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Novel Fluorescent Coumarin Reagents Appending 15-Crown-5 Ether for the Rapid and Self-Catalytic Derivatization of Carboxylic Acids

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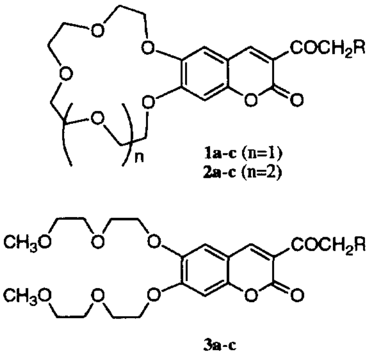
Novel fluorogenic coumarin derivatives appending 15-crown-5 ether as more active catalytic site were designed and synthesized for the fluorescence derivatization of carboxylic acids. From the stability constants (K_s) for complexation with metal acetates in methanol, it was found that their catalytic abilities were superior to the corresponding benzo-15-crown-5 ether type reagent. However, the reactivity of these reagents in the derivatization of lauric acid was dependent on not only the catalytic ability of the introduced crown-ether but also on the appending position of that in a reagent molecule. Furthermore, the derivatized products showed remarkably high fluorescence quantum yields of above 0.8 in methanol and acetonitrile. Allowing to hold a certain function such as catalyst in a reagent molecule should serve as a new strategy for the development of excellent analytical reagents.

The fluorescence derivatization is one of the most sensitive detection techniques for the trace analyses of environmentally and biologically relevant molecules.[1] For this purpose, various fluorescence derivatization reagents which can transform non-fluorescent molecules into fluorescent ones by the chemical reaction of functional groups between the analyte and the reagent, have been developed. They are widely utilized with the various separation techniques such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and the like. In a series of our investigations on the development of the fluorescence derivatization reagents for carboxylic acids,[2—5] the high sensitivity beyond 0.8 in fluorescence quantum yields of derivatized products has been achieved by using coumarin reagents which were designed based on the findings from the emission characteristics of original coumarin fluorophores.[6,7] In addition to the high sensitivity, the high reactivity of reagent should be also required for an excellent reagent because the harsh derivatization conditions such as high temperature and usage of strong base catalysts may cause serious damages to the analytes and the reagent itself. One approach for this requirement may be the suitable selection of a reacting group for the target molecule. Indeed, we have pointed out the superiority of a bromoacetyl group over a bromomethyl group which is used as a reacting group for carboxylic acid in the commercial fluorescence derivatization reagents.[3] There has been no definitive approach for the development of the derivatization reagents with both the high sensitivity and the high reactivity.



a; R=H, b; R=Br, c; R=OCO(CH$_2$)$_{10}$CH$_3$

Chart 1. Benzo-type crowned-coumarin reagents.

Recently, we have reported new-type reagents (**1b**, **2b**, and **3b** in Chart 1) which combined benzocrown ethers and analogous moieties as catalytic sites to coumarin fluorophore to accelerate and simplify the derivatization reactions for carboxylic acids using conventional esterification.[8,9] Then, the derivatization reactions with these reagents proceeded smoothly without crown ether catalysts to afford the corresponding esters such as **1c**, **2c**, and **3c**, which also kept the high fluorescence quantum yields beyond 0.8. As expected, their reactivities in the derivatization reaction of carboxylic acids were dependent upon the metal-binding ability of benzocrown ether moieties in reagent molecules. Interestingly, serious difference in the reactivity of a benzo-15-crown-5 (**B15Cr5**) type reagent, **1b** was observed between two bases, that is, the reactions were significantly accelarated by addition of $KHCO_3$ but not by that of $NaHCO_3$, suggesting the 2 : 1 complex of two **1b** with one K^+ ion. These results showed that the reactivity of such "functionalized reagents" was affected by not only the metal-binding ability of a crown-ether catalytic site in a molecule but also by the complexation mode. It is well known that crown-ethers selectively form the complexes with metal cations fitted in their ring sizes, and also that the aliphatic and aromatic crown-ethers such as 15-crown-5 (**15Cr5**) and **B15Cr5** ethers have the same crown-ring structures but the former is nevertheless greater in the complexing abilities with metal cations like Na^+ than the latter because of the flexible ring conformation of **15Cr5**.[10] At the stage of reagent design, combining the complexing characteristics of crown ethers with the emitting characteristics of fluorophores should facilitate the development of excellent reagents with both the high sensitivity and the high reactivity.

