Synthesis and Evaluation of Novel Crowned Coumarins as Self-Catalytic Fluorescence Reagents

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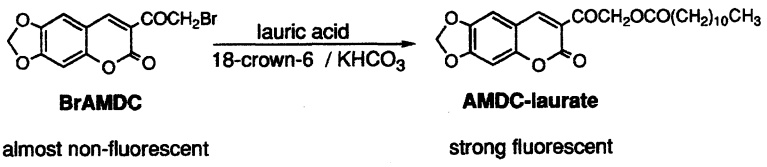
Three coumarin reagents carrying crown-ether moieties as catalytic sites for the fluorescence derivatization of carboxylic acids were designed and synthesized. The catalytic abilities of these reagents were evaluated based on the stability constants (K_s) for complexation with metal acetates in methanol. The derivatization reactions of carboxylic acids with these reagents proceeded self-catalytically without crown-ether catalysts, and gave the corresponding coumarin esters in good yields. It was found that their reactivities significantly depended upon the metal-binding ability of the reagent molecules from kinetic treatments of the reactions. The derivatized products showed remarkably high fluorescence quantum yields of above 0.8 in methanol. These results suggested that the functionalization of reagents was a quite useful approach for the development of new-type analytical reagents.

Fluorescence reagents have been widely used as molecular tools for the microanalysis of biological materials or the elucidation of physiological function. In the development of such reagents, both high sensitivity and reactivity should be considered first as factors required for excellent reagents. The sensitivity could be enhanced to some extent by a reagent design based upon detailed information concerning primary fluorophores, which includes an understanding of their emission mechanism. We have already studied the fluorescence characteristics of a series of methoxycoumarin fluorophores, and then found that the structural features of coumarins for intense fluorescence establish diether bonds at the 6- and 7-positions and an electron-withdrawing group at the 3-position on the coumarin ring, as shown in 3-acetyl-6,7-dimethoxycoumarin with a quantum yield of 0.52.[1)]

Now, the reactivity of the reagents is considered to be equally significant for a non-destructive analysis of bioactive samples and a simplification of the handling in such derivatization reactions. A number of approaches for this purpose were predominantly appropriate conversions of the reacting groups on fluorophores.[2)] Recently, studies concerning functional molecules have been successfully undertaken for the construction of biofunctional substances in the field of "Supramolecule Chemistry."[3)] We have planned to introduce the concept of "functionalization" into the reagent design in order to develop a new-type reagent with a certain function, such as a self-catalytic ability, which may increase its reactivity. In this paper we describe the design, synthesis and reactivity of some coumarin reagents carrying additional crown-ether moieties, which may be useful for derivatization reactions of carboxylic acids (ester-forming reaction).

Results and Discussion

Reagent Design. We first note the fluorescence derivatization reactions of carboxylic acids with 3-bromoacetyl-6,7-methylenedioxycoumarin (**BrAMDC**) in the presence of 18-crown-6 ether and $KHCO_3$, because of the strong fluorescence of the derivatized products,[4)] as can be seen in Scheme 1. The reagent, **BrAMDC**, was synthesized, of course, under consideration of the structural requirements for intense fluorescence. Although the reagent, itself, was shown to be almost non-



Scheme 1.

