20.46.

Syntheses of MPAC-Esters (9a—i): General Procedure A mixture of MPAC-Br (**8**, 116 mg, 0.3 mmol), a carboxylic acid (C_2—C_{18}) (0.3 mmol), 18-crown-6 (79 mg, 0.3 mmol) and KHCO$_3$ (300 mg, 3.0 mmol) in acetonitrile (30 ml) was refluxed for 1 h. The solvent was evaporated *in vacuo* and the residue was chromatographed on silica gel using AcOEt–hexane (1 : 6, v/v) as the eluent to give the corresponding MPAC-ester (**9a—i**). Yields, physical properties and spectral data for

MPAC-esters are listed in Tables 2 and 3.

Derivatization Procedure and HPLC Conditions Stock solutions of carboxylic acid (0.1 mM), MPAC-Br (**8**, 1.0 mM), 18-crown-6 (1.0 mM) and internal standard (0.1 mM) were prepared in acetonitrile. A mixture of the four stock solutions (100 μl each) and $KHCO_3$ (10 mg) in a reaction vial was heated at 60 °C for 30 min and diluted with MeOH to 5 ml, and then an aliquot (10 μl) of the mixture was injected into the liquid chromatograph. The eluent from the column was monitored with a fluorophotometric detector at an excitation wavelength of 403 nm and an emission wavelength of 474 nm. The eluent flow-rate was 1.0 ml/min.

Calibration Curve for Formation of 9f Stock solutions of lauric acid (0.05 μM—0.5 mM), MPAC-Br (**8**, 1.0 mM), 18-crown-6 (1.0 mM) and internal standard (0.25 μM—0.25 mM) were prepared in acetonitrile. The derivatization reaction and detection were carried out according to the standard procedure described above.

Simultaneous Separation of Saturated Fatty Acids Stock solutions of nine carboxylic acids (C_4—C_{18}) (0.01 mM each), MPAC-Br (**8**, 0.80 mM) and 18-crown-6 (0.80 mM) were prepared in acetonitrile. The derivatization reaction and detection were carried out according to the standard procedure described above. Simultaneous separation was attained by gradient elution with MeOH–H_2O (MeOH concentration in the mobile phase: 90%, 0—5 min; 90—95%, 5—10 min; 95%, 10—15 min; 95—100%, 15—20 min).

Synthesis of 4-(7-Methoxy-2-oxo-2*H*-1-benzopyran-3-yl)phenylmethyl Acetate (11) i) A mixture of the 2-hydroxy-4-methoxybenzaldehyde (4.26 g, 28 mmol), methyl 4-carbomethoxyphenyl acetate[6] (7.76 g, 37 mmol) and piperidine (1 ml) in pyridine (43 ml) was heated at 100 °C for 18 h. The pyridine and piperidine were evaporated off, and the residue was treated with $CHCl_3$ and H_2O. The organic layer was washed with 1 N HCl and H_2O, dried over $MgSO_4$, and evaporated to dryness. The resultant mixture was purified by column chromatography on silica gel using AcOEt–hexane (1 : 2, v/v) as the eluent to give methyl 4-(7-methoxy-2-oxo-2*H*-1-benzopyran-3-yl)benzoate (**12**, 3.71 g, 43%). Pale yellow needles (from AcOEt), mp 229—230 °C (lit.[19] mp 233—234 °C). IR (Nujol): 1720, 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 3.90 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 6.8—6.9 (2H, m, ArH), 7.46 (1H, d, $J=8$ Hz, ArH), 7.79 (2H, d, $J=8$ Hz, ArH), 7.84 (1H, s, ArH), 8.10 (2H, d, $J=8$ Hz, ArH). MS *m/z*: 310 (M^+). *Anal.* Calcd for $C_{18}H_{14}O_5$: C, 69.67; H, 4.55. Found: C, 69.17; H, 4.58.

ii) A suspension of the ester (**12**, 1.67 g, 5.4 mmol) in 7.5% KOH EtOH–H_2O solution (4 : 1, v/v, 36 ml) was slightly warmed to aid dissolution. The mixture was stirred at room temperature for 30 min, then acidified with 10% HCl. The resulting precipitates were collected by filtration and recrystallized from *N*,*N*′-dimethylformamide to give 4-(7-methoxy-2-oxo-2*H*-1-benzopyran-3-yl)benzoic acid (**13**, 1.34 g, 84%). Pale yellow needles, mp > 300 °C. IR (Nujol): 1720, 1685 cm^{-1}. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 3.89 (3H, s, OCH_3), 7.00 (1H, dd, $J=9$, 2 Hz, ArH), 7.05 (1H, d, $J=2$ Hz, ArH), 7.72 (1H, d, $J=9$ Hz, ArH), 7.86 (2H, d, $J=8$ Hz, ArH), 8.01 (2H, d, $J=8$ Hz, ArH). MS *m/z*: 296 (M^+).

iii) Ethyl chloroformate (2.6 ml, 27 mmol) and triethylamine (3.6 ml, 26 mmol) were added to a solution of the carboxylic acid (**13**, 2.31 g, 7.8 mmol) in THF (300 ml) at room temperature, and the whole was stirred for 1 h at the same temperature. Next, a solution of $NaBH_4$ (1.98 g, 52 mmol) in H_2O (5.0 ml) was added to the above solution during 30 min, and the mixture was stirred for an additional 1 h. After acidification of the reaction mixture with acetic acid, the THF was evaporated and the residue was treated with $CHCl_3$ and H_2O. The organic layer was washed with H_2O, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed on silica gel using $CHCl_3$–MeOH (20 : 1, v/v) as the eluent to give 3-[4-(hydroxymethyl)phenyl]-7-methoxy-2*H*-1-benzopyran-2-one (**14**, 1.21 g, 55%). Pale yellow needles, mp 130—132 °C. (from AcOEt–hexane). IR (Nujol): 3240, 1715 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.6—1.8 (1H, br s, OH), 3.89 (3H, s, OCH_3), 4.75 (2H, s, $C\underline{H}_2OH$), 6.8—6.9 (2H, m, ArH), 7.44 (2H, d, $J=8$ Hz, ArH), 7.70 (2H, d, $J=8$ Hz, ArH) 7.77 (1H, s, ArH). MS *m/z*: 282 (M^+).

iv) A mixture of the alcohol (**14**, 282 mg, 1.0 mmol), sodium acetate (164 mg, 2.0 mmol) and acetic anhydride (2.0 ml, 21 mmol) was refluxed for 30 min. After cooling to room temperature, the reaction mixture was poured into ice-water and neutralized with K_2CO_3. The aqueous solution was treated with $CHCl_3$ and the organic layer was washed with H_2O, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was recrystallized from AcOEt–hexane to afford **11** (300 mg, 93%). Pale yellow needles, mp 137—139 °C. IR (Nujol): 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 2.21 (3H, s, $COCH_3$), 3.88 (3H, s, OCH_3), 5.26 (2H, s, CH_2O), 6.34 (2H, s, ArH), 6.8—7.0 (4H, m, ArH), 7.41 (2H, d, $J=8$ Hz, ArH). MS *m/z*: 324 (M^+). *Anal.* Calcd for $C_{19}H_{16}O_5$: C, 70.36; H, 4.98. Found: C, 69.99; H, 5.24.

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