In this paper, we present the synthesis of two novel reagents appending **15Cr5** residue, so called "Lariat-type Crowned Reagents", at the different positions of coumarin skeleton as a more active catalytic site for the fluorescence derivatization of carboxylic acids. Besides the synthesis of reagents, the fundamental reagent properties including the reactivity and the sensitivity are evaluated through the derivatization reaction with a model compound, lauric acid.

Experimental

General. The melting points were measured with a Yanagimoto micro-melting point apparatus, and are uncorrected. The absorption and fluorescence spectra were taken with a Hitachi U-3210 spectrophotometer and a Hitachi F-4000 fluorescence spectrophotometer, respectively. Fluorescence quantum yields were determined according to the method of Parker and Rees,[11] and the value (0.55) for quinine sulfate in 0.5 M H_2SO_4 ($1\ M = 1\ mol\ dm^{-3}$) was used as the standard. ^1H NMR spectra were obtained on a JEOL JNM-GSX 500FT-NMR spectrometer using tetramethylsilane as an internal reference. The following abbreviations are used: s, singlet; t, triplet; m, multiplet. Mass spectra (MS) were recorded on a JEOL JMS-DX303 spectrometer. HPLC was carried out on a Hitachi 6556A-12 high-performance liquid chromatograph equipped with a Hitachi F-1000 fluorescence spectrophotometer 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.

3-Bromoacetyl-6-[(15-crown-5)-2-ylmethoxy]-7-methoxycoumarin (5a). To a solution of 3-acetyl-6-[(15-crown-5)-2-ylmethoxy]-7-methoxycoumarin (5.5 mmol, **4a**)[7] in dichloromethane (100 ml) was added tetrabutylammonium tribromide (TBA-Br_3, 5.5 mmol).[12] After the mixture was stirred at room temperature overnight, the solvent was evaporated to dryness under reduced pressure. Resulting residues were submitted to column chromatography ($CHCl_3$) on alumina. Recrystallization from ethanol gave yellow needles. Mp 162—164 °C. ^1H NMR ($CDCl_3$) $\delta = 3.64$—4.20 (21H, m, C_6–OCH_2–15Cr5), 3.96 (3H, s, C_7–OCH_3), 4.77 (3H, s, C_3–$COCH_2Br$), 6.84 (1H, s, C_5–H), 7.09 (1H, s, C_8–H), 8.59 (1H, s, C_4–H). MS *m*/*z* 544 (M^+), 546 ($M^+ + 2$).

3-Bromoacetyl-7-[(15-crown-5)-2-ylmethoxy]-6-methoxycoumarin (5b). 3-acetyl-7-[(15-crown-5)-2-ylmethoxy]-6-methoxycoumarin (**4b**)[7] could be used in the same manner as that of **5a** to give yellow needles. Mp 135—137 °C. ^1H NMR ($CDCl_3$) $\delta = 3.66$—4.28 (21H, m, C_7–OCH_2–15Cr5), 3.90 (3H, s, C_6–OCH_3), 4.77 (3H, s, C_3–$COCH_2Br$), 6.93 (1H, s, C_5–H), 6.97 (1H, s, C_8–H), 8.60 (1H, s, C_4–H). MS *m*/*z* 544 (M^+), 546 ($M^+ + 2$).

Standard Samples. A mixture of **5a** or **5b** (0.55 mmol), lauric acid (2.75 mmol), and $KHCO_3$ (2.75 mmol) in acetone (300 ml) was stirred at room temperature for 2 h. After the solvent was evaporated off under reduced pressure, the residue was submitted to column chromatography on alumina ($CHCl_3$). Products **6a** and **6b** were purified by recrystallization from ethanol and stored as standard samples.