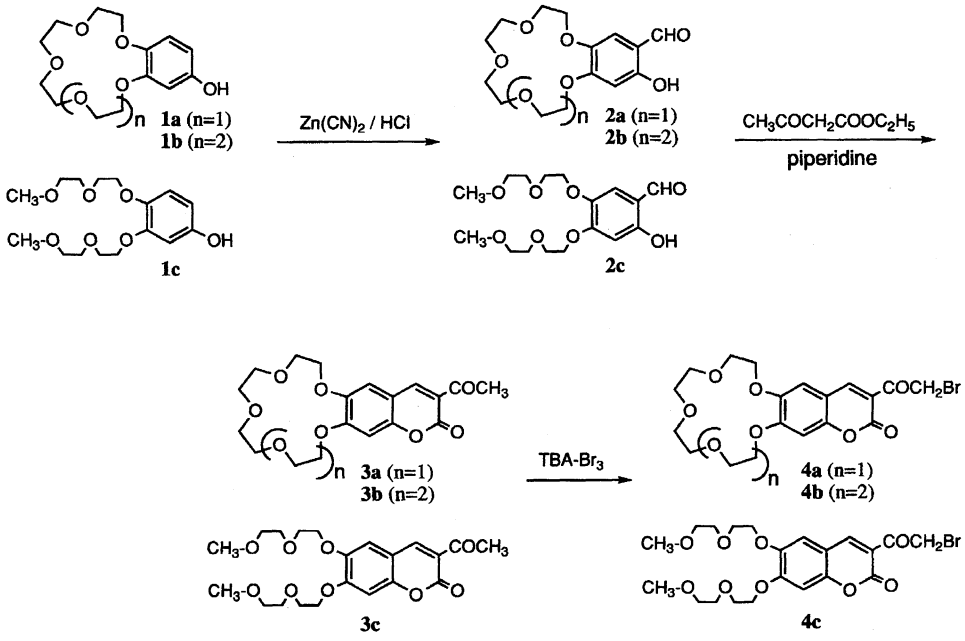
fluorescence, the derivatized products were strongly fluorescent, suggesting that their fluorescence may be controlled by both the heavy-atom effect[5] of the bromine atom in **BrAMDC** and the electronic effect, as speculated based on Hammett's substituent constants of the bromine and ester groups.[6]

Thus, the reagent (**4a**) carrying coumarin fluorophore along with the benzo-15-crown-5 (B15Cr5) moiety, instead of using a crown catalyst while keeping such strong fluorescence, was designed and synthesized as an attempt to obtain functionalized reagents.[7] The derivatization reaction of lauric acid with **4a** was smoothly proceeded without a crown catalyst, as expected. The ester of the lauric acid derived from **4a**, which satisfied the fluorescence requirements, also gave high fluorescence quantum yields of 0.8 or above.[4,7]

In addition, the reagent (**4b**) carrying the benzo-18-crown-6 moiety (B18Cr6), which generally has a stronger binding ability for K^+ than that of B15Cr5,[8] was synthesized in expectation of a higher reactivity, together with an acyclic polyether analog (**4c**) for a comparison. The high hydrophilicity of crown-ether sites in these reagents may also serve to increase the solubility in polar solvents, such as methanol and acetonitrile, which are frequently used for high-performance liquid chromatography (HPLC) or other analyses.

Synthesis of Reagents. In general, 3-substituted coumarins have been conveniently synthesized by the Knovenagel condensation of salicylaldehydes with active methylene compounds, such as malonic esters and β-keto esters, both with and without a basic catalyst.[9] Reagents **4a**—**c** were synthesized from **1a**—**c** obtained by using the method of Wada et al.[10] through three steps, as shown in Scheme 2. Namely, the Gattermann formylation[11] of **1a**—**c** in dry ether gave **2a**—**c** as intermediate compounds, which were used for the next step without further purification. Compounds **2a**—**c** were subsequently condensed with ethyl acetoacetate in the presence of piperidine in absolute ethanol to give 3-acetyl coumarins **3a**—**c** in good yields. Finally, the bromination[12] of **3a**—**c** with tetrabutylammonium tribromide (TBA-Br_3) in dichloromethane gave the desired **4a**—**c**. The structures of **4a**—**c** were confirmed by ^{1}H NMR, MS spectral and elemental analysis data, which were consistent with the expected structures.

Catalytic Ability of Crowned Coumarins. Although a number of determination methods concerning the complexation ability of crown ethers with metal ions have been reported,[8] we estimated their ability spectrophotometrically using **3a**—**c**, instead of reagents **4a**—**c**, because the latter have a highly reactive bromoacetyl group. Metal acetates were also used for measurements by considering the solubility in organic solvents and the derivatization reaction for carboxylic acids. The complexation ability, that is, catalytic ability of crown sites in the reagent molecules, was at first investigated from absorption measurements of **3a**. Although decreases in the absorbance were observed along with a blue shift of the absorption maximum at around 370 nm upon adding metal acetates to methanolic solution of **3a**, its degree was too slight to estimate the catalytic ability. On the other hand, the fluorescence responses for **3a** showed a great decrease in the fluorescence intensity, along with a blue shift of the fluorescence maximum, as shown in a previous paper.[7] The same behaviors as that of **3a** were observed for **3b** and **3c**, indicating the complexation of the crown sites of these compounds with metal ions. Table 2 shows