6a: Mp 89—92 °C. ^1H NMR ($CDCl_3$) $\delta = 0.88$ (3H, t, –CH_3), 1.22—2.49 (20H, m, laurate), 3.64—4.21 (21H, m, C_6–OCH_2–15Cr5), 3.96 (3H, s, C_7–OCH_3), 5.37 (2H, s, C_3–$COCH_2$–), 6.84 (1H, s, C_5–H), 7.07 (1H, s, C_8–H), 8.57 (1H, s, C_4–H). MS *m*/*z* 664 (M^+).

6b: Mp 94—96 °C. ^1H NMR ($CDCl_3$) $\delta = 0.88$ (3H, t, –CH_3), 1.27—2.49 (20H, m, laurate), 3.66—4.29 (21H, m, C_6–OCH_2–15Cr5), 3.90 (3H, s, C_6–OCH_3), 5.37 (2H, s, C_3–$COCH_2$–), 6.91 (1H, s, C_5–H), 6.93 (1H, s, C_8–H), 8.57 (1H, s, C_4–H). MS *m*/*z* 664 (M^+).

Stability Constants.[13—15] Measurements for the stability constants (K_s) were made on a methanol solution of **4a** or **4b** (1.0×10^{-5} M, $1\ M = 1\ mol\ dm^{-3}$) and sodium or potassium acetate (1.0×10^{-6}—5.0×10^{-5} M). The K_s's were estimated by the usual treatment of Benesi–Hildebrand plots obtained from the changes in the fluorescence intensities (E_x 387 nm, E_m 477 nm).

Rate Constants.[8,9] Coumarin reagents (**5a,b**) and lauric acid were dissolved in acetonitrile to prepare at 5.0×10^{-5} M and 5.0×10^{-3} M in concentration, respectively. The derivatization reaction proceeded by the addition of $KHCO_3$ (2 mg) into a mixed solution (1 ml each of the above solutions). With a constant stirring at 30 °C, an aliquot (10 μl) of the reaction mixture was then injected into a HPLC at two minutes intervals, and the fluorescence intensities (E_x 392 nm, E_m 485 nm) were measured at the first stage under the pseudo-first-order kinetics conditions (experimental errors: ca. ± 5%). **5c**[5] (5.0×10^{-5} M) and the corresponding **15Cr5** (5.0×10^{-5} M) were also prepared in the same solvent and then treated with the same manner as the cases of **5a** and **5b**.

Results and Discussion

Synthesis of Lariat-Type Crowned Coumarin Reagents. The synthetic routes of novel fluorescent coumarin reagents, **5a** and **5b**, are shown in Chart 2. The starting 3-acetylated derivatives, **4a,b**, were prepared according to the method described in our recent paper.[7] Subsequent bromination of the acetyl groups with TBA-Br_3[12] in dichloromethane gave the desired reagents **5a** and **5b** in good yields as well as those of benzo-type crowned coumarins; **1b**, **2b**, and **3b**. The structures of **5a** and **5b** were fully characterized by mass spectrometry (MS) and ^1H NMR spectroscopy. For example, in reagent **5a**, a pair of isotope ion peaks due to a bromine atom was observed at *m*/*z* 544 and 546 corresponding to M^+ and $M^+ + 2$ in the MS spectrum, respectively. Furthermore, a new singlet peak for a bromoacetyl group appeared at 4.77 ppm, instead of that of the acetyl group of **4a** at 2.71 ppm in the ^1H NMR spectrum. As reference reagents without crown ether catalytic site, 6,7-dimethoxycoumarin derivatives, **4c**, **5c**, and **6c**, were also prepared.[5] It is noteworthy that all the crowned coumarin derivatives used in this study had good solubility in common organic solvents compared with 6,7-dimethoxycoumarin derivatives because of the high hydrophilicity of crown ether residues introduced in the reagent molecules.