Scheme 2.

that the stability constants (K_s) for complexation with Na^+ and K^+ revealed remarkable responses compared with other metal ions. The stability constants of **3b** for both ions were 3.24 and 4.21, respectively, which were definitely larger than those of **3a**. In contrast, smaller values were obtained for **3c** because of a weak complexation of the flexible acyclic polyether site with metal ions. Although the stability constants of **3a—c** were slightly small compared with the values of analogous B15Cr5 and B18Cr6, these compounds were found to have the ability to bind metal ions into the crown cavities.[8] These results, therefore, suggest that **4a—c** should also have a metal-binding ability, and, hence, show a good self-catalytic function in derivatization reactions for carboxylic acids. Reagent **4b** was particularly expected to increase its reactivity.

Reactivity of Reagents. The reactivity of **4a—c** was actually examined by derivatization reactions with some kinds of carboxylic acids (acetic, benzoic, and lauric acids), as shown in Scheme 3 to elucidate the expectation from the metal-binding ability of **3a—c**. The progress of the reaction was monitored by an HPLC equipped with a fluorescence detector (E_X 392 nm, E_m 485 nm); $NaHCO_3$ and $KHCO_3$ were used as catalysts for each reaction. The derivatization of carboxylic acids with **4a—c** automatically proceeded at room temperature only upon the addition of bases in the absence of crown-ethers. The reaction rates of derivatization with **4a** and **4b** were greater than those with **BrAMDC** in the presence of the corresponding benzocrown ethers, as described for **4a** in a previous communication.[7] The differences in the reactivity between **4a—c** and **BrAMDC** were primarily considered to depend upon the metal-binding ability, as shown by the K_s values (Table 1). Further, serious differences in the reactivity were observed between two bases; that is, the reactions were definitely accelarated by addition of $KHCO_3$, but not $NaHCO_3$. Thus, the complexation mode may also affect the reactivity, because **4a** forms a 2:1 complex with K^+ and a 1:1 complex with Na^+ as well as B15Cr5, so that the access of two **4a** molecules favors a reaction with carboxylate ions.[8] Therefore, the reaction of **4a—c** with lauric acid in the presence of K^+ was examined in detail. Table 2 shows the relative rate constants for the reactions of lauric acid obtained under the conditions of pseudo-first-order kinetics. The effects of accelaration in the reactivities of **4a** and **4b** were about 1.6- and 3.9-times that of **BrAMDC** upon adding B15Cr5, respectively. On the other hand, the stability constant of **3c** for K^+ was smaller than that of B15Cr5, as shown in Table 1, but the k_{rel} of **4c** in the derivatization of lauric acid was comparable with that of the **BrAMDC**–B15Cr5 reaction system. The reactivity between **4a—c** was increased with the magnitude of the K_s values for K^+, indicating that the rate-limiting step of the reaction of carboxylic acids with **4a—c** is at the complexation with metal ions. The limiting data suggest the superiority of combined reagents with a catalytic site in a molecule and the great utility of **4b** as a derivatization reagents for carboxylic acids.

Table 1. Stability Constants (K_s) of **3a—c** with Alkaline Metals

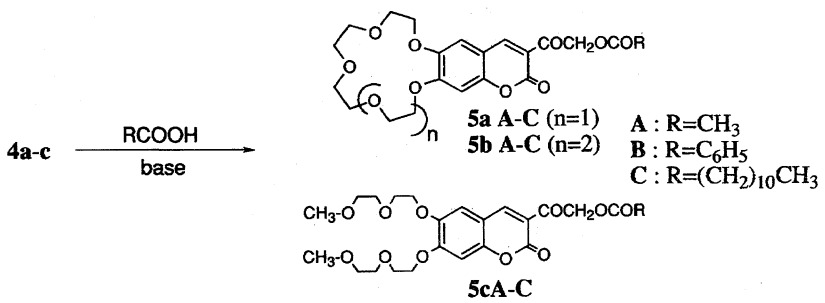
Compound	log K_s	
	Na^+	K^+
3a	1.98	2.24
3b	3.24	4.21
3c	0.69	1.54
B15Cr5	3.05	2.8 (3.15)[a]
B18Cr6	4.50	5.20

a) Value for the 2:1 complex.

Table 2. Relative Rate Constants for **4a—c**

Compound	k_{rel}
4a	163
4b	392
4c	85
BrAMDC (+B15Cr5)	100
BrAMDC (+B18Cr6)	124

Fluorescence Characteristics of Derivatized Products. Since the derivatized products obtained by using **4a—c** showed a marked spectral resemblance to each other, the spectral behaviors were discussed by exemplifying lauric esters **5a—cC**, in connection with biological substances. Figure 1 shows the fluorescence spectra of **5aC** and highly fluorescent **AMDC–laurate** in methanol for a comparison. The fluorescence maximum of **5aC** was observed in a longer wavelength region by ca. 10 nm than that of **AMDC–laurate**, suggesting



Scheme 3.

Table 3. Absorption and Fluorescence Spectral Data of Coumarin-Laurates in Some Solvents

Compound	UV λ_{max} (nm)			F λ_{max} (nm) (Quantum yield)								
	Acetonitrile			Methanol			Dichloromethane			Benzene		
5aC	391	471	(0.89)	392	482	(0.82)	395	466	(0.60)	394	461	(0.42)
5bC	391	470	(0.88)	391	483	(0.81)	395	466	(0.64)	394	461	(0.37)
5cC	390	470	(0.88)	392	485	(0.80)	393	466	(0.67)	393	461	(0.41)

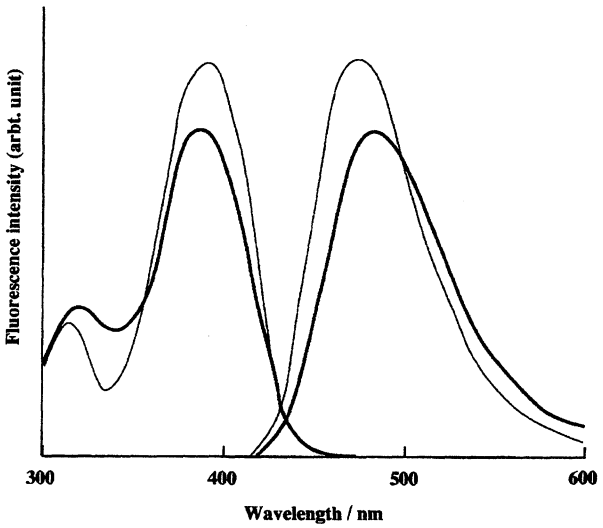


Fig. 1. Excitation and emission spectra of **AMDC–laurate** and **5aC** in methanol. (—): **AMDC–laurate**; (——): **5aC**; concentration; 1.0×10^{-6} mol dm^{-3}.

a slight increase in hydrophilicity of the products. The spectral data of **5a**—**cC** in various solvents are summarized in Table 3. The absorption and fluorescence maxima of **5a**—**cC** were shifted to longer wavelength regions with increasing polarity of the solvents, and their fluorescence quantum yields were quite large in polar solvents, such as acetonitrile and methanol (Table 3). Such a tendency in the fluorescence properties was also observed in those of all other compounds examined. Futhermore, these products also showed strong fluorescence, regardless of the kinds of carboxylic acids, as exemplified by their quantum yields (near to 0.8 for **5aA–C**, Table 4). This supports the validity of the reagent design based on the structural features for the strong fluorescence described above.

In conclusion, three reagents, **4a**—**c**, carrying crown-ether moieties in their molecules could be synthesized, which expressed the self-catalytic ability in fluorescence derivatization reactions for carboxylic acids. The reactivity was accelerated according to the complexation ability with metal ions, particularly K^+ in this case. Derivatized products still have the strong fluorescence expected based on the reagent design, as shown by their high quantum yields (Table 3). These results suggest that the functionalization of reagents is quite useful as an approach for the development of new-type reagents.