Catalytic Ability of Lariat-Type Crowned Coumarin Reagents. Because of the higher reactivity of the bromoacetyl group in the reagent molecules, the catalytic ability of

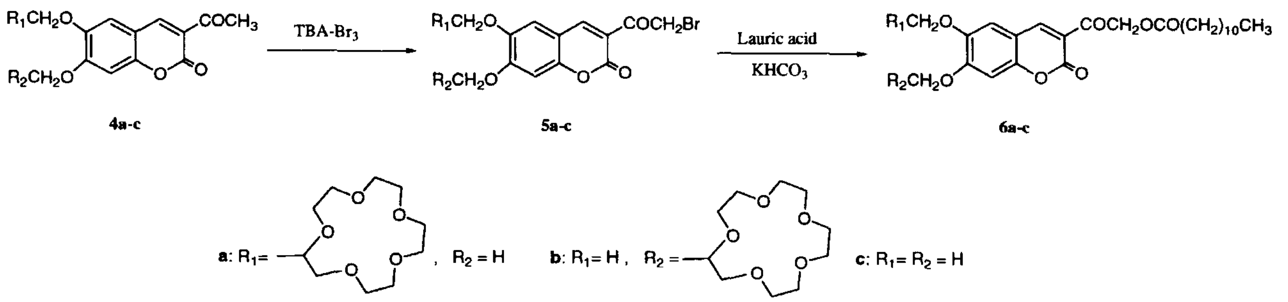


Chart 2.

5a and **5b** was estimated from the complexation ability of 3-acetyl derivatives, **4a** and **4b** with metal cations according to the method described in our previous paper concerning benzocrown-type coumarin reagents.[8,9] Sodium and potassium acetates as metal cation sources were used in this study by considering the derivatization reaction for carboxylic acids, and methanol was selected as measurement solvent for its good solubility of these salts. Table 1 shows the stability constants ($\log K_s$) for the complexation of **4a** and **4b** with Na^+ and K^+ in methanol together with those of **15Cr5** and benzocrown-type coumarin, **1a**, as reference compounds. The complexation abilities ($\log K_s$; 3.03—3.46) of **4a** and **4b** for Na^+ and K^+ were comparable with those of **15Cr5** itself ($\log K_s$ = 3.30 and 3.34 for Na^+ and K^+, respectively) as shown in Table 1. In contrast, the significant difference in $\log K_s$ values between lariat-type **4a** and **4b**, and benzo-type **1a** ($\log K_s$ = 1.98 for Na^+ and 2.24 for K^+) was observed. These results suggest that the reactivities of **5a** and **5b** are higher than that of the benzocrown-type reagent, **1b** in the derivatization of carboxlic acids.

Reactivity of Lariat-Type Crowned Coumarin Reagents. At first, the derivatization reactions of lauric acid with crowned reagents **5a** and **5b**, and a non-crowned **5c** for a comparison was actually attempted to compare the reactivities between these reagents. The reactions were carried out in the presence of base catalysts ($KHCO_3$ or $NaHCO_3$) in acetonitrile to give the corresponding lauric acid esters, **6a**, **6b**, and **6c**. These reactions were followed by a HPLC equipped with a fluorescence detector. Figure 1 shows the time-courses for the derivatization of lauric acid with **5b**, together with **5c** for a comparison. With both reagents, the significant acceleration of the reaction was observed in the presence of $KHCO_3$ as base catalyst as in the case of **1b**.[8,9] We had believed that the great differences in the reactivity of between K^+ and Na^+ ions should be attributed to the formation of a 2 : 1 sandwich complex of **B15Cr5** moiety as catalytic sites in **1b** with K^+ ion. However, this estimate may not be conclusive judging from the facts that the complexation abilities of **4a** and **4b** for Na^+ and K^+ were comparable with each other and were higher than that of **1a** for K^+, as shown in Table 1. For clearing up this doubt, it is necessary to investigate several factors such as the solubilities of base catalysts and their complexes with crown ethers in organic solvents and the nucleophilicities of naked carboxylate anions.[10]