Further investigations concerning the development of other reagents, such as lariate-type crowned coumarins, are currently under way for higher reactivity.

Table 4. Fluorescence Quantum Yields of **5aA**—**C** in MeOH

Derivative	Quantum yield	F λ_{max} (nm)
5aA	0.79	484
5aB	0.79	483
5aC	0.82	485

Experimental

Apparatus and Measurements. The melting points were measured with a Yanagimoto micro-melting point apparatus, and are uncorrected. The absorption and fluorescence spectra were taken on a Hitachi U-3210 spectrophotometer and on a Hitachi F-4000 fluorescence spectrophotometer, respectively. The fluorescence quantum yields were determined according to the method of Parker and Rees;[13] the value (0.55) for quinine sulfate in 0.5 M H_2SO_4 was used as the standard (1 M =1 mol dm^{-3}). ^{1}H NMR were obtained with a JEOL JNM-GSX 500 FT-NMR spectrometer using tetramethylsilane as an internal standard. The following abbreviations are used: s, singlet; t, triplet; m, multiplet; Ar, aromatic. The mass spectra (MS) were taken with a JEOL JMS-DX303 spectrometer. HPLC was carried out on a Hitachi 6556A-12 equipped with a Hitachi F-1000 fluorescence spectrometer using a stainless-steel column (150×4.6 mm i.d., GL-Science Co., Ltd.) packed with Inertsil ODS-2 (5 μm) at room temperature. Methanol was used as the mobile phase at a flow rate of 1.0 ml min^{-1}.

Stability Constants: Measurements for the stability constants (K_s) were made on a methanol solution of acetyl compounds (**3a**—**c**: 1.0×10^{-5} mol dm^{-3}) and sodium and potassium acetates (1.0×10^{-6}—5.0×10^{-1} mol dm^{-3}). The K_ss were estimated by the usual treatment of Benesi–Hildebrand plots[14] obtained from the changes in the fluorescence intensities (E_x 373 nm, E_m 474 nm).

Relative Rate Constants: Coumarin reagents (**4a**—**c**) and lauric acid were dissolved in acetonitrile to prepare at 5.0×10^{-5} mol dm^{-3} and 5.0×10^{-3} mol dm^{-3} in concentration, respectively. The reaction was started by the addition of $KHCO_3$ (2 mg) to a mixed solution (1 ml each) prepared as mentioned above in a reaction vessel at 30 °C, then injected into a HPLC at two-minute intervals, followed by measurements of the fluorescence intensities (E_x 392 nm, E_m 485 nm) at the first stage under the conditions of pseudo-first-order kinetics. **BrAMDC** (5.0×10^{-5} mol dm^{-3}) and the corresponding B15Cr5 or B18Cr6 (5.0×10^{-5} mol dm^{-3}) were prepared in the same solvent, and then treated in the same manner as cases **4a**—**c**.

Synthesis. Formylation:[11] Dry hydrogen chloride

was bubbled into a mixtue of the phenol **1** (8.0 mmol) and zinc cyanide (12.0 mmol) in dry ether (100 ml) for 2 h under vigorous stirring. After the solvent was evaporated off, the residue was heated with water (30 ml) at 80 °C for 20 min. After the reaction mixture was extracted with chloroform, evaporation of the solvent gave folmylated compounds **2**.

2a: Yield 73%; ^{1}H NMR ($CDCl_3$) δ=3.73—4.15 (16H, m, $-OCH_2CH_2O-$), 6.45 (1H, s, ArH), 7.05 (1H, s, ArH), 9.66 (1H, s, CHO), 11.34 (1H, s, OH); MS m/z 312 (M^+).

2b: Yield 70%; ^{1}H NMR ($CDCl_3$) δ=3.66—4.23 (20H, m, $-OCH_2CH_2O-$), 6.44 (1H, s, ArH), 7.05 (1H, s, ArH), 9.67 (1H, s, CHO), 11.35 (1H, s, OH); MS m/z 356 (M^+).

2c: Yield 69%; ^{1}H NMR ($CDCl_3$) δ=3.39 (6H, s, OCH_3), 3.56—4.23 (16H, m, $-OCH_2CH_2O-$), 6.45 (1H, s, ArH), 7.05 (1H, s, ArH), 9.66 (1H, s, CHO), 11.34 (1H, s, OH); MS m/z 358 (M^+).