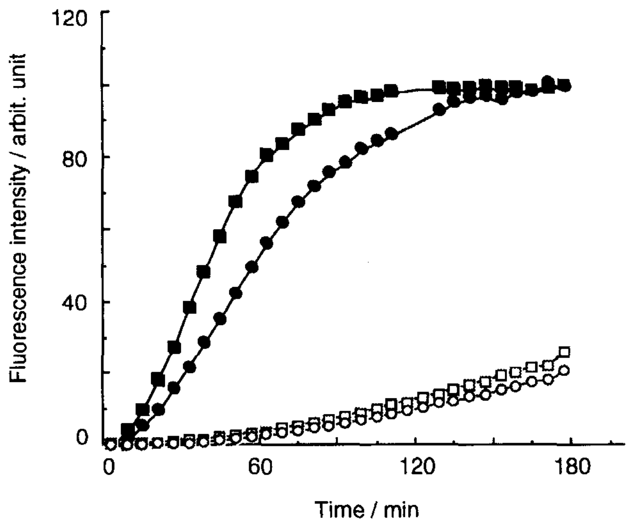


Fig. 1. Time course of the derivatization of lauric acid with **5b** and **5c** in acetonitrile at 30 °C. [**5b** and **5c**] = 2.5×10^{-5} mol dm^{-3}, [**15Cr5**] = 2.5×10^{-5} mol dm^{-3}, [lauric acid] = 2.5×10^{-3} mol dm^{-3}, [base] = 2 mg in a reaction solvent (2 ml). ■ : **5b**+$KHCO_3$; ● : **5c**+**15Cr5**+$KHCO_3$, □ : **5b**+$NaHCO_3$; ○ : **5c**+**15Cr5**+$NaHCO_3$.

Table 1. Stability Constants (K_s) of **4a,b** and Related Compounds with Na^+ and K^+ Ions in Methanol

Compound	$\log K_s$	
	Na^+	K^+
4a	3.03	3.36
4b	3.11	3.46
15Cr5	3.30[a]	3.34[a]
1a	1.98[b]	2.24[b]

a) Ref. 10c. b) Ref. 7.

Therefore, the reactivity of **5a** and **5b** were estimated only in the presence of $KHCO_3$, not that of $NaHCO_3$, together with other reagents, **1b** and **5c**. Table 2 shows the observed rate constants (k_{obsd}) obtained under the condition of pseudo-first-order kinetics. The effects of acceleration in the reactivity of **5a** and **5b** with lauric acid were about 1.7 and 1.2-times that of a reference compound, **5c**[5] upon adding **15Cr5**. The k_{obsd} of **5a** was also about 1.2-times that of **1b**. On the other hand, the k_{obsd} of **5b** was slightly smaller compared with that of **1b**. Furthermore, a large difference in the k_{obsd}s between **5a** and **5b** was observed in spite of their comparable stability constants for K^+ (Table 1). This suggests that the appending position of crown-ether catalytic sites in the

Table 2. Rate Constants for the Derivatization of Lauric Acid with Crowned Reagents in Acetonitrile

Crowned reagents	k_{obsd} / 10^4 M s^{-1}
1b	8.65
2b	20.80
3b	4.51
5a	10.66
5b	7.61
5c (+ **15Cr5**)	6.31

reagent molecules greatly affected their reactivity in addition to the catalytic ability. Perhaps the favorable distance and configuration between a bromoacetyl reactive site at C_3-position and a crown catalytic site at C_6-position of **5a** makes it easy to react with carboxylate anion. In contrast to **5a**, the separation of a crown catalytic site at C_7-position of **5b** is a little long from a reactive site at C_3-position, so the k_{obsd} of **5b** was slightly lower than that of **5a**, and also **1b** in spite of the higher catalytic ability than that of **1b**. Unfortunately, direct evidence for above estimate is not obtained even from their NMR spectroscopic or fluorescence spectrophotometric studies. On the other hand, the logarithmic plots of the k_{obsd} and the K_s for the same derivatization reactions of lauric acid with all crowned coumarin reagents,**1b**, **2b**, **3b**, and **5a, b** gave a good straight line (Fig. 2). This interesting result suggests that the complexation process of the reagents with metal ion governs the rate of derivatization reactions, namely the rate-determining step. This quantitative relationship between the k_{obsd} and the K_s also suggests that the molecular design based upon both the discerning and the stationing of individual components with various functions in the reagent molecule is essential for the development of "Supramolecular Analytical Reagent"[16] in addition to an understanding of the fluorescence characteristics of fluorophores used.[6,7]

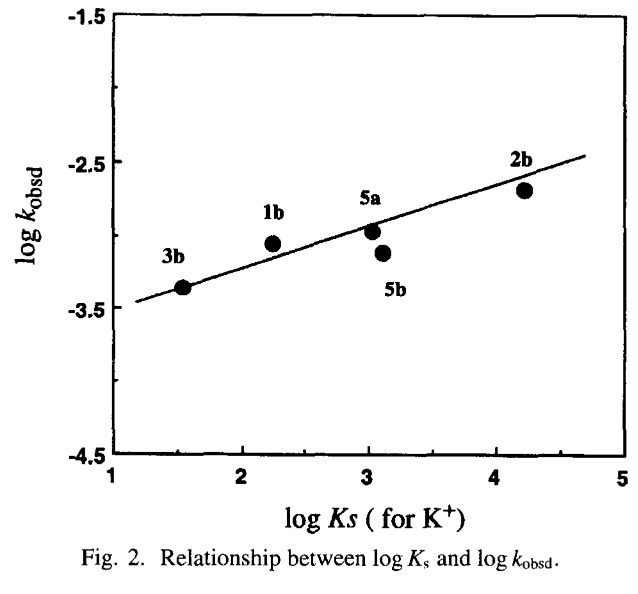


Fig. 2. Relationship between log K_s and log k_{obsd}.

Fluorescence Characteristics of Derivatized Products. The fluorescence spectra of **6a,b** obtained by derivatizing lauric acid with **5a,b** were measured in methanol and acetonitrile which are frequently used as mobile phases for the HPLC analysis. Their spectral data are shown in Table 3 together with those of the other compounds, **6c** and **1c**, for comparison. The fluorescent behaviors of derivatized products, **6a** and **6b** resembled those of **6c** and **1c**. Small difference between in methanol and in acetonitrile in fluorescence quantum yields of these esters seem to be attributable to the solvent polarity (for example E_T = 55.5 for methanol, E_T = 46.0 for acetonitrile) supposing the emission mechanism of coumarin derivatives to be based on the intramolecular charge-transfer.[6,7] It was confirmed that the strong fluorescence beyond 0.8 in fluorescence quantum yields still held in **6a** and **6b** as expected from their structural features for fluorescing.[6,7] Besides the excellent fluorescence spectral properties of such derivatized carboxilic acid products, their structures carrying a very hydrohilic crown ether site and a hydrophobic site such as different long alkyl side-chain originated from fatty acids on a coumarin skeleton at the same time may serve an unique eluting properties in the separation analysis such as HPLC or CE. In practice, the satisfactory separation between a crowned reagent **5a** and its derivatized product of lauric acid **6a** was achieved in a reversed HPLC analysis using a short ODS column and methanol only as a mobile phase.

In conclusion, two fluorescence derivatizing reagents, **5a** and **5b**, appending **15Cr5** at different positions in their molecules could be synthesized. They possessed excellent self-catalytic reactivity, compared with those of non-crowned types. Especially, **5a** showed a greater reactivity than the corresponding benzocrown-type reagent **1b** and its isomeric type **5b** in the fluorescence derivatization of lauric acid. Fluorescence quantum yields of derivatized products with these reagents were over 0.8 in the polar solvent like methanol and acetonitrile, as shown in Table 3.