Coumarin Ring Formation:[9] To a mixture of the formyl compound **2** (5.8 mmol) and ethyl acetoacetate (5.8 mmol) in absolute ethanol (30 ml), two drops of piperidine were added; the solution was then refluxed for 10 min. After cooling, the resulting precipitates were collected by filtration and recrystallized from ethanol to yield the crowned coumarins **3**.

3a: Yield 94%; mp 194—196 °C; ^{1}H NMR ($CDCl_3$) δ = 2.71 (3H, s, $COCH_3$), 3.75—4.21 (16H, m, $-OCH_2CH_2O-$), 6.80 (1H, s, ArH), 6.97 (1H, s, ArH), 8.46 (1H, s, ArH); MS m/z 378 (M^+).

3b: Yield 88%; mp 180—182 °C; ^{1}H NMR ($CDCl_3$) δ= 2.71 (3H, s, $COCH_3$), 3.69—4.24 (20H, m, $-OCH_2CH_2O-$), 6.82 (1H, s, ArH), 6.98 (1H, s, ArH), 8.46 (1H, s, ArH); MS m/z 422 (M^+).

3c: Yield 80%; mp 134—136 °C; ^{1}H NMR ($CDCl_3$) δ=2.71 (3H, s, $COCH_3$), 3.40 (6H, s, OCH_3), 3.54—4.27 (16H, m, $-OCH_2CH_2O-$), 6.87 (1H, s, ArH), 7.07 (1H, s, ArH), 8.45 (1H, s, ArH); MS m/z 424 (M^+).

Bromination:[12] A mixture of the acetyl compound **3** (5.5 mmol) and TBA-Br_3 (5.5 mmol) in dichloromethane (400 ml) was stirred at room temperature for 12 h. After evaporation of the solvent, the crude product was submitted to column chromatography ($CHCl_3$) on alumina, and then purified by recrystallization from ethanol to yield the desired reagents **4**.

4a: Yield 85%; mp 188—190 °C; ^{1}H NMR ($CDCl_3$) δ = 3.75—4.23 (16H, m, $-OCH_2CH_2O-$), 4.77 (2H, s, $COCH_2Br$), 6.81 (1H, s, ArH), 6.99 (1H, s, ArH), 8.58 (1H, s, ArH); MS m/z 456 (M^+), 458 (M^++2). Found: C, 50.66; H, 4.72%. Calcd for $C_{19}H_{21}BrO_8$: C, 49.91; H, 4.63.

4b: Yield 84%; mp 174—176 °C; ^{1}H NMR ($CDCl_3$) δ = 3.68—4.26 (20H, m, $-OCH_2CH_2O-$), 4.77 (2H, s, $COCH_2Br$), 6.83 (1H, s, ArH), 6.99 (1H, s, ArH), 8.58 (1H, s, ArH); MS m/z 500 (M^+), 502 (M^++2). Found: C, 50.31; H, 5.03%. Calcd for $C_{21}H_{25}BrO_9$: C, 50.49; H, 5.08.

4c: Yield 81%; mp 143—145 °C; ^{1}H NMR ($CDCl_3$) δ=3.40 (6H, s, OCH_3), 3.57—4.28 (16H, m, $-OCH_2CH_2O-$), 4.77 (2H, s, $COCH_2Br$), 6.89 (1H, s, ArH), 7.10 (1H, s, ArH), 8.58 (1H, s, ArH); MS m/z 502 (M^+), 504 (M^++2). Found: C, 50.11; H, 5.41%. Calcd for $C_{21}H_{27}BrO_9$: C, 49.83; H, 5.47.

Derivatization of Carboxylic Acids: A mixture of the bromoacetyl reagent **4** (0.55 mmol), carboxylic acid (2.75 mmol) and $KHCO_3$ (2.75 mmol) in acetone (300 ml) was stirred at room temperature for ca. 2 h, and then filtered. After evaporation of the solvent, the residue was submitted to column chromatography ($CHCl_3$) on alumina. Products **5**, except **5cA** and **5cB**, were purified by recrystallization from ethnol and stored as standard samples.

5aA: Yield 68%; mp 203—205 °C; ^{1}H NMR ($CDCl_3$) δ = 2.22 (3H, s, $OCOCH_3$), 3.75— 4.23 (16H, m, $-OCH_2CH_2O-$), 5.37 (2H, s, $-COCH_2-$), 6.81 (1H, s, ArH), 6.98 (1H, s, ArH), 8.56 (1H, s, ArH); MS m/z 436 (M^+).

5bA: Yield 69%; mp 173— 175 °C; ^{1}H NMR ($CDCl_3$) δ=2.22 (3H, s, $OCOCH_3$), 3.69—4.25 (20H, m, $-OCH_2CH_2O-$), 5.37 (2H, s, $-COCH_2-$), 6.83 (1H, s, ArH), 7.00 (1H, s, ArH), 8.56 (1H, s, ArH); MS m/z 480 (M^+).

5cA: Yield 72%; ^{1}H NMR ($CDCl_3$) δ = 2.22 (3H, s, $OCOCH_3$), 3.40 (6H, s, OCH_3), 3.75—4.23 (16H, m, $-OCH_2CH_2O-$), 5.37 (2H, s, $-COCH_2-$), 6.89 (1H, s, ArH), 7.10 (1H, s, ArH), 8.56 (1H, s, ArH); MS m/z 482 (M^+).

5aB: Yield 72%; mp 218—220 °C; ^{1}H NMR ($CDCl_3$) δ = 3.72—4.24 (16H, m, $-OCH_2CH_2O-$), 5.62 (2H, s, $-COCH_2-$), 6.83 (1H, s, ArH), 6.99 (1H, s, ArH), 7.46—8.15 (5H, m, benzoate), 8.56 (1H, s, ArH); MS m/z 498 (M^+).

5bB: Yield 70%; mp 154—156 °C; ^{1}H NMR ($CDCl_3$) δ = 3.69—4.26 (20H, m, $-OCH_2CH_2O-$), 5.62 (2H, s, $-COCH_2-$), 6.84 (1H, s, ArH), 6.99 (1H, s, ArH), 7.46—8.15 (5H, m, benzoate), 8.58 (1H, s, ArH); MS m/z 542 (M^+).

5cB: Yield 65%; ^{1}H NMR ($CDCl_3$) δ=3.40 (6H, s, OCH_3), 3.56—4.29 (16H, m, $-OCH_2CH_2O-$), 5.61 (2H, s, $-COCH_2-$), 6.90 (1H, s, ArH), 7.10 (1H, s, ArH), 7.45—8.15 (5H, m, benzoate), 8.58 (1H, s, ArH); MS m/z 544 (M^+).

5aC: Yield 70%; mp 172—174 °C; ^{1}H NMR ($CDCl_3$) δ=0.88 (3H, t, CH_3), 1.26—2.49 (20H, m, laurate), 3.76—4.22 (16H, m, $-OCH_2CH_2O-$), 5.37 (2H, s, $-COCH_2-$), 6.81 (1H, s, ArH), 6.98 (1H, s, ArH), 8.56 (1H, s, ArH); MS m/z 576 (M^+).

5bC: Yield 71%; mp 173—175 °C; ^{1}H NMR ($CDCl_3$) δ=0.88 (3H, t, CH_3), 1.27—2.49 (20H, m, laurate), 3.68—4.25 (20H, m, $-OCH_2CH_2O-$), 5.37 (2H, s, $-COCH_2-$), 6.83 (1H, s, ArH), 6.97 (1H, s, ArH), 8.56 (1H, s, ArH); MS m/z 620 (M^+).

5cC: Yield 64%; mp 115—117 °C; ^{1}H NMR ($CDCl_3$) δ=0.88 (3H, t, CH_3), 1.27—2.47 (20H, m, laurate), 3.40 (6H, s, OCH_3), 3.56—4.27 (16H, m, $-OCH_2CH_2O-$), 5.36 (2H, s, $-COCH_2-$), 6.88 (1H, s, ArH), 7.08 (1H, s, ArH), 8.55 (1H, s, ArH); MS m/z 622 (M^+).

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