A series of our investigations on the development of derivatizing reagents would make it predictable the future generation of such reagents, in which 18-crown-6 ether or [2.2.2]cryptand residues would serve as novel and potential

Table 3. Spectral Properties of Crowned-Coumarin Laurates

Laurate	Methanol		Acetonitrile	
	UV λ_{max} / nm	F λ_{max} / nm (Φ)	UV λ_{max} / nm	F λ_{max} / nm (Φ)
6a	393	485 (0.82)	390	486 (0.85)
6b	390	478 (0.83)	390	474 (0.85)
6c	391	485 (0.81)	391	471 (0.85)
1c	393	485 (0.83)	391	477 (0.89)

catalytic components in a reagent molecule.

References

1 "Handbook of Derivatives for Chromatography (in Japanese)," ed by K. Blau and J. Halket, 2nd ed, Maruzen-Wiley, Tokyo (1996); "Modern Derivatization Methods for Separation Sciences," ed by Toyo'oka, Wiley, Tokyo (1999).

2 A. Takadate, T. Masuda, C. Tajima, C. Murata, M. Irikura, and S. Goya, *Anal. Sci.*, **8**, 663 (1992).

3 A. Takadate, T. Masuda, C. Murata, C. Haratake, A. Isobe, M. Irikura, and S. Goya, *Anal. Sci.*, **8**, 695 (1992).

4 T. Masuda, A. Isobe, C. Murata, A.Takadate, M. Komai, and S. Kimura, *Tohoku J. Agr. Res.*, **43**, 111 (1993).

5 A. Takadate, T. Masuda, C. Murata, A. Isobe, T. Shinohara, M. Irikura, and S. Goya, *Anal. Sci.*, **13**, 753 (1997).

6 A. Takadate, T. Masuda, C. Murata, T. Tanaka, M. Irikura, and S. Goya, *Anal. Sci.*, **11**, 97 (1995).

7 A. Takadate, T. Masuda, C. Murata, M. Shibuya, and A. Isobe, *Chem. Pharm. Bull.*, **48**, 256 (2000).

8 A. Takadate, T. Masuda, C. Murata, T. Tanaka, H. Miyahara, and S. Goya, *Chem. Lett.*, **1993**, 811.

9 A. Takadate, T. Masuda, C. Murata, T. Tanaka, and S. Goya, *Bull. Chem. Soc. Jpn.*, **68**, 3105 (1995).

10 For example, see: a) E. Hiraoka, "Crown Compounds-their characteristics and applications-," Elsvier, Tokyo (1982). b) "Host Guest Complex Chemistry I—III (*Top. Curr. Chem.*, **98**, **101**, **121**)," ed by F. Vogtle and E. Weber, Springer-Verlag, Berlin (1985). c) R. M. Izatt, J. Bradshow, S. A. Nielsen, J. D. Lamb, and J. J. Christensen, *Chem. Rev.*, **1985**, 811.

11 C. A. Parker and W. T. Rees, *Analyst (London)*, **85**, 587 (1960).

12 S. Kajigaeshi, T. Takinami, T. Okamoto, and S. Fujisaki, *Bull. Chem. Soc. Jpn.*, **60**, 1159 (1987).

13 S. Fery-Forgues, M. T. LeBris, J. P. Guett, and B. Valeur, *Chem. Commun.*, **1998**, 384.

14 S. Fery-Forgues, M. T. LeBris, J. P. Guett, and B. Valeur, *J. Phys. Chem.*, **92**, 6233 (1988).

15 J. Burson and B. Valeur, *J. Phys. Chem.*, **93**, 3871 (1989).

16 J. -M. Lehn, *Angew. Chem., Int. Ed. Engl.*, **27**, 89 (1988); J. -M. Lehn, *Angew. Chem., Int. Ed. Engl.*, **29**, 1304 (1990); J. -M. Lehn, "Supramolecular Chemistry," VCH, Weinheim (1995).